GENE THERAPY AND MOLECULAR MEDICINE

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March 26-April 1, 1995; Steamboat Springs, Colorado

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Genetic Disorders

C6-001 GENE THERAPY FOR METABOLIC DISORDERS & CANCER, Savio L.C. Woo, Ph.D., Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas.

The liver is the major organ for metabolism and there are dozens of known genetic disorders secondary to a variety of hepatic deficiencies in man. The development of technologies to deliver functional genes into hepatocytes in vivo would permit gene therapy for these disorders in the future. Using recombinant retroviral vectors to deliver the canine factor IX gene into the liver of Hemophilia B dogs, we were able to achieve sustained partial correction of the bleeding phenotype in vivo. In addition, we have demonstrated that recombinant adenoviral vectors are extremely efficient for hepatic gene delivery in vivo. Complete restoration of whole blood clotting time to normal was achieved in the Hemophilia B dogs, although the therapeutic effect did not persist much beyond 3 weeks. Therapeutic levels of factor IX expression in the treated hemophilia dogs however, persisted for more than 3 months if the animals were immuno-suppressed by cyclosporin A administration, suggesting that the lack of persistence was due to rejection of the adenoviral transduced hepatocytes by the host immune system in vivo. The recombinant adenoviral backbone is being further modified to reduce viral antigen expression in order to achieve sustained therapeutic gene expression in vivo.

The lack of persistence in gene expression after recombinant adenoviral vector delivery in vivo is not a particular concern for cancer treatment, as the objective here is to eliminate the cancer cells within a few days. The therapeutic efficacy of adenovirus-mediated HSV-tk gene transduction of rat glioma cells followed by ganciclovir (GCV) administration was studied in tumors generated in immunocompetent syngeneic Fisher rats. Tumors were generated by stereotaxic intracerebral injection of 9L glioma cells. After 8 days of tumor growth, ADV/RSV-tk viral particles were directly injected into the tumors and the animals were subsequently treated with GCV for 6 consecutive days. Tumor size in untreated and treated animals was compared at 20 days after tumor cell implantation. The mean cross-sectional area and the tumor volume were drastically reduced in the treatment group. Furthermore, the treated animals survived for more than 120 days without relapse while the control animals all died of brain tumors by 21 days after glioma cell inoculation. These results demonstrate unambiguously that the recombinant adenoviral vector can function as an efficient suicide gene delivery vehicle for the treatment of solid tumors by in vivo gene therapy.

We have also investigated the efficacy of combination therapy for metastatic tumors in the liver with a suicide gene and a cytokine gene. Tumor in the liver was generated by intrahepatic injection of a colon carcinoma cell line (MCA 26) in syngeneic BALB/c mice. Recombinant adenoviral vectors containing various control and therapeutic genes were injected directly into the solid tumors, followed by treatment with ganciclovir. While the tumors continued to grow in all animals treated with a control vector or a mouse interleukin 2 vector, those treated with a Herpes Simplex Virus thymidine kinase vector, with or without the co-administration of the mouse interleukin 2 vector, exhibited dramatic necrosis and regression. However, only animals treated with both vectors developed an effective systemic anti-tumoral immunity against challenges of tumorigenic doses of parental tumor cells inoculated at distant sites. The anti-tumoral immunity was associated with the presence of MCA26 tumor specific cytolytic CD8+ T-lymphocytes. The results suggest that combination suicide and cytokine gene therapy in vivo can be a powerful approach for treatment of metastatic tumors.

Stem Cell Biology and Tissue Remodeling

C6-002 DIFFERENTIATION AND GROWTH CONTROL IN MYOBLASTS: APPLICATIONS IN GENE THERAPY, Helen M. Blau, Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA 94305-5332.

Skeletal muscle has many advantages for gene therapy. Gene delivery can be achieved either by direct injection of DNA or by implantation of genetically engineered muscle precursor cells. DNA injection leads to high level localized gene expression that can be effectively regulated over several orders of magnitude by exogenous agents such as tetracycline. Alternatively, genetically engineered myoblasts can be used for systemic delivery of recombinant proteins. Myoblasts appear to be advantageous over other cell types because following injection they become integrated into the multinucleated myofiber of the host, are in contact with the circulation, and sustained by neuronal activity. Recently developed methods for isolating and purifying primary neonatal mouse myoblasts may now allow myoblasts to be isolated from any strain including a wide range of mouse models of human disease created by homologous recombination. The behavior of such mutant and genetically engineered myoblasts can be characterized in vitro and following transplantation into mice. Molecules of particular interest include cell adhesion proteins and untranslated RNAs (riboregulators) with a role in growth control and tumor suppression. Given these properties, muscle is an attractive target for the treatment of inherited and acquired myopathies and nonmuscle disorders including hemophilia, heart disease, and cancer which are being actively explored in mouse models of human disease.

C6-003 EVALUATION OF PROCEDURES FOR GENE TRANSFER INTO PRIMITIVE HUMAN HEMATOPOIETIC CELLS USING THE SCID-REPOPULATION ASSAY. John E. Dick, Josef Vormoor, Andre Larochelle Dept. of Genetics Hospital for Sick Children and Dept of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario, Canada, M5G1X8.

One of the major problems in assessing the efficiency of retrovirus-mediated gene transfer into human pluripotent stem cells is the absence of experimental assays for these cells. Although progenitor cells can be assayed in vitro, stem cells can only be measured by their ability to re-populate the entire blood system after transplantation. It has clearly been shown that gene transfer into progenitors is very efficient, but this may not be relevant for more primitive cells. Although murine stem cells are easily infectable, the infection frequency into stem cells from other species such as primates and dogs is much lower. In order to study gene transfer into primitive human cells, we have taken advantage of the system we have developed for transplanting human hematopoietic cells into immune-deficient mice. Our past and present studies strongly suggest that primitive cells (more immature than progenitors) engraft the murine microenvironment. The bone marrow contains large numbers of $CD34^+CD38^-Thy^+$ cells. The mice can also be transplanted with purified $CD34^+$ cells. These immature cells proliferate and differentiate into multiple myeloid, erythroid, and B cell lineages resulting in extensive repopulation of the murine bone marrow. Although we do not yet know the exact relationship of the SCID-repopulating cell with the human stem cell, this system does provides the foundation for an assay for primitive human cells. Using a similar transplantation approach, we have also identified and characterized leukemic stem cells in AML. We have recently established an hemoglobinopathy model in SCID mice that permits both the assay of primitive cells but also a means to monitor correction of the erythroid cellular defect in thalassemia and sickle cell anemia. Using common methods for retrovirus gene transfer, we have been able to assess two important parameters; the maintenance of primitive cells during in vitro cultures and the efficiency of gene transfer. Human cord blood cells were co-cultivated for 1-2 days with packaging cells producing a NEO vector or an ADA vector. The cells were prestimulated with a cocktail of IL-3/IL-6/SCF. The cells were then transplanted into SCID mice. Interestingly, there was a striking difference in the level of engraftment between the 1 and 2 day co-cultured cells-the 1 day group had high levels of human cells (>10%) while the 2 day group had lower levels (~1%). This indicates that primitive cells are lost during the in vitro cultures. All subsequent experiments were done with a more immune-deficient NOD/SCID mouse which can be engrafted with lower numbers of stem cells and contain high levels of human cells even after 2 days of culture. With the lower titer (~10^5 pfu) NEO vector, the efficiency of gene transfer into CFU-C progenitors was 4% after 1 day and 14% after 2 days co-culture. The higher titer ($5x10^6$pfu) ADA vector resulted in much higher frequencies (>60%). In contrast to the efficient gene transfer into CFU-C, only low levels of genetically manipulated cells could be detected in the mice (<1% by PCR) using the NEO vector. Experiments are in progress with the ADA vector. These results establish that the SCID system is an important tool in the development of gene transfer protocols directed to primitive cells. Our results imply the the current procedures that lead to efficient gene transfer into progenitors are very inefficient for primitive cells. The reason for this difference is not known but may be due to our lack of understanding of the culture conditions required for stem cells or the stem cells may not express the retroviral receptor.

Gene Expression and Regulation Vectors

C6-004 EXPLOITING PROKARYOTIC ELEMENTS FOR THE CONTROL OF GENE ACTIVITY IN HIGHER EUKARYOTICS, Manfred Gossen[1], Sabine Freundlieb[1], Gabi Bender[1], Andreas Kistner[1], Udo Baron[1], Gerhard Müller[2], Wolfgang Hillen[2], and Hermann Bujard[1], [1] ZMBH, Universität Heidelberg, Germany, [2] Institut für Mikrobiologie, Universität Erlangen, Germany.

The high specificity of regulatory elements of the *E.coli* tetracycline (Tc) resistance operon as well as the well known pharmacological properties of Tc and many of its derivatives appeared promising prerequisites for the development of a regulatory system which would allow to specifically control the transcription of individual genes in higher eukaryotic systems. By fusing the Tet repressor with the activating domain of Herpes simplex virus protein 16, a tetracycline controlled transcriptional activator (tTA) was obtained which strongly activates minimal RNA polymerase II promoters fused downstream of multiple tet operator sequences. This activation is abolished by low concentrations of Tc (1μg/ml) in the medium. In HeLa cells producing constitutively tTA and containing appropriate reporter units stably integrated, an up to 10^5 fold range of regulation was observed depending on the concentration of Tc in the medium. This regulatory system has been transferred into transgenic plants (tobacco) and mice where again tight control and a wide range of regulation in dependence of Tc can be achieved. By genetically altering the Tet repressor moiety, we have reversed the phenotype of tTA. The resulting rtTA now requires the binding of a ligand (certain tetracycline derivatives) for function. Thus, addition of a Tc-derivative such as anhydrotetracycline will lead to activation of transcription via rtTA. Again, this system allows to tightly regulate a transcription unit in HeLa cells over a range of more than 3 orders of magnitude, and its function in transgenic mice has been demonstrated as well. While complementing the previous tTA system, transcription control via rtTA has two distinct advantages: (a) to turn on transcription by the addition of an effector molecule is a kinetically favorable process; (b) to keep a gene of interest inactive, the continuous presence of Tc is not required. The latter properties are of particular advantage for studies *in vivo* such as in transgenic animals or in gene therapy. Moreover, proper combination of the two systems may open up perspectives for the temporal control of more than one transcription unit via Tc and its derivatives.

C6-005 A REGULATORY SYSTEM FOR USE IN GENE TRANSFER, Bert W. O'Malley, Yaolin Wang, and Sophia Y. Tsai, Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX, 77030.

We recently have demonstrated that a C-terminal deletion mutant of the human progesterone receptor (hPRB891) fails to bind to progesterone but can bind RU 486 and other progesterone antagonists. Most significantly, this mutant receptor activates transcription of a reporter gene containing the progesterone response element in the presence of these antagonists. Taking advantage of this finding and the modular nature of functional domains of steroid receptors, we constructed a chimeric regulator (pGL-VP) by fusing the ligand-binding domain of human progesterone receptor hPRB891 to the yeast transcriptional activator GAL4 DNA-binding domain and the herpes simplex virus protein VP16 activation domain. We demonstrated that this chimeric regulator activates target genes containing the GAL4-binding sites in transient transfection assays in response to RU 486. In addition, this regulatory system has been validated by *ex vivo* transplantation of a stable cell line containing both the regulator and a reporter gene into rats. The dosage of RU 486 used is significantly lower than that required for antagonizing progesterone action. A second generation of vectors has been constructed recently which allows greater versatility relative to basal levels and induction ratios of expression of the target genes. The gene-switch system reported here represents a regulatory system, which could be applicable for gene-transfer studies involving animals, as well as humans, in which the delivered gene(s) can be specifically turned on/off in response to an exogenous compound.

C6-006 REGULATORY ELEMENTS OF THE AVIAN α-SKELETAL ACTIN DIRECT HIGH LEVELS OF TISSUE SPECIFIC EXPRESSION: APPLICATION FOR GENE THERAPY, Robert J. Schwartz, Michael Coleman, Louis Smith, and Francesco DeMayo, Department of Cell Biology, Baylor College of Medicine, Houston.

Gene therapy may be useful in non-genetic diseases by the introduction of novel genes into an individual to circumvent a defective biological function or to reverse the pathological process. An excellent example would be the increased expression of IGF-I from muscle to serve as a therapeutic neurotrophic factor for long term treatment of diabetic peripheral neuropathies. Elevated IGF-I expression from muscle might also play an important role in stimulating muscle growth and regeneration. A myogenic vector system, MyVS, based on the skeletal α-actin gene was developed for postnatal gene therapy, which will be used to increase localized levels of human insulin like growth factor in muscle. We report here that the proximal 411 bp promoter region, together with the first intron and 2.3 kb of 3' flanking sequence of the chicken α-skeletal actin gene direct high level and tissue-specific expression of a hIGF-I cDNA in stably-transfected C_2C_{12} myoblasts and transgenic mice. Transgene expression in stably-transfected C_2C_{12} cells was negligible in replicating myoblasts and up-regulated significantly with the onset of myogenesis as determined by Northern blotting and RIA analysis of conditioned media. This resulted in increased levels of myogenic factor and contractile protein mRNAs and myotube hypertrophy. The 3' UTR of the chicken α-skeletal actin gene was found to be necessary for restricting expression of the transgene to skeletal muscle in mice. Expression of the single copy transgene in mice resulted in concentration of IGF-I in skeletal muscle being 10- to 20-fold higher for transgenic animals as compared to littermate controls. This resulted in pronounced hypertrophy (i.e., an approximate 70 to 80% increase in cross-sectional diameter) of both type I and type II myofibers. Direct DNA delivery in muscle results in rather long-term expression of DNA expression vectors as a non-integrated, episomal DNA. However, a major stumbling block for implementing gene therapy in muscle is the poor efficiency of naked DNA uptake and stability following muscle injection. We will present our progress in developing novel synthetic carriers to improve DNA vector uptake and stability in adult muscle.

Results of Human Gene Therapy Trials

C6-007 RETROVIRAL VECTOR GENE TRANSFER INTO DONOR PERIPHERAL BLOOD LYMPHOCYTES FOR IN VITRO SELECTION AND IN VIVO IMMUNOMODULATION OF DONOR ANTI-TUMOR IMMUNITY AFTER ALLO-BMT. Simona Verzeletti, Chiara Bonini, Catia Traversari, Silvano Rossini, Giuliana Ferrari, Nadia Nobili, Paolo Servida, Fulvio Mavilio, and Claudio Bordignon, Istituto Scientifico H. S. Raffaele, Milan, Italy.

The infusion of donor's lymphocytes after allogeneic BMT is a potentially useful therapeutic tool for relapse of hematologic malignancies and for other complications related to the aggressive immunosuppression regimens. However, severe graft-vs-host disease (GvHD) may result from this therapeutic approach. In order to circumvent this complication we constructed and tested at preclinical and clinical levels retroviral vectors for transfer of the HSV-tk gene. To this purpose, we designed a series of retroviral vectors carrying the "suicide" gene for the selective elimination of the infused lymphocytes. The vectors carried either a tk-neoR fusion gene, coding for a chimeric protein for both negative and positive selection, or the HSV tk gene alone. The latter construct produced higher sensitivity to ganciclovir as result of a higher TK activity. This was shown in vitro by complementation of LTK- cells in HAT medium. Ganciclovir sensitivity in transduced cells was shown in vitro in the packaging cell line, as well as in human melanoma cell lines, human lymphocytes, and in the murine lymphoma cell line YC8. In vivo, ganciclovir infusion resulted in reduced survival of transduced cells in a syngeneic recipient murine model.

A crucial prerequisite for the application of this strategy to the clinical contest is the transduction of all infused donor lymphocytes. For this purpose, we devised a simple protocol based on vector-mediated gene transfer and expression in transduced cells of a modified (non-functional) cell surface marker not expressed on human lymphocytes, followed by positive immunoselection of the transduced cells (1). This results in virtually 100% gene-modified PBL. An additional advantage of this strategy is related to the possibility of PBL infection during antigen stimulation of effector cells. Since retroviral vectors infect almost exclusively dividing cells, positive selection of transduced cells allows a significant enrichment of antigen-specific lymphocytes. Based upon the preclinical data described above, a clinical protocol was developed for the use of donor lymphocytes transduced by the SFCMM retroviral vector for transfer and expression of two genes: 1- the HSV thymidine-kinase gene that confers to the transduced PBL in vivo sensitivity to the drug ganciclovir for in vivo specific elimination of cells potentially responsible for GvHD; 2- a modified (non functional) form of the low affinity receptor for the nerve growth factor gene (ΔLNGFR), for in vitro selection of transduced cells and for in vivo follow-up of the infused donor lymphocytes. After approval by the Ethical Committee, escalating doses (beginning at $1x10^6$/kg) of donor PBL were infused into six patients affected by hematologic malignancies who developed severe complications (two with an EBV limphoproliferative disease, two in CML relapse, and two in AML relapse) following a T-cell depleted BMT from their HLA-identical related donors. After infusion, the transduced lymphocytes could be detected in the blood of recipients by FACS and PCR analyses. No correlation was observed in this small series between the number of infused PBL and the appearance of GvHD. However, GvHD was observed in the two patients who showed higher levels and persistence of circulating "marked" cells. A progressive increase of the infused marked lymphocytes up to 13,4% (98% CD3+, CD8+ with high proliferation index) of total PBL was observed in one patient affected by a EBV-BLPD and was accompanied by a complete clinical response. A patient with CML showed a less pronounced increase in the infused lymphocytes up to 4.7% of circulating PBL. During the two months following the administration of gene-modified donor PBL, the two patients showed clinical signs of skin and liver GvHD. In both cases the administration of multiple (2-4) doses of ganciclovir (10 mg/Kg/die) quickly resulted in elimination of marked donor PBL, and near resolution of all clinical and biochemical signs of acute GvHD.

1 - F. Mavilio et al., Blood 83:1988,1994

AIDS

C6-008 GENETIC INTERVENTIONS FOR CANCER AND AIDS IN HUMANS, Gary J. Nabel[1], Clive Woffendin[1], Elizabeth G. Nabel[2], Zhi-yong Yang[1], Keith Bishop[2], Magda Marquet[3], Philip Felgner[3], David Gordon[2], Udaykumar[2], Ling Xu[2], Ning-Sun Yang[4], Michael Sheehy[4], Alfred E. Chang[2], [1]Howard Hughes Medical Institute, Ann Arbor, MI; [2]University of Michigan Medical Center, Ann Arbor, MI; [3]Vical, Inc., San Diego, CA; [4]Agracetus, Inc., Middleton, WI.

Genetic instability within malignant cells gives rise to mutant proteins which can be recognized by the immune system. Recognition of tumor-associated antigens by T lymphocytes could thus contribute to the elimination of neoplastic cells. We have developed a molecular genetic intervention for the treatment of malignancies based upon the knowledge that foreign major histocompatibility complex (MHC) proteins expressed on the cell surface are efficient at stimulating an immune response. Foreign MHC genes were introduced directly into malignant tumors, *in vivo,* in an effort to stimulate an immune response and the subsequent rejection of the tumor cells. Expression of this foreign MHC gene within tumors induced cytotoxic T cell response to the introduced gene and to tumor-specific antigens on unmodified tumor cells as foreign. Our laboratory has conducted two clinical studies to determine the safety and efficacy of this treatment in humans. Specifically, a DNA-liposome complex containing the gene encoding the MHC HLA-B7 protein was introduced into HLA-B7-negative patients with advanced melanoma. Gene transfer, recombinant gene expression, safety, and potential toxicity were measured. In both cases, plasmid DNA was detected in treated tumor nodules, and all patients produced the recombinant HLA-B7 protein. Immune responses to autologous tumors were also induced. There were no antibodies to DNA. The molecular and immunologic analysis of this response will be described. These studies suggest that direct gene transfer provides a safe and feasible approach for the treatment of human cancer. Since the acquired immunodeficiency syndrome (AIDS) has been refractory to traditional pharmacologic interventions, alternative approaches have also been developed. We have begun to develop approaches to inhibit HIV replication in $CD4^+$ cells. We have evaluated the efficacy of a transdominant mutant protein, Rev M10, against cloned and primary HIV isolates in human peripheral blood lymphocytes (PBL's) and describe novel methods of gene transfer into PBLs from HIV-infected individuals. Gold microparticles can mediate stable Rev M10 gene transfer into these cells, and provide comparable protection to retroviral vectors. Introduction of Rev M10 by these techniques conferred resistance to HIV infection *in vitro* to cloned and clinical isolates without altering normal T cell function. Nonviral delivery of HIV protective genes will facilitate the development of gene therapy for AIDS and the analysis of viral and cellular gene expression in human T lymphocytes. A human clinical study has been initiated to assess its potential to protect against HIV infection in T cells of AIDS patients. This trial and other novel antiviral strategies will be described.

Neurological Disorders

C6-009 EXTENDING GENE DELIVERY IN THE BRAIN USING VIRUS VECTORS, Xandra O. Breakefield[1], Miguel Sena-Esteves[1], Christof Kramm[1], Peter Pechan[1], Maureen Chase[1], James Livermore[1], Guicinan Nilaver[2], Leslie L. Muldoon[2], Antonio E. Chiocca[1], Edward Kaye[1], and Edward Neuwelt[2], [1]Massachusetts General Hospital, Charlestown, MA, 02129, [2]Oregon Health Sciences University, Portland, OR.

Most current schemes for gene delivery to the postnatal rodent brain involve stereotactic introduction of cells or vectors into a focal region, which limits the target range of transgene influence. We have employed several strategies in an attempt to extend this range. Replication-defective retrovirus vectors, in which the transgene is under the control of the strong mammalian "housekeeping" promoter for phosphoglycerate kinase, have been used for delivery to newborn astrocytes. These cells have the potential to migrate in the brain and to mediate sustained expression of the transgene. Replication compromised herpes simplex virus type 1 (HSV) vectors have also been employed to extend the range of delivery to tumor cells in the brain. Replication-conditional mutants defective in the thymidine kinase (TK) or ribonucleotide reductase (RR) loci are able to propagate only in dividing cells, and either enter latency or are degraded in non-dividing cells. Virus replication kills tumor cells and toxicity can be increased by including drug-enhancing genes, such as the HSV-TK/ganciclovir combination, which produce nucleotide analogues that are toxic to dividing cells. When the virus is inoculated directly in the tumor mass replication spreads in a saltatory mode, extending out to cells which have migrated short distances from the mass. Virus delivery can also be achieved through intracarotid injection of the virus combined with osmotic shock to temporarily open the blood-brain-barrier. This approach has the potential to allow the vector to reach newly forming tumor foci throughout the brain. These new strategies can be employed both to extend the delivery range of diffusible molecules, like growth factors and lysosomal enzymes, and to reach new loci of tumor formation in the postnatal brain.

C6-010 PROTECTIVE EFFECTS OF INCREASED EXPRESSION OF CuZn-SUPEROXIDE DISMUTASE IN THE CENTRAL NERVOUS SYSTEM OF TRANSGENIC MICE. Charles J. Epstein[1], Ting-Ting Huang[1], Elaine Carlson[1], Pak H. Chan[1], Patrik Brundin[2], Naoyuki Nakao[2], EvaFrodl[2], Håkan Widner[2], and Jean L. Cadet[3], [1]University of California, San Francisco, CA 94143, [2]University Hospital, S-22185 Lund, Sweden, and [3]NIH/NIDA Addiction Research Center, Baltimore, MD 21224.

There is considerable interest in the use of CuZn-superoxide dismutase (SOD), the first enzyme in the pathway of oxygen radical detoxification, as a therapeutic agent, and many therapeutic studies have been carried out using exogenously administered CuZnSOD. These have been problematic in the CNS because of difficulties in introducing the enzyme across the blood-brain barrier. However, transgenic mouse technology permits the constitutive alteration of intracellular CuZnSOD activity, and we have generated a series of transgenic mice carrying between 2 and 8 copies of the human CuZnSOD gene under control of the native promoter. Transgenic mice with 3 to 5 times increased CuZnSOD in erythrocytes, fibroblasts, and neurons are protected to various degrees in vivo against a variety of acute and chronic insults to the central nervous system in which oxygen free radicals (O_2^-) and/or nitric oxide (NO·) are believed to play a pathogenic role. These insults, both physical and chemical in nature, include cold injury, blunt trauma, ischemia and reperfusion (stroke), and the toxic effects on dopaminergic neurons of MPTP, methamphetamine, and methamphetamine derivatives (which produce models of Parkinson disease). Furthermore, when dopaminergic neurons derived from the mesencephalon of transgenic mouse embryos were transplanted into the striatum of adult hemiparkinsonian rats, the survival of the transplanted neurons was four times greater than that of nontransgenic neurons, the graft-derived reinnervation was increased 6.5 fold, and the functional response was more rapid. The transplantation findings suggest that oxidative stress may play a crucial role in the death of transplanted dopaminergic neurons in rodents and, by inference, in humans. Taken together, our results indicate that relatively small changes in CuZnSOD activity can have profound effects on the cellular response to a variety of stimuli that cause damage to the CNS through the generation of oxygen free radicals.

C6-011 **GRAFTING GENETICALLY MODIFIED CELLS TO THE BRAIN FOR NEUROLOGICAL DISEASE,** Fred H. Gage, The Salk Institute, La Jolla, CA 92037, USA

Intracerebral grafting has emerged as a useful experimental tool to address a variety of questions in neurobiology. Furthermore, the ability to restore function through neuronal grafting in the CNS has suggested a potential approach to CNS therapy through the selective replacement of cells lost as a result of disease or damage. Independently, the development of genetic tools has begun to suggest genetic approaches to the treatment of human diseases. A combination of these two techniques, namely the intracerebral grafting of genetically modified cells, has been shown to be a useful tool to address biological issues, and an advanced approach toward the restoration of function in the damaged or diseased CNS.

Neurotrophic factors are molecules that support the survival of selective neuronal processes during development. These molecules have also been shown to protect adult and aged neurons from undergoing degeneration. Furthermore, these factors have been shown to promote the growth of intact and damaged axons. When trophic factors are delivered to the brain via somatic cell transplantation, evidence for functional regeneration has now been established. The anatomical and physiological results of intracerebral grafting of cells genetically modified to secrete NGF, BDNF, NT-3, and FGF-2 will be presented. Data will be presented that show that 1) the local delivery of these factors can result in cell specific neuron survival, neuronal hypertrophy, and axonal regeneration; 2) the biological effects of the implants in a specific brain region can have a long lasting effect on the behavior of the whole organism; 3) the biological effects are predictable and consistent between rodent and non-human primate models of human neurological disease; 4) different cells perform and function differently, though carrying the same gene, dependent on the area of the brain that they are implanted, and finally that 5) grafting combinations of genetically engineered cells with defined neuronal populations or with other cells that are engineered to secrete neurotransmitters, can reveal more specifically the function of specific micro circuits in the brain.

C6-012 **CNS PROGENITOR & "STEM-LIKE" CELLS AS GENE DELIVERY VEHICLES & MEDIATORS OF REPAIR,** Evan Y. Snyder[1], JD Macklis[1], JH Wolfe[2], EI Ginns[3], Moncef Jendoubi[4], RL Sidman[5], A Tessler[6], RL Proia [7], DI Gottlieb[8], T Friedmann[9], BD Yandava[1], JD Flax[1], S Aurora[1], CH Yoon[1], RL Mozell[1], Z-H Pan[1], RM Taylor[2], C McKinney[3], HD Lacorazza[4], CM Rosario[5], B Kosaras[5], D L Kitchens[8], L Baum[9] [1]Dept. of Neurology, Harvard Med. School, Boston MA; [2]Dept. Med. Genetics, Univ. of PA. School of Vet. Med., Philadelphia PA; [3]NIMH, Bethesda MD; [4]NEI, Bethesda MD; [5]New England Regional Primate Center, Southborough MA; [6]Dept. of Anatomy, Med. College of PA, Philadelphia PA; [7]NIDDK, Bethesda MD; [8]Dept. of Anatomy & Neurobiology, Washington U, St. Louis MO; [9]UCSD Sch. of Med, La Jolla CA

We have demonstrated that immortalized multipotent clonal neural progenitors can, following transplantation, integrate in a cytoarchitecturally-appropriate, non-tumorigenic manner into recipient mouse CNS (from embryo to adult) & differentiate into a wide range of neurons & glia throughout the neuraxis, presumably responding to region- & developmental stage-specific microenvironmental signals. Donor progenitors intermingle non-disruptively with endogenous progenitors & respond to the same spatial & temporal cues in the same manner as host progenitors. The immortalization process does not subvert the ability of these progenitors to respond to normal cues (e.g., withdraw from the cell cycle, differentiate, interact with host cells). Some transplant-derived neurons receive synapses. No brain tumors are ever seen. Engrafted progenitors are recognized by expression of a retrovirally-transduced reporter gene (*lacZ*) (which often remains robust for prolonged periods).

These data suggest the feasibility of the transplantation of immortalized neural progenitors expressing gene products of therapeutic &/or developmental importance (either intrinsically or following *ex vivo* genetic manipulation) as a strategy for the sustained delivery of such factors directly to the CNS as integral members of the cytoarchitecture. With delivery targeted & restricted to CNS (perhaps with a regulated release by virtue of their integration as neural tissue), the systemic side effects from peripheral interventions (drugs, bone marrow transplantion) might be avoided. Furthermore, progenitors might also replace injured cell types &/or provide nondiffusible factors (myelin, "bridges", cell-cell contact signals) which might allow the injured host to reform its own connections. In fact, such CNS-derived progenitors might intrinsically provide as-yet-unrecognized substances beneficial to the host. Our studies have, in fact, suggested successful gene transfer throughout the brains of mouse models of neurovisceral diseases with single gene defects, e.g. *β-glucuronidase* into the *Mucopolysaccaridosis VII* mouse & *β-glucocerebrosidase* into the *Gaucher's Disease* mouse. In the former, we achieved rescue of the mutants' neuropathology (i.e., cross-correction of host neural tissue with elimination or decrease of lysosomal storage.) *Hexosaminidase A* has also been successfully expressed throughout brain. Other studies have suggested repletion of selectively *degenerated neurons in adult neocortex*. Other studies have suggested replacement of oligodendrocytes throughout the brains of mutants (*Shiverer*) with *diffuse white matter disease*. Other studies are exploring the utility of progenitors for replacing motorneurons in segments of mouse *spinal cord rendered depleted of motorneurons*. We hypothesize that the basic biology & inherent properties of neural progenitors make them appealingly suited to be ubiquitous, economical, efficient, & multifaceted vehicles for cell replacement, repair, & gene transfer in CNS dysfunction. Such lines may also provide models for commitment, differentiation, & plasticity of neural progenitors, lending developmental insights.

DNA Delivery Systems

C6-013 CATIONIC LIPOSOME-MEDIATED, *IN VIVO* GENE TRANSFER AND EXPRESSION, Wendy Zhong[1], Denny Liggitt[2], Yong Liu[1], Tim Heath[3], Karin Gaensler[1], Guanghuan Tu[1] and Robert Debs[1], [1] University of California, San Francisco, CA,94143-0128, [2]University of Washington School of Medicine, Seattle, WA. 98195, and [3]University of Wisconsin, Madison WI., and.

We are investigating cationic liposome-based *in vivo* gene and transfer expression both to create somatic cell transgenic animals and to develop efficient and safe approaches for gene therapy. Currently, we are attempting to maximize the level, duration and cellular-specificity of gene expression produced by cationic liposome-mediated gene delivery into rodents. We have determined the effects of varying multiple different parameters: including cationic liposome formulation, expression vector construction, formation of the DNA:liposome complex and the host milieu on the level of gene transfer and expression in animals. We have found that by selectively altering these parameters, we can increase the level of tissue luciferase activity up to than five orders of magnitude, following intravenous (iv) injection of human cytomegalovirus (CMV)-luciferase expression plasmid:cationic liposome complexes into mice. We have produced significant levels of reporter gene expression in various tissues following iv, intraperitioneal, aerosol, subcutaneous, or lateral ventricle (brain) administration into adult mice, or by injecting DNA:liposome complexes directly into fetuses in pregnant rats. Administration of appropriate expression plasmid:cationic liposome complexes can also be used to express a variety of biologically and therapeutically relevant genes, including the cystic fibrosis transmembrane conductance regulator (CFTR) gene, the human glucocerebrosidase gene and the mouse granulocyte-macrophage colony stimulating factor gene. While iv injection of DNA:liposome complexes produces transgene expression in essentially every tissue, and a variety of cell types, their administration by the other routes mentioned above can produce tissue-specific patterns of gene expression. We have also produced some degree of tissue- and cell type-specific targeting in mice by administering cationic liposomes complexed to the chloramphenicol acetyltransferase (CAT) gene linked to 3.8 kb of the human CFTR promoter region, suggesting that tissue-specific promoters also can be used to target transgene expression. Currently, we are exploring methods for targeting the delivery and the expression of delivered genes to circulating immune effector cells and tissue-based phagocytic cells.

C6-014 *Abstract Withdrawn*

C6-015 RECEPTOR - MEDIATED GENE TRANSFER AND ENDOSOMAL RELEASE, Ernst Wagner[1,2], Michael Buschle[2], Karl Mechtler[1], Antoine Kichler[1], Christian Plank[1], Wolfgang Zauner[1], Dieter Blaas[3], Ernst Küchler[3], Kurt Zatloukal[1], Matt Cotten[1], and Max L. Birnstiel[1]; [1]Institute of Molecular Pathology, and [2]Bender/Boehringer Ingelheim, [3]Vienna Biocenter, Vienna, Austria.

Gene transfer systems have been developed which use the receptor-mediated endocytosis route to import DNA into mammalian cells. Gene constructs have been complexed with polylysine-conjugated ligands (such as transferrin, EGF, or anti-CD3 antibodies) for uptake into endosomes. Accumulation of these complexes in intracellular vesicles however strongly reduces the efficiency of gene transfer.

Viruses have acquired mechanisms to release their genome from endosomes into the cytoplasm. The endosomal acidification process specifically activates viral surface proteins that trigger destabilization of the endosomal membrane. This has led to the development of virus-like gene transfer complexes consisting of DNA complexed with a cell-binding ligand and polylysine-conjugated, endosome-disruption agents (such as adenoviruses or rhinoviruses) which allow cytoplasmic entry of the DNA. Transferrin-polylysine / DNA complexes linked to replication-defective and methoxypsoralen /UV-inactivated adenoviruses have been applied for the expression of high levels of genes (factor VIII, cytokines) in a large proportion of target cells (up to >80% in primary fibroblasts, primary myoblasts or human melanoma cell isolates).

Synthetic versions of the gene transfer system have been generated by replacing the whole virus by small synthetic peptides similar to sequences occurring in the hemagglutinin of influenza virus or in the VP-1 protein of rhinoviruses. At at acidic, endosomal pH these peptides adopt the active structure of an amphipathic helix that can interact with the lipid membrane and destabilize it. DNA complexes containing endosome-disruptive peptides were used for transfection of several cell lines or primary human melanoma cells. The great enhancement of gene expression mediated by the peptides strongly correlated with their capacity to lyse erythrocytes in an acidic environment.

Structural and charge properties of DNA complexes strongly influence the cell targeting specificity, endosomal release, and overall gene transfer efficiency. The destabilization of lipid membranes is dependent on the composition of the membrane. Using optimized DNA complexes containing transferrin and polylysine, and compounds that transiently change the cell membrane properties, efficient and non-toxic gene transfer to cultured primary fibroblasts was obtained in the absence of membrane-active viruses or peptides.

Recombinant Viral Vectors

C6-016 GENE THERAPY APPLICATIONS USING HERPES SIMPLEX VIRUS VECTORS

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Herpes simplex virus type 1 (HSV-1) naturally establishes latency in neurons of the peripheral nervous system with the concommitant expression of the latency associated transcripts (LATS) and the loss in lytic gene functions. Recent evidence indicates that the mutant viruses lacking the ability to replicate can persist in a variety of post mitotic cells in an episomal state. We have attempted to exploit these features to develop HSV vectors for gene transfer to brain and other tissues. However two impediments to using this virus for gene delivery have to be overcome: namely viral cytotoxicity and the design of promoter/regulatory systems to maintain foreign gene expression during latency in different cell types *in vivo*. To address the first problem, virus mutants deleted for the essential immediate early (IE) genes (ICP4 and ICP27) responsible for initiating the lytic gene program were constructed and propagated on specifically engineered complementing cell lines. Nonessential genes encoding the host shut off function and the viral ribonucleotide reductase large subunit were also eliminated. The multiply deleted virus was greatly reduced in cytotoxic potential relative to currently used HSV vector strains and host cell protein synthesis was not affected upon infection. To solve the second problem, a variety of viral, cellular and recombinant promoters was recombined into defective HSV vectors and tested for their ability to continuously express reporter gene RNA and protein in rat hippocampus following stereotaxic inoculation. These included first, the natural HSV-1 latency promoters LAP1 and LAP2 and a recombinant autocatalytic promoter driving expression of a GAL4-VP16 transactivator. The HSV-1 LAPs remained active in brain and were capable of expressing the *E. coli lacZ* gene and ß-galactosidase product but at reduced levels compared with their ability to express this reporter gene in the peripheral nervous system. Studies are underway to use the latency promoter system to express nerve growth factor *in vivo*. Second, an antogene vector carrying a recombinant yeast GAL4:VP16 fusion gene was capable of transactivating promoters containing the Gal4 binding site. This transcription system was used to effect vector mediated tyrosine hydroxylase (TH) expression resulting in the production of both L-DOPA and dopamine in neuronal cells in culture and in rat brain following stereotaxic infection into cortex and striatum. Finally, HSV vectors carrying the natural thymidine kinase gene and various cytokine and immunomodulatory genes under control of strong nonHSV promoter are being tested for debulking and immunization against skin and brain tumors in animal model systems.

C6-017 REDUCING THE IMMUNE RESPONSE AGAINST ADENOVIRAL VECTORS. Martin G Lee, Hedi Haddada, Michel Perricaudet. Viral Oncogene Genetics Laboratory, URA 1301 CNRS, Institut Gustave Roussy PR2, 94805 Villejuif, France

The immune response against cells infected by gene therapy vectors may be a major hindrance for gene therapy. Gene therapy vectors are explicitly designed to express foreign genes in cells. Antigens expressed by the exogene and antigens from the vector itself provoke a specific and non specific immune response, resulting in inflammation and limiting the length of exogene expression. Adenoviruses and many other pathogens have evolved strategies for escape from immune surveillance, including the gp19k gene found in the adenovirus E3 region. This region being dispensable for *in vitro* viral replication is deleted in many current adenoviral vectors in order to increase the cloning capacity. The inclusion in vectors, adenoviral and otherwise, of genes such as gp19k or genes from other pathogens known to act on the immune system may reduce the capacity of the immune response to lyse infected cells and reduce inflammation following vector administration. We are evaluating the effects on the immune response of *in vivo* administration of several adenoviral vectors expressing genes known to reduce immune functions. Further localised immune supression is being investigated by the concurrent administration of monoclonal antibodies to neutralise immune effectors at the site of injection. These methods may be preferable to the induction of systemic immuno-suppression to reduce inflammation and rejection of infected cells. Further deletions in the adenoviral genome and the tailoring of virus promoters are being undertaken in order to further reduce the immunogenicity of the virus and to introduce further levels of securtiy to prevent the propagation of recombinant virus.

Cancer

C6-018 MOLECULAR SURGERY FOR SOLID TUMORS. Kenneth W. Culver, Jesse Lamsam, Jamie Stratton, Monte Bennett, and Tatiana Seregina, Molecular Immunology Laboratory, Human Gene Therapy Research Institute, Des Moines, IA 50309.

Targeting features of the genetic basis of malignancy may allow the selective destruction of tumor cells, sparing normal tissues. Therefore, we have focused on the selective transfer of the Herpes Simplex-thymidine kinase (HS-tk) gene into cancer cells in vivo. The transfer of the HS-tk gene confers a sensitivity to the anti-viral drug, ganciclovir (GCV). In order to achieve selective gene transfer into tumor cells, we have utilized the requirement for cell proliferation as a means to selectively deliver murine retroviral vectors into tumor cells. We have demonstrated in selective gene transfer into tumors growing in the brain, liver, skin and peritoneal cavity, with gene transfer efficiencies that range from 10-55%. Interestingly, the treatment of animals bearing HS-tk positive tumors with GCV resulted in complete tumor ablation in more than 50% of animals if ≥ 10% of the cells contained the HS-tk gene. This phenomenon has been termed the "bystander" tumor killing effect. Further investigations into the bystander effect have shown that there is substantial variability between human tumors. In cell cultures that are 10% HS-tk positive, there are substantial differences in the percent inhibition of proliferation that generally correlates with the rate of proliferation. Cell lines that have a doubling time of 20-24 hours have >90% inhibition, while cells with doubling times of ≥40 hours are inhibited only 60-80%. These findings suggest that regardless of the delivery system, the particular histologic type and/or their inherent rate of proliferation may have a significant impact of the antitumor efficacy in vivo. A phase I human clinical trial using the stereotactic injection of PA317/ G1TkSvNa.53 VPC (Genetic Therapy Inc.; GTI) into the gadolinium-enhancing portion of the tumor began in December, 1992 at the National Institutes of Health. Fifteen patients with a relatively diverse group of tumors have been enrolled. No acute toxicities related to the injection of xenogeneic VPC or GCV have been noted, with some of the patients demonstrating evidence of an anti-tumor effect. Based upon these phase I results, efforts to improve gene delivery were made leading to the initiation of a phase I/II multicenter trial in July, 1994. The new trial uses a new GTI vector, PA317/G1Tk1SvNa.7, that has a 2-fold higher titer and a markedly increased antitumor efficacy in animals. The intratumoral delivery method has also been altered. First, the tumor is maximally resected and then VPC are injected into the surrounding brain, where infiltrating, nonresectable tumor cells reside. Repeated injections of the VPC without surgery are made through an Ommaya reservoir. The inclusion criteria have also been narrowed to include only the most aggressive form of glioblastoma multiforme. Hopefully these trial design changes will result in enhanced antitumor efficacy.

C6-019 PRECLINICAL STUDIES TOWARD GENE THERAPY OF PROSTATE CANCER, Johannes Vieweg[1], David Snyder[1], David Boczkowski[1], Kathryn Roberson[2], Cary Robertson[2], Mohan Philip[3], Ramila Philip[3], and Eli Gilboa[1], [1]Department of Surgery, [2]Division of Urology, Duke University Medical Center, Durham, NC and Applied Immunosciences, Santa Clara, CA.

Cancer of the prostate is the most commonly diagnosed cancer in men and is the second most common cause of cancer death in the Western civilization. The majority of cancers are discovered once they have already metastasized, and there is no effective therapy for prostatic cancer at this stage. Using the Dunning rat R3327-MatLyLu prostatic tumor model we have shown that IL-2 secreting, irradiated, tumor cell preparations were capable of curing animals with subcutaneously established tumors, and induced immunological memory that protected animals from subsequent tumor challenge. Immunotherapy was less effective when tumors were induced orthotopically, but nevertheless led to improved outcome, significantly delaying, and occasionally preventing, recurrence of tumors after resection of the cancerous prostate. Granulocyte-macrophage colony stimulating factor secreting tumor cell preparations were less effective, and interferon-gamma secreting cells had only a marginal effect.

To test the clinical feasibility of using genetically modified tumor vaccines (GMTV) for the treatment of prostate cancer, we have explored the use of a simplified gene delivery system based on liposomes to introduce and express the human interleukin 2 (IL-2) gene in the Dunning rat R3327 MatLyLu prostatic tumor cell line (MatLyLu), and in short-term cultures of primary human prostatic tumor cells. Liposome-DNA complexes containing the adeno-associated virus (AAV) inverted terminal repeats (ITR) exhibited 3-10 fold higher levels of gene transfer and IL-2 expression than liposome complexes with non-AAV containing plasmids. Single transfections resulted in IL-2 expression for an extended period of time that exceeded several fold the amount of IL-2 secreted from retrovirally transduced MatLyLu cells. X-irradiation of cells (4,000 Rads) prior to transfection did not affect cytokine secretion, indicating that liposomal-mediated gene transfer does not depend on cell proliferation. High levels of gene transfer and IL-2 expression were also achieved in short term cultures of primary human prostatic tumor cells established from tumor specimen obtained following radical prostatectomy of cancer patients. IL-2 levels secreted from the human prostatic tumor cells were comparable to the levels of IL-2 secreted from retrovirally transduced MatLyLu cells which induced antitumor immunity in the rat model. The ability to culture and expand ex vivo human prostatic tumor cells, and the use of simple and highly efficient gene transfer method to generate GMTV sets the stage for clinical exploration of gene-based immunotherapy of prostate cancer.

Late Abstract

GENE THERAPY FOR VASCULAR SMOOTH MUSCLE CELL PROLIFERATION AFTER ARTERIAL INJURY, Elizabeth G. Nabel, Takeshi Ohno, David Gordon, Hong San, Vincent J. Pompili, Michael J. Imperiale, Gary J. Nabel. University of Michigan, Ann Arbor.

Arterial wall injury induces the synthesis of gene products that stimulate smooth muscle cell migration and proliferation, leading to intimal hyperplasia. This process contributes to the pathogenesis of many cardiovascular disorders, including vascular proliferative diseases and atherosclerosis. Molecular approaches to the inhibition of smooth muscle cell (smc) proliferation could potentially limit intimal expansion following vascular injury. This problem was approached by introducing adenoviral vectors encoding the herpesvirus thymidine kinase (tk) gene into porcine arteries that had been injured by balloon angioplasty. We first defined the kinetics of smc proliferation in the intima. Proliferation was observed within 24 hours after injury; incorporation of BrdC was maximal 4-7 days after the injury and subsided by 14 days. The efficacy of herpesvirus tk in limiting porcine vascular smc growth was assessed after gene transduction and exposure to ganciclovir (GC) in vitro where a bystander effect was demonstrated. To determine whether gene expression could be achieved at the appropriate sites in injured arteries, adenoviral vectors encoding a reporter gene, human placental alkaline phosphatase, were introduced into injured porcine arteries by catheter. Gene expression was observed in smc in the intima and lumenal region of the media, suggesting that genetic modification of relevant cell types could be achieved in vivo and used to modulate the response to injury. Adenoviral vectors encoding a tk gene or no cDNA insert were introduced into porcine arteries immediately after balloon injury, and a course of ganciclovir or saline was initiated. Three and six weeks later, a significant reduction in the intima to media area ratio (54-59%) was observed in tk/+GC treated animals compared with control groups. A 40% reduction in intimal cell proliferation was observed in tk/+GC animals compared with control animals 7 days after gene transfer. These data suggest that expression of a enzyme that catalyzes the formation of a cytotoxic drug locally within an artery may limit smc proliferation following balloon injury.

Genetic Disorders; Stem Cell Biology

C6-100 GENE THERAPY IN THE FUCOSIDOSIS DOG. Donald S. Anson*, Teresa Occhiodoro*, Margaret L. Ferrara#, John J. Hopwood* and Graeme J. Stewart#. * Department of Chemical Pathology, Women and Childrens Hospital, Adelaide, S. Australia 5006 and # Clinical Immunology, Westmead Hospital, Westmead, NSW 2145, Australia.

The potential for gene therapy for the CNS disease associated with many of the lysosomal storage disorders has been demonstrated by allogeneic bone marrow transplantation experiments in the fucosidosis dog. The fucosidosis dog displays a variety of symptoms, the vast majority of which are due to CNS pathology. Somatic pathology has only a peripheral effect on the development of overt symptoms. Early allogeneic BMT leads to an almost complete cessation in disease development.

We are now using this animal model to develop gene therapy for the lysosomal storage disorders. We have generated retroviral vectors and demonstrated correction of fucosidosis fibroblasts in culture (1). We have now generated simplified retroviral vectors and used these to transduce bone marrow cells in allogeneic transplantation experiments using fucosidosis donors and recipients. Two dogs have shown preliminary evidence of successful gene transfer into bone marrow cells and are being monitored for persistence of proviral sequences and expression of fucosidase enzyme.

1. Occhiodoro, T., Hopwood, J.J., Morris, C.P. and Anson, D.S. (1992) Human Gene Therapy 3:365-369

C6-101 CENTRIFUGATION PROMOTES RETROVIRAL TRANSDUCTION OF CD34+ ENRICHED HEMATOPOIETIC CELLS. Alfred B. Bahnson, Maya T. Nimgaonkar, Bora Baysal, Jane Mannion-Henderson, James Dunigan, Trina Mohney, Robert W. Atchison, Edward D. Ball and John A. Barranger. Department of Human Genetics, Graduate School of Public Health and Division of Hematology/Bone Marrow Transplantation, University of Pittsburgh, Pittsburgh, PA 15261.

Gene therapy protocols involving viral vectors may benefit from improved *ex vivo* transduction using centrifugation. (This approach improves detection of many types of viruses in clinical specimens.) We are conducting preclinical studies of retroviral-mediated transfer of the normal human glucocerebrosidase (GC) gene into CD34+ cells for gene therapy of Gaucher disease. Using three 2-hour centrifugations at 2400xg following one day of prestimulation in IL-3, IL-6, and SCF, cord blood CD34+ cells were transduced with an efficiency of 95% based upon CFU-GM analysis by PCR. Nonadherent cells from long term cultures (LTC-IC) after 4 to 6 weeks yielded CFU-GM transduction frequencies of 17 to 20% by PCR. CFU-GM colonies from control nontransduced cells and from cells transduced at 1xg in this experiment were negative. GC activity from the MFG-GC vector was about 6 times the normal levels in nontransduced cells following expansion in cytokine-containing medium for 2 to 3 weeks after infection. Expression in nonadherent cells from LTC-IC was 2 times control levels after 6 weeks of culture. Additional experiments with CD34+ enriched samples are underway.

The parameters affecting centrifugal enhancement of retroviral transduction have been investigated using a nonadherent human hematopoietic cell line, TF-1. Transduction efficiency was directly related to centrifugation time up to 7 hours and to relative centrifugal force up to 10,000xg. Transduction efficiency was inversely related to cell number per tube, and was higher in round-bottom tubes in comparison to conical-bottom tubes. No adverse effect on cell viability was observed after a 2-hour centrifugation at 20,000xg. Further improvement of CD34+ transduction efficiency may be obtained using optimized conditions.

C6-102 **GENE TRANSFER STRATEGIES FOR LYSOSOMAL STORAGE DISEASES,** John A. Barranger, Department of Human Genetics, University of Pittsburgh, Pittsburgh, PA 15261

Lysosomal storage disorders result from mutations in genes encoding glycoprotein enzymes translocated across the membrane of the endoplasmic reticulum to reside finally within the lysosome. Failure to catabolize natural substances because of these enzymatic deficiencies is the cause of more than 20 different lysosomal storage disorders. The physical findings in these disorders are protean. Animal models exist or have been developed for some of these disorders. The most prominent signs for a particular disorder determine the tissue or cell which might result in the greatest benefit if a genetic correction were accomplished. This can be quite different among the lysosomal storage disorders. For example, in Type 1 Gaucher disease, the bone marrow is the most appropriate target cell. However, in lysosomal disorders involving the nervous system, gene transfer to bone marrow may not be sufficient to achieve a satisfying clinical outcome. Data will be presented on the use of different vector systems and expression in different target cells that encompass several strategies in the application of gene transfer to lysosomal storage disorders. Included will be the development of retroviral and adeno-associated viral vectors and transfer of the genes for glucocerebrosidase and arylsulfatase A to bone marrow cells, muscle cells, and cells of the central nervous system. The results of transplantation will be discussed.

C6-103 TRANSPLANTATION OF CD34-POSITIVE PERIPHERAL BLOOD PROGENITOR CELLS FOLLOWING HIGH-DOSE CHEMOTHERAPY FOR PATIENTS WITH ADVANCED MULTIPLE MYELOMA, J. Berenson, G. Schiller, R. Vescio, C. Freytes, G. Spitzer, M. Lee, A. Lichtenstein, M. Lill, J. Hall, and R. Berenson, UCLA & DVA WLA; CellPro, Inc., Los Angeles, CA and Bothell, WA.

A multi-institutional study of purified CD34-selected peripheral blood progenitor cell (PBPC) transplantation was conducted in 37 patients with advanced multiple myeloma (MM), receiving myeloablative chemotherapy. Fourteen days after intermediate-dose cyclophosphamide, prednisone and G-CSF, a median of 3 leukaphereses yielded $9.8x10^8$ mononuclear cells/kg (range 3.7-28.3). The adsorbed fraction contained $5.9x10^6$ cells/kg (range 1.6-25.5) with $4.65x10^6$ CD34 cells/kg (range 1.2-23.3). Using Poisson distribution analysis of positive PCR reactions with patient-specific complementarity-determining regions (CDR) 1 and 3 Ig gene primers, tumor was detected in 7 of 13 unselected leukapheresis products and ranged from $2.95x10^4$-$2.14x10^6$ malignant cells/kg. After CD34 selection, tumor was detected in only three patients' products (range 83-2,000 cells/kg). Overall, a > 2.7 to > 4.5 log reduction in contaminating MM cells was achieved. CD34-PBPC were infused one day after busulfan (14 mg/kg) and cyclophosphamide (120 mg/kg), and GM-CSF was used posttransplant. The median time to both neutrophil and platelet recovery was 12 days. Patients receiving fewer than $2x10^6$ CD34 cells/kg had significantly prolonged platelet recovery and an increased erythrocyte and platelet transfusion requirement. Thus, CD34 selection of PBPCs markedly reduces MM contamination and provides effective hematopoietic support after myeloablative therapy.

C6-104 STRUCTURE/FUNCTION STUDIES OF DYSTROPHIN: FUNCTIONAL ANALYSIS OF MINI DYSTROPHIN GENES, Jeffrey S. Chamberlain, Michael A. Hauser, Jill A. Rafael, Kathleen Corrado, Andy Amalfitano, and Rajendra Kumar-Singh, Dept. of Human Genetics, University of Michigan, Ann Arbor, MI 48109

Duchenne muscular dystrophy (DMD) is caused by defects in the dystrophin gene. The phenotype of patients with milder, BMD-causing deletion mutations have often been studied with the goal of understanding the functional domains of the dystrophin protein. However, these studies have been hampered by several factors. First, deletion of various domains of the dystrophin protein often lead to production of a protein with greatly reduced stability, complicating efforts to distinguish between phenotypic symptoms resulting from proteins with impaired function and symptoms that result simply from lower than normal levels of dystrophin. Second, some portions of the dystrophin gene are much less commonly deleted than others, limiting study of functional domains to those that are missing from multiple patients. Finally, the precise function of dystrophin is not entirely clear, making it difficult to ascribe phenotypic features of the disease to defects in structural domains whose role is not entirely clear.

One approach to treating DMD is by gene therapy, in which dystrophin is delivered to muscle cells by a virus. However, current generation viral vectors have a limited cloning capacity that precludes insertion of even a full-length dystrophin cDNA (>14 kb). If truncated dystrophin clones could be designed that produce a small, but functional protein, it might be possible to apply gene therapy for DMD patients using modifications of current generation viral vectors. We have explored several domains of dystrophin to study the effects that deletions would have on the dystrophic phenotype. By expressing deletion constructs in transgenic *mdx* mice under the control of a strong, muscle-specific promoter, animals have been obtained with high levels of truncated proteins that might not accumulate to physiological levels when regulated from the endogenous muscle dystrophin promoter. We will describe the characterization of *mdx* mice that express a variety of levels of full-length dystrophin, and compare the symptoms in these mice with other transgenic animals that express truncated dystrophins. In particular, a mini-dystrophin gene small enough for insertion into viral vectors has been shown capable of preventing virtually all symptoms of muscular dystrophy in mice. The results suggest that low levels of dystrophin can eliminate dystrophic symptoms, and that various deletions of the gene can lead to production of highly functional proteins when expressed at moderate to high levels.

C6-105 SUSTAINED EXPRESSION OF PHYSIOLOGICAL LEVELS OF FUNCTIONAL HUMAN FACTOR VIII IN MICE, Sheila Connelly, Joann M. Gardner, Alan McClelland, and Michael Kaleko, Genetic Therapy, Inc., Gaithersburg, MD 20878

Hemophilia A results from subnormal levels of blood coagulation factor VIII (FVIII), and is an attractive target for gene therapy. We have generated and characterized two adenoviral vectors encoding human FVIII, and demonstrate expression of physiological levels of functional human FVIII in mice for at least three months post vector administration. The recombinant adenoviral vector, Av1ALH81, contains a modified version of the mouse albumin promoter directing expression of a FVIII cDNA encoding the human FVIII protein from which the B-domain had been deleted (BDD FVIII). The second vector, Av1ALAPH81, contains, in addition, an untranslated exon and intron positioned upstream from the FVIII coding region. Comparison of FVIII expression in immune competent mice injected with each vector at a dose of 4×10^9 pfu revealed that Av1ALAPH81 mediated the expression of significantly greater levels of human FVIII in the mouse plasma (up to 2000 ng/ml) than did Av1ALH81 (300 ng/ml), as measured by a sensitive human FVIII-specific ELISA. Analysis of mouse liver DNA and RNA by Southern and RNAse protection assays revealed that both vectors transduced the mouse livers to a similar extent, and that higher levels of FVIII-specific RNA were detected in Av1ALAPH81-treated mouse liver samples. With both vectors, FVIII expression slowly decreased to background levels by weeks 8 to 10. However, administration of 8 to 20-fold lower doses of Av1ALAPH81 to immune competent mice (5 or 2×10^8 pfu) resulted in expression of FVIII at physiological levels for at least three months. Analysis of vector toxicity in the mice by liver function tests and H&E staining of liver sections showed that lower vector doses are substantially less toxic. We suggest that vector toxicity damages transduced hepatocytes, resulting in loss of vector from the liver, and decreased FVIII expression over time. Therefore, lower vector doses induce less hepatocyte damage, allowing long-term persistence of FVIII expression. The finding that an adenoviral vector can mediate long-term high level FVIII expression provides the foundation for the development of gene therapy for hemophilia A.

C6-106 GROWTH, CELL CYCLING AND TRANSDUCTION OF $CD34^+CD38^-$ CORD BLOOD CELLS, Gay M. Crooks, Qian-Lin Hao, Ami J. Shah, E. Monika Smogorzewska, Flavia T. Thiemann, Division Research Immunology/BMT, Childrens Hospital Los Angeles, Los Angeles CA 90027

Umbilical cord blood is the focus of increasing interest as a source of hematopoietic stem cells for gene therapy. Although the $CD34^+$ progenitor population in cord blood (CB) has been extensively studied, the immunophenotypic and growth characteristics of the hematopoietic stem cells in CB are less well understood. Previous studies have noted that the most primitive progenitors in CB are $CD34^+Dr^+$ in contrast to the most primitive cells in bone marrow (BM) which are $CD34^+Dr^-$. We report that the $CD34^+CD38^-$ immunophenotype defines a highly purified and primitive population of progenitors in CB as it does in BM. $CD34^+$ cells are less frequent in CB than in BM ($0.42 \pm 0.25\%$, mean $\pm$ SD, of mononuclear cells in CB compared with $1.18 \pm 0.35\%$ in BM). The frequency of $CD34^+CD38^-$ cells is similar however ($0.04 \pm 0.03\%$ in CB and $0.05 \pm 0.03\%$ in BM). Cell cycle analysis shows >95% of CB $CD34^+CD38^-$ cells to be in G0/G1 phase. Using four parameter Fluorescence Activated Cell Sorting (FACS) we isolated $CD34^+CD38^-$ cells and $CD34^+CD38^+$ cells from CB and cultured them on irradiated BM stroma in the presence of IL3, IL6 and Steel factor (36S) in an extended Long-Term Culture Initiating Cell (LTCIC) assay. Expansion of non-adherent cells was significantly greater from cultures initiated with $CD34^+CD38^-$ cells than with $CD34^+CD38^+$ cells and cell numbers continued to increase beyond 70 days (maximal expansion at 26 days with $CD34^+CD38^+$). CFU-C were detectable from $CD34^+CD38^-$ cultures beyond 90 days of LTBMC compared to 35–50 days in cultures initiated with $CD34^+CD38^+$ cells. Although the $CD34^+CD38^-$ immunophenotype is consistent between CB and BM in distinguishing the most primitive subpopulation of hematopoietic progenitors, CB $CD34^+CD38^-$ cells proliferate more rapidly in culture than their counterparts in BM. This rapid release from quiescence may translate into a higher efficiency of retroviral transduction. We are currently comparing the efficiency of retroviral mediated transduction of CB and BM $CD34^+CD38^-$ cells.

C6-107 HIGH INCREASE OF METHYLUMBELLIFERYL-TETRA-N-ACETYLCHITOTETRAOSIDE HYDROLASE IN GAUCHER DISEASE. Den Tandt W.R.+ and Van Hoof F.* Fac. Medicine+, Universitaire Instelling Antwerpen and *ICP, Brussels

Gaucher disease of which 3 types are known, is caused by a deficiency of the lysosomal enzyme ß-D-glucosidase and is characterized by the accumulation of ceramide-glucose.

In 14 plasma samples of Gaucher disease (all types), we have found an average increase of about 484 times of the enzyme methylumbelliferyl-tetra-N-acetylchitotetraose (MU-TACT) hydrolase of which the function is unknown (Den Tandt et al., 1988, Int. J. Biochem. 20 : 713-719).

In order to verify the specificity of the enzyme's increase, we have examined the activity of the enzyme in other lysosomal storage diseases. In the following diseases, we have also found increased levels (increase in parentheses) : GM_1-gangliosidosis (12x), Wolman disease (39x), Krabbe disease (17x). The increase in the Gaucher plasma samples is much higher than in any of the other diseases.
In the Gaucher patients, the MU-TACT hydrolase activity is also much higher than the well-known increased values of other enzymes like angiotensin converting enzyme, lysozyme, acid phosphatase or other lysosomal enzymes.

The characteristics of MU-TACT hydrolase in the plasma of Gaucher patients pH optimum (thermostability, non-binding to Concanavaline A) were not different from control samples. The search for a possible activator of MU-TACT hydrolase by dialysis of a Gaucher sample or addition of Cer-glu to a control sample was negative.

C6-108 MULTIPLE MYELOID AND LYMPHOID POTENTIAL OF HUMAN UMBILICAL CORD BLOOD STEM AND PROGENITOR CELLS. David DiGiusto, Amy Voytovich, Donna Webster, Kathy Moss, Roxanne Holmes, Robert Lee, Tom O'Toole and Jim Mule'. Progenesys, Palo Alto, California

We have established a pre-clinical model system for testing the feasibility of using human umbilical cord blood as a source of transplantable stem cells for HIV gene therapy. In vitro stromal cell cultures and SCID-hu thymus/liver animals were used to evaluate the myeloid and lymphoid potential of CD34hi/Lin- cells isolated from cryopreserved cord blood samples. Our results indicate that 1/20 CD34hi/Lin- cells gave rise to growth positive wells as early as three weeks and lasting for up to seven weeks of culture. Phenotypic analysis of individually plated cells reveals the B lymphocytic and/or multiple myeloid potential. In a small fraction of the growth positive wells, B, myeloid and CD34+ progenitor cells were detected from a single inoculating cell. This finding suggests that CD34hi/Lin- cells contain stem cells by virtue of their multilineage potential and ability to self renew. $CD34^+$ cells from primary bulk cultures were transferred to secondary cultures and gave rise to multiple lineages of progeny, consistent with the expected behavior of primitive stem cells.

To establish the T lymphoid potential of umbilical cord blood progenitor cells we examined the ability of CD34hi/Lin- cells to competitively repopulate pre-established SCID-hu thymus/liver grafts. Chimerism was observed in 32/85 grafts (3.3% - 74% donor) using cells isolated from seven different cord blood samples. When the CD34hi/Lin- cells were prestimulated with cytokines, we observed chimerism in 22/25 grafts (12% - 91% donor) using cells from six different tissues. Multiple stages of thymocyte maturation were observed in the donor-derived population with a slight increase in the CD4+/CD8-:CD4-/8+ ratio (10:1 in donor vs 2:1 in the host population). We are currently using this system to develop gene therapies for HIV.

C6-109 **GENE THERAPY FOR HEMOPHILIA B: HOST IMMUNOSUPPRESSION PROLONGS THE THERAPEUTIC EFFECT OF ADENOVIRUS-MEDIATED GENE TRANSFER.** B. Fang[1], R. C. Eisensmith[1], H. Wang[2], M. A. Kay[3], R. E. Cross[4], C. N. Landen [4], G. Gavin[4], D. A. Bellinger[4], M. S. Read[4,5], P. C. Hu[6], K. M. Brinkhous[4,5] and S. L. C. Woo[1,2]. [1]Department of Cell Biology, [2] Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas 77030. [3]Department of Medicine, Markey Molecular Medicine Center, University of Washington, Seattle, WA. Departments of [4]Pathology and [6]Pediatrics and [5]Center of Thrombosis, University of North Carolina, Chapel Hill, NC 27599.

A deficiency of blood clotting factor IX causes hemophilia B, an inherited disease that requires life-long regular infusion of factor IX concentrates prepared from human plasma. Research efforts have focussed on developing a cure for this disease using gene replacement therapy. In previous studies, we have shown that the delivery of an adenoviral vector expressing canine FIX (cFIX) to hemophilia B dogs results in a complete albeit transient phenotypic correction of these animals (PNAS 1994;91:2353). Here we report that longer term high level expression of cFIX and phenotypic correction of hemophilia B can be achieved in adult hemophilia B dogs by combining adenovirus-mediated gene transfer with cyclosporine A treatment. 1-2% of normal plasma factor IX levels, which are sufficient for partial correction of the hemophilia B phenotype, can still be detected in dogs 6 months after the treatment. Since high levels of adenovirus-neutralizing antibodies were detected in the cyclosporine A treated dogs, the humoral immune response of the host animal was not suppressed at the cyclosporine A dose used in this study (300mg/dog/day per os). Furthermore, a second administration of the cFIX-expressing adenoviral vector at 6 months after the first infusion had only a minimal effects on plasma factor IX levels in a dog that had been continuously treated with cyclosporine A. However, the prolonged expression of the transgene observed in this study indicates that immunosuppression may be applicable in attaining clinically significant long term high level expression of a target gene.

C6-110 PREVENTION OF ATHEROSCLEROSIS BY BONE MARROW TRANSPLANTATION IN APOE DEFICIENT MICE

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Apolipoprotein (apo) E deficiency causes severe hyperlipidemia and extensive aortic and coronary atherosclerosis both in men and in gene-targeted mice. Although hepatocytes represent the major contributors to plasma apoE levels, other cells are able to synthesize and secrete apoE. Based on the knowledge that tissue macrophages secrete high levels of apoE, we performed bone marrow transplantation (BMT) as a way to deliver macrophages with the normal complement of the apoE gene to apoE deficient mice. Three weeks after BMT, apoE was detectable in plasma and there was a 50% drop in cholesterol levels. By 4 weeks, plasma apoE increased to 10% of normal, cholesterol levels were reduced an additional 25%, and the lipoprotein profile normalized. In control experiments, apoE deficient mice that were transplanted with apoE deficient marrow showed no changes in cholesterol levels. Turnover studies indicated that the effect of BMT was mediated by an increased clearance of lipoproteins induced by the extrahepatic apoE. When BMT mice were fed a high-fat, high cholesterol diet for 3 months, the control group showed tremendous hypercholesterolemia and aortic atherosclerosis (as expected for apoE deficient mice). However, the apoE deficient mice that received normal marrow were partially protected from diet-induced hypercholesterolemia and had a 52-fold reduction in the extent of aortic atherosclerosis. These data demonstrate that plasma apoE of extrahepatic origin is functionally active and promotes lipoprotein clearance. Thus, BMT represents a suitable system for delivery of apoE into plasma and provides a cure for apoE deficiency. These results indicate that gene therapy of hyperlipidemias can be achieved by targeting tissues other than the liver. Due to its ability to associate with different lipoproteins and to interact with different receptors, apoE is likely to have a therapeutic function in other forms of hyperlipidemia. Transplantation of bone marrow stem cells engineered to over-express apoE might become a novel strategy for gene therapy of hypercholesterolemia and atherosclerosis.

C6-111 **CUTANEOUS GENE THERAPY FOR ADA DEFICIENCY: A MODEL APPROACH FOR INHERITED METABOLIC DISORDERS,** Elizabeth S. Fenjves[1], R. Michael Blaese[2] and Lorne B. Taichman[1]. [1]Dept. of Oral Biology and Pathology, State University of New York, Stony Brook, NY 11794. [2]National Institutes of Health, Cell Immunology, Bethesda, MD 20892.

In ADA deficiency, the lack of normal ADA enzyme results in high levels of circulating deoxyadenosine. One approach for this disorder is to create an intracellular reservoir of normal ADA enzyme in a subset of somatic cells that would deaminate the circulating deoxyadenosine and thus reduce the systemic load. The goal of this research is to determine if autologous grafts of ex vivo-modified fibroblasts and keratinocytes would function as an ADA-enzyme sink. We have examined the capacity of intact cutaneous fibroblasts and keratinocytes in culture to deaminate adenosine and deoxyadenosine present in the culture medium. Fibroblasts and keratinocytes obtained from a child with ADA-deficiency were shown to deaminate deoxyadenosine in the culture medium at a very low rate. When these cells were infected with LASN, a retrovirus encoding the normal ADA gene (A. Dusty Miller), the transduced cells were able to deaminate deoxyadenosine at a higher rate than seen in similar cells from a normal donor and at a much higher rate than seen in ADA-deficient cells prior to infection. The cells' deamination capacity was examined in two ways: (1) ADA enzyme activity was measured in cell extracts by standard procedure; and (2) intact cultures were exposed to C^{14}-deoxyadenosine and its conversion to deoxyinosine and hypoxanthine in the medium was assayed. Experiments are currently underway to determine the capacity of corrected fibroblasts and keratinocytes to deaminate deoxyadenosine in the medium and to determine if this capacity is altered when the cells are cultured as a "skin equivalent". The results of this research will help determine the potential of cutaneous gene therapy for treating ADA-deficiency as well as other inherited metabolic disorders where there is a high systemic load of toxic precursor.

C6-112 PRECLINICAL EVALUATION OF AAV VECTORS EXPRESSING THE HUMAN CFTR cDNA, Terence R. Flotte, Carol Conrad, Thomas Reynolds, Sandra Afione, Robert Adams, Sandra Allen, William B. Guggino, & Barrie J. Carter, Depts of Pediatrics and Physiology, Johns Hopkins Univ, Baltimore, MD & Targeted Genetics Corp, Seattle WA 98101

Adeno-associated virus (AAV) vectors may be useful for gene therapy of cystic fibrosis (CF) since they mediate long-term *in vivo* expression after delivery to the airway surface (*PNAS* 1993; 90: 10613-17) and have not been found to elicit airway inflammation. In the current study, doses of AAV-CFTR (from $1x10^6$ to $1x10^{11}$ particles) were administered to the bronchial epithelium of a single lobe of the lungs of New Zealand white rabbits and rhesus monkeys. Animals were sacrificed at timed intervals ranging from 10 days to 3 months. The distribution of vector in the lungs and distant organs was studied by DNA-PCR and *in situ* PCR. Vector expression was detected by RT-PCR and immunohistochemistry. Toxicity was evaluated by histopathologic examination of the lungs and other organs and by bronchoalveolar lavage (BAL) fluid analysis. Vector presence and expression were detected in the lungs of animals receiving doses of $\geq 10^8$ particles of AAV-CFTR. There was a direct relationship between vector dose and the proportion of bronchial epithelial cells transduced, with a maximum of approximately 50% transduction observed at a dose of 10^{10} particles in the rabbits and 10^{11} particles the monkeys. There was no evidence of pulmonary inflammation or neoplastic change. At the highest dose (10^{11} particles), vector was present in the liver of one of two monkeys studied, but there was no evidence of hepatotoxicity. Studies on primary bronchial cells cultured from monkeys 3 months after vector delivery indicated that AAV-CFTR was present in a rescuable form in these cells, but chromosomal integration has not been demonstrated. Taken together, these studies indicated that AAV vector delivery to the lungs of rabbits and monkeys results in high frequency gene transfer without overt toxicity.

C6-113 CFTR GENE THERAPY: IN VIVO AND IN VITRO EXPRESSION. Edward A. Fox, Elizabeth S. Roche, Molly K. McClarrinon and Cornelia M. Gorman, Department of Gene Expression and Delivery, MEGABIOS Corp. San Carlos, CA 94070

To pave the way for preclinical studies for CFTR gene therapy, we have developed *in vitro* functional assays and *in vivo* expression assays. To follow CFTR gene delivery via DNA:lipid complexes, an assay for the functional protein is to be compared to levels of expression as determined by RNA levels. To optimize delivery, reporter gene expression (β-galactosidase) following gene transfer by DNA:lipid complex *in vitro* was used. A variety of DNA:lipid ratios tested expressed detectable levels of β-gal. Ratios of 1:1 and 3:2 resulted in the strongest expression. Function was assessed independently using an anion efflux assay after transfection of *HCMV-CFTR in cells that do not* express endogenous CFTR. Radiochloride efflux measured by the sample replacement method increased by 100% in response to 10μM forskolin in cells transfected with HCMV-CFTR as compared to controls transfected with a reporter plasmid. Expression of the CFTR transgene was assayed by RT PCR. For *in vivo* expression studies DNA:lipid complexes were injected IV into the mouse tail vein. Tissues were harvested 24 hrs following injection and assayed for expression. Parameters involved in achieving efficient IV delivery were determined with the use of a marker gene prior to work with the CFTR expression vector. To specifically monitor CFTR expression *in vivo*, mRNA isolated from mouse tissue was amplified by RT PCR using a 5' primer designed from expression plasmid sequence and a 3' primer from the 5' region of CFTR cDNA. This primer pair specifically detected expression plasmid DNA and CFTR transgene RNA. The expression plasmid and RNA template yield different molecular weight RT PCR products. We have detected both plasmid DNA (471 bp) and CFTR specific transcript (129 bp) in both lung and heart following *in vivo* delivery.

C6-114 ESTROGEN TREATMENT OF OVARIECTOMIZED MICE MODULATES RECEPTOR FUNCTION AND OSTEOGENIC ACTIVITY IN MARROW DERIVED STROMAL STEM CELLS, Dan Gazit, Yoel Sadovsky, Martin W. Edwards, D.L. Cellino, Arnold J. Kahn and Steve D. Bain, Faculty of Dental Medicine , Hebrew University of Jerusalem, Israel; University of california , San Francisco; and Zymogenetics , Inc., Seattle, WA.

Ovariectomy in the mouse leads to a 50% reduction in cancellous bone volume in 4 weeks. Estrogen (E2) replacment prevents osteopenia but, in contrast to most other species , E2 treatment in the mouse also stimulates cancellous bone formation (Bain et al. JBMR 1993). As the E2 dependent increases in formation would require the proliferation of osteoblast precursors, we have hypothesized that E2 regulates osteoblast progenitor populations in bone marrow (colony forming units-fibroblastic; CFU-F). To test this hypothesis we investigated colony formation and E2 receptor function in CFU-F isolated from ovariectomized (OVX) mice treated with E2. Experimentally, 50 Swiss-Webester mice were randomized to 5 groups: a sham and OVX group treated with vehicle; and 3 groups of OVX mice treated with 2.5, 10 or 50 μg of E2 twice weekly. After 4 weeks bone marrow was isolated from femora for CFU-F culture (Friedenstein et al. Transplantation 1974). After 6 days in culture, the CFU-F in 6 replicate cultures of each treatment were stained for alkaline phosphatase (AP) activity. In a second set the CFU-F colonies were harvested and E2 receptor function was assessed using transfection of a vitellogenin A2 promoter upstream of a CAT reporter gene. The CAT activity was normalized to a cotransfected expression vector for β-hCG. Analysis of the AP stained cultures revealed an E2 dependent increase in the number of AP positive CFU-F ($P< .01$). E2 treatment decreased CFU-F numbers ($P< .01$), while colony size increased in a dose-dependent manner ($P< .01$). Change in AP staining and CFU-F morphometry were also correlated with altered E2 receptor function. The CAT activity in the CFU-F of OVX mice treated with vehicle was reduced 20%. The 10 and 50 μg doses of E2 restored CAT activity to sham levels. Taken together these results indicate that the CFU-F isolated from E2 treated mice retain a phenotype which appears to reflect their respective *in vivo* milieus. Morever, the modulation of E2 receptor function and its correlation to AP staining and differentiation are consistent with a direct role of CFU-F in the mediation of E2's anabolic effect in the mouse.

C6-115 **ADENOVIRAL GENE TRANSFER TO VENOUS SEGMENTS PRIOR TO THEIR USE AS ARTERIAL BYPASS GRAFTS.** Samuel E. George, Keith M. Channon, Michael A. Blazing. Division of Cardiology, Duke University Medical Center, Durham NC 27710.

Venous bypass graft atherosclerosis is the major cause of vein graft failure necessitating repeat coronary bypass surgery. Vein graft atherosclerosis is characterized by smooth muscle cell proliferation, loss of normal endothelium and loss of normal vasomotor function, notably loss of endothelium-dependent relaxation to acetylcholine, a response mediated by nitric oxide.

Vein grafts are ideal candidates for the application of gene transfer therapies because vectors can be directly applied during vein harvesting.

We have carried out adenoviral gene transfer to *ex-vivo* segments of rabbit carotid artery, jugular vein and vein graft from a rabbit vein graft model, using a novel adenoviral vector containing a ß-galactosidase (ß-gal) gene (see accompanying abstract by Blazing et. al. for description of vector system). Using a passive 'dwell' technique to infect the vessels, we observed extremely high-efficacy gene transfer of ß-gal in rabbit carotid artery. With an infection time of 1 hour, using a titer of 5×10^8 pfu/ml, virtually 100% of endothelial cells stained blue with X-gal, whereas there was little viral penetration to deeper levels. Significant ß-galactosidase expression was seen using titers as low as 1×10^7 pfu/ml. We also carried out *ex-vivo* gene transfer to rabbit jugular veins and to vein grafts harvested after 28 days in the arterial circulation. ß-gal expression in veins was more marked in the adventitia than in the endothelium, but demonstrated the same overall level of efficiency as seen in carotid arteries. In vein grafts with neointimal proliferation, ß-gal expression was present on the endothelial and adventitial surfaces and was able to penetrate into the neointima.

We are now making preliminary investigations using an novel adenoviral vector containing a brain nitric oxide synthase (bNOS) gene (Ad-bNOS) which we have developed in our laboratory. Ad-bNOS shows excellent bNOS expression and NO production in cultured VSM cells (see accompanying abstract by Channon et. al.). We plan to carry out gene transfer of bNOS to *ex-vivo* vessel segments, then determine bNOS protein expression and enzyme activity. We will also examine the biological effect of bNOS gene transfer on vasomotor function and on neointimal proliferation.

C6-116 RETROVIRAL GENE TRANSFER INTO BONE MARROW CD34+ CELLS AS A GENE THERAPY APPROACH FOR CHRONIC GRANULOMATOUS DISEASE.

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Chronic Granulomatous Disease (CGD) is a recessive disorder characterized by recurrent life-threatening bacterial and fungal infections. The phagocytes of CGD patients lack a functional NADPH oxidase, a multimeric enzyme complex which catalyzes the reduction of molecular oxygen to superoxide (O_2^-) upon stimulation of these cells by microorganisms (respiratory burst). The NADPH oxidase consists of at least four subunits: a membrane bound protein heterodimer (cytochrome b_{558}) containing the gp91-*phox* and p22-*phox* subunits and two cytosolic factors, p47-*phox* and p67-*phox*. Defects in the gp91-*phox* gene are transmitted as an X-linked trait and account for approximately 60-70% of all CGD cases. As an approach to somatic gene therapy of the X-linked form of CGD we have generated recombinant retroviral vectors containing the human gp91-phox cDNA. Retroviral transduction efficiencies of normal bone marrow CD34+ cells were determined by PCR analysis of individual hematopoietic colonies. 60-80% of the colonies were found to contain the transduced gene. Expression of the transgene was observed in 80-100% of the transduced colonies. Long term culture conditions were used to asses the degree of retroviral transduction into more primitive progenitor cells. After 7 weeks in long term culture, 20% of the hematopoietic colonies were found to contain and express the transduced gene. In parallel experiments, the transduced CD34+ cells were kept in suspension in the presence of GM-CSF and G-CSF to induce monocytic and granulocytic differentiation. Cell differentiation was followed by FACS analysis. After 4 weeks in myeloid differentiation media most of the cells (>70%) were found to express the CD14/CD15 antigens, alone or in combination with CD33. PCR analysis of RNA extracted from these cells showed high levels of expression of the transduced gene. These studies will provide the basis for the development of a gene therapy protocol for the X-linked form of CGD.

C6-117 AN IMPROVED MURINE MODEL OF HEREDITARY TYROSINEMIA TYPE 1, Markus Grompe*, Muhsen Al-Dhalimy*, Ken Overturf*, Ching-Nan Ou, Terry Burlingame*, N. G. Kennaway*, Sven Lindstedt and Milton Finegold, *Dept. of Molecular and Medical Genetics, Oregon Health Sciences University, Portland OR 97201

Hereditary tyrosinemia type I (HT1) is a severe autosomal recessive metabolic disease which affects the liver and is caused by deficiency of the enzyme fumarylacetoacetate hydrolase (*Fah*). We recently created a mouse strain in which the *Fah* gene was disrupted by gene targeting in embryonic stem cells. Fah deficient mice died within 12 hours after birth, from hypoglycemia and liver dysfunction. The *Fah* gene is contained within the c^{14Cos} lethal albino deletion and it has now been shown that the neonatal lethal and liver dysfunction phenotype of the *alf/hsdr*-1 albino deletion is entirely due to loss of *Fah* .

Due to the neonatal lethality, the original goal of an animal model for human HT1 was not achieved. Here we demonstrate that treatment of affected animals with NTBC, a potent inhibitor of 4-OH phenylpyruvate dioxygenase, abolishes neonatal lethality, corrects liver function and normalizes the altered expression pattern of hepatic mRNAs. The prolonged life span of affected animals resulted in a phenotype analogous to human tyrosinemia type I when the drug was discontinued at a later age. Liver failure was a consistent feature, including jaundice and elevated AST levels. α-fetoprotein levels were markedly elevated, indicating liver cell regeneration. Homozygous animals were tyrosinemic and secreted succinyl acetone. Histopathologic abnormalities were found in liver, kidney and pancreas and untreated animals died after 6-8 weeks. Long term treatment with NTBC permitted Fah deficient animals to breed and to survive. Nevertheless, 7/8 homozygotes developed macroscopic liver tumors after 9 months whereas 0/9 controls did. Tumors were of both malignant and non-malignant types and appeared to be of hepatocellular origin. We conclude that these animals will serve as useful model for studies of the pathophysiology and treatment of human HT1, including hepatic tumors.

Retroviral and adenoviral vectors for Fah gene therapy have been developed and are being tested in this animal model.

C6-118 SITE-DIRECTED MUTAGENESIS OF CFTR IN A EUKARYOTIC EXPRESSION VECTOR FOR THE DETECTION OF TRANSGENE EXPRESSION.

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The assessment of the efficacy of gene transfer protocols is based on the determination of gene expression at several levels. For cystic fibrosis these involve electrophysiological assessments for restitution of chloride transport, immunological detection and localisation of CFTR protein produced by the introduced gene and the determination of transgene transcription. This last parameter is determined by RT-PCR. In most cases of cystic fibrosis, however, the mutation associated with the disease does not prevent production of a full-length transcript from the endogenous gene. We have introduced silent point mutations into the CFTR gene one of which destroys a *Hpa*I restriction site while the other creates an *Age* I restriction site. We transfected Caco-2 epithelial cells, which produce their own endogenous CFTR, with the mutated plasmid, extracted RNA and performed RT-PCR. Gel analysis of restriction digested PCR products revealed banding patterns consistent with the presence of both endogenous and CFTR transgene expression. Transgene expression was, therefore, clearly differentiated from the endogenous background expression. This construct would be useful for gene therapy of patients with any known CF-associated mutation or could be used for the assessment of gene delivery in non-CF volunteers.

C6-119 DELIVERY OF FACTOR IX IN MICE FROM IMPLANTABLE MICROCAPSULES AS A MODEL FOR GENE THERAPY OF HEMOPHILIA B, Gonzalo Hortelano[1,2], Ayman Al-Hendy[2], Fred Ofosu[1,3] and Patricia L. Chang[2]. Canadian Red Cross Society, Blood Services[1]; Department of Pediatrics[2] and Pathology[3], McMaster University, Hamilton, Ontario L8N 3Z5 Canada; .

We have developed a non-autologous strategy for somatic gene therapy. Allogeneic recombinant cells secreting the therapeutic product of interest are enclosed in alginate-polylysine-alginate microcapsules and implanted into recipients. The non-antigenic microcapsules offer immune-protection to the enclosed cells. The perm-selective microcapsules allow the free diffusion of the recombinant product out of the microcapsules, while excluding IgG, lymphocytes or macrophages. We now evaluate the feasibility of this approach to deliver FIX in vivo for the eventual treatment of hemophilia B, caused by a deficient clotting factor IX (FIX). Mouse C2C12 myoblasts were transfected with a plasmid containing the human FIX cDNA. A recombinant clone secreting FIX (±300 ng/10^6 cells/day) was encapsulated in microcapsules. Male C57BL/6 mice where implanted intraperitoneally (IP) with encapsulated cells while control mice were injected (IP) with unencapsulated recombinant cells. Plasma from mice implanted with microcapsules showed detectable levels of human FIX for up to 14 days with a peak of ±4 ng/ml, whereas plasma from control animals showed undetectable human FIX. Beginning 2 weeks post-implantation, anti-human FIX antibodies developed at increasing titres in the treated mice but not in the controls. Microcapsules retrieved from implanted mice 4 months post-implantation were found to be mostly intact, free from inflammatory cells adhesions and continued to secrete FIX "in vitro". The enclosed myoblast cells had proliferated "in vivo" within the microcapsular space, and around 70% of the enclosed cells remained viable. In conclusion, encapsulated myoblasts can deliver human FIX in mice. Although the amounts delivered are below physiological levels they should be adequate in reducing the whole blood clotting time in hemophilia B. Further, the microcapsules can protect the cells for a long period of time and sustain the viability of enclosed myoblasts, suggesting their potential application in allogeneic somatic gene therapy.

C6-120 GENE THERAPY TOWARD HURLER'S SYNDROME

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Hurler's syndrome (Mucopolysaccharidosis I or MPSI) is a congenital mucopolysacchride disorder resulting from an inheritable deficiency of α-L- iduronidase which is required for the lysosomal degradation of glycosaminoglycans (GAG) heparin sulfate and dermatan sulfate. The gene involved in this autosomal recessive disease has been cloned and localized on chromosome 4p. Even though HLA matched heterologous bone marrow transplantation has been applied for the treatment, gene therapy via autologous BMT may be more beneficial for Hurler patients. Animal MPS models treated by allogeneic BMT or gene therapy have shown a stabilizing effect on disease progression and a marked improvement on symptoms of the Hurler-Scheie syndrome which is caused by mutations yielding minimal level of functional α-L-iduronidase. A retroviral vector containing a normal human α-L-Iduronidase cDNA, has been constructed from the LXSN Moloney Murine Leukemia virus-based vector. A high-titer clone of an amphotropic retrovirus, expressing full length cDNA has been established (PA317/L-HuID-SN). The α-L-Iduronidase activity of the cell line was measured using 4-MU-iduronide as substrate. Results show that the enzyme activity in this cell line is about sixty times higher than the control cell line (PA317/LNC11) which caries a vector without HuID. Several other vectors using the backbone of G1 and MFG with different promoters are being constructed and will be tested for their enzyme expression. Vectors with high α-L-Iduronidase expression will be transduced into murine and human marrow to quantify expression in hematopoietic cells. Iduronidase participates in GAG metabolism through endocytosis via mannose 6-phosphate receptor. The ability of the engineered hematopoietic cells to provide enzyme for intercellular metabolic cross-correction will be studied. Iduronidase expression will be monitored both at the message RNA level with Northern blot and protein level with monoclonal antibody. Our research will provide background information for future clinical trials of gene therapy for Hurler's syndrome.

C6-121 GENOMIC SEQUENCES INCREASE ADENOVIRAL VECTOR-MEDIATED FACTOR IX EXPRESSION 1,900 FOLD AND ENABLE SUSTAINED EXPRESSION *IN VIVO*

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Adenoviral vectors provide a promising modality for the treatment of hemophilia B, a bleeding disorder caused by factor IX deficiency. Our previous study demonstrated that the vector Av1H9B, which encodes the human factor IX (hFIX) cDNA, mediated expression of therapeutic levels of hFIX (> 250 ng/ml) in mice for several weeks. However, the high dose of Av1H9B required to achieve therapeutic levels was hepatotoxic. The goal of the present study was to design vectors with more potent hFIX expression cassettes that could be used at lower, nontoxic doses. The strategy was to modify the cDNA to include hFIX genomic sequences. Three new vectors were generated. Av1H9D, Av1H9E, and Av1H9F. All three contained the hFIX 5' leader sequence. Av1H9D contained the hFIX 3' untranslated region; Av1H9E contained a centrally truncated hFIX first intron; and Av1H9F contained both the 3' untranslated region and the intron. hFIX secretion was assayed in HepG2 cells transduced with each of the four Av1H9 vectors (B, D, E, and F). hFIX levels in the tissue culture medium ranged from 1 to 3 ug/ml with B, D, and E, and were significantly higher at 12 ug/ml with F. Northern analysis demonstrated that hFIX RNA levels paralleled hFIX protein levels. Nuclear run-on analysis revealed similar transcription initiation rates with all four vectors indicating that improved expression with Av1H9F was due to a post-transcriptional process which served to boost hFIX RNA accumulation. For *in vivo* evaluation, cohorts of 5 immune competent, adult mice were injected via tail vein with 10^9 pfu/mouse, a low dose which causes only mild hepatotoxicity. One week later, the level of hFIX in the plasma from mice injected with B, D, E, and F was 80, 335, 2024, and 151000 ng/ml, respectively. DNA and RNA analyses again revealed that improved expression was due to increased hFIX mRNA accumulation. Plasma levels of hFIX were followed in mice injected with B, D, and E. In mice treated with the B vector, hFIX levels returned to baseline by 10 weeks; however, in mice treated with the D and E vectors, therapeutic levels were maintained for 6-8 months. Finally, injection of a nontoxic dose (2×10^8 pfu/mouse) of Av1H9F yielded physiologic levels of hFIX.

C6-122 SELECTIVE IMMUNE INHIBITION OF RECOMBINANT ADENOVIRUS VECTORS USING SOLUBLE CTLA4Ig RESULTS IN PERSISTENT GENE EXPRESSION FROM HEPATOCYTES IN VIVO.

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The use of recombinant adenovirus for gene therapy is limited by immunologic factors. The ability to block the immune response to the vector without permanent impairment of the immune system would be of great clinical benefit. This study was designed to determine whether soluble CTLA4Ig which is known to block the co-stimulatory signal between T-cells and antigen presenting cells and may produce an anergic response to a neo-antigen, could result in persistent adenoviral-mediated gene expression from hepatocytes *in vivo*. C3H/HeJ mice were injected with $5x10^9$ pfu of the Ad/RSV-hAAT recombinant adenoviral vector (containing the human α 1-antitrypsin cDNA) on day 0 and divided into 4 groups. The first two groups received 200 ug of murine CTLA4Ig (IP) on day 2 or days 0, 2 and 10. The second two groups received L6 (control antibody) on an identical schedule. The animals were monitored periodically for serum human α1-antitrypsin (hAAT) and CTLA4Ig concentrations. The L6 control animals (n=8) had rapid fall off of serum hAAT and had undetectable (<5 ng/ml) serum levels between 3 and 7 weeks, whereas both of the CTLA4Ig treated groups (n=8) had constitutive hAAT serum concentrations (1000-5000 ng/ml) for at least 4 months. Because serum CTLA4Ig was undetectable by 8 weeks, the improvement in gene expression was not likely due to the persistent biologic function of this protein. Furthermore, in order to determine that the animals could mount an immune response against a new antigen, at 8 weeks and 12 weeks, the animals were challenged with bacteriophage phi X174 and followed for phage clearance. The results showed that the CTLA4Ig treated animals had similar rates of phage clearance as L6 controls demonstrating functional B and T cell interactions. Additional experiments include examining treated mice for differences in the presence of immune cells, CTL response, cytokines in the recipient's livers and neutralizing antibody titers at various times after adenovirus administration. Studies are underway to determine whether longevity of gene expression is the result of permanent tolerance to the vector. These results enhance the likelihood that recombinant adenoviral vectors will be useful for treatment of hepatodeficiency disorders.

C6-123 INFLUENCE OF DIFFERENT CONDITIONING REGIMENS ON ENGRAFTMENT OF GENETICALLY MARKED HEMATOPOIETIC STEM CELLS,

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Many of the current gene therapy trials in humans attempt to treat genetic diseases. Conditioning, and conditioning-related toxicities, could be avoided in these patients, if transduced hematopoietic stem cells engrafted in sufficient numbers without preceding myeloablation. We have studied the effect of different conditioning regimens on the engraftment of genetically marked hematopoietic stem cells (HSC). Peripheral blood and/or marrow cells collected after treatment with recombinant canine stem cell factor (rc-SCF) or cyclophosphamide were transduced in a retrovirus vector containing long-term culture system. Supernatants from three different vector producing cell lines with similar viral titers were used. Nineteen dogs received either no conditioning (group A, n = 5), irradiation to both humeri with 1000 cGy (group B, n = 5), a sublethal dose of cyclophosphamide 40mg/kg (group C, n = 4), a sublethal dose of 200 or 300 cGy total body irradiation (TBI) (group D, n = 3), or and an otherwise lethal dose of 920 cGy TBI (group E, n = 3), before intravenous (groups A,C,D,E) or intramedullary infusion (group B) of the transduced hematopoietic cells. Dogs were followed for at least 6 months. Peripheral blood granulocytes were obtained every two to three weeks posttransplant and analyzed by PCR for the presence of the transduced gene. The percentages of positive results in dogs more than 4 weeks after transplantation were 0% without conditioning, 5% with local irradiation, 18% with sublethal cyclophosphamide, 33% with sublethal TBI, and 17% with otherwise lethal TBI. Analyzing the influence of conditioning regimens by a generalized estimating technique, we found that the engraftment of genetically marked hematopoietic stem cells was significantly higher in dogs receiving systemic conditioning. Engraftment of transduced cells in dogs treated with sublethal TBI or sublethal cyclophosphamide was comparable to engraftment in dogs receiving otherwise lethal TBI, suggesting that complete myeloablation may not be necessary.

C6-124 INFLUENCE OF HGF ON THE REPLICATION AND RETROVIRAL VECTOR-MEDIATED TRANSDUCTION OF HUMAN HEMOPOIETIC STEM CELLS

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A long-lasting genetic correction of congenital and acquired disorders of the hemopoietic system requires transduction of pluripotent hemopoietic stem cells (PHSC). In mice and rhesus monkeys PHSC could be transduced with recombinant retroviral vectors. The low replication rate of PHSC in steady-state marrow necessitated the use of hemopoietic growth factors (HGF) during retroviral vector-mediated transduction. To elucidate the effect of HGF on human PHSC we evaluated the frequency and DNA replication of $CD34^{bright}Lin$(CD33, CD38, CD71)^{neg} cells in HGF-supplemented cultures using a three-colour FACS analysis. The $CD34^{bright}Lin^{neg}$ cells constitute approximately 0.01% of the total bone marrow population. The frequency of PHSC in cycle was determined by measuring the proportion of $CD34^{bright}Lin^{neg}$ cells engaged in S or G2/M phase of the cell cycle using 7-Aminoactinomycin D. Our assumption that the $CD34^{bright}Lin^{neg}$ population represents PHSC was supported by our finding that the frequency of these cells during culture correlates with the content of late (week 6) cobblestone area forming cells (CAFC). Between individual marrow samples from healthy donors large differences were observed in the size as well as the cycle state of the PHSC. A similar variation was found with regenerating marrow and with mobilized peripheral blood stem cells. CD34-purified bone marrow samples were cultured for 9 days in the presence of recombinant human IL-3, IL-6 and SCF either as single factors or in combination. Regardless of the HGF combination used, a transient increase in the $CD34^{bright}Lin^{neg}$ population was observed in most marrow samples between days 3 and 5. The frequency of cells in cycle during this period was elevated reaching up to 20% of the $CD34^{bright}Lin^{neg}$ population. At later time points the occurrence of PHSC declined. However, in some cultures the frequency of cells synthesizing DNA remained high, indicating that extended exposure to HGF stimulates differentiation rather than renewal of the PHSC population. Currently, transduction of CD34 enriched marrow cells with retroviral vectors carrying the MDR1 or ADA gene is being performed during the first 5 days of culture. Transduction efficiency is determined by PCR analysis of sorted $CD34^{bright}Lin^{neg}$ cells and by culturing late-CAFC under selective conditions.

C6-125 **GENE TRANSFER FOR GAUCHER DISEASE.** Donald B. Kohn, Wanda Krall, Pia M. Challita, Punam Malik, Susie Wells, and Jan Nolta. Childrens Hospital Los Angeles, University of Southern California School of Medicine, Los Angeles, CA 90027.

Gaucher disease is prototypic for genetic diseases of hematopoietic cells which may be treated by gene transfer into stem cells. We have been studying retroviral-mediated transduction of the human glucocerebrosidase (GC) gene to develop clinical gene therapy strategies. Expression of human GC enzymatic activity in primary murine gene transfer/BMT recipients is at levels consistent with correction of the genetic deficiency. Using the human GC protein as an immunohistochemical marker, we have documented progressive replacement of a fraction of the fixed tissue macrophages and microglial cells of murine recipients. However, serial transplants of GC-transduced marrow has shown a high frequency of expression failure from the Moloney LTR associated with DNA methylation in secondary recipients. New vectors to overcome this inactivity are being studied. One series of vectors employs modifications of the LTR to remove negative-acting *cis*-elements or to add sequences to inhibit methylation. Among these vectors, those containing multiple alterations have been shown to be transcriptionally active in ES and EC cells. A second set of vectors use promoters from genes active in myelomonocytic cells. A retroviral vector containing the CD11b integrin promoter shows lineage-specific expression of human GC in HL-60 cells at levels similar to those from the MoMuLV LTR. We have developed an *in vivo* model to study long-term human hematopoiesis, by co-transplantation of immune deficient mice with human CD34+ cells and primary human stroma expressing human IL-3. These studies have demonstrated that the presence of a stromal underlayer during supernatant exposure greatly increases transduction of the human progenitor cells active for over nine months from CD34+ cells from bone marrow, with a smaller effect on peripheral blood CD34+ cells. A protocol to study transfer of the human GC cDNA into CD34+ cells from patients with Gaucher disease has been approved by the NIH RAC and will be performed coordinately with Drs. Karlsson and Dunbar at the NIH. These studies represent initial steps towards application of gene therapy for Gaucher disease.

C6-126 DEVELOPMENTAL DELAY AND FAILURE-TO-THRIVE IN ISOLATED PANCREATIC LIPASE DEFICIENCY, L.J. Krueger, D.I. Mehta, Departments of Pediatrics, Hahnemann University Philadelphia, PA 19102 & Cooper Hospital/UMDNJ Camden, NJ 08103

Insufficiency of human pancreatic lipase (HPL; triacylglycerol hydrolase; EC 3.1.1.3) is seen in newborn infants. Through alternate lipolytic pathways, approximately 70% of normal fat digestion and occurs. In almost all infants, mature levels of HPL are reached by the first year. In the rare autosomal recessive disorder (#246600), enzyme levels continue to be significantly reduced or absent. We report three cases, mean age 25.5 mo range 18-48 month, of HPL deficiency with very low secretin-stimulated HPL (mean 18.2 SEM 11.9 μmol/min/mg protein), as compared to normal levels (greater than 102.7 μmol/min/mg protein), while other pancreatic activities, e.g. amylase and proteases, as well as pancreatic juice pH were normal. Clinical indications of HPL deficiency found in the patients include steatorrhea and growth impairment. Developmental delay was documented in this disorder for the first time. On subsequent annual follow-up, very low HPL activity with otherwise normal pancreatic function was demonstrated. Based on the previously described sequence of the HPL gene, intron primers for 9/13 of the exons were synthesized and used for PCR amplification of control DNAs. Several amplified exons were analyzed by single-stranded conformational polymorphic and heteroduplex analysis, and by direct sequencing of the PCR-products. To identify functional loss, and to identify critical sequence changes, we plan to reengineer these changes into the pVL1392, a baculovirus transfer vector containing the entire coding region of HPL and to co-transfected with *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA into *Spodoptera frugiperda* (Sf9) cells. The isolated recombinant HPL protein will be analyzed for the properly matured-protein, molecular weight, and enzyme activity. Other molecular characteristics will include immunologic-identity, interfacial activation, inhibition by bile salts and activation by colipase in the presence of bile salts. In this way, studies of the genetics of HPL-deficiency and the correlations to failure-to-thrive and developmental delay will be begun.

C6-127 MYOBLAST TRANSFER: GENE THERAPY FOR MUSCULAR DYSTROPHY, P.K. Law, T.G. Goodwin, Q. Fang, M.B. Deering, V. Duggirala, C. Larkin, J.A. Florendo, L.M. Li, T. Quinley, T.J. Yoo, T.H. Krahn, and R.L. Holcomb, Cell Therapy Research Foundation, Memphis, TN 38117

In myoblast transfer therapy (MTT), *in vivo* transfer of the normal genome contained within the donor myonuclei occurs spontaneously through cell fusion of host and donor cells. This leads to the orderly replacement of dystrophin and related proteins in Duchenne muscular dystrophy (DMD). Donor myoblasts also form normal fibers to replenish degenerated fibers. Begun in February 1990, MTT is the first clinical trial of gene therapy in humans. Using cyclosporine as an immunosuppressant, the injection of 8 million myoblasts into a foot muscle was found safe in 11 DMD boys. Phenotype correction 3 mo after MTT showed dystrophin, and structural and function improvement in the myoblast-injected muscles not seen in the sham-injected controls (Acta Cardiomiologica 3:283, 1991). In the next study 5 billion myoblasts were injected into 22 lower body muscles of each of 32 DMD boys aged 6 to 14. Of the 180 muscles tested in total, 88% of the ankle plantar flexors, 49% of the knee flexors, and 45% of the knee extensors showed either a mean 50% increase in strength or did not show continuous loss of strength 9 mo after MTT (Cell Transplantation 2:485, 1993). The current whole body treatment (WBT) protocols, permitted by the FDA with an IND, involves injecting 25 billion myoblasts into 64 muscles. To date, one infantile facioscapulohumeral dystrophy (IFSH) and six DMD boys have received WBT and 16 more DMD boys have received 12.5 billion myoblasts into the upper or the lower body with no adverse reaction. The 16-year-old IFSH subject showed a 22% increase in forced vital capacity and a 31.7% increase in maximum voluntary ventilation 6 months after WBT. The DMD subjects showed similar improvement in respiratory function in addition to strength increase and behavioral improvement (Transpl Proc 26:3381, 1994). Dystrophin was found in the biopsies of the myoblast-injected biceps 9 mo after MTT but not in the sham-injected controls.

C6-128 TRANSCRIPTIONALLY ACTIVE ALU REPEAT SEQUENCES IN THE BREAKPOINT REGION OF THE HUMAN ALL-1 GENE, K. Löchner, I. Nilson, G. Siegler, S. Luschnig, J. Greil‡, J. D. Beck‡, R. Marschalek, and G.H. Fey, Department of Genetics, University of Erlangen-Nuernberg, Staudtstr. 5, 91058 Erlangen, Germany, ‡Department of Paediatrics, University of Erlangen-Nuernberg, Loschgestr. 15, 91054 Erlangen, Germany.

The preB cell leukemia derived cell line SEM carries a reciprocal t(4;11)(q21;q23) translocation. It was used to clone and map the breakpoint region of the wild-type acute lymphoblastic leukemia 1 gene (ALL-1). Using overlapping genomic clones an 8.5 kb BamH1 segment of this gene was sequenced and mapped for repetitive elements and putative protein binding sites. The cloned region codes for the exons 5 to 11 of the wild-type ALL-1 gene, and subsequently for introns 5 to 10. A total of 8 Alu-repeat sequences was identified within intron 6, 8 and 9. These Alu repeat sequences were positioned in the same orientation as the ALL-1 gene. Furthermore, all chromosomal breakpoints analyzed in patient material in different laboratories were found to reside either in Alu elements or less than 800 nucleotides 3' of such elements. To test the hypothesis that transcription of Alu elements may cause genomic instability, the transcriptional activity of selected Alu elements was analyzed. Initial data will be presented in support of this hypothesis.

C6-129 MOLECULAR ANALYSIS OF THE CHROMOSOMAL BREAKPOINT AND BREAK-REGION TRANSCRIPTS IN THE NOVEL ACUTE LYMPHOBLASTIC SEM CELL LINE WITH CHROMOSOMAL TRANSLOCATION t(4;11), R. Marschalek, J. Greil‡, K. Löchner, I. Nilson, G. Siegler, I. Zweckbronner, J. D. Beck‡, and G.H. Fey, Department of Genetics, University of Erlangen-Nuernberg, Staudtstr. 5, 91058 Erlangen, Germany, ‡Department of Paediatrics, University of Erlangen-Nuernberg, Loschgestr. 15, 91054 Erlangen, Germany.

The chromosomal breakpoint and break-region transcripts of the novel pre B leukemia-derived SEM cell line carrying a t(4;11) translocation were analyzed. The chromosomal breakpoint from derivative chromosome der4 was cloned and sequenced. The crossover site was localized in intron 7 of the ALL-1 gene on chromosome 11q23 and in a large intron of the AF-4 (FEL) gene on chromosome 4. RNA transcripts from both wild-type genes and both hybrid genes were detected by reverse transcriptase polymerase chain reaction (RT-PCR) assays. In addition, alternatively spliced mRNA species derived from the der4 chromosome were found. They were generated by using the exon 5' of the breakpoint on der4 as a common splice donor site and the 5' boundaries of exons 8 or 9 of the ALL-1 gene as alternative splice acceptor sites. The hypothesis is proposed, that selective pressure operates to maintain the presence of both derivative chromosomes as important elements in the leukemogenic process.

C6-130 DELIVERY AND EXPRESSION OF HUMAN FACTOR IX USING MICROREACTORS CONTAINING GENETICALLY MODIFIED FIBROBLASTS, Joanne P. Marsh[1], Robert P. Lanza[1], Lin Chen[2], David M. Nelson[2], Richard A. Morgan[2], William L. Chick[1], [1]BioHybrid Technologies Inc, Shrewsbury, MA 01545, and [2]National Center for Human Gene Research, NIH, Bethesda, MD, 20892

Factor IX deficiency or dysfunction (hemophilia B) occurs in 1 in 100,000 male births. There is hope that by encapsulating gene-modified cells to prevent immune rejection it may prove possible to treat these patients. We have developed allo- and xenogeneic gene therapy models for hemophilia B whereby primary rabbit fibroblasts were transduced with human Factor IX (FIX) using either the Moloney Murine Leukemia Virus (MoMLV) or the Myeloproliferative Sarcoma Virus (MPSV) linked to the LTR promoter. Both constructs secrete human FIX at a rate of 1 $\mu g/10^6$ cells/day. After encapsulation, the transduced cells were implanted in nude and C57BL/6J mice (0.4-1.0 x 10^6 cells/animal). Human FIX in plasma (measured by ELISA) ranged from 6.4-7.5ng/ml and 0.4-2.24 ng/ml at day 3, respectively. By day 4, however, FIX was undetectable with either construct. When the number of implanted cells was increased to 5 x 10^6/C57 mouse, FIX levels rose to 12.9 ng/ml within 24 hours. Plasma FIX was detectable through day 4 (1.2-2.2 ng/ml). No FIX was detected at day 7. Administration of cyclosporine (CsA; 10, 20, and 30 mg/kg/day) did not affect human FIX plasma levels. Implantation of encapsulated FIX-engineered fibroblasts into Lewis rats (6 x 10^6 cells; CsA 20 mg/kg/day) was less successful. Plasma FIX levels reached 3.1-3.2 ng/ml one day postimplantation. No FIX was detected at day 3. In the allogeneic model, encapsulated fibroblasts (2 x 10^7 cells) were implanted into Dutch Belted rabbits. Plasma levels of FIX were detected for 1 week, reaching a peak level of 10.4 ng/ml on day 4. Preliminary analysis of anti-human FIX antibodies in the sera of the rabbit recipients showed a significant increase in antibody titer directed against FIX during the first month. Histologic examination of the encapsulated cell implants 1 month postimplantation revealed viable cells. The explanted cells also continued to secret FIX in culture. These results suggest that encapsulated allo- and xenogeneic cells modified genetically to produce a desired therapeutic gene product can be used as a living drug delivery system.

C6-131 A CANINE MODEL FOR *IN VIVO* GENE THERAPY OF HEMOPHILIA B. A.E. Mauser[1], K.M. Whitney[1], S.A. Goodman[1], M. Kaleko[2], T.A.G. Smith[2], A. McClelland[2], and C.D. Lothrop Jr.[1], [1]Scott-Ritchey Research Center, Auburn University College of Veterinary Medicine, Auburn, AL 36849, [2]Genetic Therapy Inc., Gaithersburg, Maryland 21046.

Hemophilia B is a bleeding disorder caused by a deficiency of factor IX (FIX). We have established a model of hemophilia B in Lhasa Apso dogs to evaluate gene therapy strategies of hemophilia B. In sequencing the cDNA of FIX deficient dogs we found a five base pair deletion, (^{5}'GC-CCC^3' nucleotides 548-549 and 551-553) which causes a premature termination codon in the coding sequence for the activation peptide. The mutation has been verified by sequencing genomic DNA and is distinct from the mutation described in the University of North Carolina dog colony. Adenoviral vectors expressing the LacZ gene or the normal human FIX cDNA have been used to develop non-surgical *in vivo* gene delivery strategies. Cephalic vein and portal vein injections of the LacZ adenovirus (10:1 MOI) were compared in 4 dogs ($2X10^{10}$ pfu/kg) to determine if peripheral cephalic vein delivery of adenovirus to hepatocytes was comparable to surgical portal vein administration. Approximately 8.8% $\pm$ 0.9 and 8.6% $\pm$ 1.2 at 2 days, 1.8% $\pm$ 0.4 and 1.4% $\pm$ 0.2 at 7 days, 1.4% $\pm$ 0.2 and .8% $\pm$ 0.2 at 30 days of hepatocytes expressed the nuclearly located β-galactosidase in the cephalic and portal vein dogs, respectively. The non-invasive cephalic vein injection gave equivalent numbers of positive staining hepatocytes as compared to the surgically invasive injection. To determine if gene expression was prolonged in neonatal dogs with immature immune systems four dogs 2 to 4 weeks old were treated with the LacZ or the FIX adenoviral vector by cephalic vein injection. Similar to the studies with the adult dogs gene expression plateaued at low levels by 1 week. These studies indicate that non surgical adenoviral vector gene delivery to hepatocytes is possible but that improvements in long term gene expression are necessary to optimize clinical protocols for gene therapy of hemophilia.

C6-132 LIPOSOME-MEDIATED GENE TRANSFER INTO PRIMITIVE HUMAN HEMATOPOIETIC STEM CELL POPULATIONS, Philip Musk, Michael J.P. Lawman, Adrienne R. Burges and Patricia D. Lawman, Walt Disney Memorial Cancer Institute, Division of Cancer Molecular Biology, 12722 Research Parkway, Orlando, FL 32826

Gene replacement therapy followed by autologous bone marrow re-infusion will greatly improve treatments for genetic disorders of the hematopoietic system. However, the efficacy of such therapies will be dependent upon efficient gene transfer into hematopoietic stem cells, and their subsequent selection and expansion under conditions that maintain their primitive phenotype. DNA complexed with cationic lipid vesicles has been shown to efficiently transfect a wide variety of eukaryotic cells. Using lipid transfection reagents, we have identified conditions necessary for the efficient transfection of human hematopoietic stem cells. The 10.4kb pXT-1 eukaryotic expression vector, which contains the neomycin-resistance gene, and the 7.5kb pWE16 cosmid vector, which contains the dihydrofolate reductase gene and an SV40 promoter to drive its expression in eukaryotic cells, were used in these experiments. These vectors carry genes conferring resistance to G418 and methotrexate (MTX) respectively, and were chosen to allow us to quantitate the efficiency of gene transfer, and to test our ability to select cells using these genetic markers. Target cell populations used in these studies include KG-1, i.e. a $CD34^+$ acute myelogenous leukemia cell line, and three continuously growing $CD34^+$ cell lines established from stem cell proliferation factor-expanded $CD34^+$ cells (Lawman *et al.*, 1992, Blood 80: 149a), previously isolated from normal human bone marrow. Prior to gene transfer, the toxicity of lipid transfection reagents, G418 and MTX were determined to optimize conditions for gene transfer and selection. Successful gene transfer into these cells has been confirmed by Southern analysis and polymerase chain reaction, and maintenance of stem cell characteristics after gene transfer has been monitored by flow cytometry.

C6-133 DIRECT *IN VIVO* RETROVIRAL-MEDIATED GENE TRANSFER INTO HEMATOPOIETIC PROGENITOR CELLS. David M. Nelson[1], Mark Metzger[2], Robert Donahue[2], and Richard A. Morgan[1]. [1]National Center for Human Genome Research, [2]National Heart, Lung and Blood Institute, NIH, Bethesda MD.

Many gene therapy efforts have focused on effecting gene transfer into either mature (PBMC) or progenitor (CD34+) hematopoietic cells *ex vivo*. Targeting progenitor cells provides the theoretical advantage of sustained delivery of therapeutic molecules without the need for repeated treatments. *Ex vivo* efforts to target progenitor cells have been encouraging but may have limitations as *ex vivo* manipulation may alter the transducibility or natural character of the cell. We have developed a procedure to effect gene transfer into hematopoietic progenitor cells in their natural environment, the marrow cavity (*in vivo*). This approach is designed to overcome some of the limitations with *ex vivo* manipulations and to eliminate the extensive preconditioning treatments required to reintroduce these cells back into the patient. Different doses of lethally irradiated human FIX expressing retroviral vector PA317 producer cells were injected into a hole drilled into femurs of recipient rabbits (n=4). Animals received between 5×10^7 and 2×10^8 cells total with a maximum of 1×10^8 cells/leg. Two animals have been extensively characterized. PCR analysis revealed the presence of cells harboring the provirus in different hematopoietic fractions in both animals. Proviral-containing peripheral blood mononuclear cells (PBMC) and were consistently detected at levels between 0.1-3.% in both animals and have been maintained through our latest time point (10 months). Proviral containing cells have also been detected in the granulocyte fraction at levels between 0.2% and 7% to 10 months. RT-PCR analysis revealed detectable proviral message in both rabbits out to 5 months post-procedure. A dose-dependent antibody response against the retrovirus was detected starting 2 weeks post-surgery reaching a peak at about one month which was sustained to a least 6 months. No human FIX was detected in serum of the animals. No unusual pathology was observed as a result of the procedure in control animals. This work serves to demonstrate the feasibility of effecting gene transfer into hematopoietic progenitor cells *in vivo*.

C6-134 ANALYSIS OF T LYMPHOCYTES FROM PATIENTS WITH ADENOSINE DEAMINASE (ADA) DEFICIENCY TRANSPLANTED WITH ADA TRANSDUCED CORD BLOOD CELLS. Robertson Parkman, Bernadette Barrantes, Kenneth I. Weinberg, Linda Heiss, and Donald B. Kohn. Childrens Hospital Los Angeles, Ca. 90027.

Three patients with ADA deficiency were transplanted with ADA transduced autologous CD34+ cord blood cells without the administration of marrow ablative therapy. PEG-ADA therapy was initiated between 1-7 days following birth. Patients were monitored for 1) the phenotypic presence of T lymphocytes, 2) PHA induced blastogenesis, and 3) antigen specific (tetanus toxoid) T lymphocyte blastogenesis. All patients developed phenotypic T lymphocytes (20, 33, 35%; normal = 72 ± 14), PHA responsiveness (36,000; 146,000; 94,000 cpm: normal > 75,000), and antigen specific blastogenenesis (7,727; 3,745; 67,646 cpm: normal =>3,000 cpm). PHA induced T lymphocyte clones were sequentially established from the patients. Clones were evaluated for their phenotype. The clones from one patient were predominantly CD4+ while clones from a second patient were exclusively CD4+. Clones are being analyzed for the presence of the transduced ADA gene to determine if the transduced T lymphocytes have a selective advantage as compared to the PEG-ADA dependent T lymphocytes.

C6-135 ADENOVIRUS-BASED GENE THERAPY FOR CITRULLINEMIA USING A MOUSE MODEL. Gerald Patejunas, Frank L. Graham*, Arthur L. Beaudet, William E. O'Brien. *McMaster University, Dept. of Biology, Hamilton, Ontario, Canada L8S 4K1, and Dept. of Human and Molecular Genetics, Baylor College of Medicine, Houston, TX, 77030

Argininosuccinate synthetase (AS) forms argininosuccinate from citrulline and aspartate and is a participant in the urea cycle. Lack of expression of AS in humans results in citrullinemia, a disease characterized by elevated levels of blood citrulline and ammonia. In severely affected newborns, ammonia can quickly accumulate to neurotoxic levels resulting in convulsions, coma and death. Survival can be prolonged by the administration of drugs which promote nitrogen excretion and by restricting dietary protein intake.

We have used homologous recombination in embryonic stem cells to generate mice having a targeted disruption of the AS gene. Mice that are heterozygous for the disrupted gene have half normal enzyme activity and develop normally. Homozygous mutant animals have essentially no AS enzyme activity. Their blood citrulline level rises more than 60-fold higher than that of normal animals, they become hyperammonemic, and they die within twenty-four hours after birth.

We are now conducting experiments aimed at treating these mice using an adenovirus vector to deliver a functional copy of the human AS gene. Delivery of up to 10^8 pfu/gram by intraperitoneal injection gives only a modest increase in survival time. Experiments using a β-galactosidase-expressing adenovirus suggest that intravenous injection may bring about more efficient uptake of virus in the liver.

C6-136 ACID α-GLUCOSIDASE DEFICIENCY: A MODEL FOR GENE TRANSFER INTO MUSCLE. Daniel Pauly, Barry Byrne, David Johns, Elizabeth Deyesu, Matthew Lowery, Terry Flotte, John Lawrence, Paul Kessler. Departments of Medicine and Pediatrics, Johns Hopkins University, Baltimore, MD 21205.

The lysosomal enzyme, acid α-glucosidase (GAA), catalyzes the breakdown of glycogen. Pompe's disease, an autosomal recessive glycogen storage disorder is characterized by an absence of this enzyme. Affected individuals accumulate glycogen within striated muscle, and develop hypotonia, marked cardiomegaly, and respiratory insufficiency that leads to death within the first year of life. The goals of our studies are to create a cell culture model for infantile Pompe's disease and to test if reconstituting enzyme activity will reverse the sequelae of lysosomal glycogen accumulation in muscle. Toward these aims, mutant fibroblasts, isolated from patients with infantile Pompe's disease, and normal human fibroblasts were infected with a recombinant retrovirus that encodes the muscle determination factor *myoD* (gift of A.D. Miller). Forced expression of *myoD* can induce myogenic expression in a variety of cell types. Following selection, converted fibroblasts recapitulate an aspect of the Pompe's disease phenotype in striated muscle, a paucity of myofilaments. Second, an expression construct coding the full length DNA was generated based on a previously described sequence (Martiniuk *et al.* DNA and Cell Biology, 1990). The activity of expressed GAA, as assessed by the cleavage of the glycogen analog 4-methylumbelliferyl-glucoside, in cell extracts prepared from transfected Cos-1 cells was 228 ± 44 nmol/mg/hr, versus 43 ± 4 for cells transfected with a control plasmid (N=4). Third, we have generated three vectors for gene transfer, stable C2C12 myoblasts that express GAA, and recombinant adenoviral and adeno-associated vectors that encode GAA under the control of RSV and CMV promoters respectively. We are currently evaluating the ability of these vehicles to reconstitute GAA activity in mutant fibroblasts and myoD-transformed GAA deficient cells in tissue culture. This may be a useful system to test strategies for gene transfer into myopathic muscle *in vivo*.

C6-137 TRANSFER OF THE CFTR cDNA TO CYSTIC FIBROSIS PATIENTS' AIRWAYS BY AEROSOL-MEDIATED DELIVERY OF A RECOMBINANT ADENOVIRUS, Andrea Pavirani and Gabriel Bellon, Transgene, 11 Rue de Molsheim, 67000 Strasbourg, France and Centre Hospitalier Lyon-Sud, Clinique Medicale Infantile A, 69310 Pierre-Benite, France

The present day concept of gene therapy for cystic fibrosis (CF) relies on the correction of the fatal pulmonary manifestations of the disease. However, the ultimate goal in treating CF individuals is not only reversion of the airways complications, but an actual prevention therapy to be applied to young patients in which pulmonary exacerbations are not yet manifest. We have previously determined that aerosolization of recombinant adenovirus (Ad.CFTR) carrying the human cystic fibrosis transmembrane conductance regulator (CFTR) cDNA to Rhesus monkey lungs is efficient and safe. In terms of future CF gene therapy the inhalation route might be more favorable in terms of therapeutic efficiency, safety and commodity with respect to the bronchofiberoptic way of administration. We have therefore designed a Phase I clinical trial involving 6 CF patients. The study is open, monocentric and is designed to assess whether single escalating doses (starting from 10^7 pfu) of Ad.CFTR, when aerosolized to patients' lungs, are clinically tolerable and safe during a follow-up period of 3 years. Aerosol administration is firstly preceeded by nasal instillation of the vehicle and subsequently of 1/100 of the actual Ad.CFTR dose to be aerosolized to the lungs. A secondary goal of the study consists in evaluating biological efficacy and chronicity of the CFTR delivery by monitoring the presence of Ad.CFTR DNA and RNA, as well as exogenous CFTR protein in airway cells. At present the clinical trial is ongoing and related progress and results will be reported. This work is partially funded by the French Cystic Fibrosis Association (AFLM).

C6-138 EFFICIENT ADENOVIRUS-MEDIATED IN VIVO GENE TRANSFER TO THE DIAPHRAGM OF ADULT DYSTROPHIC (MDX) MICE, Basil J. Petrof, Gyula Acsadi, Agnes Jani, Bernard Massie, Johanne Bourdon, Neola Mastusiewicz, and George Karpati; Respiratory Divison, Royal Victoria Hospital, and Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada H3A 1A1.

The protein dystrophin is absent in muscles of patients with Duchenne muscular dystrophy (DMD) as well as mdx mice. In the mdx mouse, the diaphragm is the only muscle demonstrating persistent myofiber necrosis, extensive fibrosis and a major loss of strength with age. Thus, the mdx mouse diaphragm closely resembles the human DMD phenotype. The present study was undertaken in order to determine the feasibility of employing the mdx diaphragm as a model for future studies of dystrophin gene transfer via direct intramuscular injection of recombinant adenovirus. Using two different reporter genes (firefly luciferase and bacterial β-galactosidase), we compared the efficiency of adenovirus-mediated gene transfer in the diaphragm of young adult normal and mdx mice. The level of adenovirus-mediated gene expression in the mdx diaphragm was approximately 2-fold greater than that obtained in normal control diaphragm for both reporter genes. In the case of β-galactosidase, the mean number and percentage of transduced fibers within the injection area amounted to 644 ± 79 (± SE) and 41 ± 4 %, respectively, for the mdx group. These values were significantly higher ($p < 0.01$) than those observed in the normal group of mice, in which the number and percentage of transduced fibers amounted to 217 ± 45 and 18 ± 4 %, respectively. In both mdx and normal diaphragms, there was preferential transduction of small fibers (cross-sectional area $< 500\mu m^2$). The results indicate that the mdx mouse diaphragm may serve as an appropriate model system for testing the ability of adenovirus-mediated gene transfer to prevent or mitigate muscle damage in DMD.

C6-139 IL-2 RECEPTOR γ CHAIN MUTATIONS CAUSING HUMAN X-LINKED SCID ARE VARIABLE; SOME MAY BE DOMINANT NEGATIVES WHEN COEXPRESSED WITH WILD TYPE, Jennifer Puck, Judith Isakov, Amy Pepper, Fredric Rosenberg, Laboratory for Gene Transfer, National Center for Human Genome Research/NIH, Bethesda, MD 20892

The IL-2 receptor γ chain gene (IL2RG) in human Xq13.1 is mutated in males with X-linked severe combined immunodeficiency (XSCID). Retrovirus mediated correction of IL2RG defects in stem cells of XSCID patients is a potential therapeutic approach for this disorder. IL2RG is normally widely expressed in developing bone marrow lineages; moreover, obligate female carriers of IL2RG mutations have skewed X inactivation, reflecting a selective advantage for cells expressing normal γ chains in lymphoid, but not myeloid, lineages. Detection of specific mutations in 40 unrelated XSCID males has made possible correlation of genotype with clinical data, identification of critical protein domains, and establishment of a basis for planning therapeutic approaches. Widely varied mutations have been found in each of the 8 IL2RG exons, all due to small changes at the nucleotide level. Only 2 frequently mutated sites have emerged, CG dinucleotides with C to T transitions at cDNA 690 and 879, together accounting for around 20% of XSCID. About half of XSCID mutations have low mRNA levels and/or mutations which would prevent normal splicing or translation of γ chains, e.g. mutated methionine start codon or frame shift in signal peptide. The remaining mutant genes have the capacity to produce an altered or truncated protein, which has in some cases been detected at the cell surface. In these latter mutations dominant negative effects may complicate correction of the defect by gene transfer. Because of Lyonization, only a single IL2RG mRNA species is naturally expressed per cell even by XSCID carriers. Coexpression studies *in vitro* will address interference in cytokine receptor function by mutant chains.

C6-140 **Hereditary haemochromatosis: physical and genetic mapping of the candidate disease region.**
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Dept. Medicine, University of Cambridge, Cambridge CB2 2QQ, U.K.

Hereditary haemochromatosis is a disorder of iron metabolism characterised by increased absorption of dietary iron, and damage to parenchymal organs. The disease is inherited as an autosomal recessive condition linked to the HLA class I region of chromosome 6, at 6p21.3. Current genetic data indicate that a large section of 6p21.3 tends to be inherited in disequilibrium in haemochromatosis patients, with few recombinants described. Several highly polymorphic CA repeat loci are available(1) which map to the region, and these have been used to demonstrate allelic associations in unrelated patients compared with controls(2,3). Some of these markers have been placed on YACs, but a completed physical map of this zone of linkage disequilibrium has not been described; existing evidence indicates that it is quite large (2-3Mb), extending telomerically from the HLA class I region.

We have analysed a YAC contig. of the telomeric end of the HLA class I region by Southern blotting and demonstrated that it conforms to current mapping data. We have used Alu-PCR to generate novel single copy probes and have used this procedure to analyse YACs at the telomeric marker D6S105. We have analysed YACs at this locus ranging in size from 1.7Mb to 250kb, and have used this material to map the histone H1.5 gene and to place the microsatellites D6S306 and D6S464 on either side of D6S105. The physical order of these markers is: cen-HLA-D6S306-(D6S105-H1.5)-D6S464-tel. These YACs do not contain HLA related sequences.

We have reported an HLA haploidentical family showing independent segregation of haemochromatosis(4). This family and a number of unrelated patients and controls have been investigated further by the use of microsatellites at HLA F, D6S306, D6S105 and D6S464. Allelic association with disease was present at all loci. Analysis of the pedigree revealed segregation of disease independently of D6S105 and D6S464, which thus defines the telomeric limit of the disease gene.

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3.Worwood, M et al (1994) Br. J. Haem. 86: 863-866
4.Dokal, I et al (1991) Hum. Genet. 88: 209-214.

C6-141 HIGH LEVEL EXPRESSION OF RECOMBINANT CFTR IN HETEROLOGOUS CELLS LEADS TO INCREASED CELL VOLUME AND G2/M GROWTH ARREST. S.C. Schiavi, A.E. Smith, J.M. McPherson, S.H. Cheng, and H. Hoppe. Genzyme Corp., One Mountain Road, Framingham, MA 01701.

An inducible gene amplification system was utilized to study the effects of overexpressing the cystic fibrosis transmembrane conductance regulator (CFTR) *in vitro*. A monkey kidney cell line expressing a temperature-sensitive simian virus 40 (SV40) Large-T antigen was stably transfected at the non-permissive temperature with a plasmid containing an SV40 origin of replication and the cDNA for either the wild-type CFTR or the defective chloride channel mutant, G551D. Shift of the isolated cell lines to the permissive temperature resulted in induction and accumulation to high levels of the CFTR plasmid, mRNA and protein. Interestingly, high level expression of CFTR protein was transient in both BTS-CFTR and BTS-G551D cells. Maximal CFTR protein concentrations were typically observed between 4 and 6 days post-induction but were reduced to low levels by day 9 at the permissive temperature. Quantitation of CFTR DNA and mRNA levels further revealed that the decline in CFTR expression was due to a decrease in steady state levels of CFTR mRNA. SPQ analysis indicated that some Cl^- channel activity was retained in BTS-G551D cells although it was much lower than the Cl^- channel activity observed in BTS-CFTR cells. This argued that down-regulation was induced by either the physical presence of high amounts of CFTR protein or of some low threshold level of Cl^- channel activity. Examination of cell growth properties revealed a correlation between high wild-type CFTR expression and growth arrest of the cells. Cytofluorographic DNA analysis indicated that cells expressing high levels of wild-type CFTR had abnormal cell cycle profiles characterized by an enriched G2 cell population. However, similar induction of the G551D-CFTR mutant showed only a slight growth inhibition and little enrichment of cells at G2/M. Cytofluorographic analysis further revealed that BTS-CFTR cells were significantly larger than parental BTS or BTS-G551D cells at all stages of the cell cycle, suggesting that the BTS-CFTR cell volume increase was not simply a reflection of these cells arresting at G2. Taken together, these results indicate that minimal levels of CFTR may induce down-regulation whereas higher levels of Cl^- channel activity may lead to increased cell volume and subsequent growth abnormalities.

C6-142 GENE THERAPY FOR AUTOSOMAL RECESSIVE CARBAMOYL PHOSPHATE SYNTHETASE (CPSASE I) DEFICIENCY, J. Paul Schofield, C. Thomas Caskey, Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030

The hepatic enzyme CPSase I catalyses the first step of the urea cycle: the formation of carbamyl phosphate from ammonia and carbon dioxide. The gene is highly conserved during evolution, and maps to human chromosome 2p.

Autosomal recessive deficiency of CPSase I is mostly fatal in the early neonatal period from hyperammonaemic brain damage. A more rare group of delayed onset patients are due to a partial CPSase I deficiency. These patients self-select a low protein diet, though hyperammonemic crises may result in coma and death during times of catabolic stress, e.g. childbirth, infection or surgery.

As a first step towards gene therapy I am developing a mouse model by homologous recombination. I have cloned mouse liver CPSase I cDNA by RT-PCR, using primers designed to hybridise evolutionary conserved amino acid residues. This sequence will be used to screen a mouse genomic DNA library (129/Sv), as a precursor to the construction of a gene targeting vector.

Estimates of the CPSase I gene size of 70-100 Kb in human and rats is likely to be similar in the mouse. As the CPSase I enzyme is composed of 1500 amino acids the majority of the gene is therefore occupied by non-coding DNA sequence. To facilitate the identification of tissue-specific and other conserved regulatory DNA sequences I am sequencing the CPSase gene of another vertebrate species, that of the Japanese puffer-fish *Fugu*. This approach is based on the observation that the *Fugu* genome is highly homologous to that of humans, except that the non-coding DNA is ~10 fold compressed. This is confirmed by my provisional sequencing data of around 10 Kb for the *Fugu* CPSase complete gene. Furthermore, the intron-exon organisation of *Fugu* CPSase appears to be identical to that of rat CPSase I, and should facilitate a rapid approach to the characterisation of the as yet undetermined human CPSase I gene structure.

Sequence data will be utilised to design a more rational gene transfer vector, with particular attention to the 5' non-coding sequence. This may include tissue-specific regulatory elements as well as intron(s) to help stabilise expression of the correcting CPsase I gene.

Acknowledgements: J.P. Schofield is supported by The Wellcome Trust, London, UK, C.T. Caskey is an Investigator of The Howard Hughes Medical Institute.

C6-143 **SELECTIVE EXPRESSION OF PROTEIN TYROSINE PHOSPHATASES IN EARLY HUMAN HEMATOPOIETIC PROGENITOR CELLS.** Gunter Schumann, Hubertus Kohler*, Dagmar Wider, Roland Mertelsmann and Lothar Kanz, Div. of Hematology Oncology, Dep. of Medicine, Freiburg University Medical Center and *Max-Planck Institute for Immunobiology, Freiburg, Germany.

Processes regulating proliferation and differentiation of human pluripotent hematopoietic stem cells are not well defined. Protein Tyrosine Kinases (PTK) and Protein-Tyrosine Phosphatases (PTP) have been shown to mediate regulatory processes in a variety of cell types. In order to study the role of PTP in a human hematopoietic progenitor population enriched for pluripotent stem cells we examined the selective expression of PTP in peripheral CD34+CD38-HLA-DR- cells from cancer patients. These progenitor cells were mobilized from the bone marrow by standard dose chemotherapy plus G-CSF and harvested by leukapheresis. Selection of CD34+ cells by immunoaffinity columns was followed by staining with anti-CD34, CD38 and HLA-DR mAbs and sorting by FACS. RT-PCR was performed with degenerate oligonucleotid-primers specific for conserved PTP-domains. DNA-fingerprinting with various restriction enzymes was used to compare CD34+ CD38+ HLA-DR+ cells with the CD34+ CD38- HLA-DR- subpopulation. Using this approach we identified the selective expression of cDNA encoding for PTP in early hematopoietic progenitor cells. Presently we are investigating the functional significance of these PTP in hematopoiesis.

C6-144 RETROVIRAL GENE TRANSFER TO XID BONE MARROW CELLS. AN EXPERIMENTAL MODEL FOR GENE THERAPY IN X-LINKED AGAMMAGLOBULINEMIA.

C. I. Edvard Smith, Lars J. Brandén, Donald B. Kohn and Kleanthis G. Xanthopoulos. Center for BioTechnology, Karolinska Institute, NOVUM, 141 57 Huddinge, Sweden and Division of researsh Immunology, Childrens Hospital, Los Angeles, CA, USA.

X-linked agammaglobulinemia is an immunodeficiency disorder caused by a B lymphocyte differentiation block resulting in the absence of B lymphocytes and plasma cells. The defect is caused by mutations in the newly identified protein.tyrosine kinase, Btk (*Nature* 361:226, 1993). A mutation in the same gene causes the X-linked immunodeficiency, XID, in mice (*Science* 261:355, 1993). We have constructed retroviral plasmids containing the human Btk cDNA. One vector construct also harbours a selectable marker, making it possible to identify Neomycin-resistant clones. Integration of the vector plasmid into the packaging cell line PA317 has allowed us to produce retroviral supernatants. The supernatants have been used to transduce bone marrow cells from Xid mice to analyze whether the genetic defect in these mice can be reverted. Genecorrected mice are presently being investigated by immunoglobulin serum levels and B lymphocyte markers.

C6-145 EXPRESSION OF HUMAN APOLIPOPROTEIN E BY AN ADENOVIRAL VECTOR RESULTS IN A SUSTAINED CHOLESTEROL LOWERING EFFECT IN A HYPERCHOLESTEROLEMIC MOUSE MODEL, Susan C. Stevenson, Jennifer Marshall-Neff, and Alan McClelland, Department of Molecular and Cell Biology, Genetic Therapy, Inc., Gaithersburg, MD 20878

To investigate the use of apolipoprotein E (apoE) in gene therapy of hyperlipidemias, an adenoviral vector was constructed which contains the human apoE3 cDNA under the control of the RSV promoter (Av1RE). Intravenous injection of apoE-deficient mice with $0.5x10^{11}$, $1x10^{11}$, or $5x10^{11}$ Av1RE vector particles resulted in expression of human apoE in mouse plasma at levels of 0.7, 2.1, and 4.3 μg/ml respectively, 7 days after injection. A significant reduction in total plasma cholesterol levels was observed in all of the treated mice, while values were unchanged in mice injected with 5×10^{11} particles of a control vector, Av1LacZ4. The reduction in the average plasma cholesterol concentration for each treatment group was related to the vector dose and the resulting level of human apoE expression. The plasma cholesterol concentrations were reduced 1.5 fold in the low dose treatment group from 869 to 562 mg/dl, 2 fold in the medium dose group from 901 to 426 mg/dl, and 7 fold in the high dose group from 914 to 127 mg/dl 7 days after vector administration. Reduced plasma cholesterol concentrations were maintained for at least 2 months after vector administration, consistent with sustained expression of human apoE over this time period. To assess the effect of apoE gene delivery on the development of atherosclerosis, quantitative analysis of lesions is being performed. Correction of the hyperlipidemic condition in the apoE knockout mouse model by direct *in vivo* gene transfer establishes the potential of this approach for treatment of hyperlipidemia due to apoE deficiency or malfunction in human disease.

C6-146 *IN VIVO* RECOVERY OF HUMAN FACTOR IX IN SYNGENEIC RECIPIENT RATS AFTER TRANSPLANTATION OF HEPATOCYTES BEARING CMV AND MULV LTR PROMOTER-DRIVEN HUMAN FACTOR IX RETROVIRAL VECTORS. Hui Tang, Roberto Salazar, Erlinda M. Gordon. Childrens Hospital Los Angeles, Los Angeles, CA.

Hemophilia B is a bleeding disorder caused by a deficiency of functional clotting factor IX. To evaluate the potential of gene transfer as treatment of hemophilia B, we compared the expression of CMV and MuLV LTR promoter-driven factor IX and *lacZ* retroviral vectors in primary fetal and adult rat hepatocytes, and report *in vivo* recovery of human factor IX in recipients transplanted with hepatocytes transduced with factor IX vectors. Northern analysis showed endogenous factor IX and vector transcripts of the expected sizes in total RNA prepared from transduced cells. More endogenous factor IX mRNA was noted in adult than in fetal hepatocytes, attesting to immaturity of fetal liver. In contrast, more vector mRNA was noted in blots from transduced fetal than transduced adult hepatocytes. Western analysis showed that secreted factor IX migrated to the region of human plasma factor IX at 68 kDa. Transduced fetal hepatocytes produced more factor IX than transduced adult hepatocytes. Further, the human transgene was detected in recipient rats' circulation for as long as 4 weeks after transplantation with hepatocytes bearing factor IX vectors. At sacrifice, vector sequences were detected in liver and spleen of animals transplanted with hepatocytes transduced with factor IX vectors. These data emphasize the potential of hepatocytes as targets for gene therapy of hemophilia B.

C6-147 AN IN VITRO ASSAY OF HUMAN HEMATOPOIETIC STEM CELL TRANSDUCTION, Flavia T. Thiemann, Qian-Lin Hao, E. Monika Smogorzewska, Ami J. Shah, Gay M. Crooks, Division of Research Immunology/BMT, Childrens Hospital Los Angeles, Los Angeles CA 90027

The major obstacle to successful hematopoietic cell gene therapy is the inefficient transduction of human hematopoietic stem cells (HSC). Transduction levels of Colony-Forming Unit cells (CFU-C) and Long Term Culture Initiating Cells (LTCIC) do not reflect the low levels of transduction of HSC seen in clinical gene therapy trials. The development of in vitro assays which accurately reflect transduction of HSC is a crucial step toward the development of transduction conditions and vector strategies which achieve high efficiency transduction. Using highly purified HSC, we have developed an extended LTCIC assay which more closely reflects in vivo HSC transduction levels. CD34+CD38- cells are isolated from normal adult bone marrow (BM) by Fluorescence Activated Cell Sorting (FACS), transduced over 4 days with the retroviral vector LN, and transferred to pre-established, allogeneic, irradiated human BM stroma in medium containing IL3, IL6 and Steel Factor (36S) for long term culture. Aliquots of non-adherent cells are replated at 2 weekly intervals into semi-solid media to measure Colony Forming Unit-Cells (CFU-C). Gene transduction is measured by PCR detection of the neo^R gene in individual CFU-C. CD34+CD38- cells, over 95% of which are in G0/G1 phase prior to transduction, are able to produce CFU-C for over 100 days. Transduction efficiency into CFU-C from the first 5–8 weeks of culture (ie standard LTCIC assay) is 30–60%. Gene transfer drops to 0–5% in CFU-C produced from 12–14 week old cultures. In this extended LTCIC assay, the fall in transduction levels of CFU-C which arise later in culture suggests that progeny from a originally more quiescent sub-population of CD34+CD38- cells is being assayed at time points beyond 8 weeks. The extended LTCIC assay may therefore be a more accurate reflection of transduction of the total target HSC population, most of which is quiescent during transduction, than standard LTCIC or short term progenitor assays.

C6-148 UROKINASE EXPRESSION IN TRANSDUCED VASCULAR ENDOTHELIAL CELLS, L.E. Townsend, K.T. Trevor*, M.S. Seymour, S.G. Ward, D. Reitz-Vick, B. Ellmers and J.G. Glover,

Wm. Beaumont Hospital Research Institute, Royal Oak, MI 48073 and *Center for Molecular Medicine and Genetics, Wayne State University Medical School, Detroit, MI 48202

There is currently no suitable vascular prosthesis to bypass obstructed small diameter vessels. In addressing this problem, we have shown vascular graft patency is increased when the interior surface of a synthetic graft is lined with endothelial cells (EC). Further improvement of patency may occur by adding a gene for the plasminogen activator, urokinase, to EC increasing thrombolysis. Thus we have constructed an LXSN-type retrovirus vector that contains a unique human urokinase cDNA (gift of Dr. A. Bollen, Univeristy of Brussels, Belgium). Preliminary work using NIH3T3 fibroblasts indicated that the recombinant retrovirus was capable of transduction; expression and secretion of urokinase could be demonstrated by RT-PCR, immuno-staining and ELISA techniques. For the purpose of graft implantation into our experimental canine model, dog endothelial cells were transduced at an MOI of 40 CFU/cell. Selection with G418 resulted in cultures that were 100% expressive of urokinase as determined by immunohistochemsitry. Canine EC were grown in defined, serum free medium for 24 hr; ELISA assay indicated high secretion of urokinase into the culture medium. A spectrophotometric assay for urokinase determined that active enzyme is being produced. Parallel studies on human umbilical cord EC produced the same results. Thus, we have shown transduction, selection for 100% transduced cells and determined enzyme activity for a unique prourokinase retroviral vector construct in both dog and human EC. Supported by a grant from the Am. Heart Assn, Mich. and Wm. Beaumont Hosp. Funds.

C6-149 DOWN-REGULATION OF FIBRILLIN EXPRESSION BY ANTISENSE HAMMERHEAD RIBOZYMES, P Tsipouras (1), MW Kilpatrick (1), M Velinov (1), , M Godfrey (3), G Wu (4) and LA Phylactou (1,2), Departments of Pediatrics (1) and Internal Medicine (4), University of Connecticut Health Center, Farmington CT 06030, University of Nebraska Medical Center, Omaha NE (3) and Department of Clinical Genetics, University of Birmingham UK (2).

Mutations in the fibrillin gene on chromosome 15 (FBN1) are responsible for Marfan syndrome (MFS), a systemic genetic disorder of the connective tissue. Many such FBN1 mutations appear to act in a dominant-negative fashion, raising the possibility that reduction of the amount of product from the mutant FBN1 allele might be a valid therapeutic approach for MFS. As the first step in the development of such an approach, we have investigated the ability of antisense hammerhead ribozymes to down-regulate the FBN1 gene product. A DNA fragment coding for an antisense hammerhead ribozyme (FBN1-RZ1) specific for the 5'-end of the FBN1 mRNA has been cloned into the vector pBSIISK+ to allow large scale *in vitro* synthesis of the ribozyme. The FBN1-RZ1 ribozyme can efficiently cleave its target *in vitro*, in a magnesium dependent manner, at both 50°C and 37°C. To determine the effect of FBN1-RZ1 on cultured cells, the ribozyme was bound to a transferrin-polylysine conjugate and delivered by receptor-mediated endocytosis into cultured dermal fibroblasts. RNase protection was used to demonstrate the presence of the FBN1-RZ1 ribozyme in total RNA isolated from fibroblasts harvested 24 and 48 hours following transfection. To demonstrate the effect of FBN1-RZ1 on fibrillin production, cultured fibroblasts were stained with an anti-fibrillin antibody following transfection with FBN1-RZ1 transferrin-polylysine complex. Visualisation of the antibody by fluorescence microscopy demonstrated that transfection with FBN1-RZ1 dramatically reduces the amount of fibrillin produced by cultured fibroblasts.

C6-150 A TRANSGENIC MOUSE MODEL FOR FRAGILE X SYNDROME P.J. Willems[1], C.E. Bakker[2], E. Reyniers[1], C. Verheij[2], K. De Boulle[1] and B.A. Oostra[2], [1]Department of Medical Genetics, University of Antwerp, Antwerp, 2610, Belgium and [2]Department of Clinical Genetics, University of Rotterdam, Rotterdam, 3000 DR, The Netherlands

Fragile X syndrome is the most frequent genetic form of mental retardation. Male patients with fragile X show mental retardation, macroorchidism and phenotypic abnormalities of the face. Although the fragile X gene (FMR1) has been isolated three years ago, hardly anything is known about its physiologic function and the mechanisms leading to the clinical abnormalities.

To gain more insights into FMR1 function we knocked out the Fmr1 gene in transgenic mice with embryonic stem cell technology. By introduction of a neomycine cassette in exon 5 of the Fmr1 gene, FMR1 protein synthesis was severely reduced, a situation comparable with the human condition. The transgenic mice show macroorchidism and abnormalities in cognitive function and behaviour. Macroorchidism develops slowly with age and adult mice age have significantly ($p < 0.001$) greater testes than their normal littermates. Pathology of testes and brain was normal. Learning was tested in the Morris water maze and shows significant ($p=0.0001$) delay for the mutant knockout mice to find the hidden platform in reversal trials.

Apart from the learning deficits mutant mice also show behavioural abnormalities such as increased exploratory activity in the exploratory behaviour test, and hyperactivity in the motor activity test.

As the knockout mice show abnormalities comparable to human fragile X patients, they offer a good animal model to study mental retardation, behaviour and macroorchidism, and experimental designs can now be made to start drug and gene therapy for fragile X syndrome.

C6-151 CYTOKINE GENE EXPRESSION IN HUMAN CORD BLOOD DERIVED CD34+ STEM/PROGENITOR CELLS FOLLOWING PARTICLE-MEDIATED GENE TRANSFER, Zi-Qing Ye†, Ping Qiu*, Joseph K. Burkholder*, Joel Turner*, Jerilyn Culp*, Tim Roberts*, J. S. Malter‡, Nasrollah T. Shahidi† and Ning-Sun Yang*‡, *Cancer Gene Therapy, Agracetus, Inc., Middleton, WI, †Hematology/Oncology Division, Dept. of Pediatrics, University of Wisconsin Medical School, ‡Dept. of Pathology and Laboratory Medicine, University of Wisconsin Medical School.

We previously reported the successful transfer of luc and LacZ reporter genes into CD34+ cells using a particle-mediated gene delivery technique. Up to 5.6 ± 1.7% CD34+ cells bombarded with pCMV-LacZ DNA expressed high level transgenic ß-gal activity at the cellular level. Here we report that four candidate therapeutic cytokine genes (hGM-CSF, hIFN-γ, hIL-2 and hTNF-α) were transferred into both freshly isolated and growth factor-stimulated CD34+ cells. Expression levels for transgenic proteins defined as pg/10^6 CD34+ cells-24 hours were observed as follows:

Transfected cells	hGM-CSF	hTNF-α	hIL-2	hIFN-γ
Freshly isolated	216±59	41±16	109±34	41±19
4 day stimulated	4001±104	150±19	210±41	181±97

The expression kinetics of hGM-CSF transgene was determined in a time course study. Detectable levels of the transgenic protein lasted between 4 to 7 days. The turnover rate of transgenic GM-CSF was shown to be significantly lower than that of the recombinant GM-CSF protein exogenously supplemented to the culture medium, exhibiting a half-life (t1/2) of 96hr and 36hr, respectively. Similar results on expression kinetics of hIFN-γ, hTNF-α and hIL-2 transgenes were observed. Using a firefly luciferase gene cassette vector, we determined the relative strength of a number of cellular and viral promoters in CD34+ cells at different time points post plating in primary culture. A human elongation factor 1α promoter was identified as the strongest cellular promoter tested, exhibiting a similar strength as the commonly employed CMV i/e gene promoter enhancer. These results suggest that a combination of the particle-mediated gene transfer technique and the CD34+ cell culture system can offer a unique approach for gene transfer studies and gene therapy using human hematopoietic stem/progenitor cells. Application of the current CD34+ gene transfer system to gene therapy of Fanconi anemia and to cancer therapy using multidrug resistance gene are being evaluated.

C6-152 A DUPLICATION IN THE LYSYL HYDROXYLASE GENE MAY BE A COMMON MUTATION IN A SUBSET OF PATIENTS WITH EHLERS DANLOS SYNDROME TYPE VI. HN Yeowell, LC Walker, MK Marshall and SR Pinnell, Duke University Medical Center, Durham, North Carolina 27710

Lysyl hydroxylase (LH) is an essential enzyme in collagen biosynthesis that catalyzes the formation of hydroxylysine required for intermolecular crosslinking of collagen. Dermal fibroblasts from patients with Ehlers Danlos Syndrome type VI (EDS VI), an inherited connective tissue disorder, express decreased levels of LH activity. These patients have clinical features of joint laxity, kyphoscoliosis and fragile skin. To date, clinical diagnosis of EDS VI has been confirmed by assay of LH activity in dermal fibroblasts . In the present study, we have measured LH activities and levels and sizes of LH mRNAs in dermal fibroblasts cultured from 24 EDS VI patients. LH activities in these patients (normalized to prolyl hydroxylase) ranged between 10-30% of control. A 1.2 Kb cDNA from the coding region of LH hybridized to a 3.4 Kb mRNA for LH in patients; however in 13 patients sharply decreased levels of LH mRNAs were observed. Interestingly, in the remaining 7 patients, the 3.4 Kb mRNA for LH was replaced by an abnormally large mRNA of 4.2 Kb. This has been recently characterized, in a report of two sisters with EDS VI (Genomics, 15: 399-404, 1993), to be the result of a large duplication rearrangement in the LH gene. Preliminary PCR analyses of the cDNAs isolated from these seven patients using primers covering this region indicate that the 4.2 Kb mRNA may be the result of a similar duplication event. It therefore appears that a subset of EDS VI patents may have a common mutation in their LH gene that results in the transcription of an oversized mRNA. These results should enable us to develop a reliable PCR-based diagnostic test for this particular mutation.

Supported in part by research grants AG 10215 from NIA and March of Dimes Birth Defects Foundation FY 94-0901.

C6-153 CLEAVAGE OF α1-ANTITRYPSIN (α1-AT) RNA BY A SEQUENCE-SPECIFIC HAMMERHEAD RIBOZYME, Mark A. Zern, Iwata Ozaki, and Ling-Xun Duan. Department of Medicine, Jefferson Medical College, Philadelphia, PA 19107

α1-AT deficiency is one of the most common hereditary disorders that mainly affects the liver and lung. Liver involvement is thought to be caused by the accumulation of the abnormal α1-AT protein in hepatocytes. One approach to reduce this accumulation of the abnormal protein is to inhibit α1-AT gene expression. As a first step in formulating gene therapy for the disease, we attempted to develop a hammerhead ribozyme that would cleave α1-AT mRNA at a specific site.

METHODS: The computer-derived α1-AT mRNA secondary structure modeling system was selected to design the hammerhead ribozyme for targeting the different putative GUX sequences. The ribozymes were synthesized by PCR with specific oligonucleotides and cloned into the R7Bleu(R) vector. Full-length α1-AT cDNA was amplified by RT-PCR and inserted into the pGEM-T vector. Ribozyme RNA and α1-AT RNA were then transcribed *in vitro* by T7 RNA polymerase with P^{32}-UTP. Purified ribozyme RNA and α1-AT RNA were then incubated and the RNA catalytic reaction was analyzed by denaturing 6% polyacrylamide gel electrophoresis.

RESULTS: α1-AT RNA was specifically cleaved into two fragments by the hammerhead ribozyme at the designed site. Based on the computer-modeling RNA structure and *in vitro* cleavage reaction, the best target sequence was selected at position 206. This ribozyme cleaved α1-AT RNA immediately after incubation with the ribozyme, and this occurred efficiently even in nM concentrations of the ribozyme.

CONCLUSION: This successful in vitro cleavage of α1-AT RNA is an initial step in designing a novel therapy for the liver disease caused by α1-AT deficiency. The transduction of the ribozyme into hepatic cells employing a retroviral vector system is underway.

Tissue Remodeling; Gene Expression and Regulation Vectors

C6-200 KERATINOCYTE-SPECIFIC EXPRESSION OF FACTOR IX FOR GENE THERAPY FOR HAEMOPHILIA B, Sanjay I. Bidichandani, M. Yvonne Alexander and Rosemary A. Akhurst, Dept. of Medical Genetics, University of Glasgow, Glasgow, G3 8SJ, UK

Haemophilia B is a candidate disease for somatic cell gene therapy. Keratinocytes are a particularly advantageous cell type to effect somatic gene therapy by both *ex vivo* and *in vivo* approaches. In order to investigate the role of keratinocytes as a target cell type we have linked the factor IX cDNA (FIX) to a strong cytokeratin gene promoter. We have used the bovine counterpart of the human cytokeratin 10 (K10) promoter to achieve suprabasal keratinocyte-specific expression. To improve the translation efficiency of the basic K10-FIX gene construct, two sequence modifications were performed. A favourable sequence context was introduced surrounding the translation initiation codon (K10-FIX-G4) and synthetic oligonucleotides were used to lengthen the 5' leader sequence thus closely resembling the natural K10 gene (K10RB-FIX and K10RB-FIX-G4). Since K10 is a differentiation-specific keratin and not expressed in conventional monolayer cultures, it was not possible to assess *in vitro* factor IX expression using primary keratinocytes. A spontaneously transformed, non-tumorigenic, human keratinocyte cell line (HaCaT) was used to measure absolute and relative factor IX expression by the various gene constructs. The HaCaT cell line is known to express a wide variety of keratins in monolayer cultures, including low levels of K10 at confluence. The keratinocytes were transfected using a cationic liposome preparation followed by selection with G418. PCR was used to assess the genomic integration of the various gene constructs. A ~2.5 kb recombinant factor IX transcript was detected by Northern blot analysis. Factor IX that was γ-carboxylated and secreted into conditioned medium was detected using a double antibody (A5/A7) ELISA. A maximum expression of 35 ng/10^6 cells/24 hours was achieved by the K10-FIX-G4 construct. It is known that approximately 5-10% of HaCaT cells express K10, and accounting for this, the observed level of expression can be considered comparable to those achieved by other investigators. The implications and future prospects of this strategy are discussed.

C6-201 GENE THERAPY FOR INSULIN-DEPENDENT DIABETES MELLITUS BY THE HEPATIC EXPRESSION OF INSULIN

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Ketoacidosis is a potentially fatal consequence of insulin dependent diabetes mellitus (IDDM), and results from low serum insulin and high serum glucagon levels. Since β-cells are destroyed in IDDM, any attempt to reconstitute insulin gene expression must be directed to an ectopic organ. Our objective was to determine if ketoacidosis can be prevented in severely diabetic rats by the constitutive expression of the insulin gene from the liver. A recombinant retroviral vector, LX/rINS, encoding the rat insulin 1 gene was constructed and used to transduce hepatocytes in vivo by portal vein infusion 24 hours after a 70% partial hepatectomy. IDDM was induced in the rats with streptozotocin, which selectively destroys the insulin producing β-cells. Control rats had high serum ketone levels of 63±21 mg/dl, a 25% weight loss in 3 days, and 18/18 died within 6 days of the induction of diabetes due to ketoacidosis. In contrast, rats transduced with the insulin expression vector had low or no significant serum ketone levels, no net weight change, and 13/16 survived for 21 days. The treated animals exhibited normo-glycemia during a 24 hour fast, with no evidence of hypoglycemia. Histopathological examination of the liver showed no apparent abnormalities. Thus, the liver is an excellent target organ for ectopic expression of the insulin gene as a new treatment modality for type 1 diabetes mellitus by gene therapy.

Under non-fasting conditions the experimental rats exhibited hyperglycemia, despite immunoreactive insulin levels 3-5 fold higher than normal. It is likely that the insulin produced in the liver is not fully matured. Insulin expression by transducing rat liver will be enhanced with higher titers of recombinant retrovirus and to use viruses encoding engineered insulin which will allow processing in the liver to mature insulin. Using a retrovirus encoding beta-galactosidase we have demonstrated that increasing virus titers 10 fold, by sedimentation of the virus by centrifugation, increases hepatocyte transduction efficiency 4-6 fold in vivo. We have also constructed insulin gene mutants that will allow insulin processing by furin: this should greatly enhance insulin activity as compared with the partially processed insulin. By enhancing insulin activity glucose homeostasis may be better achieved.

C6-202 **EXPRESSION OF HUMAN FACTOR IX IN RATS AND MICE FROM CELLS IMPLANTED WITHIN AN IMMUNOISOLATION DEVICE.** **J. Brauker, V. Dwarki*, V. Carr-Brendel, T. Nijjar*, C. Vergoth, R. Chen*, L. Cohen*, O. Danos*, R. Mulligan*, R. C. Johnson, Baxter Healthcare Corp., Round Lake, IL 60073 and *Somatix Therapy Corp., Alameda, CA 94501.**

Immunoisolation is a unique approach for gene therapy involving the implantation of cells within a membrane-bound device to sequester the cells from the immune system of the patient. The approach improves the safety of gene therapy by segregating the implanted cells from the host tissues. Moreover, if an allogeneic cell line is used, the cells will be rejected if they escape from the device. We employed a ported immunoisolation device to implant a human fibroblast cell line in athymic rats and mice. The cells were transduced with the MFG retroviral vector containing human factor IX, without a selectable marker. About 60% of the cells were positive for human factor IX by immunohistological analysis. The aggregate cell population expressed about 1 mg/10^6 cells/day of human factor IX. Devices were loaded with 2 X 10^7 cells and 4 devices were implanted in athymic rats, two subcutaneously and two in the epididymal fat pads. Two devices were implanted subcutaneously in athymic mice. Control animals received implants with non-transduced human cells. Factor IX was assayed in the plasma of the animals using an ELISA. Initial rat samples were taken at day 11, and the mean factor IX levels were 64.4 ng ml of plasma for the four rats receiving transduced cells. Initial mouse levels were taken at day 12 and the average level was 22 ng/ml of plasma. Values for both rats and mice rose continuously for over 50 days, reaching levels of 182 ng/ml in the rat and 492 ng/ml in the mouse. No detectable factor IX was observed in controls. After six weeks, devices were removed from one rat and one mouse of each condition. Five days later the animals were assayed and no detectable plasma levels of factor IX were observed in the animals that were formerly positive. Their devices were examined immunohistologically and heavy staining of factor IX was observed in cells within the device. The remaining animals are being assayed regularly. The levels of factor IX observed in the rat are about 10% of normal values found in human plasma. These results indicate that significant amounts of human factor IX can be delivered to animal blood from an immunoisolation device implanted in soft tissues.

C6-203 ADENOVIRUS MEDIATED GENE THERAPY FOR RESTENOSIS AND OTHER VASCULAR PROLIFERATIVE DISORDERS, Mark W. Chang*, Eliav Barr*, Jonathan Seltzer*, Min-Min Lu*, Gary J. Nabel¥¶, Elizabeth G. Nabel¥, Michael S. Parmacek*, and Jeffrey M. Leiden*†, Departments of Medicine* and Pathology†, University of Chicago, Chicago, IL 60637, and Department of Medicine¥ and the Howard Hughes Medical Institute¶, University of Michigan, Ann Arbor, MI 48109

Vascular smooth muscle cell (SMC) proliferation in response to injury is an important etiologic factor in a number of vascular proliferative disorders including atherosclerosis and restenosis following balloon angioplasty. Thus, a better understanding of the molecular mechanisms that regulate SMC proliferation is important for the rational design of novel therapies for these diseases. We have examined the role of the retinoblastoma gene product (Rb) in regulating SMC proliferation both *in vitro* and *in vivo*. Rb is present in the unphosphorylated and active form in quiescent primary arterial SMCs, but is rapidly inactivated by phosphorylation in response to growth factor stimulation *in vitro*. To directly assess the role of Rb in regulating SMC cell cycle progression, we constructed a replication-defective adenovirus encoding a non-phosphorylatable, constitutively active form of Rb. Infection of cultured primary aortic SMC with this virus inhibited growth factor-stimulated proliferation of these cells *in vitro*. More importantly, localized arterial infection with this virus at the time of balloon angioplasty significantly reduced SMC proliferation and neointima formation in both the rat carotid and porcine femoral artery models of restenosis. Taken together, these results demonstrate an important role for Rb in regulating smooth muscle cell proliferation and suggest a novel gene therapy approach for vascular proliferative disorders associated with arterial injury.

C6-204 GENE THERAPY FOR HEMOPHILA-A & B: RETROVIRAL AND ADENOVIRAL MEDIATED GENE TRANSFER, Varavani J. Dwarki, Paula Belloni*, Tarlochan Nijjar , Linda Couto, Richard Chin, Srinivasa Shankara, Kaye Spratt, Lawrence Cohen, Olivier Danos, Richard Mulligan, Somatix Therapy Corporation, 850 Marina Village Parkway, Alameda, CA 94501, USA. • Syntex Discovery Group, Palo Alto CA 94303, USA.

Continuous delivery of FVIII and FIX protein in hemophiliacs by gene therapy will represent a major clinical advance over the current practice of infrequent administration of purified protein. We report retroviral-mediated gene delivery of B-domain deleted human FVIII using the MFG retroviral vector system. High levels of FVIII (0.5-1.0μg/10^6cells/24hr) were produced by a variety of transduced primary cells (human fibroblasts, myoblasts and endothelial cells) *in vitro*. Upon transplantation of 30X10^6 transduced primary fibroblasts into the peritoneal cavity of mice therapeutic levels of FVIII were detected in the circulation for over a week. The initial levels as measured by ELISA on day 2 ranged between 60-120ng/ml in the plasma. The capacity of primary cells to deliver the FVIII into the circulation was strongly dependent on the site of implantation. The only route by which the protein reached the circulation was by intraperitoneal route. Transplantation of transduced myoblasts into the muscle or fibroblasts underneath the skin failed to deliver the protein into the circulation.

We have demonstrated that transplantation of 1-2X10^7 primary mouse/human myoblasts transduced with MFG Canine FIX retrovirus leads to long term expression (100days) of FIX in the circulation of nude mice. The levels in the circulation were in the range of 5-10ng/ml. We are also pursuing direct delivery of FIX protein through adenoviral vectors. Our efforts to overcome the immunological problems with the adenoviral vectors will be presented.

C6-205 **EVALUATION OF HUMAN ß-GLOBIN GENE THERAPY CONSTRUCTS BY EXPRESSION IN SINGLE-COPY TRANSGENIC MICE.** J. Ellis[1], K.C. Tan-Un[2], A. Harper[3], N. Yannoutsos[4], P. Fraser[4], D. Michalovich[4], S. Philipsen[4], and F. Grosveld[4]. [1]Hospital for Sick Children, Toronto, Canada; [2]S.P.A.C.E., University of Hong Kong; [3]St. Thomas' Hospital, London, U.K.; [4]Erasmus University, Rotterdam, The Netherlands.

Effective gene therapy constructs based on retrovirus or adeno-associated virus vectors will require regulatory elements that direct expression of genes transduced at single copy. Most ß-globin constructs designed for gene therapy of ß-thalassemia are regulated by the 5'HS2 component of the Locus Control Region (LCR) but it is unclear whether 5'HS2 can reproducibly activate an integrated single-copy ß-globin gene. We have therefore evaluated expression from potential gene therapy constructs in erythroid cells of single-copy transgenic mice. Such a procedure is a good model for gene therapy because the transgene is already present in hematopoietic stem cells but must be expressed in erythroid cells derived from them.

We show that multiple 5'HS2 core elements or the complete 5'HS2 fragment (1.5 kb) do not direct reproducible ß-globin expression from independent single-copy transgenic mouse lines, indicating that 5'HS2 is not ideally suited for regulating gene therapy vectors that integrate at single copy. In contrast, full expression is obtained from independent single-copy ß-globin transgenes regulated by the microlocus LCR construct (6.5 kb) composed of all four hypersensitive site. The 1.9 kb 5'HS3 fragment expresses significant but reduced levels from single-copy transgenes. Our findings suggest that 5'HS3 contains a dominant chromatin opening activity, but that full expression by the microlocus LCR is most suitable for gene therapy applications.

C6-206 DYSREGULATED EXPRESSION OF GATA-1 FOLLOWING RETROVIRAL TRANSFER INTO MURINE HEMATOPOIETIC STEM CELLS INCREASES ERYTHROPOIESIS. S.F. Farina[1], L.J. Girard[1], E.F. Vanin[2], A.W. Nienhuis[2] and D.M. Bodine[1]. [1]Hematopoiesis Sec., NCHGR/NIH, Bethesda, MD. 20892. [2]St Jude Children's Research Hospital, Memphis, TN. 38101.

Retrovirus mediated gene transfer was used to study the effects of dysregulated expression of the transcription factor GATA-1. A retroviral vector was constructed with the murine GATA-1 gene linked to the human phosphoglycerate kinase (PGK) promoter (PGK-GATA-1). Expression of GATA-1 was demonstrated in transduced GATA-1 negative CTLL cells by gel shift analysis, and super-shift analysis using an antibody against murine GATA-1. For *in vivo* studies, bone marrow cells were transduced *in vitro* and transplanted into recipient animals. Southern blot analysis performed on DNA extracted from bone marrow, spleen and thymus 16 to 48 weeks post transplantation demonstrated the presence of the PGK-GATA-1 provirus. The animals which received cells transduced by the GATA-1 virus maintained a significantly lower white blood cell count and a higher red blood cell count than control animals which received cells transduced with a virus containing a neo^r gene. Erythropoiesis was stimulated in GATA-1 and control animals by phlebotomy. The GATA-1 animals required more phlebotomy to reach a hematocrit of 20, and returned to normal hematocrit levels faster than control animals. GATA-1 animals had an average hematocrit of 34.3 after 48 hours of recovery, compared to an average hematocrit of 27.0 for the control animals. The hematocrits of the GATA-1 animals had returned to normal by 72 hours compared to 96 hours for the control animals. The effect of twice daily injections of 10 units of recombinant erythropoietin (epo) was also examined in two experiments. The hematocrit of the GATA-1 animals (n=10) showed a more rapid and elevated response to epo(55.2 vs. 50.2, p=.008, on day 5, 61.4 vs. 56.0, p=.05, on day 9) than the hematocrit of control animals (n=8). These data suggest that dysregulated expression of GATA-1 in primitive hematopoietic cells enlarges the pool of epo dependent erythroid progenitor cells.

C6-207 GENE TRANSFER INTO PRIMARY MYOGENIC CELLS BY RETROVIRAL VECTORS.

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Gene transfer into muscle tissue has a number of potentially relevant applications in medicine, including somatic cell gene therapy of muscular dystrophies and systemic delivery of recombinant proteins for correction of genetic or acquired disorders. We have developed an in vivo model for testing the expression of foreign genes into human adult muscle fibers after retroviral vector-mediated gene transfer into primary myogenic cells. Satellite cells isolated from adult muscle biopsies are infected by co-culture with a high-titer, MoMLV-derived retroviral vector expressing the β-galactosidase gene under LTR control (LBSN). Gene transfer efficiency averages 50%, and the reporter gene is stably integrated, faithfully inherited, and expressed at significant levels in myogenic cells for at least 10 generations under clonal growth conditions, and throughout the culture life span upon differentiation into myotubes. Transduced human satellite cells are injected into regenerating muscle of immunodeficient mice, where they formed new muscle fibers in which expression of the reporter gene is detectable for more than 2 months. These results show that retroviral vectors can be used to transfer foreign genes with high efficiency into primary human myogenic cells, leading to stable expression into mature muscle. The human-mouse model described here will allow direct in vivo testing of the function of genetically modified muscle fibers, and therefore pre-clinical evaluation of a number of gene therapy strategies based on muscle tissue. We developed a new vector system in order to drive a regulated expression of the transgene in the muscle tissue.The retroviral vector for expression of β-galactosidase was manipulated by inserting the muscle creatine kinase (mck) enhancer into the viral LTR. Cell lines of different origin, including fibroblasts,melanoma, hematopoietic and myogenic cells were transduced with the mck-LBSN and LBSN retroviral vectors. The reporter gene of mck-LBSN vector was never expressed in all the tested cell cultures and in undifferentiated murine and human satellite cells.The transgene expression was obtained after induction of differentiation of myoblasts to myotubes, and timely correlated with the synthesis of skeletal myosin.Transduced human myoblasts were trasplanted into immunodeficient mice where activation of β-galactosidase expression was observed only after the in vivo fusion to myotubes. These results show that retroviral vectors, suitably manipulated, may be used to target tissue- and differentiation- specific expression of the transduced genes into the muscle tissue in vivo.

C6-208 DIRECT INJECTION OF RECOMBINANT ADENOVIRAL VECTORS INTO PORCINE MYOCARDIUM PROVOKES LEUKOCYTIC INFILTRATION

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Although the direct injection of recombinant adenovirus (Ad5) into rodent hearts has been characterized, the feasibility of this approach in large mammals has not been elucidated. We therefore injected replication-deficient Ad5 vectors carrying either the luciferase or *LacZ* reporter genes directly into the ventricular myocardium of 7 adult swine. Regional myocardial function as assessed by systolic wall thickening (ultrasonic crystals) was unaffected. Luciferase activity was detected 3 d after injection, increased markedly at 7 d, and declined progressively at 14 and 21 d. Luciferase production was comparable in the right and left ventricles, and increased with the dose of virus, reaching 61 $\pm$ 21 ng at 3.6×10^9 pfu. The injection of 200 µg of plasmid DNA (pRSVL) produced levels of luciferase comparable to 1.8×10^8 pfu of recombinant Ad5; however, when normalized to the number of genes injected, the adenovirus was 140,000 times more efficient than plasmid DNA. Histochemical analysis of ß-galactosidase activity produced by the *LacZ* reporter virus showed that >95% of the stained cells were cardiomyocytes and that the percentage of infected cardiomyocytes was quite high in microscopic regions adjacent to the needle track (up to 75% in fields of 60-70 cells). However, despite the use of high titer (1.4×10^9 pfu) injections, Ad5-infected cells were rarely observed farther than 5 mm from the injection site. Importantly, the Ad5 vector induced pronounced leukocytic infiltration which was far in excess of that seen following injection of vehicle alone. **Conclusions:** Direct myocardial injection of Ad5 can be used to program recombinant gene expression in the cardiomyocytes of a large animal species with relevance to human physiology. However, the marked immune response and the limited distribution of reporter gene expression indicate that less immunogenic vectors and more homogeneous administration methods will be required before Ad5 can be successfully used for widespread phenotypic modulation.

C6-209 ADENOVIRUS VECTOR CYTOKINE GENE TRANSFER TO LUNG TISSUE, Jack Gauldie, Frank Graham, Zhou Xing, Todd Braciak and Ronan Foley, Department of Pathology, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

We have developed a number of recombinant human Adenovirus 5 vectors encoding various cytokine genes and have determined the effect of specific cytokine overexpression in bronchial epithelial cells. Cytokine cDNA's, including IL-6 (1.3 kb), an immunomodulatory cytokine, MIP-2 (0.40 kb), a C-X-C chemokine, chemotactic for neutrophils, and RANTES (0.30 kb), a C-C chemokine, chemotactic for mononuclear cells including CD4, T Cells, were inserted into the E1 region of Adeno5 viral vectors under control of the human cytomegalovirus promoter (hCMV). Control virus included E1 delete, LacZ in E1 and wild type intact virus. 2.5×10^8 pfu of virus, purified by ultracentrifugation were instilled IT to rats. Lavage fluid, lung tissue and serum samples were collected at 24h, 48h, 72h, 7 days and 14 days. Ad-IL-6 caused no change at 24-48h. At 7 days there was massive lymphoproliferation, with expansion of B and T cell subsets in both parenchymal tissue and bronchial associated lymphoid tissue and seen in BAL fluids. By 14 days, resolution had occurred with no evidence of residual fibrosis. Several fold enhancement of IgA anti-Adenovirus was seen with IL-6 virus treatment. Ad-MIP-2 induced massive neutrophil accumulation into parenchyma and BAL fluids at 24-48h. By 72h neutrophilia had resolved and by 14 days there was normal lung appearance and no evidence of fibrosis. Ad-RANTES induced pronounced mononuclear accumulation in the tissue with related, but not identical, changes in the BAL cells at 24-48h. These changes also resolved by 7 days and at 14 days there was no evidence of fibrosis. These vectors caused cytokine-specific changes in lung parenchyma and indicate that accumulation of inflammatory cells to the lung does not by itself lead to chronic tissue damage (fibrosis). The expression system is well-suited to transient-tissue restricted overexpression of biologically important genes and the lung is a well-defined environment in which to examine cytokine specific effects. (Supported by MRC Canada, Terry Fox Foundation and London Life)

C6-210 DETECTION OF HORMONE-REGULATED ENHANCERS USING AN ENHANCER TRAP WITH POSITIVE AND NEGATIVE SELECTION, Robert W. Harrison and Janice Dusel, Endocrinology/Metabolism Unit, U. of Rochester School of Medicine and Dentistry, Rochester, NY 14642

Glucocorticoid regulation of gene expression is accomplished by interaction of the steroid-activated receptor with enhancer-like DNA elements termed glucocorticoid regulatory elements (GREs). Although the GRE responsible for transcriptional stimulation has been well described, negative regulatory elements are less common and not well understood. Additional negatively-regulated enhancers are needed for study to help clarify the mechanism of negative regulation. In the enhancer trap strategy, a selectable gene such as hygromycin phosphotransferase, is driven by a promoter made ineffective by excision of the upstream enhancer elements that drive promoter activity. When transfected, this gene will be actively transcribed only if the transgene inserts near ("traps") an endogenous enhancer. To identify regulated enhancers, we constructed a plasmid in which the fused hygromycin phosphotransferase and Herpes Simplex thymidine kinase genes are driven by a promoter containing only a TATA box. AtT-20/D1 cells were transfected with this construct and the transfectants selected in hygromycin. Only cells in which the promoter of the transfected gene was activated by an endogenous enhancer could survive selection in hygromycin. The resulting hygromycin-resistant clones were next selected in 10^{-6}M dexamethasone and 2.5μM ganciclovir. Since expression of the Herpes Simplex thymidine kinase gene would render ganciclovir lethal, only those cells in which the tagged enhancer was negatively regulated by dexamethasone could survive. Several cell clones have been isolated in this way and negative glucocorticoid regulation of the transgene confirmed. These results show that regulated enhancers can be readily identified when the enhancer trap strategy is used with a fusion gene that permits both positive and negative selection.

C6-211 TRANSIENT GENE EXPRESSION IN EPIDERMIS FOLLOWING INJECTION OF NAKED DNA, Ulrich R. Hengge, Edward F. Chan, Ruth A. Foster, Patricia S. Walker, Jonathan C. Vogel, Dermatology Branch, NCI, NIH, Bethesda, MD 20892

Epidermis is an attractive target for genetic manipulations because it is readily accessible and easily monitored. Expression of genes, such as cytokines, in the epidermis would have both therapeutic and experimental uses. We have developed an in vivo method to introduce and express genes in epidermis by directly injecting naked plasmid DNA into the upper dermis of miniature swine. Plasmids containing either ß-galactosidase (ßGal) or human IL-8 cDNAs driven by CMV promoters were used. Following injection, the pCMV:ßGal plasmid is taken up and expressed throughout the epidermis, most prominently above the dividing basal layer. ßGal enzyme can be detected for 3 weeks in the epidermis. However, ßGal mRNA expression is transient and declines over 1 week from initially high levels, probably due to the lack of plasmid integration and loss of plasmid DNA from the epidermis as evidenced by Southern analysis of total cellular DNA and Hirt extracts. Quantitation of ßGal expression using a chemiluminescent assay of ßGal activity showed that approximately 30 ng of ßGal enzyme were produced per injection and that a dose response relationship existed for 0.5-12.5 µg of injected plasmid. Injection of pCMV:IL-8 resulted in the production of functional IL-8 protein causing increased accumulation of neutrophils in the upper dermis. This simple technique holds promise for genetic immunizations and local treatment of dermatologic diseases.

C6-212 DESIGNING PLASMID DNA VECTORS TO EXPRESS THERAPEUTIC LEVELS OF PROTEIN *IN VIVO* P. Hobart, X. Liang, M. Margalith, K. Barnhart, A. Abai, A. Kuwahara-Rundell, M. Nolasco, M. Sawdey, J. Hartikka, M. Manthorpe, J. Marrow, J. Norman. Vical, Inc. San Diego, CA 92121.

Direct tissue injection of plasmid DNA has proven to be an effective means of expressing antigen proteins at levels sufficient to manifest a protective vaccination from infectious viral, bacterial, and parasitic agents. However, the use of plasmid DNA to deliver therapeutic levels of proteins required to treat cancer and metabolic diseases necessitates the development of new vectors able to express proteins at significantly higher levels. To address this need, a systematic study of *in vitro* and *in vivo* expression levels was made with plasmids that use either the Rous Sarcoma Virus LTR (3') (RSV) or the Cytomegalovirus Immediate Early Gene (CMV-IE) promoter. Levels of expression were compared by measuring intracellular reporter proteins (i,e chloramphenicol acetyl transferase and luciferase) as well as a secreted protein (factor IX). The results show four principle findings: (1) relative to RSV, the CMV-IE promoter expresses > 10-fold protein in most cells transfected in vitro and when administered directly into murine muscle and lung tissue; (2) plasmid based transcription is enhanced by deleting sequences unnecessary for eukaryotic expression or bacterial cell replication; (3) CMV-IE promoter activity can be increased by deletions and/or modifications of sequences within the enhancer domain; (4) co-expression of a heterologous transcription factor, such as the bacterial tet-repressor/herpes VP16 fusion protein, can be used to further modulate (both up and down) the CMV-IE promoter when specific response elements are inserted within the promoter sequence. This study has shown that reporter gene expression can be increased up to 100-fold both in cells *in vitro* and *in vivo*.

C6-213 REGULATABLE GENE ACTIVATION / INHIBITION USING A POWERFUL GENETIC SWITCH, Rob Hooft van Huijsduijnen, R. Pescini and J.F. DeLamarter, GLAXO Institute for Molecular Biology, Chemin des Aulx, 1228 Geneva, Switzerland

Recently a powerful regulatable gene induction system was developed by Bujard and co-workers (Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA*, 89, 5547-5551). This system is based on a tetracycline-repressor/VP16 designer transcription factor which is regulated by the antibiotic tetracycline (Tc). We are using this system for regulatable and tissue-specific gene activation or inhibition. One application in genetic therapies would be as a means to dose therapeutic gene products. Regulatable promoters also offer a refined tool to study gene function in transgenic animals where "inducible knock-out" mice can be generated. In tissue culture we have found that the "Tc-system" allows amplification of weak, tissue-specific promoter activity, in addition to providing regulation by tetracycline. In one example we were able to demonstrate regulatable inhibition of IL8-receptor using expression of the antisense message. In another example we showed regulatable inhibition of BDNF (brain-derived neurotrophic factor). Transgenic mouse lines have been obtained carrying anti-sense BDNF under a promoter that is recognized by the tet-repressor. These mice will be bred with transgenics that express the designer factor under various tissue-specific promoters. Since BDNF null-mutants die soon after birth, this approach allows studying the role of BDNF in adult animals. These results also illustrate the potential of the tet-system as a "control switch" for transgene expression in gene therapy.

C6-214 GENE THERAPY IN 3 CHILDREN WITH ADENOSINE DEAMINASE DEFICIENCY.

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Retrovirus mediated gene transfer into bone marrow stem cells of 3 children with Severe Combined Immune Deficiency (SCID) due to Adenosine Deaminase (ADA) deficiency was performed. Bone marrow stem cells were purified by CD34+ cell enrichment using AIS CD34 Cell Separation devices. The enriched stem cells were co-cultured with a virus-producing cell line shedding an amphotropic retrovirus carrying the human ADA gene. *Following co-cultivation, the stem cells were infused* into the patients. No myeloablative therapy was given. Two patients continued treatment with bovine ADA linked to polyethylene glycol (PEG-ADA). PEG-ADA treatment in the third patient was started 4 months after gene therapy.

Following co-cultivation, 5, 8 and 12% of CFU-C's showed ADA expression. Short term presence of the gene was *seen in mononuclear* cells and granulocytes; the recombinant ADA gene could not be demonstrated in the cultured T-cells. In 1 patient the ADA gene was present in the bone marrow 6 months after gene transfer; the marrow of all 3 patients was negative at 1 year after gene transfer.

To study whether the absence of myeloablative therapy contributed the the lack of stable presence of the gene, we performed gene transfer into non-conditioned monkeys and mice conditioned with low-dose TBI. Stable presence of the gene could not be detected in the unconditioned monkeys; the murine studies reveiled a dose effect relation between the presence of the ADA gene and the amount of TBI. These studies suggest that the absence of long-term presence of the gene in our 3 patient may be related to the absence of conditioning.

C6-215 PHYSIOLOGIC ASSESSMENT OF ANGIOGENESIS BY ARTERIAL GENE THERAPY WITH VASCULAR ENDOTHELIAL GROWTH FACTOR, Isner J.M., Takeshita S., Bauters C., Asahara T., Zheng L.P., Rossow S.T., Kearney M., Barry J.J., Ferrara N., Symes J.F., St. Elizabeth's Medical Center, Tufts University School of Medicine, Boston, MA 02135

We have previously demonstrated angiographic and necropsy evidence of augmented collateral artery development and capillary density following direct arterial gene transfer of vascular endothelial growth factor (VEGF) in a rabbit model of lower limb arterial insufficiency. To evaluate the physiologic response to VEGF gene transfer, 400 μg of plasmid phVEGF (cytomegalovirus promoter) was applied to the polymer coating of an angioplasty balloon and delivered percutaneously to iliac artery of rabbits in which femoral artery had been excised to cause limb ischemia. Limb perfusion was evaluated pre- and post-papaverine (2 mg, intra-arterial) at 30 days post-transfection using a Doppler wire to measure flow velocity, and quantitative angiography to measure iliac artery diameter at site of wire placement. Results were compared to those of rabbits treated with single intra-arterial bolus of VEGF protein (500 μg) or saline. Flow (ml/min), calculated assuming circular lumen geometry, is shown below:

	Gene (n=7)	Protein (n=11)	Saline (n=6)
Pre-papaverine	28.1±2.6**	23.8±1.1*	17.5±1.0
Post-papaverine	62.3±6.6***	52.7±2.4**	30.9±2.6

(*p<0.05, **p<0.01, ***p<0.001 vs saline control using ANOVA)

These physiologic findings complement previous anatomic studies by showing that VEGF gene transfer improves resting and maximum flow at the level achieved by bolus administration of VEGF protein.

C6-216 ADENOVIRAL-MEDIATED EXPRESSION OF A VOLTAGE-GATED POTASSIUM CHANNEL: A MECHANISM FOR MODIFYING CARDIAC EXCITABILITY, David C. Johns, Brian Ramza, Eduardo Marban, John H. Lawrence, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, 21205

The action potential duration (APD) is a critical parameter that influences cardiac excitability and propensity to cardiac arrhythmias. APD is determined by the relative balance of small inward and outward currents active during the plateau phase which is therefore an attractive target for modulation. For example, the heterologous expression of a delayed rectifier-type potassium channel in a cardiac cell would be expected to shorten the APD and if a sufficient number of cells could be modified *in vivo*, the electrical substrate of the heart would also change. Toward this end, we have constructed a recombinant adenovirus (Ad5-ShK) by homologous recombination into the E1 region of an insert encoding a constitutive promoter (the LTR of RSV) and an inactivation-deficient *Shaker* Kv1.1 potassium channel. The *Shaker* channel had previously been engineered (T449Y; Yellen *et al.*) to be sensitive to the blocker tetraethylammonium (TEA). In HEK293 cells, following infection with Ad5-ShK, transcription of a specific 1.8 Kb mRNA was confirmed by Northern analysis. Patch clamp recordings revealed expression of a rapidly activating, noninactivating TEA-sensitive outward current up to 3 nA in amplitude. In 3T3 cells infected with Ad5-ShK at an moi of 10-100, outward currents with amplitudes up to 1.2 nA were measured at 48 hrs. Similarly infected rat neonatal cardiomyocytes in culture exhibited ranging from 0.8-2.0 nA of TEA-sensitive outward current in 10 consecutive patches. Following intravascular injection of 10^8 pfu Ad5-ShK, Northern analysis in liver and heart was positive and explanted hepatocytes in culture had up to 1 nA of outward current. These findings demonstrate the feasibility of an adenoviral vector for achieving expression of voltage-gated ion channels *in vitro* and *in vivo* and suggest that this system may be able to modify cardiac excitability through direct effects on the action potential.

C6-217 ECTOPIC EXPRESSION OF THE MITOCHONDRIAL UNCOUPLING PROTEIN GENE IN WHITE FAT,

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While mammalian white fat is specialized for storing nutritional energy in triglycerides, brown fat is equipped for energy dissipation. Thermogenesis in brown fat depends on expression of the mitochondrial uncoupling protein gene (*Ucp*). This expression is specific for brown fat where it is thought to be essential during exposure to cold and possibly as a mechanism for energy balance during overfeeding. We are interested in relationship between brown fat thermogenesis and obesity. In order to test the potential role of *Ucp*-directed thermogenesis in energy balance, we created mice that have ectopic expression of *Ucp* in white fat, by introducing a transgene which carried *Ucp* under control of the enhancer region of the aP2 gene. This enhancer will direct expression of *Ucp* to both brown fat and white fat. As predicted expression of the *Ucp* was detected in both subcutaneous and abdominal fat depots as well as in brown fat. This pattern of ectopic *Ucp* expression reduced only marginally the total body weight of the transgenic mice on the normal C57BL/6J background, but did result in about a 20% reduction in body weight when the transgene was combined with A^{vy}, a gene which causes obesity in mice. Also development of obesity in mice fed by a high fat containing diet, or due to a chemically induced hypothalamic lesion, was largely abolished in the transgenic animals. Expression of the transgene was always associated with a redistribution of fat depots. The subcutaneous fat was reduced several fold, while gonadal fat depots were correspondingly increased. Our data indicate a possibility for gene therapy of pathological obesity in humans in future.

C6-218 NOVEL STRATEGIES FOR PLASMA CHOLESTEROL REDUCTION WITH HEPATIC GENE TRANSFER IN LDL RECEPTOR-DEFICIENT ANIMALS, Karen F. Kozarsky[1], Federico Giannoni[2], D. K. Bonen[2], T. Funahashi[2], M. Donahee[1], J. Strauss[3], J. M. Wilson[1], and N. O. Davidson[2]. [1]The Institute for Human Gene Therapy and Department of Molecular and Cellular Engineering, and [3]Department of Obstetrics and Gynecology, University of Pennsylvania, and [1]The Wistar Institute, Philadelphia, PA; [2]Department of Medicine, University of Chicago, Chicago, IL.

Previously, we have shown that introduction of a recombinant adenovirus encoding the human low density lipoprotein (LDL) receptor cDNA into the livers of LDL receptor-deficient Watanabe heritable hyperlipidemic (WHHL) rabbits resulted in large though transient reductions in plasma cholesterol. Two alternative gene therapy strategies utilizing recombinant adenovirus have been designed. One involves the gene which encodes the rat apolipoprotein B mRNA editing protein, APOBEC-1, which converts the apoB100 mRNA into apoB48 mRNA. This method circumvents the LDL receptor defect by directing hepatocytes to synthesize lipoproteins enriched in apoB48 and apoE which are taken up by receptors other than the (defective) LDL receptor. Cholesterol levels in WHHL rabbits dropped, with a reduction in the VLDL/LDL fraction, and plama apoB48 was detected. The second strategy utilizes the human very low density lipoprotein (VLDL) receptor cDNA to obtain hepatic uptake of VLDL and IDL, the lipoprotein precursors to LDL. Introduction of this virus into hepatocytes of WHHL rabbits and LDL receptor knockout mice resulted in lowered plasma cholesterol levels. These studies demonstrate the power of gene therapy in elucidating the *in vivo* function of these recently cloned genes in models of human familial hypercholesterolemia.

C6-219 LONG-TERM EXPRESSION OF THE BIOLOGICALLY ACTIVE GROWTH HORMONE IN GENETICALLY MODIFIED FIBROBLASTS AFTER IMPLANTATION INTO A HYPOPHYSECTOMIZED RAT

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We employed the hypophysectomized rats of which the pituitary glands were surgically removed as an animal model to explore the feasibility of using fibroblast cells for growth hormone gene therapy. An internal ribosome entry site (IRES)-directed bicistronic retroviral vector, PSN, which contained a porcine grwoth hormone (pGH) cDNA at the first cistron and a Neo^r gene at the second cistron as well as a N5 vector control were used to infect primary rat embryo fibroblast (REF) cells prepared from the inbred Brown Norway rats. The infected cells were directly injected intraperitoneally (i.p.) ($5x10^6$ cells/rat) into the hypophysectomized rats. We demonstrate that the implanted PSN-infected REF cells could secrete biologically active pGH *in vivo*, leading to significant growth of the tibia and an increase in body weight as observed at day 15 and day 57 post-transplantation; N5-infected REF cells could not produce this growth effect. We further treated the PSN-infected REF cells with collagen to form a tissue-like structure. The skin-like discs were grafted underneath the skin on the back of rats, and retrieved at different times. Using two criteria, semiquantitative reverse transcription-polymerase chain reaction on the RNA extracted from the explants and G418 resistance conferred from the explanted cells, we demonstrate that pGH was expressed persistently in the implanted fibroblasts up to 70 days. These results suggest that fibroblast cells are capable of persistently expressing the foreign genes *in vivo*; thus may provide a great potential alternative for gene therapy.

C6-220 ENHANCED AND SWITCHABLE EXPRESSION SYSTEMS FOR GENE-TRANSFER, Xiaowu Liang, Ann Kuwahara-Rundell and Peter Hobart, Vical Inc., 9373 Towne Centre Drive Suite 100, San Diego, CA 92121, USA.

Two of the main thrusts for current gene-therapy research are a) enhancing expression in order to deliver therapeutic levels of gene products, and b) regulating the expression of transferred exogenous genes. As a step towards this, we have sought to utilize an artificial transcriptional regulation system involving a *tet*-repressor(R)/VP16 fusion protein and the promoters containing *tet*-operator elements, a system first described by Gossen and Bujard (Gossen, M. and Bujard, H., 1992, *Proc. Natl. Acad. Sci. USA.*, **89**, 5547-5551). Modifications have been made to both the tetracycline (tet)-responsive promoter construct and the *tet*R/VP16 trans-activator in an effort to improve expression in mammalian cells grown *in vitro* and cells *in vivo*. The improved system showed a significantly higher (> 10 fold) level of expression of reporter genes when compared to constructs using the human cytomegalovirus immediate-early (CMV IE) gene promoter plus intron A. The expression was down-regulated by the antibiotic tetracycline. In addition, a new chimeric promoter has been constructed enabling up-regulation by tetracycline. By placing *tet*-operator sequences 3' to the TATA box of CMV IE promoter, we found this new chimeric promoter retained high level of reporter gene expression and that expression was greatly repressed when cells were co-transfected with DNA encoding the *tet*R-containing transcription factor. When co-transfected cells were grown in the presence of tetracycline, reporter gene expression was restored to unrepressed levels. Using this new tet-inducible construct, a novel expression system has been established in which two genes can be differentially regulated by expression of *tet*R/VP16 and that expression can be reversed by addition of tetracycline. Such tight regulation of expression of one or more gene products by a small effector will be of great importance for the regulated expression of therapeutic proteins in gene therapy.

C6-221 ADENOVIRUS-MEDIATED UROKINASE GENE TRANSFER INDUCES LIVER REGENERATION AND ALLOWS FOR EFFICIENT RETROVIRUS TRANSDUCTION OF HEPATOCYTES IN VIVO. A.Lieber, M.J. Vrancken-Peeters, J.D. Perkins and M.A. Kay. Departments of Medicine (RG-25) and Surgery (RF-25), University of Washington, Seattle, WA 98195.

Direct retroviral-mediated gene transfer into hepatocytes *in vivo* requires a surgical partial hepatectomy that leads to a brief period of regeneration in which the virus can be infused for permanent gene transfer. Disappointingly low levels of gene transfer have been achieved by this approach. We have eliminated the need for surgical hepatectomy by treating mice first with a recombinant adenovirus vector that expresses urokinase (Ad.RSV-upa), a protein that when produced in hepatocytes leads to their degeneration and induces a regeneration stimulus. To quantitate liver regeneration, animals subjected to infusion of Ad.RSV-upa or control adenovirus or to 2/3 partial hepatectomy were monitored for liver regeneration by measuring the ^{3}H-thymidine incorporation into DNA and by autoradiography. Administration of the Ad.RSV-upa vector led to high rates of hepatocyte labelling extending over a period of more than one week. At different time points after Ad.RSV-upa administration the animals were infused with about 2×10^6 cfu of recombinant retrovirus encoding either βgalactosidase or human α1-antitrypsin (hAAT). Some animals were given three retrovirus infusions over a 5 day period via an indwelling portal vein catheter. Multiple retrovirus transfusions after Ad.RSV-upa treatment resulted in gene transfer into about 5% of the hepatocytes compared to 1% in the partially hepatectomized animals. Similarly, the serum hAAT concentrations were about 5 to 10-fold greater in the Ad.RSV-upa animals compared with partial hepatectomized animals and were stable over the analyzed period of three 3 months. The recipients of the Ad.RSV-upa virus had transient serum upa levels of up to 400 ng/ml (100 x normal) which resulted in elevations of the prothrombin time. To circumvent this problem, the urokinase cDNA was modified such that after adenovirus-mediated gene delivery, the protein product remained intracellular, did not increase the prothrombin time, but still induced the same level of hepatic regeneration. This approach offers a means by which to transduce retrovirus in the liver at a greater efficiency and without the need of surgical hepatectomy. Future studies will determine whether gene transfer can be increased by using higher titers of retrovirus and persistent infusion of virus over a several day period. This development may be important for applications requiring permanent gene transfer into the liver.

C6-222 INHIBITION OF RECURRENCE OF BACTERIAL CELL WALL-INDUCED ARTHRITIS IN RATS BY GENE TRANSFER OF THE INTERLEUKIN-1 RECEPTOR ANTAGONIST. S.S. Makarov[1], J.C. Olsen[2], W.N Johnston[3], J.H. Schwab[3], S.K. Anderle[3] R.R. Brown[3] and S. Haskill[1,4], *Lineberger Cancer Research Center[1], Division of Pulmonary Diseases[2], Department of Microbiology and Immunology[3], Department of Obstetrics and Gynecology[4], University of North Carolina School of Medicine, Chapel Hill, NC 27599*

Rheumatoid arthritis (RA) is a devastating, autoimmune disease in which almost every arm of the inflammatory response is activated. Gene therapy may provide an attractive alternative to conventional approaches for treating RA. Based on previously developed protocols for the retroviral gene transfer to inflamed synovium, we examined feasibility of the antiinflammatory gene therapy in an animal model of arthritis. Using *ex vivo* gene transfer, the human secreted receptor-antagonist (hsIL-1ra) cDNA was expressed intraarticularly in the model of streptococcal cell wall (SCW) arthritis in rat. Primary synovial cells from syngeneic animals with SCW arthritis were infected in culture with a retroviral vector carrying the cDNA of hsIL-1ra and a *neo*- resistance gene. Following drug selection, secretion of hsIL-1ra was about 200 ng per million cells per day. To induce reactivation model of arthritis, animals were pre-injected with subarthropatic dose of peptidoglycan-polysaccharide (PG-APS) complexes from SCW in both ankle joints. Four to six weeks later, 10^5 hsIL-1ra$^+$ cells were engrafted in one joint. In a control group, the animals received an equal number of cells transduced with reporter genes *LacZ* and *neo*. One day after engraftment, arthritis was reactivated by the i.v. injection of PG-APS. Severity of arthritis was evaluated measuring joint swelling. The acute recurrence reached a peak 3 days following i.v. injection of PG-APS. Both contralateral joints and those injected with *LacZ*$^+$ cells developed similar levels of inflammation. In striking contrast, joints with engrafted sIL-1ra$^+$ cells were almost unaffected by reactivation. The average increase of joint diameter in control groups was 17±4 % vs. 5±2 % in group with hsIL-1ra$^+$ joints. Eight days after transplantation, the engrafted cells continued to express transferred genes. The experiment was repeated with similar outcome. We conclude that the gene transfer of the hsIL-1ra to synovium can suppress inflammation in an experimental model of arthritis relevant to human disease.

C6-223 INTRATRACHEAL TRANSFECTION OF LUNG WITH FACTOR IX PLASMID DNA LEADS TO SECRETION OF FACTOR IX PROTEIN INTO BLOOD, Marston Manthorpe, Michal Margalith, Jane Morrow, Anna M. Abai, Gary Rhodes, Peter Hobart, VICAL INCORPORATED, San Diego, CA 92121

All current approaches for the treatment of hemophilia using gene therapy require invasive methods, such as needle-based injection of expression vectors or surgical transplantation of genetically modified cells. Such methods induce hemorrhaging that must be controlled using systemic pre-administration of recombinant clotting factors. Here, we have infused canine Factor IX plasmid DNA into the mouse lung using an olive-tipped cannula. For comparison, mouse skeletal muscles were injected with the same DNA. Factor IX protein and anti-Factor IX antibodies were monitored in blood samples periodically collected for two months following lung infusion or muscle injection. Intramuscular administration of a CMV-driven FIX DNA results in the detection of up to 12 ng FIX protein per ml of blood. FIX protein levels in blood were lower after lung intubation than after intramuscular injection, but both routes of DNA administration yielded similar time course profiles. Canine Factor IX protein levels in blood rose to peak about a week after transfection and declined thereafter. The decline in Factor IX levels in blood correlated with a rise in blood anti-canine Factor IX antibodies. The lung route of administration for blood clotting factor DNA may eventually be preferred over more invasive gene therapy methods for the treatment of hemophilia.

C6-224 FUNCTIONAL EXPRESSION OF THE HUMAN INSULIN GENE IN A HUMAN HEPATOMA CELL LINE (HEP G2). G. Marshall[a], A. M. Simpson[c], B. E. Tuch[c], M. A. Swan[c], J. Tu[c].
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The identification of an appropriate (non-islet) cell type that has the ability following the insertion of the insulin gene and a suitable promoter to synthesise, process, store and secrete insulin is of foremost importance in developing a somatic gene therapy system for treatment of Type I diabetes. A cDNA for insulin (pC_2) was inserted into the constitutive expression vector RcCMV and introduced into a human hepatoma cell line HEP G2 by electroporation. Human insulin (hI) and proinsulin (hPI) were measured by radioimmunoassay, 74% of hPI being detected by the hI antibody (Ab) and no hI by the hPI Ab. More hPI than hI was released daily by the stably transformed cell line (HEP G2ins) (78 ± 4 pmol hPI/10^6 cells vs 13 ± 3 pmol hI) (n=5). The reverse was seen in acute stimulation with the secretogogue 5 mM 8-Br-cAMP: 0.6 ± 0.2 hPI/10^6 cells vs 4 ± 0.2 pmol hI. The cells did not respond to 20 mM glucose. Examination of acid/ethanol extracts of the cells confirmed hI was preferentially being stored: 29 ± 5 pmol/10^6 cells vs 7 ± 1 pmol hPI. Immunohistochemical analysis of the cells using a polyclonal hI Ab confirmed hI + hPI was being stored. Electron microscopy revealed large membrane-bound vacuoles containing electron-dense material, which were not seen in control cells. Five min pulse-chase labelling of the HEP G2ins cells with ^{3}H leucine confirmed hI synthesis in the presence of 20 mM glucose and 5 mM 8-Br-cAMP. After a 60 min lag period release of hI was detected; this was stimulated sig. ($P<0.05$) ($n=3$) in the presence of glucose (33 ± 1 cpm x 10^3/μg DNA) and 8-Br-cAMP (42 ± 1), compared to control cells (27 ± 0.6). A dose response curve for hI synthesis was also generated in response to different concentrations of glucose with a half V_{max} of 4.9 mM. The V_{max} (90 ± 2 nmol/min/mg protein) and K_m 1.5 ± 0.3 mM (n=3) for glucokinase at different glucose levels (0.03 - 100 mM) were not significantly different from untransformed hepatoma cells. Similarly, albumin secretion (53 ± 5 μg/10^6 cells/day) for those cells was unaltered from controls. In summary our results show that the introduction of insulin cDNA into a liver cell line results in synthesis, storage and acute regulated insulin release. However, chronic insulin release was constitutive and the cells did not secrete insulin in response to glucose.

C6-225 MODELS OF GENE THERAPY FOR MYOCARDIAL ISCHEMIA, Patricia McDonald, Martin N. Hicks [1], Stuart M. Cobbe[1] and Howard Prentice. Department of Genetics and Department of Medicine and Therapeutics, University of Glasgow and [1]Department of Medical Cardiology, Royal Infirmary, Glasgow, UK.

Gene therapy for myocardial disease will depend on the selection of effective vector systems and the ability to appropriately regulate expression of foreign gene products. The direct DNA injection technique has been valuable in the assessment of tissue specific and inducible promoter activity in heart and skeletal muscle. By comparing levels of transcriptional activity for troponin C fast and cardiac troponin C promoter constructs in heart and skeletal muscle it was found that these promoters retained their tissue specific patterns of activation following direct DNA injection. Recent experiments employing direct DNA injection to target cardiomyocytes *in vivo* in ischemic/reperfused heart resulted in high level transcription from ubiquitously active promoters. We are investigating the activities of muscle specific promoter constructs by direct DNA injection into rat heart in vivo after a 15 minute coronary ligation and reperfusion. Our findings indicate that the promoter for the skeletal α-actin gene is active in ischemic heart. The promoter regions for this and other tissue specific genes will be used to drive expression of potentially therapeutic gene products in ischemic myocardium. In a second model of myocardial gene transfer aimed at targeting genes to non-myocytes we have injected a β-galactosidase expressing retrovirus into the wall of the ischemic left ventricle. In rabbits subjected to permanent coronary ligation, injection of retrovirus into the area of infarct resulted in significant β-galactosidase expression at 7 days post-infarct. We are currently developing replication defective adenovirus and retroviral constructs to direct high level expression of foreign genes in mildly ischemic heart and within a myocardial infarct. The transfer of viral constructs into cardiomyocytes and non-myocytes in ischemic heart should afford the opportunity to create phenotypic changes that affect cardiac remodelling and angiogenesis.

C6-226 EXPRESSION OF FACTOR VII *IN VITRO* AND *IN VIVO* FOLLOWING DIRECT GENE TRANSFER INTO MUSCLE: A MODEL FOR HAEMOPHILIA. Gaynor Miller[1,2], Ricarda A. Steinbrecher[1,2], Paul J. Murdock[1], K. John Pasi[1], Edward G.D.Tuddenham[3], Christine A Lee[1] and Geoffrey Goldspink[2]. [1]Katharine Dormandy Haemophilia Centre,Departments of Haematology, [2]Anatomy and Developmental Biology, Royal Free Hospital and School of Medicine and [3]Haemostasis Research Group, Royal Postgraduate Medical School, London, UK.

Direct gene injection of plasmid DNA has been proposed as a method of effecting somatic gene therapy. Plasmid constructs are taken up, maintained episomally and are expressed by muscle fibres following a singular injection of plasmid DNA. This approach avoids the unpredictable effects of random integration. Such plasmids can be maintained for up to a year after gene transfer. Coagulation factor plasmid constructs are candidates for gene therapy of haemophilia by an intramuscular route. In this study the coagulation factor VII (FVII) gene has been chosen as a model for testing such a system. FVII is used as a reporter gene as it is a small, highly expressed gene that has a relatively stable gene product. We have produced a construct comprising myosin heavy chain promoter, artificial ribosomal binding site, FVII cDNA, polyA signal and skeletal muscle specific enhancer element of myosin light chain gene, ensuring muscle specific expression. Following calcium phosphate transfection of the construct into C2 myoblast culture, FVII antigen was secreted into the culture supernatant (230ng/10^6cells/24 hours). Expression persisted at similar levels for the duration of culture maintenance. Secreted FVII was functional in a PT based FVII bioassay, showing effective terminal gamma carboxylation. Following direct injection of the construct into quadriceps muscle of mice, circulating human FVII antigen was detected in plasma after 4-6 days. Anti-human FVII antibodies have also been detected in animals 7 days after injection of the construct. From this encouraging data it appears that direct gene transfer, using skeletal muscle as an *in vivo* expression system, offers a potential method of effecting gene therapy for the haemophilias.

C6-227 MESOTHELIAL CELL-MEDIATED DELIVERY OF HUMAN GROWTH HORMONE: TOWARDS THE TREATMENT OF MALNUTRITION IN PATIENTS ON CHRONIC DIALYSIS, Janice A. Nagy‡, Ty R. Shockley*, Catherine M. Hoff*, and Robert W. Jackman‡, ‡Departments of Pathology, Beth Israel Hospital and Harvard Medical School, Boston, MA 02215 and *Renal Division, Baxter Healthcare Corporation, McGaw Park, IL 60085

Protein and caloric malnutrition is common in patients on chronic dialysis. Recombinant human growth hormone (rhGH), a protein with anabolic properties, has been proposed for the treatment of malnutrition. Previous studies in dialysis patients have shown that short-term rhGH therapy induces a net decrease in total (i.e., blood plus urine) urea nitrogen appearance and an increase in IGF-1 levels, consistent with the anabolic effects of hGH; however, long-term studies, to define the steady-state effects of hGH administration on nutritional indices, are still needed. One potential strategy for the sustained systemic delivery of hGH would involve a gene therapy approach. To this end, we evaluated the feasibility of hGH delivery to the circulation by *ex vivo* mesothelial cell (MC)-mediated gene therapy. Rat primary peritoneal MCs, isolated from parietal peritoneum by enzymatic digestion, were stably transfected, using strontium phosphate DNA co-precipitation, with the plasmids pSVTKgh, containing the gene for hGH, and pCDneo, as the selectable marker. Following G418 selection, hGH-transfected MCs secreted hGH into the medium at a rate of ~ 1700 ng per 10^6 cells per day. Such hGH-transfected MCs, tagged with DiO, a fluorescent lipophilic dye used for long-term tracing of transplanted cells were reseeded on the denuded peritoneal surface of syngeneic recipients, and remained attached there for > 90 days. PCR analysis, using primers specific to hGH recombinant sequences also demonstrated persistence of the reimplanted MCs for at least 12 weeks. Moreover, these genetically-modified MCs continued to express hGH *in vivo* (i.e., hGH was detected by RIA in the plasma of immunocompetent animals for ~ 4 days and in the plasma of dexamethasone-treated rats for ~ 2-3 weeks). Furthermore, a sensitive immunoblotting technique demonstrated that plasma from immunocompetent rats contained increasing titers of hGH-specific antibodies, indicating indirectly that hGH secretion persisted in these animals for at least 12 weeks. Our results demonstrate the ability of genetically-modified MCs to deliver recombinant proteins such as hGH to the systemic circulation, and suggest the potential of *ex vivo* MC-mediated gene therapy to deliver hGH for nutritional therapy.

C6-228 DIRECT *IN VIVO* GENE TRANSFER INTO HEALING PATELLAR LIGAMENT, Norimasa Nakamura, Shuji Horibe, Norinao Matsumoto, Tetsuya Tomita, Yukiyoshi Toritsuka, Yasufumi Kaneda*, Konsei Shino**, Department of Orthopaedic Surgery, Osaka University Medical school, *Institute for Molecular and Cellular Biology, Osaka University, 2-2, Yamada-oka, Suita, Osaka, 565 JAPAN, **Department of Orthopaedic Sports Medicine, Osaka Rosai Hospital, Sakai, Osaka, 591 JAPAN

Accelerating the healing of injured ligaments is one of the greatest concerns for orthopaedic surgeons. In this context, proper delivery of key molecules for wound healing into the wound site may potentially make it possible to manipulate the healing process. Recently, we established a highly effeicient method for *in vivo* gene transfer with hemagglutinating virus of Japan (HVJ) conjugated liposomes. In the present study, we demonstrated the introduction of reporter genes into healing rat patellar ligaments using this technique. The mid-portion of the medial half of the patellar ligament was transversely cut with a scalpel in 14-week-old male Lewis rats. HVJ-liposomes suspension containing SV40 large T antigen or β-gal cDNA was injected directly into the injured site and beneath the fascia covering the injured site at 5 days postoperatively. At 3 days following injection, the reporter gene was detected immunohistochemically in 8 % of the cells in the injured area. At 21 days following injection, the reporter gene was detected in 7 % of histiocyte or fibroblastic cells in the injured area, while in 8 % of the fibroblastic cells in the adajcent ligament substance. Although most of the transfected cells in the injured area at 3 days following injection were ovoid and undifferentiated, some of them became more flattened and elongated (fibroblastic) within several days, and such cells remained in the injured area as well as in the adjacent ligament substance at 3 weeks following injection. These results suggest that the transfected cells took part in the early process of ligament healing and regeneration along with their differentiation. Since the transfered gene is not integrated into the host genome, our method seems suitable for the studies that must allow transient expression of foreign genes, such as the study of ligament healing as well as of graft remodeling. Although reporter gene-bearing cells remained in the injured patellar ligaments at least as long as 3 weeks following injection, longer follow-up should allow us to determe the precise duration of transgene expression in this model.

C6-229 ADENOVIRUS-MEDIATED DNA TRANSFER TO RAT SUBMANDIBULAR GLAND IN VIVO AND ANALYSIS OF GLUTAMINE-GLUTAMIC ACID RICH PROTEIN REGULATION. · Brian C. O'Connell, Kelly TenHagen, Lawrence A. Tabak and Bruce J. Baum. CIPCB, NIDR, NIH, Bethesda, MD and Dept. of Dental Research, University of Rochester, NY

The internalization of unmodified DNA and its transport to the nucleus can be facilitated by adenovirus infection. By this means large or multiple genes may be transferred without the construction of viral vectors. Using the replication-deficient adenovirus Add1312 and a plasmid-based firefly luciferase gene as a reporter, we have optimized the uptake and expression of DNA in rat submandibular glands *in vivo*. Retrograde ductal infusion of the DNA-virus mixture confines the gene transfer to the salivary gland itself. The expression of luciferase peaked at about 18 hours after infection and was undetectable after 5 days. Luciferase activity increased with the amount of plasmid administered (up to 200 μg), but was maximum at 10^9 to 10^{10} plaque forming units of Add1312 per gland. These results were used to examine a set of plasmids containing deletions of the glutamine-glutamic acid rich protein (GRP) promoter linked to a chloramphenicol acetyltransferase (CAT) reporter. The luciferase plasmid served as an internal control. Constructs with 9.1, 6.1, 2.5 and 0.009 kilobase pairs of sequence upstream of the transcriptional start site gave relative CAT activities of 100, 23.2, 4.7 and 26, respectively. These data suggest the presence of multiple regulatory elements in the GRP upstream region and can be used as a basis for further analysis. Given the difficulties in developing a relevant transgenic model and the absence of acinus-derived cell lines, the present method of *in vivo* gene transfer should prove useful in the further study of gene regulation and cell type-specific gene transfer in salivary glands.

C6-230 CHARACTERIZATION AND GRAFTING OF PROGENITOR CELLS CULTURED FROM THE ADULT RODENT BRAIN, T.D. Palmer, J. Ray, H.K. Raymon, H.G. Kuhn, D.A. Peterson, J. Suhonen, L.J. Fisher, and F. H. Gage. The Salk Institute, La Jolla, CA 92093

Genetic models for the treatment of neurodegenerative disease focus on delivering genes into postmitotic neurons *in situ* or on moderating a disease phenotype by grafting engineered non-neuronal cells. While intervention and prophylaxis will be key to effective treatment, reversal of advanced disease may also require replacement of lost neurons. Until recently, fetal tissues were considered the only source of neuronal progenitors suitable for replacement strategies, however, mounting evidence suggests that immature neuronal progenitors reside in several regions of the adult mammalian brain. These cells may provide a valuable alternative to fetal tissues if they can be efficiently recovered, genetically modified for a specific function, and successfully grafted to affected regions of the diseased brain. In adult rodents, proliferative cells in the subventricular zone normally give rise to oligodendrocytes and astrocytes, however, epidermal growth factor-responsive cells cultured from this region give rise to both neurons and glia *in vitro*. Likewise, we have observed that cells with characteristics of glial and/or neuronal progenitors can be recovered from the adult rat hippocampus, striatum, substantia nigra, septum, and subventricular zone using defined medium supplemented with basic fibroblast growth factor. When cultured progenitors are genetically marked and grafted to the adult rat striatum or hippocampus, many of the marked cells differentiate into astrocytes or oligodendrocytes, however, some cells show characteristic markers and morphology of mature neurons. Moreover, adenovirally-manipulated cells continue to express the LacZ transgene product for up to twelve weeks *in vivo*. This suggests that transplanted FGF-responsive adult progenitors reconstitute several neural lineages in the adult rodent brain and that ex *vivo* engineered progenitors may provide an effective source of replacement neurons for the treatment of neurodegenerative disease.

C6-231 OVEREXPRESSION OF THE β_2 ADRENERGIC RECEPTOR IN THE RAT HEART THROUGH ADENOVIRUS MEDIATED GENE TRANSFER

Karsten Peppel, Mark Drazner, Carmelo Milano, Greg Heintz and Robert J. Lefkowitz, HHMI @ Duke University Medical Center, Durham, NC 27710.

Overexpression of the β_2 adrenergic receptor (β_2AR) in transgenic mice in which the gene is transcribed from a heart specific promoter enhances myocardial function (Milano et. al., Science **264** (1994), 582-586).

Since chronic heart failure is correlated with a profound loss of myocardial ß adrenergic receptors we studied the feasibility of β_2AR-gene transfer into the myocardium of adult animals. We constructed recombinant adenoviruses transcribing either the β_2AR gene or a reporter gene, beta galactosidase (ß-gal) from the constitutive CMV promoter. Direct injection of 2 x 10^9 PFU of reporter virus into the myocardium of adult rats reveals strong ß-gal gene expression around the site of injection and in isolated patches throughout the heart after four days. Injection of 2 x 10^9 PFU of β_2AR directly into the left ventricular muscle leads to at least 10 fold overexpression of β_2AR four days after infection in the area surrounding the site of injection. In addition, infection of isolated myocytes with β_2AR adenovirus *in vitro* leads to over 10 fold overexpression of the receptor after 24 hours. These data demonstrate that the β_2AR can be significantly overexpressed in myocardial cells through adenoviral gene transfer.

C6-232 STRUCTURE-BASED DESIGN OF TRANSCRIPTION FACTORS WITH NOVEL DNA-BINDING SPECIFICITIES

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Computer modeling experiments that superimposed the crystal structures of the zif268-DNA and Oct-1 POU domain-DNA complexes suggested that zinc fingers and a homeodomain could be connected to create a DNA-binding domain with novel sequence specificity. A fusion protein, ZFHD1, that contains zinc fingers one and two from zif268, a short polypeptide linker, and the homeodomain from Oct-1, bound optimally to a sequence (5'-TAATTANGGGNG-3') composed of adjacent homeodomain (TAATTA) and zinc finger (NGGGNG) subsites. ZFHD1 displayed DNA-binding specificity distinct from that of either parent protein *in vitro* and, when fused to a transcriptional activation domain, could regulate promoter activity *in vivo* in a sequence-specific manner. Modeling suggests that many other DNA-binding domains could be fused in a similar fashion, providing powerful new reagents for biological research and gene therapy.

C6-233 GLUCOSE REGULATED HEPATIC PRODUCTION OF HUMAN INSULIN IN TRANSGENIC MICE, Michael E. Ripps[1] and Jon W. Gordon[2,3], [1]Brookdale Center for Molecular Biology, [2]Department of Obstetrics, Gynecology, and Reproductive Science, [3]Department of Geriatrics and Adult Development, Mount Sinai School of Medicine, New York, NY 10029

Diabetes mellitus is a chronic metabolic disorder resulting from a deficiency in pancreatic insulin secretion and/or a decrease in the peripheral response to insulin. Pancreatic beta cells normally increase insulin production in the post-absorptive state and decrease insulin production during periods of fasting, thus contributing to the maintenance of normal blood glucose levels. A potential strategy for the treatment of beta cell loss or dysfunction would be to engineer similar control mechanisms in other cell types. We are exploring the possibility of gene therapy for diabetes mellitus by engineering glucose regulated insulin production from hepatocytes. We have generated transgenic mice using a hybrid gene construct consisting of the promoter region of the rat L-type pyruvate kinase (L-PK) gene fused to the coding portion of the human insulin gene. The L-PK promoter directs gene expression predominantly to the liver, with lower levels of expression in intestine and kidney. Since transcriptional activity of the L-PK promoter is stimulated by glucose and inhibited by glucagon, the L-PK:insulin gene construct may provide a two-way control system for hepatic insulin production that is regulated in a manner similar to the pancreatic beta cell. We have detected human insulin mRNA expression in transgenic liver, intestine, and kidney using a RT-PCR assay, and have found human insulin/proinsulin protein in serum using an ELISA specific for human C peptide. The production of human insulin in response to glucose and glucagon administration will be examined. Insulin production appears to be appropriately regulated since transgenic mice have normal fasting glucose levels and significant increases in glucose tolerance within 30 minutes after an oral glucose load. The ability of extrapancreatic insulin production to compensate for beta cell loss will be assessed.

C6-234 **RETROVIRAL VECTORS FOR ENHANCED GENE EXPRESSION *IN VIVO* AND *IN VITRO*,** Paul B. Robbins and Donald B. Kohn, University of Southern California Dept. of Microbiology and Children's Hospital of Los Angeles, Los Angeles, CA 90027

Recombinant retroviruses have been used effectively to introduce and express exogenous genes in many cell types. Transduction of stem cells is well documented but sustained high level gene expression has proven elusive. To address this problem, a series of retroviral vectors were constructed incorporating promoter and/or enhancer sequences from genes/viruses which have demonstrated methylase protective or transcriptional enhancement activity in cells normally refractory to their expression. These fragments include elements from the Thy-1 gene, Myeloproliferative sarcoma virus and the Moloney dl587 revertant. The chloramphenicol acetyl transferase gene (CAT) or the neomycin phosphotransferase gene (Neo), serve as the reporters for the vectors. Virions were packaged using GP+E-86 and PA317 cell lines and used to transduce the F9 embryonal carcinoma cell line as well as the CCE stem cell line. Cell lysates were assayed for CAT expression by CAT diffusion assay, CAT ELISA and by Northern blot. Modifications incorporated into the vectors enhance gene expression in both stem cell models compared to the parental MoMLV vector. The murine bone marrow transplant model is being utilized to assess the expression capabilities of the vectors *in vivo*. Primary and secondary transplants of mice with transduced bone marrow stem cells are underway and will likely yield useful information regarding the effectiveness of these alterations. Data will be presented or I won't ski. These findings should be applicable to current protocols in retroviral based gene therapy.

C6-235 MUSCLE SPECIFIC PROMOTERS IN GENE THERAPY OF HAEMOPHILIA AND MUSCULAR DYSTROPHY. M.Skarli, A.Kiri, G.Vrbova* and G.Goldspink. Department of Anatomy and Developmental Biology, Royal Free Hospital School of Medicine, Rowland Hill, London NW3 2PF, UK
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Human genetic diseases are diverse. Two examples of disease where the defective genes have different functions in the normal state are muscular dystrophy where dystrophin, a stuctural protein of skeletal and cardiac muscle is defective and the haemophilias, where the defective genes code for clotting proteins of the circulation.

It has been shown that skeletal muscle has the ability to retain plasmids episomatically for long periods of time. This raises the possibility of using muscle fibres as sites of production of proteins that are secreted in the circulation if the constructs administered contain the signal sequence of a protein such as IGFI which is normally secreted by skeletal muscle.To obtain strong expression of the gene, a muscle specific promoter should be used to drive its expression. Since the exression of many highly expressed muscle genes is controlled by the physiological state of the muscle, it is neccessary to examine the effect of parameters like stretch and electrical stimulation on the expression of the inserted gene.

We have constructed expression vectors bearing muscle specific promoters and enhancers, used to drive the expression of chloramphenicol acetyltransferase (CAT). Direct plasmid injection of supercoiled plasmids in fast and slow muscles of normal and mdx mice showed strong expression of the constructs in skeletal muscle, in some cases comparable to that of the viral promoter SV40 used as a reference. Further experiments where fast (tibialis anterior) and slow (soleus) muscles were stretched after plamid injection showed alterations of levels of expression by different constructs. Thus it appears that the physiological state of the muscle may influence expression of an inserted construct.

C6-236 ENHANCED ANGIOGENIC PROPERTIES OF RETINAL PIGMENT EPITHELIAL CELLS AFTER RETROVIRAL VECTOR MEDIATED TRANSFER OF THE UROKINASE-TYPE PLASMINOGEN ACTIVATOR GENE. Christine Spee, Taiji Sakamoto, Zorica Scuric, Erlinda M. Gordon, David R. Hinton, W. French Anderson, and Stephen J. Ryan, Doheny Eye Institute and the Departments of Ophthalmology, Biological Chemistry and Pediatrics, and Pathology, USC School of Medicine, Los Angeles, CA.

Retinal pigment epithelial (RPE) cells play an important role in the modulation of ocular angiogenesis. This study was designed to determine whether angiogenic properties of RPE cells could be enhanced by retroviral vector-mediated gene transfer. Human RPE cells were transduced with vectors bearing the urokinase type plasminogen activator (u-PA) or tissue type plasminogen activator (t-PA) genes. Ten weeks after transduction, u-PA vector-transduced RPE cells (u-PA-RPE cells), t-PA vector-transduced RPE cells (t-PA-RPE cells), or normal RPE cells were co-cultured with human umbilical vein endothelial cells (HUVECs) by contacting and non-contacting co-culture methods. For as least 18 weeks, u-PA-RPE cells secreted large amounts of biologically active u-PA (50.2 $\pm$ 9.7 ng/ml). t-PA-RPE cells released functional t-PA (8.7 $\pm$ 3.9 ng/ml), while non-transduced RPE cells did not release either t-PA or u-PA. Further, u-PA-RPE cells stimulated HUVEC proliferation in contacting cell cultures while neither t-PA-RPE cells, normal RPE cells nor exogenous u-PA had any effect on HUVEC proliferation. In "wound healing" assays, u-PA-RPE cells in contacting co-culture and exogenous u-PA stimulated wound healing of HUVECs, while non-contacting u-PA-RPE cells, t-PA-RPE cells and normal RPE cells did not exhibit such effect. These data indicate that RPE cells transduced with vectors bearing the u-PA gene may be used to induce ocular angiogenesis and wound healing, and emphasize the potential of the RPE cell as a target for gene therapy of ocular diseases.

C6-237 MANIPULATING GENE EXPRESSION, SIGNALLING AND CELL DEATH WITH SYNTHETIC LIGANDS, David M.Spencer[1], Steffan Ho[1], Leslie Holsinger[1], Isabela Graef[1], Pete Belshaw[2], Stuart L.Schreiber[2] and Gerald R.Crabtree[1], [1]The Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305, and [2]The Department of Chemistry , Harvard University, Cambridge, MA 02138

Regulated protein interactions are the basis of essentially all cellular processes. This form of regulation includes not only ligand-mediated crosslinking and activation of cell surface receptors, but also protein-protein interactions which are induced as a result of the covalent or *noncovalent* modifications of intracellular proteins. These protein modifications, like phosphorylation, are crucial for propagating receptor-triggered signaling cascades.

A general procedure that uses lipid soluble, synthetic ligands was previously described that permits controlled intracellular dimerization or oligomerization of proteins. These dimeric ligands are based on the immunosuppresive drug FK506 and its receptor FKBP. Following chemical crosslinking of FK506, interaction with calcineurin, and therefore immunosuppression, has been eliminated. Previous results demonstrated that multiple, distinct signaling receptors, including CD3 ζ from the T cell receptor complex, and the apoptosis-triggering fas receptor, fused to the FK506 binding protein, FKBP12, could be activated with dimeric FK506, eliciting a response indistinguishable from ligand or antibody-mediated crosslinking.

These results have now been extended to the regulation of multiple intracellular signaling molecules controlling distinct signaling pathways, including the src kinases, and the ras-family G proteins. In these cases drug-based, membrane targeting of signaling molecules initiates their responses. The ability of the dimerization reagents to activate these signaling cascades is complemented by the ability of monomeric forms of the reagent to rapidly inactivate the pathways.

Lastly, data will be presented on the further development of a dimer-based transcriptional switch. Ligand-regulated activation and termination of signaling pathways controlling gene transcription or ligand-regulated apoptosis should find application in both basic research and medicine.

C6-238 HUMAN CAPILLARY ENDOTHELIAL CELLS FROM ABDOMINAL WALL ADIPOSE TISSUE: ISOLATION USING AN ANTI-PECAM ANTIBODY AND THEIR POTENTIAL USE IN GENE THERAPY, Jeremy P. Springhorn, Joseph A. Madri, and Stephen P. Squinto, Department of Molecular Development, Alexion Pharmaceuticals, Inc. and Department Pathology, Yale University School of Medicine, New Haven, CT 06511

Successful isolation and culture of pure syngeneic human capillary endothelial (huCE) cells is the first step in assessing their use in somatic cell gene therapy. Liposuction is a rich source for huCE cells and is a relatively non invasive procedure that is not gender restricted. Therefore, it could be compatible with a therapy based on autologous cells. We compared the use of either *Ulex Europaeus* Agglutinin (UEA) lectin or anti-PECAM antibody conjugated to magnetic beads for the ability to isolate and maintain pure cultures of huCE cells. During increasing passages there appeared to be a decrease in the percentage of UEA selected cells scavenging LDL or expressing vWf as compared to α-PECAM isolated cells. HuCE cells isolated using the α-PECAM antibody method maintained these endothelial cell-based biochemical properties for up to twelve passages. Furthermore, while both techniques yielded cells that bind UEA at passage six, only α-PECAM selected cells expressed the normal pattern of endothelial-specific cellular adhesion molecules. When placed in three dimensional cultures of type I collagen only the α-PECAM selected huCE cells formed tube-like structures within 2 days, while the UEA selected cells did not. Anti-PECAM isolated huCE cells were transduced with a retroviral vector containing the hGH cDNA and were found to secrete growth hormone from both two and three dimensional cultures. We propose that α-PECAM antibodies linked to a solid support provide a highly specific step in the isolation of huCE cells from abdominal wall liposuction remnants.

C6-239 RETROVIRAL-MEDIATED GENE TRANSFER OF C-FGR TYROSINE KINASES INDUCES INDUCES NON-SPECIFIC ESTERASE (NSE) AND FACTOR-INDEPENDENCE IN A MYELOID CELL LINE. Kristin Steffgen and Cheryl Willman, Depts. of Pathology and Cell Biology, Univ. of NM School of Medicine, Albuquerque, NM 87131.

Our previous studies have determined that c-fgr, a member of the src family of intracellular tyrosine kinases, is expressed in myeloid cells. The murine c-fgr cDNA encodes two proteins through alternative translation initiation: pp59c-fgr contains the amino terminal, SH3, SH2, and kinase domains while pp53c-fgr lacks the amino terminal domain and its associated myristylation sequences. pp59c-fgr associates with the plasma membrane and actin cytoskeleton while pp53c-fgr is located in the nucleus. (J. Cell Biochem. 16B:219). Using the MPZenSVNEO retroviral vector, c-fgr proteins were introduced into the FDCP-1 IL-3-dependent myeloid progenitor cell line. Similar to previous experiments in fibroblasts, expression of the monocytic-associated marker NSE (non-specific esterase) was induced in FDCP-1 clones which expressed the highest levels of wt or activated pp59c-fgr; however, other morphologic and immunophenotypic characteristics of differentiation were not seen. Relative to pp53c-fgr, FDCP-1 selected clones expressing pp59c-fgr contained a unique set of proteins phosphorylated on tyrosine including a prominent 95 kd protein which has been associated with signal transduction from the IL-3 receptor. Factor-independent clones were relatively easily isolated from FDCP-1 clones containing wt or activated forms of pp59c-fgr. Additional immunoblotting studies with anti-phosphotyrosine antibodies also identified a 62 kd phosphoprotein in FDCP-1 clones containing the activated pp53c-fgr and pp59c-fgr proteins. Using a GST-c-fgr SH3 domain fusion protein, p62 was also shown to associate with the SH3 domain of both pp59c-fgr and pp53-c-fgr in lysates from FDCP-1 clones containing one or the other of these activated c-fgr kinases. Western blots of the proteins eluted from these beads confirmed that this p62 protein was the GAP-associated protein p62 which is frequently phosphorylated in cells transformed by oncogenic src family kinases. Further studies to examine the association of factor-independence with phosphorylation of the 95 kd and p62 proteins is in progress.

C6-240 **CHARACTERIZATION OF NEW PROMOTER ELEMENTS FROM MYOSIN HEAVY CHAIN GENE ISOFORMS. MUSCLE SPECIFIC REGULATORY ELEMENTS COMBINED TO A STRONG EXPRESSION CASSETTE FOR DIRECT GENE INJECTION INTO MUSCLE.** Ricarda A. Steinbrecher[1,2], Elizabeth Lewis[2], Christine A. Lee[1] and Geoffrey Goldspink[2]. [1]Katharine Dormandy Haemophilia Centre, Departments of Haematology, and of [2]Anatomy and Developmental Biology, Royal Free Hospital and School of Medicine, London, UK.

The requirement of an effective gene therapy approach by direct gene injection into muscle is a strong and preferably inducible expression vector that is specifically activated in differentiated muscle. Plasmids are known to be taken up by muscle cells after direct injection and expressed and kept episomally for over a year without posing a risk of random integration into the genome. The use of muscle specific regulatory elements in such plasmids should facilitate the expression of any gene spliced into this construct. Myosin is a major contractile protein of muscle and its expression is regulated in a developmental and fibre type specific fashion. We characterized two closely related novel promoter elements of adult skeletal MyHC genes of pig and rabbit. The basic promoter elements and the 5' untranslated regions are highly conserved and showed up to 95% homology across species. *In vitro* expression studies of these and other muscle specific promoters in C2C12 myoblast cell lines were carried out, using the CAT gene as an intracellular reporter gene. If combined with a myosin light chain enhancer element, expression was increased strongly, especially for the novel rabbit MyHC promoter, the MCK promoter and the TK promoter. Compared to a CAT construct driven by viral SV40 promoter and enhancer elements, expression was 2-5 fold. These constructs are currently being tested *in vivo* as well as for the expression of the blood clotting factor VIII gene. We show that plasmid based direct gene injection into muscle utilizing muscle specific regulatory elements is a potential approach for gene therapy of haemophilias and other bleeding/thrombotic disorders.

C6-241 DEVELOPMENT OF REGULATABLE TRANSGENE EXPRESSION SYSTEMS USING MAMMALIAN AND NON-MAMMALIAN LIGANDS. Steven T. Suhr, Elad B. Gil, Peter S. Eriksson, Steven M. Lipkin, Nien-Hsien Liou, and Fred Gage, The Salk Institute, San Diego, La Jolla CA, 92093.

Recombinant viruses have proven effective vehicles for the transfer of a variety of exogenous genes to target organisms. With most viral vector systems however, transgene expression is maintained for only a short period within infected cells. In order to preserve a useful level of transgene expression with the added benefit of exogenously regulating transgene expression levels we are developing retroviral constructs which are regulatable by the invertebrate ecdysteroid Muristerone A (MurA). Using the native ecdysteroid receptor (EcR) we observe greater than 10-fold induction of MurA responsive promoters in transient transfection assays. We are exploring a number of modifications to EcR and known dimer partners of the EcR protein to increase transcriptional induction in mammalian cells. Hybrid receptors resulting in significantly greater induction of an ecdysone responsive promoter while having a minimal effect on basal, non-induced transactivation are currently being tested. Additional chimeric receptor proteins have also been examined as potential inducible transactivating factors in regulatable systems under development. The cooperative effects of endogenous steroid/retinoid elements on both native and ecdysteroid-responsive retroviral LTRs are also examined.

C6-242 *IN VIVO* DIRECT GENE TRANSFER INTO JOINTS USING HVJ-LIPOSOMES

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To develop a new and effective strategy for the treatment of arthritis, we have established an efficient and non-toxic method of transferring foreign genes directly into the knee joints using hemagglutinating virus of Japan (HVJ)-liposome. In our system, DNAs and nuclear protein, high mobility group-1, were entrapped into liposomes and the liposomes were treated with UV-inactivated HVJ to form HVJ-liposomes. In this study, we assessed the efficiency and suitability of HVJ-liposome method for transferring foreign genes directly into the knee joint. HVJ-liposomes containing SV40 large T (SVT) antigen DNA driven by chicken-beta actin promoter were directly injected into the knee joints of 6 week-old rats. At 3, 7, 14 and 21 days after injection, the expression of SVT was detected immunohisto-chemically in the chondrocytes of the knee joints. The average transfection efficiency was estimated as 28.2%. SVT antigen gene was detected for at least 21 days after injection, and expression did not decrease over time. No pathological change of inflammation, cytotoxicity or immunological reaction by HVJ-liposomes was observed in the joints. Next, 6 week-old rats were injected intradermally with bovine type II collagen to induce arthritis. One week after the onset of arthritis, HVJ complex containing FITC-labeled oligodeoxynucleotides was directly injected into the knee joints. At 3 and 7 days after injection, the fluorescences were detected in the cell nuclei of synovium in arthritic knee joints. Thus we have developed an efficient *in vivo* direct gene transfer method using HVJ-liposome. In our system, it is possible to transfer foreign genes safely into chondrocytes as well as synovial cells in the knee joints. This system may provide a new and useful form of gene therapy for arthritis.

C6-243 THE STABLE DELIVERY OF PHYSIOLOGIC LEVELS OF RECOMBINANT PROTEIN TO THE SYSTEMIC CIRCULATION BY INTRAMUSCULAR INJECTION OF REPLICATION-DEFECTIVE ADENOVIRUS, Sandeep K. Tripathy, Eugene Goldwasser, Min-Min Lu, Eliav Barr, and Jeffrey M. Leiden, The University of Chicago, Chicago, IL 60637

A number of inherited and acquired serum protein deficiencies including hemophilias A and B, diabetes mellitus, and the erythropoietin-responsive anemias are currently treated with repeated subcutaneous or intravenous infusions of purified or recombinant proteins. The development of an *in vivo* gene transfer approach to deliver physiologic levels of recombinant proteins to the systemic circulation would represent a significant advance in the treatment of these disorders. Towards this end, we constructed a replication-defective adenovirus (AdEF1hEpo) containing the human erythropoietin (hEpo) cDNA under the transcriptional control of the cellular elongation factor 1α (EF1α) promoter and the 4F2 heavy chain (4F2HC) enhancer. Neonatal CD-1 and adult SCID mice injected once intramuscularly (IM) with 10^7-10^9 plaque forming units (pfu) of this virus displayed significant dose-dependent elevations of serum hEpo levels and increased hematocrits which were stable over the four-month time course of these experiments. IM-injected adenovirus remained localized at the site of injection and there was no evidence of either systemic infection or a localized inflammatory response. In contrast to the results observed in immunocompromised and or neonatally-injected mice, intramuscular injection of immunocompetent animals resulted in only transient recombinant gene expression lasting 2-4 weeks. The loss of gene expression was due to a cytolytic T cell response that resulted in the destruction of adenovirus-infected muscle in the animals. In addition, we have observed antibody responses to the recombinant proteins encoded by IM injected adenovirus vectors. These results suggest that IM injection of recombinant replication-defective adenovirus vectors may serve as a paradigm for the treatment of human serum protein deficiencies. However, the use of this system for the treatment of human serum protein deficiencies will require the development of less immunogenic viral vectors and/or the use of immunosuppressive regimens to prolong recombinant gene expression *in vivo*.

C6-244 **QUANTITATIVE ANALYSIS OF GENE EXPRESSION AFTER REPETITIVE ADENOVIRUS-MEDIATED GENE TRANSFER INTO INJURED CANINE FEMORAL ARTERY,** Hikaru Ueno, Li Jian-Jun, Hideharu Tomita, Hiroaki Yamamoto and Akira Takeshita, Department of Cardiology, Kyushu University School of Medicine, Fukuoka, 812 Japan

We introduced a replication-defective adenovirus expressing *lacZ* into injured canine femoral artery by a percutaneous transluminal method using a double-balloon catheter. Up to 90 % of surface of the artery, and virtually all smooth muscle cells in the upper one-third media showed *lacZ* expression. High level of β-galactosidase (β-gal) activity (153.0 ± 38.9 mU/mg protein n=7) was detected 7 d after gene transfer. Significant level of gene expression retained for 4 wk. Since our delivery system allowed us to introduce the adenovirus multiple times to the same arterial site, we have quantified β-gal activity to determine whether a repetitive application of the same adenovirus could enhance gene expression. We found that repetitive infection resulted in enhanced β-gal activity, whether second infection was done at 7d, 5 wk or 8 wk after the first application. However, when adenovirus of high titer was applied at the first exposure and the second infection was done 7 d later, no further expression was observed, whereas low dose of virus was used at first, submaximal *lacZ* expression was detected following the second infection. This finding may suggest that the injured artery might have limited capacity for *lacZ* expression. The enhancement was decreased when the second infection was performed at 5 wk or 8 wk after the first one. Human adenovirus neutralizing antibody was detected in dogs and there was a rough correlation between the reduction of β-gal activity and antibody titers. However, we succeeded effective gene transfer in dogs that had high titer of antibody induced by repeated intravenous injection of virus so that some other local factors might also contribute to the reduction. Our study indicates that repetitive adenovirus-mediated gene transfer into the same arterial site through a catheter achieved enhanced gene expression.

C6-245 NON-VIRAL *EX VIVO* TRANSFECTION OF PRIMARY HUMAN SKIN FIBROBLASTS FOR GENE THERAPY,

Hendrik Veelken, Peter Kulmburg, Felicia M. Rosenthal, Andreas Mackensen, Roland Mertelsmann and Albrecht Lindemann, Department of Internal Medicine I, Freiburg University Medical Center, D-79106 Freiburg, Germany

In vivo secretion of recombinant proteins by genetically engineered somatic cells has therapeutic potential for hereditary protein deficiencies and for the induction of anti-tumor immunity in cancer patients. We are studying cultures of primary human skin fibroblasts as target cells for *ex vivo* transfection with physical methods. Using an optimized enzymatic dissociation procedure, primary fibroblast cultures with excellent growth potential were established from skin biopsies of human adults. Stable transfection of these cells was achieved by cationic lipofection of an expression construct containing an IL-2 cDNA under control of the CMV promoter and a separate neomycin resistance cassette. Under selection with G418, stably transfected clones were obtained at a frequency of $0.3\text{-}3x10^{-5}$ transfected cells. Production of up to 4000 IU IL-2/10^6cells x 24 h was observed in approximately 30% of all clones as determined by bioassay. Secretion of IL-2 was stable during expansion to at least 10^7 cells and after cryopreservation. Irradiation with 100 Gy led to a transient enhancement of IL-2 production. Transfected fibroblast clones retained their untransformed phenotype without any signs for insertional mutagenesis as assessed by *in vitro* proliferation characteristics, cytogenetic analysis, and *in vivo* testing in athymic mice. We conclude that primary human fibroblasts may be genetically modified *in vitro* with non-viral gene transfer. Secretion of a recombinant protein was stable during expansion of individual clones to cell numbers that may be sufficient to achieve therapeutic effects *in vivo*. The use of clonal populations of genetically engineered cells offers the opportunity for thorough characterization under safety aspects prior to their implantation into patients for gene therapy.

C6-246 THE EFFECTS OF CONTROLLED LIVER REGENERATION ON HEPATOCYTE TRANSPLANTATION IN THE MOUSE: IMPLICATIONS FOR EX VIVO GENE THERAPY.

M.J. Vrancken-Peeters, A.Lieber, J.Perkins and M.A.Kay. Division of Medical Genetics RG-25 and Transplantation Surgery RF-25, University of Washington, Seattle, WA 98195.

Transplantation of autologous genetically-modified hepatocytes is one potential form of therapy for metabolic diseases that result from an absence or deficiency of hepatocyte-derived gene products. Although the mouse is, for many reasons, an adequate model for studying this ex-vivo approach of gene therapy, their use has been limited for technical reasons related to their size. Hepatocyte transplantation has been limited to about 2×10^6 cells (or 2% of the liver mass) per mouse. We have developed a method which requires only one invasive surgical procedure to permit multiple hepatocyte transplantations in individual mice. This procedure in combination with a new method for inducing liver regeneration prior to hepatocyte transplantation was used to increase the proportion of genetically altered cells in the livers of transplantation recipients. To perform multiple infusions, the distal tip of a silicone catheter was cannulated into the portal vein of mice and the proximal port placed into a subcutaneous pocket. Donor hepatocytes from congenic recipients that express the beta-galactosidase reporter were transplanted via the spleen or portal vein catheter. Some animals were given two or three injections of 2×10^6 cells per transplantation via the catheter, a 3-fold improvement over previous studies. In order to give the donor cells a selective advantage for repopulation *in vivo*, hepatocyte transplantation was performed after portal vein infusion of a recombinant adenoviral vector that expressed urokinase, a protein that was selectively toxic when produced in hepatocytes thus inducing liver regeneration. The proportion of genetically modified cells that reconstituted the liver of recipients two months after the different experimental treatments will be presented. These technologies may have implications for improving ex vivo hepatic gene therapy protocols.

C6-247 SIMULTANEOUS TRANSDUCTION OF KERATINOCYTES AND FIBROBLASTS WITH TWO RETROVIRAL VECTORS *I N VITRO* .

Patricia S. Walker, Ulrich R. Hengge, Jonathan C. Vogel. Dermatology Branch, NCI, NIH, Bethesda, MD, 20892.

The ability to stably transduce a single cell with more than one gene has potential utility for gene therapy. Such methodology is relevant, as it would allow insertion of genes of particular interest as well as genes encoding growth factors, suicide genes or selectable markers utilizing independent constructs. This would alleviate the need to combine two desired genes on one vector and obviate size constraints that may preclude placement of multiple genes on a single retroviral vector. We have co-transduced porcine keratinocytes, fibroblast and NIH 3T3 fibroblasts simultaneously with two retroviral vectors; a murine moloney sarcoma virus-based vector containing the nuclear ß-galactosidase and neomycin resistance genes (MMSVnß-gal/neo^R) and a Harvey virus-derived vector containing the human multiple drug resistance gene (pHaMDR). In the pHaMDR vector the MDR gene was promoted by the Harvey virus long terminal repeats (LTR), whereas, in the MMSVnß-gal/neo^R vector the neo^R gene is promoted by the retroviral LTR and the nß-gal by an SV40 promoter. Retroviral vectors were produced in the amphotrophic helper virus free PA317 cell line and dual transductions were carried out sequentially or simultaneously using retrovirus-conditioned media and polybrene (8ug/ml). Co-transduction efficiency was determined by selection (colchicine and G418 for MDR and neo^R respectively), nuclear bluo-gal staining, FACS analysis and immunofluorescence. Simultaneous nuclear nß-gal and cell surface MDR expression within an individual cell was demsonstrated using two color immunofluorescence microscopy. When co-transducing two defective retroviruses, viral interference was not observed. In summary, we have demonstrated dual retroviral transduction in fibroblasts and keratinocyte cell lines both sequentially and simultaneously. In the future such techniques may enhance our ability to select and regulate the growth of genetically modified cells.

C6-248 TARGETED MUTAGENESIS IN MAMMALIAN CELLS MEDIATED BY INTRACELLAR TRIPLE HELIX FORMATION: A NEW APPROACH TO GENE THERAPY

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Oligonucleotides can bind to duplex DNA and form triple helices in a sequence-specific manner. We have investigated the use of triple helix-forming oligonucleotides to target mutations to selected genes within mammalian cells. By treating monkey COS cells with oligonucleotides linked to psoralen, we have generated targeted mutations in a simian virus 40 (SV40) vector within the cells via intracellular triple helix formation. Oligonucleotide entry into cells and sequence-specific triplex formation within the SV40 delivers the psoralen to the target site. Photoactivation of the psoralen by long wavelength UV light yields adducts and thereby mutations at that site. We engineered into the supF mutation reporter gene within the SV40 vector modified polypurine sites amenable to triplex formation. By comparing the ability of a series of oligonucleotides to target these new sites, we show that targeted mutagenesis *in vivo* depends on the strength and specificity of the third strand binding. Oligonucleotides with weak target site binding affinity or with only partial target site homology were ineffective at inducing mutations in the SV40 vectors within the COS cells. We also show that the targeted mutagenesis is dependent on the oligonucleotide concentration and is influenced by the timing of the oligonucleotide treatment and of the UV irradiation of the cells. Frequencies of intracellular targeted mutagenesis in the range of 1 to 2% were observed, depending upon the conditions of the experiment. DNA sequence analysis revealed that most of the mutations were T:A to A:T transversions precisely at the targeted psoralen intercalation site. Several deletions encompassing the triplex binding site were also seen. The ability to target mutations to selected sites within mammlian cells using modified triplex-forming oligonucleotides may provide a new tool for research and may eventurally lead to therapeutic applications.

C6-249 ENGINEERING JUNCTIONAL EPIDERMOLYSIS BULLOSA KERATINOCYTES TO PROVIDE SKIN GENE THERAPY, Kathy Wang, Chihiro Matsui, Charlotte Cantrell, Scott Herron, Eugene Bauer, Warren Hoeffler, Department of Dermatology, Stanford University School of Medicine, Stanford, CA 94305

Epidermolysis bullosa is a group of mechanobullous diseases of the skin and mucos membranes characterized by the development of blisters and erosions following minor trauma. We are studying a lethal form (Herlitz variety) of one of the four major types of hereditary EB, autosomal recessive junctional EB (RJEB), in which blister formation occurs in the lamina lucida of the epidermal-dermal juction. The hemidesmosomes are often hypoplastic and reduced in number or absent presumably because a glycoprotein component, laminin 5, is defective. Laminin 5 is composed of three polypeptide chains: $\alpha 3$ (200 kDa processed to 165 kDa), $\beta 3$ (140 kDa) and $\gamma 2$ (155 kDa processed to 105 kDa) and the complete heterotrimer is assembled into anchoring filaments .We have developed polymorphism detection methods to allow for the routine detection of mutations involved in the pathogenesis of RJEB.The EB patients screened so far are defective in the $\beta 3$ chain of laminin 5. These mutations result in defects on the protein level as shown by western blot analysis. We are attempting *ex vivo* gene therapy by introducing expression vectors for the normal $\beta 3$ chain into RJEB keratinocytes. The difficulties in obtaining skin biopsies from these patients and in culturing their keratinocytes has required us to immortalize these keratinocytes. Human papilloma Virus 18 E6, E7 expression vectors cotransfected with neo resistant plasmids and subsequent G418 selection were used for immortilization, a procedure that does not lead to tumorigenicity. Both normal and patient immortalized keratinocytes exhibit the same pattern of subunit composition of laminin 5 observed before the procedure. We constructed a $\beta 3$ cDNA expression vector from three overlapping partial cDNA clones and have shown $\beta 3$ expression in 293 cells when transiently transfected. We also deliver the B1 expression construct by lipofectin into the immortalized patient keratinocytes and show normal assembly and secretion of laminin 5 in culture medium. Methodologies for the grafting on the engineered keratinocytes onto human skin graft on mice are currently being pursued. The coupling of methods used for the identification of genetic lesions and the introduction of expression vectors into patient cells will provide the foundation for correcting the RJEB phenotype.

C6-250 GENE SWITCH VIA PROGESTERONE ANTAGONIST RU 486 Yaolin Wang, Bert W. O'Malley, Jr., Sophia Y. Tsai, and Bert W. O'Malley, Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

Current gene transfer protocols often yield constitutive expression of foreign gene(s). Under physiological conditions, however, most genes are regulated in response to various stimuli such as metabolites, growth factors and hormones. Utilizing a human progesterone receptor mutant (hPR891), which does not respond to progesterone agonist but rather antagonist RU 486, we constructed a chimeric regulator (pGL-VP) containing the yeast GAL4 DNA binding domain, the RU 486 binding region of hPRB891 and VP16 transcription activation domain. We demonstrated that the chimeric regulator pGL-VP can activate a reporter gene (containing the 17-mer GAL4 binding site) expression in response to RU 486. Using this gene switch system, we have been able to induce the expression of intracellular proteins (tyrosine hydroxylase, chloramphenicol acetyltransferase) as well as a secretory protein (human growth hormone). Most importantly, this RU 486 induced gene expression can occur at a concentration as low as 1 nM. We will discuss the use of different promoters to improve the gene switch system. We will also present evidence that the gene switch works not only in cell culture but also in *ex vivo* transplants. These results indicate that this inducible gene switch system works efficiently and should have wide applications for regulating target gene expression *in vivo*.

C6-251 DESIGN OF RETROVIRAL VECTORS FOR STABLE TRANSFER AND HIGH-LEVEL EXPRESSION OF HUMAN ß-GLOBIN GENE WITH TRUNCATED ERYTHROID ENHANCER, Qize Wei, Depei Liu, Jizhong Tiao, Qinghui Liu, Peichen Jia, Xuefeng Lei, Songsen Chen and Chih-Chuan Liang, National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of medical Sciences, Beijing 100005, P.R.China

Several retroviral vectors containing human ß-globin gene and truncated regulatory elements of the human ß-globin locus control region(LCR) have been constructed to optimize the transfer and expression of the human ß-globin gene. Although incorporation of a 341-bp segment of LCR in the retroviral construct carrying human ß-globin gene rendered the proviruses very unstable, we found that the retroviral constructs containing 292-bp or 341-bp fragments of LCR and the human ß-globin gene in which a 374-bp intronic A/T rich segment was eliminated were able to transmit the human ß-globin gene stably. At least one third of the PA317 cell clones examined contained correct proviruses and the highest virus titer was as high as $1.9X10^5$ CFU/ml. Furthermore, the recombinant retroviruses faithfully transferred the human ß-globin gene to infected NIH 3T3 and MEL cells. Finally, RNase protection assay revealed that 292-bp and 341-bp truncated erythroid enhancer provided up to 116% and 76.5% of human ß-globin /murine a-globin mRNA ratio respectively in MEL cells. The in vivo animal experiment is in progress.

C6-252 PCR SYNTHESIS, CLONING AND EXPRESSION OF HUMAN ORNITHINE TRANSCARBAMYLASE COMPATIBLE WITH MITOCHONDRIAL AND UNIVERSAL CODON USAGE, Vanessa C. Wheeler, Chris Prodromou,† Laurence Pearl,† Robert Williamson and Charles Coutelle, Department of Biochemistry and Molecular Genetics, St. Mary's Hospital Medical School, London. †Department of Biochemistry, University College, London.

We are investigating a novel strategy for the transfer and expression of genes in eukaryotic cells which aims to exploit the autonomous replication, transcription and translation apparatus of the mitochondrial genome. The approach invovles manipulation of the mitochondrial genome so that it contains an exogenous gene and the introduction of the manipulated mitochondrial DNA into mitochondria. To obtian correct expression of the introduced gene in mitochondria it must possess an amino acid code compatible with mitochondrial codon usage. A convenient disorder to use as a test system for such an approach is ornithine transcarbamylase (OTC) deficiency. OTC functions as a urea cycle enzyme and is encoded on the X-chromosome. It is synthesised in the cytosol as a preprotein with an N-terminal leader peptide and imported into the mitochondrial matrix where the leader sequence is cleaved, followed by trimerisation of the mature protein. As an initial step towards the translation of this protein within the mitochondrion we have overcome the problem of mitochondrial codon usage by the *in vitro* synthesis of a gene sequence coding for mature OTC by the method of recursive PCR (Prodromou and Pearl, 1992). The codon usage in this gene sequence should allow translation in accordance with the mammalian mitochondrial genetic code, whilst retaining the ability to be translated by the universal code. In order to facilitate the directed cloning into the mitochondrial genome, mitochondrial tRNA sequences flanking the *OTC* open reading frame were incorporated into the design of the gene. The synthetic *OTC* gene was cloned in an *E.coli* expression vector and shown to express authentic mature OTC as judged from its size, N-terminal sequence and immunoreactivity. However the protein forms inclusion bodies in *E.coli* and is enzymatically inactive, suggesting incorrect folding and /or trimerisation. We are presently recloning the gene to test for enzyme activity in eukaryotic cells.

C6-253 FINDING HOMOLOGY--IMPLICATIONS DRAWN FROM RECA PROTEIN-PROMOTED REACTIONS, Janet E. Yancey-Wrona and R. Daniel Camerini-Otero, Genetics and Biochemistry Branch, NIDDK, NIH, Bethesda, MD 20892-1810

Although meiosis and homologous recombination have been studied for many decades, the way in which large DNA molecules find homologous targets and pair remains largely unknown. Questions regarding this mechanism are especially pertinent, as efforts to use homologous recombination for gene therapy intensify. These fundamental mechanisms have been investigated using RecA protein, the central homologous recombination protein from *E. coli*. RecA protein is able to pair short single-stranded DNA oligonucleotides with homologous regions in duplex DNA and form stable synaptic complexes containing three DNA strands and RecA protein. These complexes are early intermediates in the strand exchange reaction, believed to mimic homologous recombination *in vivo*. Characterization of this reaction should elucidate important parameters required for the early steps of recombination including the homology search and the initiation of homologous pairing. This lab has previously shown that RecA protein could target an oligonucleotide to a unique site in the human genome *in vitro* (Ferrin and Camerini-Otero, 1991, *Science*, **254**, 1494-1497). We have investigated the kinetics governing this genome-wide search using a variety of duplex DNAs as target-containing molecules. The rate of synaptic complex formation at a unique target site depends on the concentrations of both the searching moiety, that is an oligonucleotide coated by RecA protein, and the target sites within the duplex, and is independent of the concentration of nonhomologous duplex DNA in the reaction. That is, the search for homology is not rate-limiting. A two-step scheme is proposed to explain synaptic complex formation wherein an initial fast searching and pairing reaction is followed by a rate-limiting slow step forming the final stable synaptic complex. We indicate how the kinetic constants derived from such reactions reveal significant conclusions regarding the biochemistry and biology of genome-wide searches relevant to homologous recombination. We point out that a dependence of targeting events on the concentration of targets does not indicate that the search for homology is rate-limiting. Therefore, whether the number of targeting events depends on the concentration of target sites, in yeast versus mammalian cells for example, most likely reflects differences in the components of the recombination protein machinery rather than differences in the rate-limiting nature of the search for homology.

C6-254 TRANSDUCTION OF PRIMARY HUMAN HEPATOCYTES WITH ADENOVIRAL VECTORS: FEASIBILITY STUDIES. Li Yang, Michael Skotzko, Steven Stain, Dilip Parekh, Paul Hallenbeck, Robert C. Jambou, W. French Anderson and Erlinda M. Gordon, Gene Therapy Laboratories, USC School of Medicine, Los Angeles, CA and Genetic Therapy, Inc., Gaithersburg, MD

The hepatocyte is a potential target for gene therapy using adenoviral vectors because the cell does not replicate. To study the feasibility of gene transfer by adenoviral vectors, human hepatocytes were isolated and maintained in differentiated state under serum free conditions for over 4 weeks. At that time, greater than 95% of the cells were cytokeratin 8 and 18 positive by immunostaining, and were morphologically hepatocytes. Hepatocytes were transduced with an adenoviral vector, Av1LacZ4, 24 hrs after isolation, and at 15 days in culture, when cells were not replicating, and were terminally differentiated. Expression of beta galactosidase was determined by X-gal staining, 2 and 6 days after transduction. Hepatocytes cultured on both collagen type I- or Matrigel-coated dishes were successfully transduced. Transduction efficiency was enhanced to 37%, 92%, 96% and 99% with MOIs of 2, 5, 10 and 100 respectively. Transduced hepatocytes remained viable even at an MOI of 100, indicating that the adenoviral vector used was not cytotoxic. Expression of the beta galactosidase gene was maintained for at least six days following transduction. These data indicate that gene transfer using adenoviral vectors is safe and effective, and emphasize the potential of human hepatocytes as cell targets for gene therapy with adenoviral vectors.

AIDS; CNS Disorders; DNA Delivery Systems

C6-300 *ANTISENSE* PHOSPHOROTHIOATE OLIGODEOXY-NUCLEOTIDES (S-ODN) AS POTENT INHIBITORS OF HEPATITIS C VIRUS TRANSLATION, Michael Alt, Renate Renz, Gustav Paumgartner, Peter H. Hofschneider and Wolfgang H. Caselmann, Max-Planck-Institut für Biochemie, 82152 Martinsried and Klinikum Grosshadern, Dept. of Med. II., 81366 München, Germany

The hepatitis C virus (HCV) is the major causative agent of non-A, non-B hepatitis in the world. 60% of the infected individuals develop chronic infection, often leading to cirrhosis and hepatocellular carcinoma. The genome of HCV is a positive strand RNA containing a 5′-non-coding region (NCR) of 341 nt essential for the viral translation. It is upstream of a long open reading frame encoding a polyprotein of about 3000 amino acids. Until now no satisfying therapy for HCV infection is available. In order to develop new therapeutic approaches we analysed whether the HCV translation can be inhibited by *antisense* S-ODN. We have tested seven S-ODN complementary to nucleotide stretches in the NCR and the 5′end of the coding sequence, respectively. To quantify the inhibitory effect of these S-ODN a modified viral RNA was produced *in vitro* and in cell culture. This RNA consists of the first 408 nt of a HCV type 1b genome fused to the coding sequence of the luciferase gene. It can be translated in rabbit reticulocyte lysates and in cell culture to produce an enzymatically active fusion protein between the first 22 amino acids of the core protein and the luciferase. The production of active luciferase was monitored in the presence of different concentrations of S-ODN. In the reticulocyte lysate system the best results were observed with S-ODN 4 directed against nt 326-348, comprising the start AUG of the coding sequence. With this ODN a dose-dependent effect was observed with a maximal inhibition of 95±2% at a S-ODN concentration of 4.14 μM. A sense S-ODN and a mismatch S-ODN serving as specific controls resulted in a maximal inhibition of only 46±10% and 46±1%, respectively. These results could be confirmed in cell culture. S-ODN 4 reduced the translation of the test RNA by 84±9% at a concentration of 2 μM whereas the sense S-ODN stimulated the expression by a factor of 1.8. These data suggest that HCV gene expression can be inhibited effectively by *antisense* S-ODN.

C6-301 TARGETING OF HIV-INFECTED CELLS BY CD8+ T LYMPHOCYTES ARMED WITH UNIVERSAL T CELL RECEPTORS: A GENERAL APPROACH TO IMMUNOTHERAPY

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Our goal is to develop cell-based immunotherapies for HIV-infected individuals. One approach that we are currently pursuing involves adoptive transfer of cytotoxic T cells which specifically recognize and eliminate host cells infected with HIV. Since T cell clones with native T cell receptors specific for HIV may have limited application by virtue of their inherent MHC-restriction, we have developed a strategy in which the antigenic-specificity of effector T cell populations is redirected via universal T cell receptors (URs) bearing ligand binding domains which, unlike native T cell receptors, are not MHC-restricted. Such ligand binding domains may be derived from monoclonal antibodies specific for HIV, or from the HIV receptor CD4.

We have conducted detailed pre-clinical studies designed to evaluate the therapeutic potential of this approach, involving two classes of HIV-specific URs: CD4-zeta and single-chain antibody (SAb)-zeta. Specifically, we have generated human blood peripheral mononuclear cell (PBMC)-derived CD8+ T cell populations which express high levels of both classes of HIV-URs, by employing retrovirally mediated transduction. Upon binding to specific viral antigen(s) on the surface of HIV-infected cells, such URs have the ability to activate T cell effector functions such as cytokine production and cytotoxicity in a highly efficient and specific manner.

The ability of URs to harness the effector functions of T lymphocytes in an antigen dependent manner may have far reaching consequences for the treatment of a number of diseases. The potential to "tailor-make" effector T lymphocytes that specifically recognize an antigen of choice in an MHC-unrestricted manner suggests that this approach may have broad application in the treatment of cancer and viral diseases in genetically diverse individuals.

C6-302 CLINICAL RECOVERY, GRAFT SURVIVAL AND LONG TERM GENE EXPRESSION IN PARKINSONIAN NON-HUMAN PRIMATES AFTER STRIATAL GRAFTING OF FIBROBLASTS GENETICALLY MODIFIED WITH TYROSINE HYDROXYLASE cDNA. K.S. Bankiewicz, D. Nagy, M. Emborg, R. Mandel, K. Spratt, F. Gage, S. Leff., Somatix Therapy Corporation, 850 Marina Village Pkwy., Alameda CA 94501 and UCSD LaJolla, California

Somatic gene therapy uses grafts of genetically modified autologous cells to produce and deliver therapeutic substances to specific sites within the body and eliminates the need for immunosuppression or barrier devices. We are utilizing a process that consists of harvesting autologous cells through a biopsy, followed by *ex vivo* genetic modification of these cells to produce specific therapeutically active proteins, and subsequent grafting of these cells to an appropriate site.

In idiopathic Parkinson's disease and MPTP-induced parkinsonism, symptoms of which are caused by dopamine deficiency in the striatum, this approach may provide stable and constant delivery of therapeutic levels of L-dopa to the striatum leading to reversal of parkinsonian symptoms.

7 MPTP-treated overlesioned hemiparkinsonian monkeys were used for this study. Using MRI guided stereotaxic surgery the 3 monkeys were grafted with autologous fibroblast transfected with retroviral vector (MFGS) carring a human tyrosine hydroxylase (TH2) cDNA, 2 monkeys were implanted with non-transfected fibroblasts and 2 animals were left non-implanted. Prior to the implantation all animals were behavioraly characterized for 9-12 months using parkinsonian rating scale, arm use scale and activity monitors. TH implanted monkeys showed immediate and significant clinical improvement after the implantation which was followed for 4 months, significant return of parkinsonian arm function in all 3 animals at 2 months and in 1 monkey at 4 months. Animals were sacrificed at 4 months. There was no significant clinical improvement seen in the sham implanted or control monkeys. MRI scanning detected grafts in the striatum and closure of the blood brain barrier at 2 weeks post implantation.

Histological examination of the implanted animals showed surviving fibroblast grafts, there were well vascularized and integrated in the host striatum. In all TH-fibroblasts-implanted monkeys TH mRNA was detected by *in situ* hybridization in the grafts, and in 1 animal TH-immunoreative fibroblasts were detected in the implant. Nither TH mRNA hybridization nor TH-immunoreactive staining were present in grafts in the sham implanted animals. These results validate the potential application of ex-vivo gene therapy for neurodegenerative brain disorders.

C6-303 SELECTION OF CELL-BINDING, ENDOCYTOSING PEPTIDES USING PEPTIDE-PRESENTING PHAGE

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Targeted gene delivery to specific cells *in vivo* is desired and may be required for safe and effective gene therapy. However, current gene therapy vectors cannot perform cell targeting. Peptide-presenting bacteriophage libraries were used to select peptides which bind specifically to living mammalian cells in culture. Remarkably, cell-specific peptides could also be selected which directed endocytosis of the phage. To date, peptides have been selected against mouse fibroblasts, myoblasts and myotubes. This selection protocol may permit the discovery of peptides which target cells *in vivo* to facilitate specific uptake of gene therapy vectors without any *a priori* knowledge of receptors on the target cells.

The luciferase gene was inserted into these "endocytosing" phage to mediate gene tranfer to mammalian cells. In the presence of endosome disrupting agents, luciferase activity was observed following incubation with selected peptide-presenting phage. The ease of mutating these M13-derived *semi-viruses* and their tolerance to changes allow a wide variety of features to be built into them. This and the fact that *semi-viruses* can be grown in E. coli to titers of 10^{12} virions/ml make them an attractive candidate as a gene therapy vector.

C6-304 STABLE AUTONOMOUSLY REPLICATING VECTORS FOR GENE THERAPY.

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Most current approaches to gene therapy use viral vectors or conventional plasmids to introduce correct copies of defective genes. However, these strategies are accompanied by potential difficulties including the size constraints and immunogenicity of viruses and the short lifetime of plasmid DNA. These problems could be overcome by using non-viral stable, autonomously replicating vectors.

My lab has developed a unique class of vectors that replicate autonomously and are retained for long periods of time in cells. The replication of these vectors is mediated by human genomic sequences and occurs at high efficiency and under correct cell cycle control. The vectors use a simple, effective mechanism of nuclear retention characterized by my laboratory. These vectors replicate autonomously in all human cell types tested, as well as in cells from other mammals, such as rodent cells. It has recently been demonstrated that plasmid vectors can be efficiently introduced into mammalian tissues, for example by using cationic liposomes or receptor-mediated endocytosis, opening the way for the use of non-viral DNA vectors in gene therapy.

We demonstrate the long-term expression of genes carried by our stable, autonomously replicating vectors by following expression of marker genes such as the chloramphenicol acetyl transferase gene over a time course of at least 8-12 weeks in human tissue culture cells, including dividing cells and non-dividing cells. Positive results in these studies, i.e. long-term gene expression from the vectors, suggest that this system has great promise for *in vivo* gene therapy.

C6-305 AN IMMORTALIZED BRAIN ENDOTHELIAL CELL LINE AS A VECTOR FOR GENE THERAPY OF NEUROLOGICAL DISORDERS.

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Experimental intracerebral transplantation of various genetically modified cell types, including fibroblasts, myoblasts, chromaffin cells, glial cells or neurons constitutes an approach to gene therapy of neurodegenerative diseases or brain tumors. Although brain microvessel endothelial cells are strategically located at the interface between blood and brain, the use of endothelial cells as vectors in gene therapy has never been applied to the CNS. We have therefore adopted an original strategy for nervous system gene therapy, by using a cerebral endothelial rat cell line (RBE4), immortalized by transfection of brain microvessel endothelial cells with an adenovirus E1A gene-containing plasmid. As a first step, RBE4 cells have been transfected with the reporter nls-lacZ gene (RBEZ cells) and implanted in the brain of adult rats. Interestingly, RBEZ cells integrate in the host parenchyma, with a frequent vascular localization, the lacZ product being still detected after 5 weeks post-implantation. As a model of potentially therapeutic gene, the nerve growth factor (NGF) neurotrophin has been chosen in this study for its capacity to enhance the survival of sensory, sympathetic and central cholinergic neurons, and to promote the outgrowth of cholinergic nerve fibers. Genetically modified endothelial cells (RBE/NGF cells) have been produced by infection of RBE4 cells with a replication-defective retroviral vector carrying the murine NGF gene. These cells release biologically active NGF, as demonstrated by the capacity of cell supernatant to promote neurite outgrowth *in vitro* in rat pheochromocytoma PC12 cells. They will be transplanted into the nucleus basalis of adult rat brains. Expression of the transgene will be assessed by *in situ* hybridization and biological effect of secreted NGF on central cholinergic neurons will be investigated by NGF low-affinity receptor immunolabeling. In conclusion, genetically engineered RBE4 cells can survive, integrate into the host cerebral vasculature, express transgenes and can thus be considered as a novel and valuable cellular platform for gene therapy in a wide range of neurological disorders.

C6-306 EFFECT OF THE EXPRESSION OF ANTI-HIV-1 GENES IN MOUSE BONE MARROW

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Transduction of effective anti-HIV-1 genes into bone marrow stem cells followed by autologous transplantation of the transduced cells may lead to re-population of the hematopoietic and immune systems with cells resistant to productive HIV infection. To test the re-population potential of the transduced hematopoietic stem cells, we are currently examining the expression pattern and possible hematopoietic toxicity of a variety of anti-HIV-1 genes in a murine bone marrow transplant model. Briefly, bone marrow is harvested from 5-FU treated male C57Blk/J6 male mice, transduced with retroviral vectors in the presence of IL-3, IL-6 and murine SCF and transplanted into irradiated syngeneic female recipients. After 12-14 days, animals are sacrificed and CFU-S are harvested and tested for expression of the anti-HIV-1 genes in cells derived from the trandsduction of late progenitors. Some animals will be sacrificed 3-6 months post-transplantation to test for successful transduction of early progenitors. These animals will be analyzed for their hematopoietic profile by FACS analyses and CFU-GM assays. The retroviral vectors used to transduce the bone marrow encode an anti *tat/rev* hammerhead ribozyme and an RRE decoy construct, both under the transcriptional control of the LTR. In addition to these two RNA based strategies we are also examining the effects of expression of the transdominant mutant *rev* protein, M10, on hematopoiesis. The efficacy of the above vectors has been demonstrated in T-cell lines, but more recently we have also shown them to be effective in inhibiting HIV-1 replication in the promonocytic cell line U1, which is latenly infected with HIV-1. After induction of the U1 cells we observed a 69.5% inhibition of viral replication when the cells were transduced with the vector LN-CM10 as compared to untransduced and Neo transduced cells.Similarly the cells transduced to express the ribozyme and the RNA decoy showed inhibiton of viral replication of 66.5% and 67.2% respectively as compared to the control cells. Additionally, expression of the wild type HIV-1 genes *tat* and *env* in the CNS has been associated with the neurological dysfunction in AIDS encephalopathy and we are currently examining the possible pathologic effects of *tat* and *env* expression in BM derived microglial cells in the CNS.

C6-307 NON-VIRAL GENE DELIVERY SYSTEMS FOR CANCER TREATMENT, Richard J. Cristiano and Jack A. Roth, Section of Thoracic Molecular Oncology, Department of Thoracic and Cardiovascular Surgery, M.D. Anderson Cancer Center, Houston, TX 77030.

Present adenovirus and retrovirus vectors for gene transduction into cancer cells can deliver gene constructs in vivo. These vectors have limitations resulting from potential toxicity and absence of specific cell targeting. We have developed a non-viral gene delivery system using DNA molecules complexed with ligands, which have receptors that are highly expressed on specific cell types. This system couples receptor-mediated endocytosis with an endosomal lysis vector for targeted gene delivery (1,2). We report on the development of a non-viral gene delivery system using epidermal growth factor (EGF) as a ligand for DNA delivery to cancer cells that over-express the EGF receptor. Recombinant human EGF was chemically coupled to Poly-L-Lysine (PLL), to allow for the attachment of DNA. The EGF/PLL conjugate could bind DNA and when incubated with several different cancer cell lines, showed high levels of gene expression when uptake was done in the presence of replication defective adenovirus as an endosomal lysis agent. When the EGF/DNA complex was coupled directly to the adenovirus, the levels of expression showed an even greater enhancement. Depending upon the cancer cell line, approximately 50% to 99% of the cells showed DNA uptake. A colon adenocarcinoma cell line, which does not express the EGF receptor at the cell surface, showed little or no DNA uptake. DNA/protein complexes have the capability of delivering potentially therapeutic genes to cancer cells targeted to specific receptors.

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C6-308 USE OF RETROVIRALLY TRANSDUCED ASTROCYTES TO DELIVER RECOMBINANT BRAIN DERIVED NEUROTROPHIC FACTOR (BDNF) TO MIDBRAIN DOPAMINE NEURONS IN CULTURE: IMPLICATIONS FOR GENE THERAPY IN PARKINSON'S DISEASE,

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Previous studies demonstrated that retrovirally transduced astrocytes can be used to deliver recombinant nerve growth factor (NGF) to the brain (Cunningham et al., Brain Res., *in press*). To determine whether astrocytes can be used to deliver a neurotrophic factor for midbrain dopamine neurons, type I astroctyes purified from newborn rat striatum were infected with a MoMLV-based replication defective retrovirus harboring the human prepro BDNF gene sequence under LTR transcriptional control. Neuronal cultures established from embryonic day 15 rat ventral mesencephalon were maintained for six days in normal N2 medium, medium conditioned by astrocytes infected with the BDNF retrovirus (AsBDNF-CM), or medium conditioned by astrocytes infected with a control retrovirus harboring the NGF cDNA sequence (AsNGF-CM). Exposure to AsBDNF-CM stimulated a 4-7-fold increase in the number of tyrosine hydroxylase-immunoreactive (TH+) neurons compared with controls ($p<0.01$). When the neuronal cultures were exposed to 100 ng/ml purified BDNF protein, there was a 2.5-fold increase in the survival of TH+ neurons. These studies suggest that primary astrocytes transduced with a BDNF-containing retrovirus produce biologically active BDNF, which promotes the survival of dopamine neurons in culture. Transplantation studies designed to test whether astrocytes can also be used to deliver BDNF to affect dopaminergic neurons in a rat model of Parkinson's disease are currently underway. Supported by NINDS NS32562

C6-309 INTRAMUSCULAR INJECTION OF PLASMID DNA: SPATIAL-TEMPORAL PROFILE OF REPORTER GENE EXPRESSION, Soeun G. Doh, Jukka Hartikka, H. Lee Vahlsing, Francine Cornefert-Jensen, Marston Manthorpe, VICAL INCORPORATED, San Diego, CA 92121

The ability to express genes in skeletal muscle myofiber cells after intramuscular injection of plasmid DNA represents a novel and simple approach for the treatment of various human diseases. To further understand the mechanism of myofiber transfection, we have examined in detail the time course and spatial distribution of reporter genes in the mouse rectus femoris muscle. Muscles were injected once with firefly luciferase or bacterial ß-galactosidase plasmid DNA expression vectors (CMV-driven Lux and LacZ, respectively). Average CMVLux expression was easily detected 30 minutes after injection, peaked as high as 156 ng luciferase/muscle (n=10) between 7-14 days and declined to stabilize after 30 days at a level of several hundred pg luciferase/muscle for the life of the animal. Dose response analyses showed that maximal luciferase expression was reached with 25-100 µg DNA/muscle. Expression of CMVLacZ was determined at 1, 3, 7, 14, 30 and 60 days postinjection by whole mount and serial section X-gal staining. The X-gal positive areas were restricted to myofiber cells within the targeted rectus femoris muscle. By 1 day post-injection, X-gal staining had appeared in the myotendinous junction and central myofiber areas. By 7-14 days, X-gal staining reached a maximum and appeared along the entire length of many myofibers. By 60 days, myofiber staining was considerably decreased although some fibers remained strongly stained along their entire length. Serial section analyses revealed that up to 400 (10%) out of the 4000 rectus femoris fibers were X-gal positive. The above information on CMVintLux and CMVintLacZ expression in muscle are being used as standards for comparison of novel DNA vectors and DNA delivery methods.

C6-310 DEVELOPMENT OF SUPERINFECTING - DEFECTIVE HIV VECTORS FOR USE IN THE TREATMENT OF HIV INFECTION. Boro Dropulic and Paula Pitha-Rowe, Oncology Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21231.

The cellular targets for HIV gene therapy are either CD4+ T cells, macrophages or their progenitors, the CD34+ haematopoetic stem cells. Gene therapy of these cells is problematic with current replication defective viral vectors since transduction efficiencies are not sufficient to cover such widely disseminating cells. Here we suggest an alternative approach for gene therapy of the cellular targets of HIV. We propose a superinfecting-defective (SID) HIV vector strategy. SID HIV vectors are conditionally replication competent HIV vectors that lack sequences required for the production of proteins but contain all the sequences required for expression and packaging of HIV vector RNAs in infected CD4+ T cells. They can contain ribozymes (Rz) that cleave infectious HIV RNA but not vector RNA. RzSID HIV vectors can be transduced into latently infected CD4+ T cells. Activation of infected CD4+ cells results in the production of virus particles where SID HIV RNA can be expressed and co-packaged along with infectious genomic HIV RNA into progeny virions. The outcome of infectious HIV RNA scission by RzSID HIV RNA is the production of defective HIV particles, resulting in an inhibition of virus spread. In addition, the defective particles will still contain viable RzSID HIV RNA that may infect other latently infected cells, establishing a competing defective HIV infection. Data will be presented on the feasibility of this approach.

C6-311 **ADENOVIRUS-MEDIATED GENE TRANSFER INTO DISSOCIATED AND EXPLANT CULTURES OF RAT HIPPOCAMPAL NEURONS.** R. C. Eisensmith[1], M. Wilkemeyer[1], Karen L. Smith[2], John W. Swann[2], Timothy A. Benke[1] and Kimon J. Angelides[1,3]. Departments of [1]Cell Biology, [2]Pediatrics and the Cain Foundation Laboratories and [3]Biochemistry and the Division of Neuroscience, Baylor College of Medicine, Houston, TX 77030

Many studies have now reported the successful transduction of neuronal tissues, both *in vitro* and *in vivo*, using recombinant vectors derived from herpes simplex virus, adenovirus or adeno-associated virus. However, relatively little data exists concerning the effects of these vector systems on neuronal physiology. To address this issue, a recombinant adenoviral vector expressing ß-galactosidase (Adv/RSV-ßgal) was used to infect cultured hippocampal neurons at MOIs of 1 - 100 or hippocampal explant cultures at doses of 1 x 10^7 - 5 x 10^8 viral particles/explant. A combination of fluorescent viability dyes and histochemical staining was used to determine the percentage of transduced hippocampal cells that were still viable at various time points after infection. Both viability and transduction efficiency were directly proportional to MOI. At an MOI of 1, about 80% of all dissociated cells were positive for ßgal gene expression within 3 days. Most of these cells survived for only 13 days in culture, as compared with mock-transduced cultures which survived for one month in culture. At MOIs of 10 and 100, nearly 100% of all cells were ßgal-positive, but these cultures survived for only 9 and 5 days, respectively. Electrophysiological recordings obtained from cells five days after transduction at an MOI of 10 revealed obvious changes in peak inward (Na) currents, in inactivating (K) currents, and in membrane resting potential, which may be significant at higher MOIs. Similar findings were observed in hippocampal explant cultures; extracellular field recordings of picrotoxin-induced network-based epileptiform activity was largely unaffected after transduction with 1 x 10^7 particles of Adv/RSV-ßgal, but was completely abolished at doses of 5 x 10^8 particles. These results demonstrate the potentially negative effects of adenoviral vectors on neuronal function and serve to emphasize the need to examine the full effects of these and other vectors on neuronal physiology before they can be applied to the treatment of various neurological or neurodegenerative disorders.

C6-312 GENE TRANSFER INTO MACROPHAGES BY TARGETING THE MANNOSE RECEPTOR. Thomas Ferkol, Jose C. Perales, Frank Mularo, and Richard W. Hanson. Department of Pediatrics at Rainbow Babies and Childrens Hospital, and Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio, 44106.

Gene transfer systems targeting various receptors have been developed to introduce genes into cells in culture and intact animals. A synthetic carrier, consisting of mannosylated poly (L)-lysine, that exploits endocytosis *via* the macrophage mannose receptor was constructed and bound to expression plasmids containing either the *P. pyralis* luciferase or *E. coli* ß-galactosidase (*lacZ*) reporter genes. The resultant complex was used to transfect murine macrophages isolated from peritoneal exudates *in vitro*. Significant levels of luciferase activity was found in cells transfected using the mannosylated poly (L)-lysine, while complexes consisting of the expression plasmid bound to a galactosylated poly (L)-lysine carrier resulted in no detectable transgene expression; 249739±177290 and 790±72 ILU per 10 µg protein, respectively. Maximal transgene expression was found 1 day after transfection, and luciferase activity decreased to approximately pre-treatment levels 8 days after transduction. The number of cells expressing ß-galactosidase activity 48 hours after transfection varied considerably, from 4.6 to 18.2% of all cells examined (mean 11.4 ± 5.5%). Morphologically, the blue-stained, transfected cells appeared to be mononuclear phagocytes and expressed non-specific esterase activity. Transfection was inhibited by the addition of excess mannosylated bovine serum albumin to the culture medium before treatment. Reporter genes were also introduced into intact animals using this gene transfer system. The mannosylated poly (L)-lysine carrier was used to transfer the luciferase gene into mice *via* the systemic circulation. Luciferase activity was maximal at 177866±71626 and 9461±5758 ILU per mg protein in extracts from the spleen and liver, respectively, 4 days after transfection, and decreased to lower levels by sixteen days. The transgene was found in the livers and spleens of mice as long as 16 days after transfection. Transcripts from the luciferase gene were also detected using RT-PCR in cells residing in the spleen, liver, and bone marrow of transfected mice. Transgene expression was localized to cells in the spleen and liver that conformed to the distribribution of cells that had nonspecific esterase. This DNA carrier system can be used to introduce functional genes into macrophages, and may be an approach to the treatment of diseases that affect the reticuloendothelial system.

C6-313 A PCR-BASED ANALYSIS OF LATENCY IN THE RAT TRIGEMINAL GANGLION ESTABLISHED WITH A DEFECTIVE HSV-1 VECTOR. David J. Fink, Ramesh Ramakrishnan and Myron Levine. Departments of Neurology and Human Genetics, and GRECC, VAMC, University of. Michigan, Ann Arbor, MI 48105.

Competitive quantitative PCR and RT-PCR was used to quantitate DNA and RNA from an attenuated ribonucleotide reductase (RR)-deleted herpes simplex virus type 1 (HSV-1) mutant in the rat trigeminal ganglion after peripheral inoculation following corneal scarification. Amplification of ganglionic DNA with oligonucleotide primers specific for the HSV gB gene and for the LAT gene indicated that there were approximately 2×10^5 genome equivalents per ganglion at 2, 7 and 56 days after inoculation. Amplification of ganglionic RNA with primers specific for HSV LAT indicated that there were approximately 1×10^7 LAT molecules per ganglion at 2 and 7 days post inoculation, and 1.4×10^7 LAT molecules per ganglion at 56 days. *In situ* hybridization with a digoxigenin-labeled riboprobe specific for LAT detected an average of 1-2 LAT positive cells in each positive 6 micron section of trigeminal ganglion. *In situ* PCR detection of HSV genomes in similar sections, using digoxigenin-labeled nucleotides with primers specific for HSV gB, identified 27-150 genome-positive cells per section.

These results indicate that there are approximately 50 LAT molecules per latent HSV genome in the trigeminal ganglion, compared to 15 LAT molecules per latent HSV genome in the CNS (Ramakrishnan *et al., J. Virol.*, 1994), but that cells with detectable LATs by *in situ* hybridization represent only a small proportion of those ganglionic neurons containing HSV genomes. The presence of latent HSV genomes in a large number of neurons suggests that an HSV-based vector may be more efficient in establishing the latent state than would be anticipated from previous reports.

C6-314 SUCCESSFUL TRANSFER OF YEAST ARTIFICIAL CHROMOSOMES INTO HUMAN NEUROBLASTOMA CELL LINES. Chin-To Fong, Elizabeth Fox, Tali M. Johnson Department of Pediatrics, University of Rochester School of Medicine, Rochester NY 14642

Successful transfer of yeast artificial chromosomes (YACs) into rodent cells have been reported by many investigators. However, there has been no report to date of successful transfer of YACs into human tumor cells. To explore this possibility, we have obtained YACs containing part of *MYCN* gene region commonly amplified in human neuroblastoma, as well as YACs representing other part of the genome. These YACs were retrofitted with the neomycin-resistance gene by homologous recombination in yeast, and then transferred into human neuroblastoma cell lines by spheroplast fusion. Neomycin-resistant fusion hybrids were obtained from the neuroblastoma cell lines NGP and SMS-KAN, both of which show amplification of *MYCN*. Neuroblastoma cell lines that do not amplify the *MYCN* region did not form neomycin-resistant hybrids with these YACs. The transferred neomycin genes appeared to be present in multiple copies. We also explored whether other parameters of the YACs, including their size (150 kb to 580 kb) and their physical configuration (circular or linear), affected the efficiency in forming neomycin-resistant hybrids with neuroblastoma cells. While circular YACs appeared to be more efficient in this regard, the differences were not striking.

Our results suggest that (1) YAC can be an alternative vehicle for gene transfer into human neuroblastoma cells; (2) the success in the formation of neomycin-resistant neuroblastoma-YAC hybrids may depend on the degree of genome instability in the neuroblastoma cells, as judged by the presence or absence of endogenous *MYCN* amplification; (3) amplification of the transferred sequences may have taken place. Results from this and further studies are expected to be directly applicable to designing new gene therapy approaches in cancer.

C6-315 THE POTENTIAL USE OF AN HIV-1 REV MUTANT WITHOUT NUCLEOLAR DYSFUNCTION FOR AIDS THERAPY, Rika A. Furuta, Satoshi Kubota, Toshio Hattori, Emiko Takano, and Masakazu Hatanaka, Institute for Virus Research, Kyoto University, Japan

The applications of transdominant mutants of human immunodeficiency virus type 1 (HIV-1) regulatory proteins, especially Rev mutant, have been attempted for gene therapy against acquired immunodeficiency syndrome (AIDS), because the Rev protein is essential for viral replication. The Rev protein is known to localize in nucleus/nucleolus of expressed cells and cause nucleolar deforming followed by cell death when a large amount of Rev is expressed. We have previously reported that a mutant Rev protein (dRev) lacking its nucleolar targeting signal remained out of nuclei in expressed cells and strongly inhibited the function of Rev. To investigate effects of dRev as an anti-viral molecule, we established several dRev expressing human cell lines with two different vector systems and examined virus production in these cells. Cells expressing dRev by HIV inducible vector suppressed remarkably virus replication, syncytium formation and cell death caused by HIV-1 infection. The infection was also very inefficient in dRev constitutively expressed cells. In addition, we found that dRev did not cause nucleolar dysfunction in a transient assay in contrast other transdominant mutants and wild type Rev. Since dRev can not migrate into the nuclei, it is expected not to interfere with nuclear/nucleolar functions of the host cell. We conclud that dRev is one of promising candidates as anti-viral molecule against gene therapy of AIDS.

C6-316 CATIONIC LIPOSOME-MEDIATED GENE DELIVERY *IN UTERO* IN FETAL RATS, Karin Gaensler[1], George Mychaliska[2], Andrea Metgus[2], Guanhuan Tu[3] Denny Liggitt[4], Michael Harrison[2], and Robert Debs[3], Departments of Medicine[1], Surgery[2], CRI[3], University of California, San Francisco, 94143-0724 and Department of Comparative Medicine[4], University of Washington

In order to develop cationic liposome-based methods for in vivo gene delivery *in utero*, we have injected reporter gene constructs into day 17-19 Fisher rat fetuses by several routes. Pregnant Fisher rats were anesthetized and the uterus delivered through a midline incision. Fetuses were injected with cationic liposome-DNA complexes in a 50 µl volume by intraperitoneal (IP), intrathoracic (IT), or intracardiac (IC) routes. The uterus was then replaced and the incision closed. Fetal tissues were harvested 18 hrs to 3 days later and the tissues analyzed for CAT or luciferase reporter gene expression. More than 200 fetuses have now been injected with an overall survival of greater than 75%. When injected IP, reporter gene expression was largely confined to intraabdominal organs. Levels of luciferase activity 1000 fold above background levels were detected in liver and spleen extracts following injection of CMV-luciferase expression plasmid:cationic liposome complexes. Lower levels of reporter gene activity were seen in the lungs. Similarly, high levels of CAT activity were detected in these organs following injection of CMV-CAT plasmid:liposome complexes. Significant levels of CAT activity were detected in tissues of newborn animals following injection several days earlier *in utero*. Fetuses injected by IT or IC approaches showed higher levels of expression in the lung and heart than in the liver.

IP injection of rat fetuses in utero is a good model system in which to develop approaches for *in vivo* direct gene delivery. The high level expression of reporter gene constructs seen in the liver shows promise for delivering genes of interest to fetal hepatocytes, as well as to fetal hematopoietic progenitor cells *in utero*. Immunohistochemical studies to identify the cell types that express the transfected gene products are presently underway. Potential advantages of cationic liposome-mediated approaches over existing viral vectors are that non-dividing can be transfected, complexes are non-immunogenic, and that there is no theoretical limit to the size of the DNA that can be delivered.

C6-317 KREBS CYCLE AND SYNTHESIS OF AMINO ACIDS IN THE STAPHYLOCOCCUS CELLS , CONTAINING PLASMIDS RESISTANT TO ANTIBIOTICS, Gavrilyuck V.G., Kosizkaya S.N., Vinnikov A.I. , Department of Microbiology , Dniepropetrovsk State University , Ukraine , 320625.

Activity of key-enzymes of Krebs cycle and at the same time of enzymes of some anaplerotic processes and synthesis of amino acids from intermediates of this cycle have been learned in Staphylococcus strains , keeping resistance plasmids to different antibiotics. Comparative studies showed the decrease in activity of citrate - synthase and succinate dehydrogenase by 1.2 - 1.8 times in plasmid-containing strains in comparison with plasmid-lacking strain. This is accompanied with higher level of activity of anaplerotic enzymes : malic-enzyme and malatedehydrogenase , which catalyse the reduction of oxalacetate acid in L - malate acid by 1.2 - 5 times, and at the same time higher activity of dehydrogenases , which catalyse the synthesis of amino acids : glutamic acid , alanine , aspartic acid and glycine , by 1.5 - 3.8 times in Staphylococcus cells, having genetic determinants resistant to antibiotics, has been established. In this way functioning of Krebs cycle in given strains leads to the increases the rate of anabolic processes , while the catabolic reactions of this cycle get retarded. Intensification of anabolic processes , which bring about considerable rearrangement of different intermediates in amino acids' synthesis , is the result of R-plasmid's presence in Staphylococcus cells.

C6-318 SYSTEMIC GENE THERAPY VIA DNA:LIPID COMPLEXES, Cori Gorman [1], Tim Heath [2] and Denny Liggitt [3]. 1 Department of Gene Expression and Delivery, MEGABIOS Corp, Burlingame, CA, 94010. 2. School of Pharmacy , University of Wisconsin, Madison. 3 School of Medicine, university of Washington, Seattle, WA.

In vivo gene therapy is an emerging therapeutic modality that involves delivery of genes into cells in the body. Early gene therapy systems such as viruses and naked DNA have shown promise but have failed to produce broadly applicable delivery systems. DNA-lipid complexes can provide an alternative means of gene delivery into a variety of tissues. Local delivery of DNA has been limited to tumors and muscle. In addition to direct tumor delivery we have expanded local delivery techniques to include skin grafts, intraperitoneal delivery into the spleen and intracranial delivery into the brain, we have concentrated on systemic delivery which allows intravenous delivery of DNA to various tissues including the lung and heart. We have achieved selective *in vivo* expression in various tissues with the use of tissue specific promoters. Over 2000 animals have been used in this *in vivo* system to determine the best routes of delivery for expression in various tissues. Our complexes are stable for upto 60 days and expression persists in some tissue for 60 days as well.

C6-319 SYNTHETIC VEHICLES FOR EFFICIENT GENE TRANSFER AND EXPRESSION IN MAMMALIAN CELLS. Stephen Gottschalk[2], James T. Sparrow[4], Louis C. Smith[4], and Savio L. C. Woo[1,2,3]. (1) Howard Hughes Medical Institute, Departments of (2) Cell Biology, (3) Molecular Genetics, and (4) Medicine. Baylor College of Medicine, Houston, TX 77030.

Gene Therapy for treatment of diseases requires suitable delivery systems. Our objective is to mimic viruses by using DNA complexes containing synthetic compounds that are functionally equivalent to viral components. With viruses, low endosomal pH activates lytic viral sequences to disrupt the endosome before it fuses with primary lysosomes. We have synthesized an amphipathic membrane active peptide (JTS-1) to mimic this function. This peptide lysed phosphatidylcholine vesicles as well as human erythrocytes at pH 5.0, compared to no membrane activity at pH 7.0. JTS-1 was incorporated into DNA complexes by ionic interaction using a novel synthetic poly-L-lysine analog (Ksyn), which contains a central cluster of lysine residues. DNA was condensed with Ksyn to form a positively charged DNA/Ksyn complex to which JTS-1 was added. To test the efficiency of DNA/Ksyn/JTS-1 complexes to deliver genes into cells CMV-ßgal was used as a reporter gene; 24 h after gene delivery cells were stained with X-gal. Without JTS-1 no blue cells were observed. Under optimal condition the percent positive cells was 20 to 50% in myoblast (sol 8), fibroblast (human), and colon carcinoma cells (MCA-26, HCT-116). These results indicate that peptides can be used for the rational design of DNA delivery systems. Future development of DNA/Peptide complexes hold the promise of replacing viruses as gene delivery vehicles for gene therapy.

C6-320 LONG-TERM PROTECTIVE EFFECT OF NEUROTROPHIN-3 SECRETING MYOBLASTS AGAINST KAINIC ACID-INDUCED EXCITOTOXIC LESIONS IN THE RAT HIPPOCAMPUS, Qing Guo, Ming Fan, Si D Gan, Yuan Y Mu, Ying You and Hong S Liu, Departemnt of Neurobiology, Institute of Basic Medical Sciences, P.O. Box 130(3), Beijing 100850,P.R.China.

Using eukaryotic expression plasmids as well as retrovirus vectors, we report here that the intact human neurotrophin-3(NT-3) gene, which was cloned from human genomic DNA by polymerase chain reaction, could be introduced into and expressed in cultured myoblasts isolated from adult rat skeletal muscle. The recommbinant NT-3 synthesized in the engeneered myoblasts could be co- and post-translationally modified and secreted into the culture supernatant. Matured NT-3 with full neurotrophic activity on cultured DRG neurons were produced. Upon implantation of plasmid-transfected or recombinant retrovirus-infected myoblasts into the hippocampus, long-term stable(up to six months) and high level expression of human NT-3 was achieved, as demonstrated by in situ hybridization and immunohistochemistry. Preimplantation of NT-3 secreting myoblasts significantly reduced kainic acid(KA)-induced neuronal degeneration in the ipsilateral hippocampus at both light and electron microscopic level when compared to non-NT-3 secreting myoblast grafts. This was characterized by decreased incidence of pyramidal cell atrophy, ribosome disappearance and disintegration of membrane and organelles. The early degeneration of dendritic spines and the initial portion of the dendritic shaft was also significantly reduced. If NT-3 secreting myoblasts were implanted into the both side of the hippocampus, seizure behaviour induced by KA was also significantly inhibited. It is suggested that myoblast-mediated NT-3 gene transfer has profound and long-term protective effect against excitotoxic lesions of neurons and this model may be proved to be feasible and safe for further clinical applications in the gene therapy of neuronal injury and degeneration.

C6-321 RGD-MEDIATED GENE DELIVERY AND EXPRESSION IN EPITHELIAL CELLS, Richard Harbottle[1], Rob Cooper[2], Andy Miller[2], Bob Williamson[1], Charles Coutelle[1], Stephen Hart[3], [1] Department of Biochemistry and Molecular Genetics, St. Mary's Hospital Medical School, Praed St., London W2 1PG; [2] Department of Organic Chemistry, Imperial College, London, SW3; [3] Transplantation Biology Unit, Division of Cell and Molecular Biology, Institute of Child Health, 30 Guilford St., London WC1N 1EH .

Receptor-mediated gene delivery has been demonstrated with DNA-protein complexes consisting of a DNA-binding moiety, such as polylysine, conjugated to a ligand for a cell-surface receptor such as asialoglycoprotein, transferrin and others. Cell surface integrins are exploited as receptors for internalisation by bacteria such as *Yersinia pseudotuberculosis* and many types of virus including adenovirus. In addition, sperm-egg fusion is also probably integrin mediated. Integrins are therefore widely exploited in nature for attachment to cell surfaces and subsequent internalisation. The process of integrin-mediated internalisation probably occurs by phagocytosis, which enables large structures such as individual bacteria to be internalised by eukaryotic cells. Integrin binding is a property of many proteins and peptides containing the amino acid sequence arginine-glycine aspartic acid (RGD). We have demonstrated previously that filamentous phage particles displaying high-affinity, cyclic RGD-containing peptides over their surface are internalised by Hep-2 and Caco-2 epithelial cells. We have now produced conjugates of the cyclic RGD-containing peptide with polylysine and tested them for gene delivery and expression. We report here construction and purification of the synthetic peptides and integrin-mediated gene delivery and expression.

C6-322 DETERMINATION OF TRIPLEX BINDING SPECIFICITY USING A COMBINATORIAL METHOD, Paul Hardenbol and Michael W. Van Dyke, Department of Tumor Biology, M.D. Anderson Cancer Center, Houston, TX 77030.

DNA triple helicies, in which a single strand DNA oligonucleotide sequence-specifically binds duplex DNA, are being investigated as a method to down regulate gene expression *in vivo*, an approach termed anti-gene therapy. The types of sequences known to form triplex are currently limited to homo-purine stretches, although the parameters governing triplex formation are not well understood. In this work, a novel combinatorial approach was used to identify double-stranded DNA sequences capable of binding a defined 19 base single-stranded DNA molecule through purine-motif triple helix formation. Thirty-two identified sequences were aligned with the presumed consensus sequence and the frequency of nucleotides at each position was tabulated. Statistical analysis verified that the presumed consensus binding sequence was correct. Further analysis indicated that adenine is the preferred flanking nucleotide of the purine tract of the duplex, binding of only 12 of 19 bases was required to form triplex, and the possible existence of a new base triplet G•AT. This information could help identify appropriate triplex binding sequences in potential gene targets.

C6-323 LIPID COMPLEX-MEDIATED GENE TRANSFER INTO FRESHLY ISOLATED TUMOR CELLS FOR VACCINATION/IMMUNOTHERAPY, Sana Isa, Hong-Ming Hu, John T. Vetto, Walter J. Urba, Bernard A. Fox. Molecular and Tumor Immunology Laboratory, Earle A. Chiles Research Institute, Providence Medical Center, Portland, OR, 97213 and Departments of Molecular Microbiology and Immunology, and Surgery, Oregon Health Sciences University, Portland, OR, 97201.

Recently, we reported that direct intratumoral injection of lipid complex and DNA encoding an allogeneic MHC class I molecule augmented the generation of therapeutic T cells from the tumor draining lymph nodes in mice. We have also demonstrated that direct allogeneic HLA-B7 gene transfer into human tumors in vivo led to expression of HLA-B7 protein in all patients treated. Because of the immunosuppressive activity associated with some tumors, the immune response and therapeutic efficacy of direct gene transfer may be improved by vaccinating with genetically modified autologous tumor cells at a site not involved with tumor.

Our current studies have focused on optimizing gene transfer into non-cultured, freshly isolated autologous tumor preparations for use in tumor vaccine trials. This approach has the following benefits: 1) immediate treatment of all patients without the need for developing an autologous tumor cell line; 2) optimization of gene transfer parameters for each patient; 3) maintainance of antigenic heterogeneity in tumor vaccine.

Lipid complex-mediated β-gal gene transfer was performed on 5 freshly isolated tumor preparations (3 renal, 1 melanoma, 1 adenocarcinoma) before and after cryopreservation. Variables analyzed included ratio of lipid:DNA utilized for lipofection and effect of lipid concentration on tumor cell viability. Transfection of all tumor cells was most efficient prior to cryopreservation (range 0.01% to 13% for renal); cryopreserved cells were transfected approximately one log less effectively (<0.001% to 1.04% for renal). The ratio of lipid:DNA was critical to gene transfer with ratios between 1:1 and 3.6:1 being optimal. Tumor cell viability following lipofection (at 1:1 to 3.6:1) was maintained at 47.8% (± 6.2 SD) of control for all experiments analyzed.

We conclude that lipid complex-mediated gene transfer may be a feasible method to create effective autologous tumor cell vaccines.

C6-324 GENETIC IMMUNIZATION: HOW IT WORKS AND A NEW METHOD FOR DISCOVERING VACCINES Stephen A. Johnston and Michael A. Barry, Departments of Medicine and Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75235-8573

Genetic immunization (a.k.a. DNA vaccines, Polynucleotide vaccines) is a form of gene therapy involving the direct introduction of naked, simple DNA which can express a gene or genes of interest into cells of the host such that strong humoral and cellular immune responses are produced to the gene product(s). Skin or muscle can be used as the target tissue, though for neither target tissue is the mechanism of the response understood. The following experiments were performed to understand this mechanism.

When DNA encoding the luciferase gene is introduced into the skin of the ear, 5% of total luciferase gene product activity is detected at remote skin sites, but not in any internal organs. Migration of luciferase acitivity to the skin occurs similarly following intramuscular injection of the same plasmid. Migration from the ear occurs within 5 min of gene inoculation. Removal of the inoculated area of the ear (95% of total activity) at 5 min does not affect the level of antibody titer elicited, suggesting that it is the migrating cells that are important for generating an immune response. Although luciferase activity in the ear decays within 1 week of inoculation, luciferase activity appears to remain constant or increase for longer than 1 month at distant sites, perhaps explaining the long term immune responses observed following genetic immunization.

We have used the genetic immunization technology to develop a new method for the identification of vaccines. This method is simple and does not require prior knowledge of the biology of the target pathogen. It is being applied to a bacterial (*Listeria monocytogenes*) and mycoplama (*Mycoplasma pulmonis*) pathogen. The protocol for this method and progress to date will be detailed and discussed.

C6-325 GENE THERAPY FOR HIV USING INTRACELLULAR ANTIBODIES, Susan D. Jones*, Julie Porter-Brooks*, Bridget Eberhardt*, SiYi Chen+, Abner Mhashilkar+, Wayne Marasco+ and Urban Ramstedt*, Virus Research Institute, 61 Moulton Street, Cambridge, MA 02138* and Division of Human Retrovirology, Dana Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, MA 02115+
Genes encoding single chain antibodies directed against several HIV target proteins have been synthesized using antibody engineering technology. The antibodies were localized to specific intracellular compartments using different localization signals. These genes have been cloned into a retroviral vector, and stable packaging cell lines have been used to make retrovirus stocks to transduce cultured and primary cells. Transduced cells were characterized for expression of the antibody gene using RT-PCR, immunoprecipitation, or immunofluorescence. Pools of stable transductants were then challenged with various doses of HIV, and monitored for HIV production and cell growth.One gene currently in use is a cloned antibody gene isolated from an HIV-positive human. This antibody, termed F105, is directed against the CD4+-binding domain of the HIV envelope protein gp120, and is effective at neutralizing numerous laboratory and freshly isolated strains of HIV. The single-chain antibody consists of the V_H portion of the heavy chain linked via an interchain linker $(gly_4ser_1)3$ to the V_K portion of the kappa light chain. Human SupT cells that were transduced with sFv 105, and then challenged with HIV IIIB, showed a marked growth advantage over untransduced or vector-transduced SupT cells. Twenty eight days after HIV infection, both untransduced SupT and antibody-transduced SupT show no production of HIV as measured by p24 ELISA assay. However, only the cells expressing the anti-gp120 antibody retained their cell surface CD4 expression. The anti-gp120 single chain antibody has also been introduced into primary human peripheral blood lymphocytes (PBLs), and these cells have been challenged with HIV. Additional antibodies against other HIV target proteins have been synthesized and are under evaluation in T cell lines. In summary, intracellular antibody expression may provide a novel approach to gene therapy of HIV and other infectious diseases.

C6-326 Targeting Foreign Proteins to HIV Particles Via Fusion with Vpr and Vpx. JOHN C. KAPPES[1]*, XIAOYUN WU[1], HONGMEI LIU[1], HONGLING XIAO[1], JUSTIN KIM[1], PARTHA SESHAIAH[2], JEF D. BOEKE[2] AND BEATRICE H. HAHN[1]. [1]University of Alabama at Birmingham, Birmingham, Alabama, 35294, [2]Johns Hopkins University School of Medicine, Baltimore, Maryland, 21205. The Human Immunodeficiency Virus Vpr and Vpx proteins are packaged into virions through virus type-specific interactions with Gag precursor polyproteins. We investigated whether it was possible to target heterologous Vpr- and Vpx fusion proteins to virions to interfere with virus replication. Using a T7-based expression system, both Vpr- and Vpx- staphylococcal nuclease (Vpr/Vpx-SN) fusion proteins were co-expressed with Gag in mammalian cells and were shown to be incorporated into virus-like particles (VLPs). VLP associated Vpr/Vpx-SN fusion proteins exhibited nuclease activity like purified SN protein, as determined by *in vitro* digestion of lambda phage DNA. To determine if Vpr and Vpx fusion proteins were also capable of packaging into mature virions, fusion genes were cloned into an LTR/RRE containing expression vectors and cotransfected with wild-type HIV-1 and HIV-2 proviral DNAs. Progeny virion were shown by continuous sucrose gradient analysis to contain both SN fusion and native proteins. To analyze whether virion incorporation of Vpr-SN could interfere with virus replication, the *vpr-SN* fusion was engineered into the HIV-1 genome in place of the existing *vpr* coding region. As a control, an enzymatically inactive *vpr-SN* fusion (*vpr-SN**) was included. *In cis* expression with the proviral genome produced virions containing Vpr-SN and Vpr-SN* fusion proteins. Cell free transmission to PBMC and T cell lines demonstrated up to 3 orders of magnitude decrease in infectivity compared with wild type virus. No significant differences in infectivity were noted between Vpr-SN and Vpr-SN* containing viruses. Another recombinant virus, constructed by ligating *vpr-SN* into the proviral genome in the reverse orientation was similar in infectivity to wild type virus, indicating that the incorporation of Vpr fusion proteins into virions interferes with replication. These results demonstrate the feasibility of incorporating foreign proteins into HIV virions by expression as gene fusions with *vpr* and *vpx* and suggest potential application in gene therapy based approaches for limiting HIV spread.

C6-327 **INHIBITION OF HIV-1_{JR-FL} REPLICATION IN HUMAN LONG TERM BONE MARROW CULTURES EXPRESSING ANTI-HIV-1 GENES** Karen Kearns, Ingrid Bahner, Chen Zhou, QianLin Hao and Donald B.Kohn, Division of Research Immunology, Childrens Hospital Los Angeles, 4650 Sunset Blvd., Los Angeles, CA 90027

We determined the ability of several anti-HIV-1 genes to inhibit replication of HIV-1_{JR-FL} in transduced and selected Long Term Bone Marrow Cultures (LTBMC) derived from CD34+ HIV-1-negative cord blood cells. Briefly, CD34+ cells were isolated from HIV-1-negative cordblood cells and transduced with retroviral vectors containing three different anti-HIV-1 genes: the transdominant mutant of *rev* M10, in the construct LN-CM10; an RNA decoy vector overexpressing the *rev* binding domain of the *Rev Responsive Element*, L-RRE-Neo, and a double hammerhead ribozyme targeted to the *tat* and *rev* transcript, L-TR/TAT-Neo. After transduction in the presence of IL3, IL6, SCF and in the presence of stroma, the cultures were G418 selected and subsequently challenged with HIV-1_{JR-FL} infection. The following values of viral inhibition are based on two infection experiments which were monitored post-infection for 24 and 40 days, respectively. Compared to the Neo transduced and untransduced control cultures, the L-RRE-Neo transduced culture showed 99.7% and 97.7%% inhibition of viral replication, the L-TR/TAT- Neo transduced cultures showed 90% and 83.8% inhibition, and the LN-CM10 transduced culture showed 68% and 99.9% inhibition. This preliminary study demonstrates the potential ability of these anti-HIV-1 genes to significantly suppress viral replication in primary human monocytes derived from transduced CD34+ progenitor cells.

C6-328 OVINE MAMMARY GLAND EXPRESSION OF JET-INJECTED PLASMID DNA: NORTHERN BLOT AND ANTIBODY ANALYSIS, David E. Kerr[1], Priscilla A. Furth[2] and Robert J. Wall[3], [1]AltraBio Inc., Arden Hills, MN 55126, [2]Div. of Infectious Diseases, Univ. of Maryland Medical School, Baltimore, MD 21201 and [3]Gene Evaluation and Mapping Laboratory, USDA-ARS, Beltsville, MD 20705

Plasmid DNA can be expressed when injected by needle and syringe into muscle but not in most other tissues. Previously we reported detection of β-galactosidase activity in jet-injected mammary tissue. We now report expression levels of jet-injected plasmid detectable by northern blot analysis and serum antibodies to the expressed protein.

Lactating mammary glands of four sheep were needle (21g)- or jet (Ped-O-Jet)-injected with 1 mg doses of plasmid DNA dissolved in 500μl of saline containing 1% india ink. The circular plasmid contained the CMV promoter ligated to a promoter-less human growth hormone (hGH) gene. At 48 hr post-injection the sheep were euthanized and ink-containing tissues were recovered. Northern analysis was performed on 20 μg of poly A-enriched RNA. Expression was clearly apparent in 6 of 6 jet-injected samples, while only faint signals were observed from 4 of 4 needle injected samples.

Antibody development to hGH was monitored in six groups of sheep (n=3) jet-injected into mammary gland or hind-limb muscle with 10, 100 or 1000 μg doses of CMV-hGH plasmid. Each dose of DNA, in 500 μl saline, was administered 3 times over a 6 wk period. Muscle and mammary groups responded similarly with no apparent response to the 10 μg dose, a weak response to the 100μg dose in 1 of 3 animals/group, and a stronger response to the 1000 μg dose in 2 of 3 animals/group. We have observed similar responses in mice jet-injected into muscle with 10 μg of plasmid DNA.

The results of this study demonstrate the superiority of jet- over needle injection as a means to transfect lactating mammary tissue in large animals. In a dose responsive manner, jet-injection of plasmid DNA into mammary gland or muscle can be used to elicit an antibody response.

C6-329 ANTIGEN-INDUCIBLE EXPRESSION OF AN EXOGENOUS IL-7 GENE IN HUMAN, ANTIGEN-SPECIFIC T-CELLS. Jerome H. Kim[1*], Karl V. Sitz[1], Silvia Ratto[2], Robert J. McLinden[2], Kim Davis[2], Donald S. Burke[1], R. Neal Boswell[1], Joseph D. Mosca[2], Robert R. Redfield[1], and Deborah L. Birx[1], [1]Walter Reed Army Institute of Research and the [2]Henry M. Jackson Foundation, Rockville, Maryland.

Cytokines play a key role in the complicated interaction between the immune system and HIV. We have previously shown that IL-7 augments HIV-specific immune responses; this effect is greatest at suboptimal antigen concentrations. A murine retroviral vector was used to transfer the human IL-7 gene into tetanus toxoid- (TT) and HIV envelope-specific T-cell lines. Quantitative PCR showed that the number of cells containing the neo^R gene increased to nearly 100% with serial passage in geneticin, and IL-7-specific mRNA was detected in T-cells receiving the sense (+) IL-7, but not the antisense (–) gene. Following stimulation with antigen-pulsed, irradiated, autologous PBMC, TT- and gp120-specific lines transduced with the (+)IL-7 gene produced up to 898.4 pg/ml of IL-7; mock-infected and (–)IL-7 transduced cells made no IL-7. IL-7 production was dependent upon antigenic stimulation. IL-7 became undetectable around 1 week post-stimulation. IL-7 could also be induced by PHA. All antigen-specific cell lines made IL-4, IL-6, TNF-α and γIFN, peaking 2-5 days poststimulation. There were no significant differences in cytokine profiles between T-cells with (+)IL-7 gene, (–)IL-7 gene, or mock-infected controls. Antigen-specific T-cells expressing the IL-7 gene showed higher antigen-specific proliferation than mock-infected or (–)IL-7-gene-containing cells. The effect of IL-7 expression on antigen-specific proliferation appeared greatest at levels of antigen that were sub threshold. Successful IL-7 gene transfer into the antigen-specific T-cells of HIV+ patients was also achieved. Strategies to augment immune activity against HIV by transfer of cytokine genes into antigen-specific T-cells may be useful and IL-7 may be an attractive cytokine for these studies.

C6-330 AN AMPLIFYING VECTOR FOR GENE THERAPY, Christine Kneidl[1], Friedrich Grummt[1], Claudia Müller[2], Daniel Baier[2] and Hans J. Lipps[2,3]. 1) Institut für Biochemie, Universität Würzburg, Würzburg, FRG, 2) Medizinische Klinik, Abt.II, Universität Tübingen, Tübingen, FRG, 3) Institut für Zellbiologie, Universität Witten/Herdecke, Witten, FRG

We describe the construction of a high copy number vector for human cells which can be used both for the overexpression of therapeutical important proteins, such as cytokines, or for inhibition of gene expression by antisense RNA. The vector contains a G418 resistance cassette for long range and a truncated NGF-receptor gene for immediate selection of transfected cells. The most important property of the vector is the presence of an amplification promoting sequence derived from the non-transcribed spacer region of murine rDNA (Wegner et al.: NAR 17, 9909, 1989). This sequence promotes spontaneous amplification of plasmid DNA to a copy number of over 100 copies per cell and the amount of transcript is proportional to this high copy number. This type of vector was already successfully used for stable and efficient inhibition of HIV-1 replication by antisense RNA (Meyer et al.: Gene 129, 263, 1993). At present, it is used for the inhibition of CML cell proliferation by the expression of antisense RNA against the bcr/abl fusion regions. Results from these studies will be presented.

This work was supported by the EU and the DFG

C6-331 IN VIVO GENE TRANSFER AND EXPRESSION OF HUMAN β-HEXOSAMINIDASE α-SUBUNIT INTO MOUSE BRAIN. *H. Daniel Lacorazza[1], Jonathan D. Flax[2], Evan Y.Snyder[2] and Moncef Jendoubi[1], [1]Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, MD 20892 and [2]Depts. of Neurology & Pediatrics, Harvard Medical School, Children's Hospital, Boston, MA 02115*

The implantation of multipotent neural progenitor cell lines provides an exciting possibility of gene delivery in the central nervous system to correct inherited neurological diseases. The β-hexosaminidase α-subunit (Hex-α) deficiency blocks the formation of intact hexosaminidase-A (Hex-A) isoenzyme leading to the accumulation of GM2 in lysosomes, which results in a severe neurodegenerative disorder. Mouse multipotent progenitor neural cell lines (Ryder et al., *J.Neurobiol.* 21:356-375, 1990; Snyder et al, *Cell* 68:33-51, 1992) were transduced with a retroviral vector containing the cDNA for human Hex-α under the transcriptional control of mouse PGK promoter. The expression of human Hex-A (hHex-A) was monitored in the recipient cells at the biochemical level. Transduced cells showed an increase in the enzymatic activity that was between 15- to 60-fold higher than the respective parental cell lines. Furthermore, these cells were transplanted into the brain of fetal and newborn mice, and the hHex-A expression was examined at different times up to 2 months after engraft. Only the engrafted brains showed a substantial, widespread, and sustained expression of the transgene.

Our results have general implication for the application of gene therapy to human neurological diseases and specifically for Tay-Sachs disease, since the engrafted donor cells are potentially able to provide recombinant lysosomal enzyme by intercellular metabolic cross-correction.

C6-332 **SHOCK WAVE PERMEABILIZATION AS A NEW GENE TRANSFER SYSTEM *IN VITRO***

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Lithotripter shock waves are pressure pulses of high amplitude and short duration used for the disintegration of urinary and biliary calculi *in vivo*. Recently, we discovered that shock waves, which display a temporary increase in cell membrane permeability, can be employed for the transfer of plasmid DNA into eucaryotic cells. We have further characterized the parameters determining transfection efficiency within this system. HeLa cell suspensions were mixed with two different reporter plasmids (coding for the secreted middle hepatitis B virus surface protein (MHBs) or for β-galactosidase), filled into polypropylene vials, exposed to variable numbers of shock waves generated at 25 kV with an experimental Dornier XL1 lithotripter, subsequently seeded out and analyzed 24-72 hrs later for reporter protein expression.

The efficiency of shock wave-mediated transfection was found to be directly proportional to the applied DNA concentrations over a wide range (3, 10, 30, 90 µg/ml). Increased numbers of shock waves applied (125, 250, 500) resulted in a linear increase in both MHBs and β-galactosidase expression. The absolute number of transfected cells was found to increase with the number of cells treated. Other experiments using hyperbaric pressure demonstrate that cavitation, i.e. shock wave-triggered generation and movement of gas bubbles, is responsible for the shock wave permeabilization effect.

The ability to shock wave transfect different cell lines, primary hepatocytes and CD34+ hematopoietic stem cell enriched populations employing HMG-1 condensed plasmid DNA now is under further investigation.

In vivo, shock waves can be well focused within body regions of interest. Therefore, a combined *in vivo* gene therapy approach, i.e. application of extracorporally generated shock waves (1) to organs simultaneously perfused with defined DNA solutions or (2) following direct *in vivo* injection of DNA, might open up a new possibility to achieve a regionally enhanced DNA uptake *in vivo* (supported by Deutsche Forschungsgemeinschaft grant DE531/1-1).

C6-333 PREPARATION OF ASOR-POLYLYSINE-pCMVLuc COMPLEXES WITH IMPROVED IN VIVO EXPRESSION, Charles P. Lollo, Todd C. Mockler, Mark Guido, Pat Ley, Deborah Kwoh, Dept. of Gene Therapy, The Immune Response Corporation, Carlsbad, CA 92008

Plasmid DNA can be efficiently delivered to hepatocytes in vivo by complexation with ASOR-Polylysine conjugates and subsequent injection into the tail vein of mice. Once formed, structural characterization of the DNA:protein complexes is difficult and to be pertinent must be correlated with biological assays. Investigation of various aspects of complex formation has been correlated with in vivo luciferase expression to derive an understanding of DNA delivery and methods for increasing levels of expression. Additionally, several aspects of the injection procedure were investigated. Parameters shown to be significant include degree of complexation, complex size, formulation, injection volume and pretreatment of the animals with lysosomotrophic drugs. Dose response data indicate that levels above 100 ng/ gram of liver can be achieved with 10 ug doses of pCMVLuciferase.

C6-334 PRODUCTION OF PHARMACEUTICAL GRADE PLASMID DNA FOR GENE THERAPY: Marquet, M., Horn, N., Meek, J., Budahazi, G. Vical Incorporated, 9373 Towne Centre Drive, Suite 100, San Diego, CA 92121

A process for the large scale production of VCL-1102 plasmid DNA from *E. coli* is described. VCL-1102 plasmid is a eukaryotic expression vector that codes for the human Interleukin-2 (IL-2) protein. The purification of pharmaceutical grade DNA requires the development of reproducible and scaleable processing methods that meet regulatory standards for biological derived pharmaceuticals.

The major issues of critical importance to any clinical use of plasmid DNA include those common to all drugs: identity, potency, efficacy, and safety. Plasmid purification using the standard CsCl/EtBr method is a commonly used laboratory procedure. However, because of the use of mutagenic reagents that bind to the DNA and the difficulties of scale up, this process is not appropriate for clinical production. The rapid evolution of direct gene therapy from the research laboratory to the clinical setting requires the development of alternative methods for the production of plasmid DNA.

The scaleable purification method developed for clinical use plasmid DNA is a combination of highly reproducible unit operations (cell lysis, precipitation and size exclusion chromatography). The advantages over existing methods include scaleability, improved plasmid purity and the elimination of undesirable process additives such as toxic solvents and animal derived enzymes. The overall process yield from fermentation through final column purified product is greater than 50%. *E.coli* DNA levels are reproducibly below 1% as measured by Southern analysis. Endotoxin levels are less than 0.03 endotoxin units/μg plasmid DNA and residual protein is undetectable.

The process has been used to produce plasmid DNA for drug safety studies in mice and primates and for human Phase I clinical trials

C6-335 COMPARISON OF ANTI-HIV-1 RETROVIRAL VECTORS AND THEIR USE IN AN AIDS GENE THERAPY TRIAL IN IDENTICAL TWINS. Richard A. Morgan, Marinee Chuah, Thierry VandenDriessche, Bruce Bunnell, Peter Bressler#, Robert Walker#, Clifford Lane#, and R. Michael Blaese. National Center for Human Genome Research; and National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda MD 20892.

Retroviral vectors were constructed that inhibit the HIV-1 virus at various points in the viral life cycle with the main focus on inhibiting Tat/TAR and Rev/RRE functions. The goal of this work is to develop the components of the ideal anti-HIV vector. This ideal vector would potentially attack HIV at three points in the viral life cycle. First, the vector should be able to inhibit the establishment of infection either by preventing HIV binding to cells or by stopping HIV proviral integration. Second, the vector should be capable of preventing viral protein production. This could be done early by inhibiting tat function, in the middle of the life cycle by preventing the rev-mediated production of structural RNAs, or at the end of the life cycle by the inhibition of virion assembly. As a third and final anti-viral approach, it may be possible to have the infected cell release a defective HIV particle and thus prevent a new round of replication. We have produced retroviral vectors that individually attack one or more of these points. These vectors include: sCD4 and CD4IgG binding decoy vectors, anti-sense Tat, antisense TAR, antisense Tat/Rev, Rev transdominant vectors, new HIV Gag gene transdominant vectors, a CD4-KDEL ER retention signal vector, and a series of HIV inducible vectors that synthesize diphtheria toxin, cytosine deaminase, or interferon. These vectors were assayed in T-cell lines, primary culture PBL and human CD34+ hematopoietic progenitor-enriched cells. All vectors can, to varying amounts, inhibit both laboratory strains of HIV and clinical isolates. Comparison of these different vectors demonstrate consistently better protection with Rev transdominant vectors than any other vector analyzed *in vitro*. To evaluate these anti-HIV-1 vectors *in vivo* we are proposing a clinical trial in which CD4+ PBL derived from an HIV-1 negative twin will be engineered and then infused into the HIV-1+ twin.

C6-336 PLURIPOTENT CD34+ STEM CELLS ARE NOT SUSCEPTIBLE TO HIV INFECTION, Joseph D. Mosca, Sumesh Kaushal, Tom Davis, Suzanne Gartner, Steve Kessler, Vince LaRussa, Eric Hall, Zhipeng Yu, Dave Ritchey, Jin Xu, Paul Perera, Kelvin Lee, Richard Carroll, Jerome Kim, Dan St. Louis, Carl June and Don Burke, the Henry M. Jackson Foundation for the Advancement of Military Medicine and the Military Medical Consortium for Applied Retroviral Research, 1600 E. Gude Dr., Rockville, MD 20850

Bone marrow-derived CD34+ cells are a population of cells capable of both self-renewal and differentiation into a variety of hematopoietic lineages. In culture, CD34+ cells differentiate to myeloid series into monocyte/macrophages and granulocytes. CD34+ cells have been implicated to be infectible by HIV. However, because CD34+ cells rapidly differentiate in culture, the possibility exists that infection of CD34+ progeny cells may be responsible for the positive HIV signal observed in these cultures. To address this possibility we infected purified CD34+ cells with HIV-1 Ba-L and re-selected them either before or after exposure to HIV. When double-purified CD34+ cells were infected with HIV, a HIV positive signal was observed; but when re-selection was done after HIV exposure, the CD34+ cells were negative for the presence of HIV. We conclude that the differentiated progeny cells and not the CD34+ cells are susceptible to HIV infection and responsible for the positive signal observed in cultures. These findings provide an unambiguous report that human CD34+ stem cells are not infectible with HIV-Bal, agreeing with the majority of results found in HIV-infected patients.

C6-337 **SENDAI VIRUS AS A CANDIDATE OF A NEW TYPE OF VIRAL VECTOR**

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We have developed a fusogenic liposome system using Sendai virus and showed it was an effective tool for *in vivo* gene transfer (ref.1). Now we examine the possibility to use Sendai virus itself as a new type of viral vector. Sendai virus has a lot of attractive characteristics as a viral vector. 1) the viruses are not pathogenic to human beings. 2) there is a mutant strain that infect cultured cells persistently and stably. 3) the viruses replicate and express their genes in cytoplasm without affecting host cell function. 4) the genome structure is simple and most of the genes are monocistronic. 5) negative strand genomic RNA made a complex (nucleocapsid) with proteins, which prevented the recombination of genome between different strain. 6) it is easy to prepare the virus stock with high titer and the virus suspension can be concentrate by centrifugation without loss of activity. 7) The viruses have a broad host range from mice to human beings.

We analyzed the condition that Sendai virus infects cultured cells persistently, using a ts mutant strain and found that virus M protein had a critical role in establishment of persist infection (ref.2). Our data also showed that M protein was not involved in replication or transcription.

Recently we examined the conditions to make recombinant Sendai virus, using mini genome RNA (containing 3' end of virus genome, luciferase cDNA and 5' end of virus genome) as a model. We found we can reconstruct the nucleocapsids from mini genome RNA and nucleocapsid proteins *in vivo* and *in vitro*. These nucleocapsids can express luciferase at least 3 days after transfection with helper virus.

These data encourage us to develop a new type of gene expression vector based on Sendai virus.

Ref. 1. Kato, K. et al. *J.Biol.Chem.* 266, 3361-3364, 1991.
2. Kondo, T. et al. *J.Biol.Chem.* 268, 21924-21930, 1993.

C6-338 THE OCULOCEREBRORENAL SYNDROME GENE ENCODES A PROTEIN THAT LOCALIZES TO THE GOLGI COMPLEX, Robert L. Nussbaum, Isabelle Olivos, and Pasi Janne, National Center for Human Genome Research, NIH, Bethesda MD 20892 and Department of Genetics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

The Oculocerebrorenal Syndrome of Lowe (OCRL) is an X-linked disorder characterized by moderate to severe mental retardation, congenital cataracts, and renal tubular abnormalities. The gene, known as OCRL1, was positionally cloned and found to encode a protein with striking homology to a 75 kd inositol polyphosphate-5-phosphatase. No obvious abnormality in inositol metabolism was detectable in OCRL patients' fibroblasts. Portions of the gene were expressed in E. coli and used to raise polyclonal antisera. The antisera detects a 105 kd protein that is absent in OCRL patients who lack OCRL1 message. Antibody staining of normal human fibroblasts revealed that the OCRL1 gene product localizes to the Golgi, with a pattern that is nearly superimposable on the pattern of staining seen with antibody against ß-COP, a member of the coatomer complex that is formed on non-clathrin coated Golgi vesicles. There is a growing body of evidence linking phospholipid metabolism in general, and inositol signaling in particular, to Golgi function. We hypothesize that OCRL may represent an inborn error of metabolism in some aspect of Golgi vesicle formation and in protein targeting in neural, renal, and lenticular cells.

C6-339 TARGETED INSERTION OF GENES INTO BOTH ALLELES OF A COLLAGEN GENE LOCUS IN THE HUMAN TUMOR CELL LINE HT-1080, Darwin J. Prockop, Corni Strydom, Wouter de Wet, and Arupa Ganguly, Department of Biochemistry and Molecular Biology, Jefferson Medical College, Thomas Jefferson Univ., Phila., PA 19107

We recently made several unexpected observations with a 1.9 kb fragment from the 5'-end of the human gene for the proα1(I) chain of type I procollagen (COL1A1): If the 5'-fragment is linked to the COL2A1 gene for type II procollagen and used to stably transfect a line of human tumor cells (HT-1080), it causes targeted insertion at high frequency of the exogenous gene constructs into both alleles of the endogenous locus of the COL1A1 gene. Moreover, after the targeted insertion, the exogenous gene constructs are expressed at high levels from the previously quiescent COL1A1 locus (Ganguly et al. *PNAS* **91**:7365, 1994). Here, we found targeted insertion of a much smaller hybrid gene composed of the promoter, first exon and most of the first intron of the COL1A1 gene fused to the human growth hormone gene. The COL1A1-hGH hybrid gene of 1.4 kb was linked in a head-to-head orientation to a 4 kb cassette containing a neomycin-resistance gene driven by the mouse metallothionein promoter. About 3% of the G418-resistant clone secreted high levels of the growth hormone. Southern blot analysis of the high secreting clones indicated that a recombination event has disrupted the 5'-end of both alleles of the endogenous COL1A1 gene. None of the constructs employed had homology between the ends of the construct and the targeted locus. Therefore, the mechanisms of the targeted insertion is unclear. The results demonstrate, however, that a 5'-fragment to the COL1A1 gene will drive targeted insertion of unrelated genes of the endogenous COL1A1 locus in HT-1080 cells and produce clones that express relatively high levels of the unrelated gene. Supported in part by NIH Grants AR39740 and AR38188, and grants from the Lucille B. Markey Charitable Trust and the Ernest Oppenheimer Memorial Trust.

C6-340 **GLUTAMATE DECARBOXYLASE EXPRESSION IN THE CNS USING DEFECTIVE HERPES SIMPLEX VIRUS VECTORS**

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Epilepsy, Huntington's chorea, and anxiety involve alterations in GABA metabolism in distinct, localized regions of the nervous system. Consequently, gene transfer to alter levels of GABA synthesis in specific brain regions may lead to methods of gene therapy for these and other neurologic diseases. Glutamic acid decarboxylase (GAD) is the single enzyme in the mammalian brain that converts glutamate to γ-aminobutyric acid (GABA). Mammalian GAD exists as two isoforms of 65 and 67 kD, encoded by different genes.

The amplicon-based defective herpes simplex virus (HSV) vector system offers an efficient method of expressing genes in cells of the central nervous system (CNS). Double-cassette defective HSV vectors, containing separate transcription units for both GAD (either 65 or 67 kD isoforms) and E. coli lacZ, were generated with a conditional-lethal helper HSV. Because GAD is expressed throughout the brain, the presence of the lacZ marker enables us to detect and quantify vector infection *in vivo*.

Defective vectors were used to infect primary cultures of cerebellar granule cells (primarily glutamatergic) and cortical astrocytes and discrete regions of the brain. Infection resulted in the expression of both ß-galactosidase and GAD in each of the cell types in culture. Infected cerebellar granule cells expressed GAD in the cell body as well as their neuronal processes. Expression of GAD led to the synthesis of GABA, both detected by immunohistochemistry.

These vectors should permit us to determine the effects of GABA synthesis on glutamatergic neurons and glia *in vitro*, as well as modulate local concentrations of glutamate and GABA *in vivo*.

C6-341 HIV-1 ENVELOPE-SPECIFIC CD4+ T-LYMPHOCYTE LINES AND CLONES AS TOOLS FOR GENE THERAPY,

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HIV-1 gp160- and gp120-specific CD4+ T lymphocyte lines were developed from 11 HIV-1 seropositive volunteers enrolled in a vaccine therapy trial. 9/20 of the HIV-1 envelope specific T cell lines were challenged with a panel of overlapping peptides spanning the gp120 LAI sequence in order to characterize the epitope responses. The responses were heterogeneous; the most recognized peptides were LAI 74 (aa 74-105) in the C1 region and LAI 306 (aa 306-328) in the V3 region. In order to evaluate the proliferative capabilities to heterogeneous strains of HIV-1, the envelope-specific lines were challenged against a panel of divergent HIV-1 envelopes. 55% of the lines were able to cross-react with gp120MN while only 22% cross-reacted with gp120SF2. Only the gp160 specific lines cross-reacted with gp160CM (Northern Thailand strain). Peptide LAI 74 seems to be the common epitope recognized by the cross-reacting lines. To address the functional properties of these T cell lines, cytotoxicity assays were performed. B-LCL pulsed with envelope-antigen or peptides were killed by 3/3 of the envelope-specific lines tested. This study has demonstrated the feasibility to grow, expand and functionally characterize CD4+ T cell populations from seropositive patients. This offers the potential for future expansion, genetic manipulation, and reinfusion of HIV-specific CD4 cells into patients as a therapeutic modality.

C6-342 NOVEL NON-IONIC BLOCK COPOLYMERS (POLOXAMERS) MEDIATE GENE DELIVERY.

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We have developed synthetic non-ionic block copolymers (poloxamers) that mediate gene delivery *in vitro* and *in vivo*. Poloxamers consist of single polymer chains of hydrophilic polyoxyethylene (POE) groups flanking hydrophobic polyoxypropylene (POP) groups. A series of poloxamers of different lengths and composition were synthesized and tested for gene delivery in *in vitro* assays. We found that poloxamer compounds CRL-1012, 1029 and 1190 (1-20 ug/ml), when mixed with plasmids pSVß and pCMVß (1-2 ug), encoding *E. coli* ß-galactosidase, yielded transfection efficiencies comparable to those obtained by lipofection with commercial reagents such as Lipofectin and DOTAP. Poloxamer-mediated transfections were not inhibited by medium containing serum, while transfections with Lipofectin were greatly inhibited. In addition, no cytotoxic effects were observed with these poloxamers at concentrations up to 100 ug/ml. Since these compounds are relatively non-toxic and mediate DNA transfection *in vitro*, we decided to explore their potential for *in vivo* genetic vaccination. The plasmid pATCMV-gD (50-100 ug) encoding HSV-1 glycoprotein D was mixed with poloxamers (100-750 ug) and inoculated i.p. or i.m. to BALB/c mice. After two inoculations every two weeks, mice were tested for the presence of anti-gD antibodies. It was shown by immunofluorescence and immunoprecipitation that mice inoculated with poloxamer-DNA mixtures produced strong humoral responses against HSV-1 gD in comparison to mice inoculated with naked DNA alone. Further studies will explore the efficacy of poloxamer-DNA vaccinations against HSV-1 infection in a murine model.

C6-343 CELLULAR DELIVERY BY FUSION-ACTIVE VIROSOMES

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Influenza virus, an enveloped virus, utilizes a membrane fusion strategy to deposit its genome into the cytoplasm of host cells. This fusion process occurs from within the endosomal cell compartment after cellular uptake of intact virions through receptor-mediated endocytosis. The virus depends on the mildly acidic conditions within the lumen of the endosomes for activation of its membrane fusion activity. In model systems, the virus can be induced to fuse with a variety of target membranes, including the plasma membrane of cultured cells, by lowering the pH of the medium.

Reconstitution of influenza envelopes can be achieved by solubilization of the viral membrane in an excess of a non-ionic detergent, and subsequent removal of the detergent with a hydrophobic resin. In model systems and with cultured cells, these so-called virosomes exhibit membrane fusion characteristics which are very similar to those of the intact virus.

The use of virosomes for the delivery of water-soluble substances was modeled by investigating the delivery of gelonin, a plant toxin which inactivates ribosomes, but lacks the capacity to penetrate into cells on its own right. By encapsulation in the aqueous interior of virosomes gelonin can be delivered efficiently to the cytoplasm of cells, either via the endocytic route of entry or through induction of virosome fusion with the cell plasma membrane. We have estimated that, through virosome-mediated delivery, several thousands of copies of a foreign molecule can be introduced into a single cell. Currently, we are investigating the potential of influenza virosomes to mediate delivery of encapsulated genes and antisense oligonucleotides to cells.

C6-344 LARGE SCALE PURIFICATION OF ENDOTOXIN-FREE PLASMID DNA FOR GENE THERAPY RESEARCH

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Common components of the cell wall of gram-negative bacteria are the pyrogenic lipopolysaccaride endotoxins. The production of plasmid DNA in such bacteria for therapeutic purposes, as well as for other sensitive biological applications such as transfection and microinjection, means that complete removal of these components is a necessity.

A variety of methods are available for the isolation of plasmid DNA from bacteria which yield DNA of varying qualities. The highest quality is achieved with two rounds of CsCl gradient centrifugation or with anion-exchange chromatography using a unique resin with very high surface charge density (Ehlert et al. BioTechniques (1993) 14, 546; Schleff & Heimann BioTechniques (1993) 14, 544).

For direct delivery of DNA into cells or organisms, however, the DNA should be completely free of toxic agents such as ethidium bromide, phenol, and chloroform, as well.

A procedure for endotoxin-free plasmid preparation avoiding ethidium bromide and phenol /chloroform treatment will be presented together with analysis of the purified DNA by HPLC, electron microscopy and other methods. These data demonstrate that more than 90% of the plasmid DNA is in closed circular supercoiled form and that the preparation is free of proteins, lipopolysaccarides, RNA and single-stranded DNA.

The effect of DNA quality on expression of plasmid DNA directly injected into muscle will be presented. The use of DNA purified by anion-exchange chromatography for treatment of cystic fibrosis patients is an additional demonstration of the quality and safety of DNA obtained by anion-exchange chromatography (Caplen et al. Gene Therapy (1994) 1, 139-147).

C6-345 IN VIVO MODEL FOR ANALYSIS OF EXPRESSION OF RETROVIRAL TRANSDUCED HUMAN CORD BLOOD MYELOID PROGENITORS WITH TREV, AN HIV-1 INHIBITORY GENE, Fernando Serrano, Javier Chinen, Estuardo Aguilar, John W. Belmont, Baylor College of Medicine, Houston, TX.

We have previously described TREV, a fusion gene of transdominant negative mutants of HIV-1 regulatory genes TAT and REV, that *"in vitro"* behaves like a potent HIV-1 replication inhibitor.

We made a high titer ($1X10e^6$) amphotropic TREV retrovirus on the PS_3 vector backbone previously described by us, by electroporating the $GP+AM_{12}$ packaging cell line. High titer clones were selected. Cord blood mononuclear cells were obtained by sedimentation of total cord blood on a percoll gradient. Infection was done by a 6h cocultivation of cord blood cells with an irradiated monolayer of vector producing cells. The efficiency of transduction of CFU-C colonies was measured by immunocytotochemistry for characteristic TREV nucleolar expression or by PCR of single colonies. A median of 20% CFU-C colonies showed TREV expression. Next, high numbers ($20-50X10e^6$) of tranduced cells were injected into irradiated SCID recipients. Animals were sacrificed after 1 to 2 months post transplantation and the extent of human reconstitution analized by semicuantitative PCR and flow cytometry for human CD45. 5-30% of cells in the bone marrow were found to be of human origin. We found presence of the transduced TREV gene in human cells from the SCID bone marrow by immunocytochemistry and TREV PCR.

C6-346 A NEW STRATEGY FOR GENE TRANSFER INTO NON-LYMPHOID CELLS USING HIV VECTORS, Takashi Shimada, Koichi Miyake, Takashi Tohyama, Taketo Igarashi, Eiji Shinya, Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo 113, Japan

HIV based vectors are capable of targeted and highly efficient gene transfer into CD4+ T cells. In addition, HIV vectors have several advantages compared with murine retroviral vectors. Since the HIV-LTR is inactive in target cells lacking TAT, efficient and regulated transcription from the internal promoter can be achieved without promoter interference. Another feature is that unlike the oncoretrovirus, HIV can infect and integrate into non-dividing cells. Therefore, the HIV mediated gene transfer system is potentially useful for development of gene therapy not only for AIDS but also for various genetic diseases. We have developed a new strategy for gene transfer into non-lymphoid cells using HIV vectors. A replication defective adenovirus vector containing the human CD4 gene driven by the CMV/ß-actin hybrid promoter was constructed. Using this adenovirus vector, the CD4 gene was efficiently transferred and expressed in HeLa, K562, and Raji cells. These CD4+ cells could be transduced with a HIV vector containing the neoR gene. Stable integration of the HIV vector was demonstrated by Southern blotting analysis. These results indicate that transient expression of CD4 by an adenovirus vector is sufficient to render non-lymphoid cells susceptible to gene transfer by a HIV vector. Since adenovirus can infect many types of cells including non-dividing cells, the combination of the CD4/adenovirus vector and the HIV vector might be used for stable gene transfer into non-dividing neurons or rarely dividing hematopoietic stem cells.

C6-347 *CATIONIC LIPID-BASED GENE MEDICINES: CHARACTERIZATION AND OPTIMIZATION*, Arun Singhal, Ling Gong and Alain P. Rolland, GENEMEDICINE, INC., 8080 N. Stadium Drive, Ste. 2100, Houston, TX 77054

Numerous studies have reported on the use of cationic lipids to enhance the delivery of DNA expression systems to a variety of cells both *in vitro* and *in vivo*. We have attempted to determine the optimal physical and physicochemical properties of such systems as a means of controlling the *in vivo* biological distribution of DNA expression systems used for gene therapy purposes. We report here on our studies with a number of different classes of cationic lipids, including DOTMA, either alone or in mixture with other lipids and formulation excipients. The physical and physicochemical parameters that have been examined include size, zeta potential, electrophoretic mobility and ultramicroscopic structure. The chemical stabilities of these systems have also been examined to determine optimal storage conditions of lipid-based gene medicines.

C6-348 *BIOPHYSICAL AND CHEMICAL DETERMINANTS OF EFFICIENT GENE DELIVERY BY POLYAMIDOAMINE DENDRIMERS*, Mary Tang, Carl T. Redemann, Francis C. Szoka. Jr., Graduate Group in Bioengineering and School of Pharmacy, University of California, San Francisco, 94143

Starburst© dendrimers are spherical polycations which interact electrostatically with DNA; the resulting complex is highly efficient in transfecting cells in vitro (Haensler, J., Szoka, F.C., Jr., *Bioconjugate Chemistry* 4, 372 (1993)). We have further identified key parameters determining efficient gene delivery, using the expression of transfected ß-galactosidase plasmid in CV-1 cells as the primary assay. The parameters studied are the effects of surface charge density and size and the identity of the functional end-group. Acetic anhydride titration was used to modulate surface charge density. A decrease of 25% in charge density results in a disproportionate decrease in transfection levels, even if DNA is sufficiently charge neutralized and condensed. A decrease of 50% abolishes transfection altogether. The size of the dendrimer is important, probably for geometric fit with the curvature of condensed DNA. Using size exclusion chromatography to estimate molecular weight, larger dendrimers are found to be more effective in transfection; the lower size limit is 20 kD. Heating the dendrimer under rigorously anhydrous conditions improves transfection from undetectable levels to levels rivaling the best liposomal systems. This dehydration side-reaction creates a functional end-group that is responsible for effecting transfection. This end-group is thought to be an imidazoline. Funded by the NIGMS Biotechnology Training Grant and NIH DK46052(FCS).

C6-349 GENE TRANSFER INTO NEONATAL RATS RESULTED IN SUSTAINED EXPRESSION BY HVJ-LIPOSOMES

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Although gene therapy seems to be realm, there are still many problems for human clinical trials. Of particular, transgene expression by almost current gene transfer method is transient. We have developed the highly efficient gene transfer method utilized HVJ (Sendai Virus) and liposomes. With this method we have successfully transfected marker and functional genes into various target organs such as liver, kidney and vessel wall. In these experiments, we have also observed that transgene expressions were transient and sustained up to 14 days. In this study we have focused on the duration of transgene expression. We examined to transfect genes into the liver of neonatal rats with HVJ-liposome method. Here we hypothesized that the transfected gene may be integrated into the host genome because the liver of neonatal rats are growing and continue replicating. Initially, we transfected the rat renin gene into the liver of neonatal rats of 2-day-age. We detected the increase in plasma renin activity (PRA) 4 weeks after transfection. At 6 weeks we could not detect no significant difference in PRA. However, at the same time RT-PCR showed the existence of mRNA transcription in the liver. In the control liver we could not detect at all. Next we examined to transfect the human insulin gene into the liver of neonatal rats of 2-day-age. At 8 weeks after transfection we detected human insulin in rat plasma by radioimmunoassay, which in adult rats we did not. Gene transfer into the liver of naonatal rats resulted in a sustained expression at least 6 weeks, whereas into adult rat liver it resulted in a short-term expression (<2 weeks). These information may be useful for gene therapy not only into the liver but also into other organs in future.

C6-350 EXPRESSION OF THE TYROSINE HYDROXYASE GENE IN A HUMAN FETAL ASTROCYTE CELL LINE: IN VITRO CHARACTERIZATION AND TRANSPLANTATION INTO THE RAT STRIATUM,

Carlo Tornatore[1], Belinda Baker-Cairns[1], Gal Yadid[2], Rebecca Hamilton[1] and Karen Meyers[1]. [1] Laboratory of Molecular Medicine and Neuroscience, NINDS, NIH, Bethesda, MD, 20892, [2] Bar-Ilan University, Ramat-Gan, Israel, 55900.

The grafting of fetal tissue in the treatment of neurodegenerative disorders would be greatly facilitated if a viable human fetal cell line could be substituted for primary fetal tissue. A permanent, immortalized human fetal astrocyte cell line (SVG) has been established (Major E. et. al PNAS 1985) which is anchorage dependent for growth, does not form tumors in nude mice and maintains characteristics of fetal astrocytes such as expression of the intermediated filaments vimentin and GFAP as well as MHC class 1. The cDNA for human tyrosine hydroxylase, type 2 was cloned downstream from the immediate early promoter of CMV and stably transfected into the SVG cell line, establishing a second cell line, SVG-TH. These cells have continuously expressed TH for the past eighteen months, with no appreciable change over time. HPLC analysis of the supernatant from these cells consistently found 4-6 pmol/ml/min of l-dopa produced, but only if BH_4 was added to the media. Unexpectedly, the SVG-TH were also found to secrete serotonin, which was not found in the parent SVG cells. To determine the viability of these cells in vivo, SVG-TH cells were grafted into the normal striatum of Sprague-Dawley Rats and followed over time. A panel of antibodies were used to unequivocally differentiate the engrafted cells from the host parenchyma, including antibodies to: SV40 large T antigen (expressed in the SVG-TH cells), human and rat MHC class 1, vimentin, GFAP, serotonin and tyrosine hydroxylase. While the graft was easily identified with the first week, over the course of a four week period of time the engrafted cells decreased in number. Concomittantly, rat CD4 and CD8 expression in the vicinity of the graft increased, consistent with xenograft rejection. When the SVG-TH cells were grafted to the lesioned striatum of 6-OHdopamine treated rats, rotational behavior of the rat decreased by 50% initially, then slowly returned to baseline over the next four weeks, parallelling graft rejection. Studies using cyclosporine immunosuppression of the rats are now being initiated to determine whether functional graft survival can be extended. The potential of these cells in the treatment of human neurodegenerative diseases is discussed.

C6-351 TARGETED GENE TRANSFER INTO HUMAN CD4+ CELL LINES USING MoMLV BASED, CD4-SPECIFIC PACKAGING CELL LINES, A. Umthun, T. Smith, A. Dietz, E. Kolb, G. Brockman, and E. Beecham, Human Gene Therapy Research Institute, Des Moines, IA 50309

Targeted delivery of genes to specific subpopulations of cells is essential to the development of gene therapy protocols to treat disseminated diseases. In order to develop a targetable gene delivery system for HIV infection we have constructed several MoMLV based packaging cell lines that are designed to specifically target human CD4+ cells. Several different vectors that contain subcloned portions of the HIV gp 160 envelope protein were transfected into MoMLV ecotropic Neo^r producer cell lines. Assays on the target cell specificity of these packaging lines has shown efficient and specific gene transfer into human CD4+ target cells (CEM-A). As expected, these packaging cells are also able to transfer genes into mouse cell lines (205, NIH 3T3). Some of these targeting vector producer cell lines (TVPC) also show low levels of gene transfer into CD4- cells. Further, we have found that these TVPC lines are capable of delivering genes to human CD4+ cells *in vivo*. In these experiments, 10^4, $5x10^4$, 10^5, or $5x10^5$ TVPC carrying a Neo^r transfer vector were mixed with 10^7 human CEM-A no vector cells and injected subcutaneously into nude mice. Three weeks later the CEM-A tumor cells were removed from the mice and cultured under G418 selection. Numerous Neo+ CEM-A colonies were detected in cells isolated from mice in each mixing ratio. We are currently conducting a variety of qualitative and quantitative assays to fully characterize and compare different TVPC's. We are also constructing chimeric MoMuLV based packing cell lines in which the MLV envelope has been deleted and replaced with the HIV-1 envelope and are testing these TVPCs for gene transfer.

C6-352 AUTOCRINE INTERFERON BETA SYNTHESIS ENHANCES THE RESISTANCE OF PRIMARY LYMPHOCYTES TO HIV INFECTION.

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We are developing a somatic cell gene therapy against infection with the human immunodeficiency virus (HIV), by enhancing the antiviral resistance of target cells through the constitutive production of interferon beta (IFN-β). Previous work has shown that stable antiviral expression can be obtained in human lymphocytic CEM cells transformed by the HMB-K^bHuIFNβ retroviral vector, carrying the human IFN-β coding sequence under the control of a fragment of the murine H-$2K^b$ gene promoter. The thus IFN-β-transformed CEM population displayed an enhanced resistance to HIV infection, partly due to a blocking effect of IFN-β on virus entry (1).

We have transformed peripheral blood lymphocytes (PBLs) as well as purified CD4+ T cells, from healthy seronegative donors, with the HMB-K^bHuIFNβ retroviral vector, and have obtained a transformation efficacy of about 50%. Analysis of the replication rate has revealed no difference between untransformed and IFN-β-transformed populations. We have then followed, for up to 20 days after HIV infection, the antiviral resistance of IFN-β-transformed PBLs and CD4+ T cells. We have observed an important reduction of reverse transcriptase activity in the culture medium of IFN-β-transformed populations, as compared to untransformed populations, and the cellular viability of such transformed cells was comparable to that of uninfected cells. In addition, a FACS analysis performed 3 weeks after HIV infection, revealed that the CD4/CD8 ratio of IFN-β-transformed PBLs was not modified after infection, reflecting a protection of CD4+ T cells.

We are presently analyzing the effects of IFN-β expression on the differentiation and the proliferation of human hematopoietic progenitors. First results indicate that transformation with HMB-K^bHuIFNβ results in 5 to 10 % PCR-positive GM-CFC and BFU-E colonies, without impeding their hematopoietic differentiation program.

(1) Vieillard *et al.*, Proc. Natl. Acad. Sci. USA, **91**, 2689-93 (1994).

C6-353 **OPTIMIZED GENE TRANSFER INTO CYSTIC FIBROSIS AIRWAY EPITHELIAL CELLS USING NOVEL AND IMPROVED CATIONIC LIPIDS.**

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Cationic lipids are able to mediate transfection of a variety of different cell types, are simple to prepare, may have favorable immunogenicity and toxicity profiles, and are able to accommodate and deliver genes of unlimited size. However, the efficiency of gene transfer is less than for viral-based approaches. To address this limitation, we have systematically evaluated several factors that may affect the efficiency of cationic lipid-mediated gene delivery. Using a high-throughput 96 well-based cytofection assay and the CF immortalized airway cell line CFT-1, we have evaluated over 50 novel cationic lipids for their ability to mediate high efficiency gene transfection *in vitro*. Several new cationic lipids and formulations with improved transfection efficiencies (greater than 5-fold over DMRIE or DC-Chol) were identified. Numerous eukaryotic expression vectors containing a variety of different heterologous introns, promoters, enhancers, polyadenylation signals, and other regulatory elements were constructed. A vector (pCMVHI-CAT) containing the CMV enhancer/promoter, a hybrid intron, and the SV40 polyadenylation sequence was found to express high levels of CAT in airway cells both *in vitro* and *in vivo*. Using the optimal conditions determined, transfection of greater than 80% of airway cells *in vitro* could be achieved. Transfection of CF airway epithelial cells using a CFTR-encoding plasmid restored cAMP-stimulated chloride channel activity as assessed using the SPQ assay and corrected the cAMP-mediated chloride current using the Ussing chamber assay. To address the efficacy of these formulations *in vivo*, lipid:DNA complexes were also instilled transtracheally into the lungs of BALB/c mice. Using a formulation composed of lipid #53 and pCMVHI-CAT, expression of up to 100 ng of CAT enzyme per mouse lung was achieved. These levels are 90 fold over what could be attained using free DNA alone. Although expression decreased markedly after a few days, CAT activity could be detected up to 14 days post-instillation. These results suggest that with further optimization, cationic lipid-mediated gene transfer may be clinically efficacious for the treatment of cystic fibrosis.

C6-354 **EXPRESSION OF FUSION PROTEINS BETWEEN CD4 AND LYSOSOME TARGETING DOMAINS INHIBITS HIV-1 REPLICATION IN HUMAN T LYMPHOCYTES.**

Chen Zhou*, Sui Fang Wen*, Xinli Lin, Karen Kearns*, Jordan Tang, and Donald B. Kohn*. * Division of Research Immunology /BMT, Childrens Hospital of Los Angeles, California. Protein Studies Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma.

Fusion proteins consisting of soluble CD4 and lysosome targeting domains, from Lamp1, Lamp2, lysosome acid phosphatase and procathepsin D, have been shown to divert the gp160 of HIV-1 to the lysosome and abolish syncytium formation. In order to develop the strategy for AIDS gene therapy, four retroviral vectors (L-L1-SN, L-L2-SN, L-HAP-SN and L-PCaD-SN) have been constructed using MoMuLV LTR to express the fusion proteins: sCD4-Lamp1, sCD4-Lamp2, sCD4-HAP and sCD4-PCaD. The vectors were transfected into amphotropic packaging cell line PA317. G418 resistent pool were used to transduce the human T cell line CEM cells by co-cultivation. RT-PCR analysis of transduced CEM cells demonstrated that the fusion protein genes are expressed in cells. However, the steady state levels of the fusion proteins were below the limits of detection. HIV-1 challenge experiments were performed to check whether the expression of the fusion proteins can inhibit virus replication and prevent virus from spreading among cells. Separate sets of transduced CEM cell pools were prepared and two different procedures were carried out to see the time course and dose dependance of virus infection. It was found that CEM cells transduced with the fusion genes showed delayed virus outgrowth compared to the parental CEM cells. CEM cells transduced with L-L1-SN showed the highest inhibition of HIV-1 replication by about 90%. In dose response experiments, L-L1-SN transduced cells again showed the highest inhibition of HIV-1 replication by 80-90% with an MOI of 0.04. Using supernatant collected on 7 days after infection to measure HIV produced, the supernatant from fusion gene transduced CEM cells showed 5-10 times lower TCID50 than from parent CEM cells. Immunostaining of HIV-1 infected cells showed that less HIV-1 envelope proteins are expressed on the surface of cells transduced with sCD4-lamp1 and sCD4-lamp2 fusion genes though the expression of gag proteins was not affected. Our work suggests that the fusion proteins can divert the gp160 to lysosomes to be degraded and prevent virus from spreading among cells by blocking the formation of infectious virus. This strategy may be useful for AIDS gene therapy.

Recombinant Viral Vector Systems

C6-400 ENHANCED RETROVIRAL TRANSDUCTION INTO HUMAN HEMATOPOIETIC PROGENITOR CELLS USING GIBBON APE LEUKEMIA VIRUS PSEUDOTYPED RETROVIRAL VECTORS AND FETAL LIVER STROMA.

Raphael G. Amado, Joyce Tan, J.P. Morgan, and Joseph D. Rosenblatt. Division of Hematology-Oncology, Department of Medicine and UCLA AIDS Institute, Los Angeles, CA 90024.

Gene therapy approaches of genetic and acquired diseases affecting hematopoietic cells require effective and stable means of gene transfer into pluripotent precursors. Infection of stem cells by retroviral vectors has been achieved with low efficiency in primates and humans using amphotropic envelopes. We isolated CD34+ cells from peripheral blood apheresis products and bone marrow, as well as Long Term Culture Initiating Cells (LTCIC) from long term bone marrow cultures. These hematopoietic progenitors were transduced using either the amphotropic LNL6 retroviral vector or a modified MoMLV based vector pseudotyped with Gibbon Ape Leukemia Virus (GaLV) envelope. We found that the use of GaLV pseudotyped vectors markedly enhanced transduction of CD34+ cells and LTCICs as measured by quantitative DNA PCR for Neo^R gene and by clonogenic assay in the presence of G418. Transduction efficiencies of 30 to greater than 80% were achieved in purified CD34+ cells and in LTCICs. Since the liver is the primary hematopoietic organ between the third and sixth months of fetal life, we hypothesized that the use of fetal liver stroma would enhance retroviral infection by inducing cell replication. Infections were carried out in the presence and absence of a fetal liver stroma cell culture. Up to a 10 fold improvement in retroviral transduction was observed with either vector when fetal liver stroma was used, even in the absence of exogenous hematopoietic growth factors. Incubation of CD34+ cells with irradiated fetal liver stroma did not adversely affect stem cell differentiation. GaLV pseudotyped vectors, in combination with fetal liver stroma provide an improved method of retroviral mediated gene transfer and may allow for more efficient hematopoietic reconstitution with transduced cells.

C6-401 DEVELOPMENT OF RETROVIRAL VECTORS FOR USE IN GENE THERAPY OF LEUKOCYTE ADHERENCE DEFICIENCY, Thomas R. Bauer, Jr., A. Dusty Miller, and Dennis D. Hickstein, Medical Research Service, Seattle Veterans Affairs Medical Center, Seattle, WA 98108, Program in Molecular Medicine, Fred Hutchinson Cancer Research Center, Seattle, WA, 98104 and Department of Medicine, University of Washington School of Medicine, Seattle, WA 98195

Leukocyte adherence deficiency or LAD, an inherited immunodeficiency disease, is due to defects in the CD18 subunit, and transduction of the CD18 subunit into hematopoietic cells from children with LAD represents a potential therapy for this disorder. To improve transduction efficiency and expression of the CD18 subunit, we constructed the retroviral vectors LgCD18SN and LgCD18, which contain modified tRNA binding sites, and produced these vectors by using the amphotropic packaging cell line PA317 or the gibbon ape leukemia virus packaging cell line PG13. By infecting K562 / CD11b cells (containing CD11b on an episomal vector) over a three day period with the LgCD18SN retroviral vector produced by the PG13 cells, 24% of the K562 cells expressed surface CD11b/CD18 and 20% of the LAD EBV B-cells expressed surface CD11a/CD18 by FACS analysis. In contrast, by transducing cells with the retroviral vector produced by using PA317 producer cells, less than 2% of the K562 cells and less than 4% of the LAD EBV B-cells expressed the CD11 / CD18 heterodimer on the cell surface. The increase in transduction with PG13 vectors correlated with Northern blotting and RT-PCR studies which indicated that both K562 cells and the LAD EBV B-cells express transcripts for the gibbon ape leukemia virus receptor at higher levels than for the amphotropic virus receptor. A study using the PG13 / LgCD18SN retroviral vector to transduce primary human bone marrow cells also showed a high transduction efficiency as assayed by CFU-GM assays. These findings indicate that the transduction efficiency of retroviral packaging cell lines correlates with receptor gene expression in the target cells, and that the retroviral vectors described here may be efficacious for gene therapy for LAD.

C6-402 **A NOVEL ADENOVIRAL GENE TRANSFER VECTOR THAT MAXIMIZES OPTIONS FOR GENE INSERTION AND CONTROL OF RECOMBINANT GENE EXPRESSION.** Michael A. Blazing, Keith M. Channon, Kevin E. Potts, & Samuel E. George, Depts. of Medicine and Pharmacology, Duke University Medical Center, Durham NC 27710.

We have developed a novel replication deficient adenoviral gene transfer vector derived from the mutant type 5 adenovirus strain HIn340. Construction of an E1 gene insertion site free of any viral DNA regulatory elements is the key advantage of using this strain. To increase the vector's E1 cloning capacity, we deleted the nonessential E3 region and inserted a unique restriction site. Thus, this modified virus, In340-E3D, has the capability to accommadate a marker gene in the E3 region in series with a gene of interest cloned into the E1 region.

To facilitate generation of recombinant virions and cloning, we modified a viral recombination plasmid as follows: 1) we placed three unique restriction sites next to the recombination sequence to provide an option to use ligation or recombination for E1 gene insertion; 2) we placed an insert into the plasmid which allows promoters and cDNAs to be individually cloned into and out of the vector in a convenient cassette format.

We inserted a CMV promoter and a nuclear localizing ß-galactosidase gene (for blue/white screening) into this plasmid and used recombination with the In340-E3D virus in 293 cells to generate the replication deficient vector, In340-E3D-Gal. We and others have used this vector to generate a substantial number of new recombinant virions, including a nitric oxide synthase adenovirus that shows excellent expression characteristics (see accompanying abstract).

In conclusion, we have developed a novel adenoviral vector with the following properties: 1) an E1 gene insertion site free of viral regulatory elements; 2) a cloning capacity of 8000 bp; 3) gene insertion by recombination or ligation into the E1 or E3 region; and 4) blue /white screening for non-recombinant virions. Additional modifications are in progress to further enhance the utility of this system.

C6-403 OPTIMIZATION OF TRANSDUCTION EFFICIENCY OF HUMAN CD4+ PERIPHERAL BLOOD LYMPHOCYTES, Bruce A. Bunnell and Richard A. Morgan, Clinical Gene Therapy Branch, NCHGR, NIH Bethesda, MD 20892

Gene transfer into lymphocytes is being tested as a method of treatment for both genetic and acquired viral diseases. For example, HIV-1 predominantly infects cells of lymphoid and myeloid origin, such as CD4+ lymphocytes and monocyte/macrophages. Thus, gene therapy approaches targeted to the CD4+ cells may prove to be beneficial at inhibiting replication of the HIV-1. CD4+ cells are well-suited for use in gene therapy because they are easily obtained, and the transduced cells can be selected and expanded in tissue culture. Despite the obvious advantages of the use of CD4+ lymphocytes for gene therapy, the transduction efficiency of the cells with amphotropic retroviruses using producer cell supernatant remains low, approaching 10% under the best of conditions. In an attempt to improve transduction conditions of human CD4+ peripheral blood lymphocytes, the tissue culture and transduction conditions were optimized. To this end, transduction marking experiments were performed using two types of retroviruses, murine amphotropic (ampho) and gibbon ape leukemia virus (GALV). Under standard transduction conditions, the GALV producer transduced cells with 2 to 5 fold greater efficiency than ampho, approaching 15%. Alternative transduction conditions were investigated including centrifugation of the cells at 2500 rpm at 32°C followed by incubation at 32°C during the transduction. Under these conditions the transduction efficiency with both types of retroviral supernatants dramatically improved, with the ampho approaching 15% and the GALV approaching 25%. It has recently been described that the gibbon ape leukemia virus and murine amphotropic receptors are inducible sodium dependent phosphate symporters(Kavanaugh, M.P., et al, 1994, PNAS, 7071). Exposure of the CD4+ lymphocytes to phosphate-free RPMI 1640 for 12 hours prior to transduction with the retroviral supernatant dramatically increased the transduction efficiency, with both retrovirus types approaching 40-50% transduction efficiency. The transduction efficiencies can be achieved repeatedly with different donors. The optimized transduction conditions may obviate the requirement for selection following the introduction of anti-HIV-1 gene therapy vector into the cells. This potential is currently being investigated *in vitro*.

C6-404 **ADENOVIRAL GENE TRANSFER OF THE BRAIN ISOFORM OF NITRIC OXIDE SYNTHASE TO VASCULAR SMOOTH MUSCLE CELLS.** Keith M. Channon, Michael A. Blazing, Kevin E. Potts, Samuel E. George. Division of Cardiology, Duke University Medical Center, Durham NC 27710.

The free radical nitric oxide (NO) has key regulatory roles in the cardiovascular system. The nitric oxide synthase (NOS) isoforms have therefore been targeted as potential therapeutic agents for gene transfer. To investigate this potential we have constructed a recombinant adenoviral vector containing the brain isoform of NOS (bNOS). The rat bNOS gene was cloned into a plasmid that enabled direct ligation with the 'body' of a novel ß-galactosidase containing adenoviral vector (Ad:PacßGal) which was developed in this laboratory. The recombinant virus (Ad.bNOS) was recovered and plaque purified on 293 cells.

Using immunoblotting, we have demonsrated bNOS protein expression in a variety of cell types following Ad.bNOS infection, including 293 cells, an endothelial cell line, the A10 embryonic rat smooth muscle cell (SMC) line, and human, rabbit and pig primary SMC cultures. In addition, we have shown that the expressed protein has NOS enzyme activity in cultured SMC, by measuring the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline by 2'-5' ADP-binding fractions of cell lysates. The NOS activity was greatly increased following Ad-bNOS infection compared with Ad:PacßGal infection. NOS activity was strictly calcium-calmodulin dependent. In addition, we showed specificity of NOS activity by demonstrating augmentation of activity by the tetrahydrobiopterin precursor, L-sepiapterin (L-sep, 100µM) and by inhibition of activity in the presence of N-methyl-L-arginine (NMA,1mM), a specific NOS inhibitor.

In conclusion, we have generated a recombinant adenovirus containing a bNOS gene. We have demonstated both protein expression and NO production in cells infected wtih this virus. This virus wll have broad applications for *in vitro* and *in vivo* assessment of the biological effects of NO production and has potential as a therapeutic modality.

C6-405 **DEVELOPMENT OF NEW SAFER RETROVIRUS PACKAGING SEQUENCES AND CELL LINES,**
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The development of helper-free retrovirus packaging cell lines was a major advance that made gene therapy as well as reliable analysis of hematopoietic lineages feasible. One of the concerns in using helper-free retrovirus as a vehicle for gene-delivery has been the generation of actively replicating wild-type retrovirus during the process of retrovirus production. The second generation retrovirus packaging cell lines eliminated most of the problems. Because of the widespread clinical application of the retrovirus vector systems anticipated in the future, however, we have undertaken to further improve such lines from a safety standpoint. Towards this end, we constructed new retrovirus packaging sequences with altered codon usage to minimize the recombination event between the packaging sequences and the recombinant retrovirus vector sequences. In addition, we developed a new nonretroviral mammalian cell expression vector that is suitable for stably expressing the retrovirus packaging sequences.

C6-406 A PACKAGING CELL LINE TO GENERATE HIGH TITER RECOMBINANT RETROVIRUS OF VARYING PSEUDOTYPES, Jennifer L. Davis, Paul R. Gross, and Olivier Danos, Vector Biology Department, Somatix Therapy Corp., Alameda, CA 94501

Classical cell lines for packaging recombinant retroviral vectors have typically been based on the murine NIH3T3 cell line and constitutively express the Moloney murine leukemia virus *gagpol* and amphotropic *env* genes. Although recombinant viruses derived from these cell lines are able to transduce a variety of human cell types *ex vivo*, vectors pseudotyped with envelopes from other viruses or hybrid envelopes may improve the efficiency of gene transfer into human cells that remain refractory to transduction, such as bone marrow stem cells, and may impart to the recombinant viruses other desirable properties such as increased particle stability or targeting to specific cellular receptors. In addition, NIH3T3 cells may not be the ideal host cell because of safety concerns and other issues related to *in vivo* gene delivery. Thus, we have stably introduced the pCRIPenv- plasmid into the human embryonic kidney 293 cell line. This plasmid is one of the constructs used to generate the NIH3T3 based CRIP packaging cell line and expresses gagpol from the MoMLV LTR. Reverse transcriptase levels produced by the 293 line are at least as high as those obtained from the CRIP line. An MFGlacZ retroviral vector was stably introduced into the 293gagpol line and used to generate high titer virus of various pseudotypes (in particular, the Gibbon Ape Leukemia Virus envelope, Vesicular Stomatitis Virus G protein, and MLV xenotropic and amphotropic envelopes) by transient transfection of env expression plasmids. The 293gagpol line was also used for the construction of stable packaging lines. These lines yielded viral titers following transient transfection of retroviral expression vectors at least as high as, and, depending on the retroviral expression plasmid employed,10 - 30 fold higher than those obtained from stable CRIP lines as measured by transgene expression in transduced cells. The results of these studies and those with various pseudotyped vectors will be presented.

C6-407 DIFFERENTIAL SENSITIVITY OF RETROVECTORS TO HUMAN SERUM INACTIVATION, N. DePolo, C. DeJesus, J. Respess, S. Chang, D. Jolly, and S. Mento, Viagene, Inc., 11055 Roselle Street, San Diego, CA 92121
A number of studies (e.g. 1,2) originally reported that sera from primates, but not that from a variety of lower mammals or birds, inactivates retroviruses by an antibody independent complement lysis mechanism. This activity is non-selective for a variety of distantly related retroviruses, including MoMLV. Retroviruses of avian, murine, feline, and simian origin were all inactivated and lysed by normal human serum. Similarly, in a recent study by Cornetta et. al. (3), the in vivo fate of amphotropic murine leukemia retrovirus was studied in rhesus monkeys, and extremely rapid clearance following i.v. injection was found. Based on parallel in vitro serum inactivation results, this clearance is likely to be caused partially or predominantly by a heat labile serum component, presumably complement. This effect is a potential barrier to gene transfer by direct injection of retroviral vector into humans or other primates.
Beta-galactosidase expressing retrovectors with a variety of envelope tropisms have been tested for sensitivity to human sera inactivations. Factors affecting the sensitivity of these retrovector preparations to human complement will be discussed.

C6-408 **IN VIVO VALIDATION OF A CANDIDATE GENE FOR THE GENOTHERAPY OF ATHEROSCLEROSIS**
Patrice Denèfle, Florence Emmanuel, Céline Viglietta°, Florence Attenot, Isabelle Magraner, Louis Marie Houbebine° and Nicolas Duverger Rhône-Poulenc-Rorer S.A., Atherosclerosis Department, Vitry. °INRA, Jouy en Josas. FRANCE.

Apolipoprotein (apo) A-I and high density lipoproteins-cholesterol (HDL-C) levels correlate inversely with the risk for coronary heart disease. In order to study the benefic effet of apoA-I gene overexpression in the protection against atherosclerosis in an animal model which develop human type lesions, a 12.5 kb DNA fragment containing the human apoA-I gene was used to generate six transgenic rabbits. Human plasma apoA-I levels ranged from 8 to 100 mg/dl whereas rabbit apoA-I levels were reduced in all transgenic rabbits. Total and HDL-C levels were dramatically increased in 3 months-old transgenic rabbits compared to controls. Highest human apoA-I expressors had very high HDL-C plasma levels and human apoA-I levels in transgenic rabbits were strongly correlated to HDL-C concentrations, indicating that human apoA-I overexpression in the rabbit results in a non atherogenic lipoprotein profiles. In addition, the overexpression of human apoA-I was assayed in Watanabe (LDL-receptor deficient) rabbits, an animal model which correspond to the Familial Hypercholesterolemia (FH) in humans. FH patients and Watanabe rabbit have high levels of non-HDL-C and very low HDL-C and apoA-I levels. ApoA-I transgenic rabbits were matted with Watanabe rabbits. Offsprings expressing human apoA-I have a very high human apoA-I (>100 mg/dL) and HDL-C plasma levels in comparison with those which do not expressed human apoA-I. The (non-HDL-C)/HDL-C ratio, considered as a risk factor is 25 fold less elevated in offsprings expressing human transgene than that of controls.
These data show that i) it is possible to restore high levels of apoA-I and HDL-C in LDL-deficient rabbits, which may contribute to a decrease of atherosclerosis development and, ii) that apoA-I may be considered as a good target of gene therapy for atherosclerosis treatment.

C6-409 PCR CLONING AND EXPRESSION OF THE HUMAN AMPHOTROPIC RETROVIRUS RECEPTOR

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The use of moloney retroviral based gene delivery systems has dominated the field of gene therapy for the last few years. However, many cell and tissue types remain difficult to transduce regardless of retrovirus titer. One factor which limits the entry of the retrovirus to the target cell is a specific cell surface receptor recognized and bound by the envelope protein. We are interested in increasing the efficiency of retroviral gene delivery by introducing the human amphotropic receptor (HAR) into cells normally difficult to transduce. The presence of the HAR correlates to the MMLV transduction of human and non-human cell lines as demonstrated with interference assays and somatic cell hybrid analysis. It has been shown that the expression of a cloned HAR can dramatically increase transduction efficiencies for moloney retrovirus to particular recalcitrant cell lines such as CHO.[1] The sequence for the HAR (GLVR-2) has recently become available. We cloned the HAR by PCR amplifying DNA derived from a Hela cell Uni-ZAP cDNA library (Stratagene). Primers were designed flanking the published coding region and used to amplify a 2.1kb fragment. The PCR product was directly cloned into a CMV driven expression vector, PCRIII (Invitrogen), or cloned after gel purification into a sequencing vector, PCRII (Invitrogen). Amplified HAR clones were confirmed by automated sequence analysis. HAR was transfected with cationic liposomes (DOTAP, Boeringer Mannhiem) and transiently expressed in CHO cells. Cells were incubated with β-gal retroviral supernatants. CHO cells transfected with HAR were transduced at a high level compared to cells not transfected with HAR. The HAR has been subcloned into an EBNA based expression vector and is being investigated to determine its ability to increase transduction efficiency in cell lines that are difficult to transduce. We are also determining if a correlation exists between transduction efficiency and HAR expression.

[1]PNAS vol.91 pp.1168-1172 (1994)

C6-410 ANALYSIS OF REPEATED GENE DELIVERY INTO ANIMAL LUNGS WITH RECOMBINANT ADENOVIRAL VECTOR, Jian-yun Dong*, Danher Wang*, Frits VanGinkle∞, David Pascrual∞ and Raymond A. Frizzell§, and Y. W. Kan*, *Department of Laboratory Medicine, University of California, San Francisco, San Francisco, CA 94143-0134 ∞Department of Microbiology, §Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, AL 35294

Adenovirus-based vectors are promising candidates for gene therapy of cystic fibrosis or lung cancers as adenoviruses naturally infect airway cells, grow to very high titers, and the transgene carried by the adenovirus is expressed at a high level. In addition, adenoviruses are relatively safe since the disease caused by wild type virus is self limiting. One disadvantage of adenovirus vectors is that the gene expression may be transient as adenoviruses do not integrate their DNA into the genome of the host cells. Delivery of the CFTR gene into the lungs is also complicated by the anatomy of the airways and the defense mechanisms of the recipient. To evaluate the feasibility of adenovirus-mediated gene therapy for cystic fibrosis a recombinant adenovirus carrying a lac-Z gene was delivered into animal lungs to study the efficiency and cellular distribution of gene transfer, the duration of gene expression, the possible pathology of the lungs after gene transfer and the efficacy of repeated administrations of the viral agent. The results of these studies indicated that (1) efficient gene transfer into animal lungs can be achieved, (2) a physically even distribution of the vectors can be achieved by airway instillation, however, the distribution of gene transfer is uneven among individual animals, (3) the pathological effects are generally mild, (4) gene expression is transient, and (5) repetitive gene transfer is achievable, but progressively less efficient, (6) repeated administering adenoviral vector through the airway provoked both humoral and cellular immune response that is specific to the adenovirus.

++ This work is supported by a grant from NIDDK/NIH (RO1DK-HL46177)

C6-411 RETROVIRAL MEDIATED GENE TRANSFER AND EXPRESSION IN MOBILIZED PERIPHERAL BLOOD MYELOID PROGENITORS. D. Douer, L. Ramezani*, A.F. Mohrbacher, W.F. Anderson. Division of Hematology, and Gene Therapy Laboratories, University of Southern California, Los Angeles, CA.

Peripheral blood progenitor cells (PBPC) can be expanded in-vivo by cytotoxic and/or growth factor treatment, and are now widely used to restore blood cell formation after myeloablative therapy instead of bone marrow (BM) cells. We wish to report the results of preclinical experiments using mobilized PBPC as potential targets for ex-vivo retroviral mediated gene transfer (RMGT). Samples were obtained from 7 leukapheresis products of 6 cancer patients (2-Breast, 3-lymphoma, 1-ALL). Apheresis was done during recovery from chemotherapy induced leukopenia (4 high dose cyclophosphamide 4-6 gm^2, 1 MINE, 1 CAF) while receiving GM-CSF 250 ug/m^2/day. Mean (±SD) white cell count on days of apheresis was $11.2\pm8.8x10^9/\ell$ (range 3.1-30.1x10^9). CD34+ cells were enriched over the CellPro avidin-biotin column and incubated with 10ngIL3+50ngIL6 plus 10% autologous plasma for 42 hrs, washed and incubated again for 6 hrs. with IL3+IL6 plus 10% autologous plasma and the retroviral vector carrying Neo^R gene (LNL6 or G1Na). The 48 hr incubation did not reduce CFU-GM colony growth (Mean CFU-GM colonies 117±29% of preincubation level). Neo^R containing cells were detected by PCR in 5 of 7 samples, and only in those mobilized by high dose cyclophosphamide. Neo^R gene was detected by PCR in 0,0,20,53,60% of individually picked CFU-GM colonies, from 5 samples grown in medium without G418. Gene expression in CFU-GM colonies, as identified by their ability to grow in medium with 1.2mg/ml G418, was found in 4 of 7 samples. Expression efficacy in the samples was 1,5, 12, 28% of the total CFU-GM. CD34+ enriched cells from 8 BM samples harvested without mobilization, were identically transduced for comparison. In 7 samples PCR detected the Neo^R gene. The gene was PCR detected in 0,0,0,13,33% of individually picked CFU-GM colonies and expressed in 1,1,3,5,0,0,26% of the colonies. Mobilized myeloid progenitors from the blood can be genetically modified by retroviral vectors at an efficacy that is comparable or possibly higher than unmobilized BM cells. Mobilized PBPC are a potential source for introducing exogenous genes into the myeloid lineage.

C6-412 **MURINE MODEL FOR GENETIC MANIPULATION OF THE T CELL COMPARTMENT**, Joseph P. Dougherty, Ming-Ling Kuo, Natalie Sutkowski, Jie Gu, and Yacov Ron, Department of Molecular Genetics and Microbiology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, NJ 08854; and the Graduate Program in Microbiology and Molecular Genetics, Rutgers University, New Brunswick, NJ 08903

The expression of exogenous genes in long-lived primary T cells is potentially beneficial for the treatment of various diseases including cancer, AIDS, genetic defects of the lymphoid compartment, and systemic enzyme deficiencies such as hemophilia. One approach for genetic modification of T cells is to introduce therapeutic genes into hematopoietic stem cells that would give rise to cells of the lymphoid lineage. However, efficient gene transfer and expression in stem cells is often problematic. A more direct approach is to introduce the genes into mature primary T lymphocytes since the transferred genes can be maintained and expressed for long periods by long-lived peripheral T cells. In this report, we describe the adoptive transfer into SCID mice of both murine and human primary T cells that have been efficiently transduced with exogenous genes. Primary murine T cells transduced with a retroviral vector containing the human adenosine deaminase (*ADA*) gene persisted for at least five months in lymphoid organs of SCID mice, continuously expressing the exogenous gene. Primary human T cells were also used as target cells for transfer of the β-galactosidase (*lacZ*) gene. Expression of the *lacZ* gene could be detected in over 20% of the transduced primary T cells before adoptive transfer into SCID mice and 6 weeks post-transfer in the peritoneal of recipient animals. Transduced human T cells were injected into SCID mice *i.p*, and the β-galactosidase activity could be detected in cells collected from peritoneal exudate washes of recipient mice 6 weeks post-injection. These results demonstrate the availability of a murine model in which the long-term effects of expression of exogenous genes in both murine and human T cells can be tested.

C6-413 POLYNUCLEOTIDE BASED GENE TRANSFER VECTOR DERIVED FROM AN ALPHAVIRUS

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A new physical gene transfer system based on Sindbis virus has been developed to enhance the utility of alphavirus based vectors for application to human gene therapy. The system involves the conversion of Sindbis virus-based vector RNA into a two-tiered DNA expression system. The first layer includes a eukaryotic expression cassette controlling the expression of a second layer, which corresponds to the Sindbis RNA vector. The Sindbis vector component retains the viral non structural genes and sequences required in cis for RNA replication and the initiation of RNA transcription at the internal viral junction region. Transcription of the heterologous gene is driven off the promoter at the viral junction region. Following introduction of the vector to cells in its DNA format, sense RNA is transcribed from the DNA layer in the nucleus and then transported to the cytoplasm using host cell gene products. Translation of this RNA into the viral RNA-dependent RNA polymerase proceeds in the cytoplasm. This polymerase preferentially transcribes sense RNA from the internal junction region promoter resulting in autocatalytic amplification of the mRNA coding for the heterologous gene. Heterologous protein expression in this system is several orders of magnitude higher than with conventional systems. This system represents the first use of an alphavirus autocatalytic amplification vector for the delivery of heterologous genes using a DNA based polynucleotide vector. Studies to demonstrate gene transfer to animal models in vivo with this physical gene delivery system will be described.

C6-414 A RETROVIRAL VECTOR BASED UPON A LOW-LEUKEMOGENIC MLV: GENETIC AND FUNCTIONAL STABILITY AFTER SELECTION FOR HIGH EXPRESSION,

Mogens Duch, Kirsten Paludan, Michael S. Sørensen, Poul Jørgensen, and Finn S. Pedersen, Department of Molecular Biology, Univesity of Aarhus, DK-8000 Aarhus C

Retroviral vectors in use for gene therapy are derived from the high-leukemogenic Moloney murine leukemia virus (MLV) harboring a strong transcriptional enhancer. We have investigated the properties of retroviral vectors derived from other MLV's.

Single-gene MLV based retroviral vectors carrying the G418 resistance gene (neo) under transcriptional control of the long terminal repeat were used to study the effect of selection on longterm vector expression in a murine lymphoid cell line, L691. Two isogenic vectors carrying either a strong or a weak transcriptional enhancerwere emploied. Effects of G418 selection were studied at the level of vector transduced cell populations and at the level of single vector transduced cell clones obtained without selection for vector expression.

Selection for vector expression prior to isolation of cell clones changed the range of vector expression levels for cell clones carrying the weak enhancer, but did not influence the long-term stability of vector expression for the two populations of cell clones.

For cell clones harboring the weaker enhancer isolated without selection, prolonged growth under selective conditions resulted in increased vector expression in some cases. The increased expression could not be accounted for by mutations in the enhancer region nor by major rearrangements.

These results point to the potential use of vectors with weak enhancers as a safety feature in ex vivo protocols.

C6-415 **EFFECTIVE CYTOKINE GENE TRANSFER TO BONE MARROW STROMAL CELLS USING ADENOVIRAL VECTORS,**

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Adenoviral cytokine gene transfer was used in human long-term bone marrow culture to demonstrate the feasibility of genetically manipulating adherent (stromal) cells to produce functional cytokine and reporter gene products over a period of days. Human bone marrow samples from healthy marrow transplant donors were maintained in modified Dexter's long-term culture from which an adherent layer representative of the marrow stroma was generated. Adherent layers were infected with adenoviral constructs designed to transfer genes for β-galactosidase and murine interleukin 6 (IL6). Adenoviral vectors were constructed with cDNA inserted into the early (E1) region of the viral genome under the transcriptional control of a human cytomegalovirus promoter (hCMV). Our results demonstrate effective gene transfer into bone marrow stromal cells and expression of functional protein over a period of at least 10 days in vitro. Levels of bioactive IL6 were in the range of micrograms per milliter of culture media. A dose response relationship between viral concentration (multiplicity of infection) and cytokine production was seen. In a canine model, the same E1 deleted vectors (10^{10} plaque forming units) were injected directly into the bone marrow space. Infection and expression of gene product locally is demonstrated. Preliminary data support the appropriate hematological response to marrow expressing increased levels of IL6. Given the important regulatory interactions between stromal cells and hematopoietic stem cells, we consider adenoviral gene transfer to stromal cells potentially useful for the modulation and possible expansion of stem cells in vitro or potentially in vivo. **(Supported by N.C.I. Canada, M.R.C. Canada and The Terry Fox Foundation)**

C6-416 STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE RECEPTOR BINDING DOMAIN OF THE ADENOVIRUS 5 FIBER PROTEIN,

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Adenovirus binds an unknown cell surface receptor via the fiber protein component of its capsid. Structurally, fiber is composed of three domains, an N-terminal tail which binds penton base, a repeating β-structure that forms the shaft, and a C-terminal globular region termed knob. We have cloned and expressed knob with a single repeat of the shaft as a soluble protein in *E. coli*. Functional analysis of knob demonstrates that it is the receptor binding domain of the fiber. Knob has been crystallized and its structure determined using X-ray diffraction and multiple isomorphous replacement. The electron density map at 1.7Å resolution reveals a trimeric arrangement of the knob protein monomers in the crystal structure. Each 196 residue monomer adopts a ten-stranded β-sandwich structure having novel strand connectivity. The receptor-binding face of the knob visually resembles a three-bladed propeller with one β-sheet from each monomer forming a blade. It also has a deep surface depression centered on the three-fold molecular symmetry axis. We compared the sequences of knob domains from different serotypes and found patterns of both highly conserved and highly variable segments on the knob surface. Comparison of the knob domains of Ad2 and Ad5, which share the same receptor, with Ad3 and Ad7, which bind a different receptor, reveals that residues lining the wall of the surface depression and in the valleys between adjacent monomers are conserved and may form the receptor binding site. Site-directed mutagenesis of knob to delineate the receptor binding region is underway.

C6-417 PROTEINS AND LIPIDS OF MEMBRANES OF STAPHYLOCOCCUS RESISTANT TO ANTIBIOTICS , Golodok L.P., Oryabinskaya L.B. , Gavrilyuck V.G., Vinnikov A.I. , Department of Microbiology , Dniepropetrovsk State University , Ukraine , 320625.

Structural changes in protein-lipid composition of membranes of Staphylococcus cells , keeping resistance plasmids to series of antibiotics , have been revealed. It has been established that the acquisition of resistance to chloramphenicol, tetracycline, eritromycin and penicillin is accompanied with changes in qualitative and quantitative protein composition of membranes of resistant staphylococcus cells which are shown by increase in protein-fractions. Analysis of protein-spectra has assured the presence of new protein in resistance strains or lack of possibility to synthesise series of proteins by sensitive strain. Studies of IR-spectra of membranes of given Staphylococcus strains showed the drastic changes in correlation between lipids and proteins during development of antibiotic resistance. Part of lipids in membrane fractions of resistant strains gets increased by 1.12 - 1.72 times. Intensification of lipogenesis deviates towards the increase cardiolipin, fraction of free fatty acids, triglicerides and carotynoid pigments shows the specific metabolism of resistant strains and increase in membranes lipid complex. These changes in lipid-protein composition of membrane may be seen as formation of compound complexes of defending mechanisms of microbial cell from antibiotic effect.

C6-418 SECOND GENERATION ADENOVIRUS VECTORS FOR GENE THERAPY, Frank L. Graham[1,2], Valeri Krougliak[1], Ludvik Prevec[1,2] and Andrew J. Bett[1], Departments of Biology[1] and Pathology[2], McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada

The construction of recombinant Ad vectors usually involves the insertion of foreign DNA into an Ad genome which has compensating deletions of viral DNA. Our studies have shown that the Ad virion has the ability to package DNA up to 105% of the wt genome length, allowing insertion of 1.8-2.0 kb of excess DNA. Deletion of 3.2 kb in E1 and 3.1 kb in E3 permits inserts of up to 8.3 kb.

Virtually all adenovirus vectors constructed for use in gene therapy carry inserts in the E1 region making them replication defective in noncomplementing cells. A problem currently being encountered during propagation of E1$^-$ vectors in complementing 293 cells is recombination with E1 sequences in these cells, generating replication competent viruses, and resulting in stocks that are inhomogeneous and unacceptable for clinical use. To avoid this we are engineering vectors with insertions in E3 combined with E1 deletions. The insertions are designed so that recombination between the left end of the vector genome and E1 sequences in 293 cells results in an Ad genome that is too large to be packaged.

A second problem with Ad vectors for gene therapy is "leaky" viral gene expression at high multiplicities of infection and in some cell types. To circumvent this and simultaneously increase the cloning capacity of Ad vectors we are attempting to delete E4.

C6-419 MASON-PZIFER MONKEY VIRUS (MPMV) AS A VECTOR FOR HUMAN GENE THERAPY. Guesdon F.M.J., Rhee S.(*), Hunter E. (**) and Lever A.M.L. Department of Medicine, Addenbrooke's Hospital, Cambridge CB2 2QQ UK (*) and (**) Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294-2170, USA

MPMV represents the prototypic virus of the type D retroviridae. This family of viruses are morphologically distinct from type C retroviruses, and the capsid assembly takes place within the cell. The assembled capsids then bud from the plasma membrane.

MPMV is a potentially useful vector for gene therapy. It infects all human cells so far tested and is non pathogenic in man.

We have identified a 5' packaging signal for MPMV, as a region immediately upstream of the major splice donor. Secondary structure analysis of this region reveals sequence and stable structures conserved among a large number of retroviruses (Harrison et al., submitted for publication). The packaging defective provirus has been stably introduced into human cell lines, and both viral protein expression and capsid formation have been characterized in these cells.

We have developed an MPMV-based vector for gene transfer studies using our above cell lines as packaging cells. We will report our results on gene transfer with the MPMV-based vectors.

C6-420 **LIGAND-DIRECTED RETROVIRAL TARGETING OF HUMAN BREAST CANCER CELLS,** Xiaoliang Han*, Nori Kasahara# and Yuet Wai Kan*#. * Howard Hughes Medical Institute and #Department of Laboratory Medicine, University of California, San Francisco, CA 94143

Her-2 receptors are the product of the Her-2/erbB-2/neu protooncogene. 20% of breast cancers overexpress Her-2 receptors and are more resistant to chemotherapy. Heregulin is a specific ligand of the Her-2 receptor. We created an ecotropic MoMLV-based retrovirus which carries human heregulin on the virus envelope to direct specific infection of human breast cancer cells which overexpress the Her-2 receptor. The chimeric envelope protein was constructed by replacing the amino-terminal portion of gp70 envelope protein with the extracellular domain of alpha or beta human heregulin. Heregulin/envelope-expressing Psi2 packaging cell lines were generated by transfection of the chimeric constructs and identified by reverse transcription and polymerase chain reaction. 16 cell ines which expressed alpha or beta heregulin/gp70 chimeric envelope protein were established from 86 hygromycin resistant colonies. Viruses were produced by transient transfection of heregulin/Psi2 packaging cell lines with a replication-defective retroviral vector containing the neomycin resistant gene and beta-gal gene. Titers of retrovirus from these 16 cell lines were measured by infection of NIH3T3 cells and averaged 1 X 10^3 by transient transfection, with the highest titer reaching 1 X 10^4. Human breast cancer cell lines MDA-MB-453 which overexpresses Her-2 receptor and MDA-MB-231 which expresses nondetectable Her-2 gene product were used as targets for retroviral infection. These human breast cancer cells and NIH3T3 cells were infected, selected in G418 (400ug/ml) and stained with X-gal. Viruses produced by packaging cell lines containing either the alpha or beta heregulin-env chimera infected human breast cancer cell line MDA-MB-453 which overexpresses Her-2 receptor, but not human breast cancer cell line MDA-MB-231 which does not express the Her-2 receptor. As the virus is ecotropic-based, it will only infect human breast cancer cells but not any human cells. This cell specific property will provide a novel approach for the treatment of breast cancer which could be applied in vivo.

C6-421 EXPRESSION OF VL30 VECTORS IN PRIMARY CELL TYPES WHICH ARE TARGETS FOR GENE THERAPY.

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Retrovirus-derived vectors are presently the preferred method for obtaining permanent insertion of genes into somatic cells. However, the LTR promoters of these vectors are sometimes transcriptionally inactivated *in vivo*, and there is a safety problem caused by homologous recombination with helper virus. Vectors derived from nonhomologous mouse VL30 retrotransposons were transduced into several important target cell types, including: human peripheral blood lymphocytes, normal human bronchial epithelial cells, mammary epithelial cells, and skeletal muscle cells. All cell types expressed moderate to abundant levels of RNA transcript originating from the VL30 (NVL3) long terminal repeat promoter. EBV-transformed lymphocytes were maintained for six months in culture to assess longevity of expression. The VL30 vector VLPPBN continued to express moderate, steady-state levels of mRNA from its LTR promoter in PBLs, compared to much lower expression from a classical murine leukemia virus vector. In mammary cells, the VL30 RNA levels were upregulated by fibroblast growth factor and insulin. Mouse embryonic stem cells also expressed the vector RNA, although at reduced levels compared to human primary cells. The VL30 vectors were expressed in primary cells at levels comparable to those observed using transformed human and murine cell lines derived from fibroblasts, intestine, and breast cancer. Thus, VL30s appear to be useful for the insertion and expression of foreign genes in several cell types which are targets for gene therapy.

C6-422 NEW GENERATION OF SAFE, EFFICIENT RETROVIRAL VECTORS AND PACKAGING CELL LINES FOR APPLICATION IN GENE THERAPY TRIALS,

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Retrovirus vectors are the most widely used gene delivery system for human gene therapy, but improvements are indispensable to limit potential risks and to augment virus titers in large scale production. A major obstacle of the currently used Moloney murine leukemia virus (Mo-MuLV) based systems is the possibility of recombination with endogenous retroviral sequences in the rodent helper cell background or with MoMuLV helper genes. This may give rise to replication competent retroviruses (RCR).

We have therefore developed a vector system based on human 293 cells which are free of endogenous retroviral sequences, and on Friend Murine leukemia virus (FrMuLV) strain FB 29 which is highly virulent in mice and is known to produce high titers. In MuLV vectors, the *psi* region required for efficient packaging extends into the gag gene and thus allows homologous recombination and mobilization of helper genes. We therefore replaced this region by a *psi* sequence derived from a rat retrotransposon virus-like 30 S RNA (VL30) which has no homology with MuLV coding sequences but has a higher packaging rate. FrMuLV-VL30 hybrid vectors replicate more efficiently than several MuLV or MSV based vectors tested.

In the new packaging cells, based on human 293 cells, FrMuLV gag/pol and amphotropic env genes, cloned in separate plasmids, are expressed under control of non-viral promotors. Virus titers obtained in these cells are 3-5 fold higher than in the commonly used very efficient PA317 and gp+am12 cells. These helper cells and the FrMuLV-VL30 hybrid vectors have no sequence homologies. The system thus allows a significant increase of both virus titer and vector safety.

Such a system therefore is ideally suited for ex-vivo gene transfer in the treatment of diseases such as AIDS and hemophilia which are currently under investigation in our laboratory.

This work is partially supported by the French AIDS research agency (ANRS)

C6-423 THE SAFETY OF INTRAHEPATIC ARTERY AND INFERIOR VENA CAVA INFUSION OF RETROVIRAL VECTOR PRODUCER CELLS IN PIGS.

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Retroviral vector-mediated transfer of suicide genes by intratumoral injection of producer cells is a safe and potentially effective treatment of malignant brain tumors. In this study, the safety of local intra-arterial or systemic intravenous route for retroviral transduction of hepatocytes was tested in 4 pigs. During an abdominal laparotomy, 8 X 10^8 producer cells bearing the LacZ gene (G1nBgSvNa) were infused, with or without collagen, over 4 min into either a branch the hepatic artery or through the inferior vena cava (IVC). Presence of producer cells in the circulation was tested, by in vitro assay, from blood drawn at serial intervals during and after producer cell infusion. Liver biopsies were obtained to determine the duration of producer cell survival in the liver and to test for organ transduction. The pigs' vital signs remained stable during producer cell infusion, and the animals survived until sacrifice with no untoward side effects. In 3 pigs, circulating producer cells were not detected at any time during or after hepatic artery infusion. In one of the 3 pigs, beta galactosidase positive producer cells were still detected, by immunostaining, in liver 5 days after infusion with collagen. In contrast, circulating producer cells were detected during infusion into the IVC but not thereafter. At sacrifice, none of the organs tested stained positive for beta galactosidase, indicating that normal tissue was not transduced by the retroviral vector using both routes. In 3 pigs receiving hepatic artery infusion, local infiltration by neutrophils, and later macrophages, was noted in the liver. These data indicate that the hepatic arterial or IVC route of retroviral gene delivery may be safe as potential treatment of unresectable HCC.

C6-424 TISSUE TROPISM OF COXSACKIEVIRUSES

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Coxsackieviruses belong to the enterovirus genus of Picornaviruses which are small, unenveloped RNA viruses. There are more than sixty enterovirus serotypes with different tissue tropism which makes them candidate vectors for gene delivery. Recently, transfer of exogenous genes into cells using enterovirus vectors has been documented.

Cellular receptors for several enteroviruses have been identified making it possible to study virus-receptor interactions at a detailed level. We have earlier reported that coxsackievirus A9 (CAV-9) differs from all the other studied enteroviruses since only this virus contains a carboxyl terminal extension to the capsid polypeptide VP1. This extension contains a conserved arginine-glycine-aspartic acid (RGD)-motif, shown by peptide blocking experiments to play a role in the the interactions of the virus with the cell surface. When this region is removed from CAV-9 by trypsin treatment, the blocking effect of the RGD-peptides disappears while the virus is still infectious indicating ability to attach to another receptor.

We have demonstrated by means of protein chemistry and blocking experiments with monoclonal antibodies that the RGD-motif interacts with vitronectin receptor in GMK cells. However, in RD cells an alternative receptor is utilized, because infection cannot be blocked by the RGD-peptides. Identification of this receptor molecule is underway. We have also made several mutations in the RGD-containing insertion in CAV-9. These give rise to a small-plaque phenotype. Analysis of the tissue tropism of theses mutant viruses in an animal model has been started. Furthermore, tissue tropism is studied by constructing recombinants between CAV-9 and other coxsackieviruses. Analysis of pathogenesis of the recombinants should reveal genomic regions responsible for different tissue tropism and thus enable us to to select suitable vehicles for gene delivery.

C6-425 DIRECT ADMINISTRATION OF RETROVECTORS AS THERAPEUTICS, D.J. Jolly, J. Robbins, M.J. Irwin, S.M.W. Chang, B. Howard, T. Fong, J. Respess, N. DePolo, S. Chada, K. Kowal, J. Barber, J.F. Warner, and S. Mento, Viagene, Inc., 11055 Roselle Street, San Diego, CA 92121-1204

Most uses of retrovectors for genetic therapy have assumed that the vectors would be used either *ex vivo* or be delivered via intact producer cells. These assumptions were based on dogma suggesting that retrovectors were sensitive to inhibition by complement and that titers were too low to allow for effective direct retrovector injection. Viagene has recently reported that direct administration of amphotropic retrovector i.m. in mice and non-human primates (at doses of 10^6 - 10^7 cfu) induces CTL responses to the antigens they encode (Irwin et al, *J.Virol* 68, 5036, 1994). These animal data were sufficiently compelling to allow Viagene to initiate several human clinical trials using directly injected HIV-1 env/ rev encoding retrovectors as an experimental therapy for treatment of HIV-1 infection.

Viagene has developed processes that reproducibly yield retrovector preparations at concentrations of greater than 10^8 cfu/ml. These high titer retrovector preparations have been tested in animal tumor models. At present, direct peritumoral injection leads to uptake of marker vectors in tumor cells at a rate of up to 9% of input vector. In addition, administration of either interferon gamma retrovectors or thymidine kinase retrovectors (followed by gangcyclovir) in or near tumor sites leads to measurable tumor regression in mouse models. Summaries of these data will be presented supporting the view that direct administration of retrovector is feasible as a therapy in a variety of clinical applications.

C6-426 TOWARDS THE DEVELOPMENT OF A NOVEL ADENOVIRAL VECTOR SYSTEM

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For gene therapy adenoviral vector systems have the advantage of a highly efficient gene transfer into replicating and non-replicating cells.

The major disadvantages of currently available adenoviral vectors are:

a) limitation in the size of DNA being transferred (maximal 8 kb of DNA at present),

b) immunogenicity of the virus proteins, at least partially caused by expression of late viral functions in those cells which have been transduced by the virus and

c) lack of integration of the transferred DNA into the cellular genome.

Our research aims in the development of technologies that would allow the transfer of large DNA fragments into eukaryotic cells *in vivo*.

We are developing adenoviral vector systems that have the potential to overcome some of the limitations of the available adenoviral vectors and present evidence that it might be possible to increase the adenoviral uptake of foreign DNA over the current maximum of about 8 kb of DNA.

C6-427 ENHANCED EXPRESSION FROM THE MFG VECTOR BACKBONE IS DUE TO INCREASED SPLICING EFFICIENCY.

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The MFG backbone has been reported to augment expression of inserted cDNAs relative to the widely used N2 backbone. We have studied the mechanism of enhanced protein production from the MFG backbone in murine spleen colonies after bone marrow transplantation using human glucocerebrosidase (GC) as a reporter. GC activity was 5-10 fold higher in spleen colonies transduced with the MGC (MFG-based) vector versus the G2 (N2-based) vector after correction for copy number. In experiments where the mean vector copy number per focus was equivalent for the two vectors, mean GC activity was 10 fold higher in the MGC vector. In Northern blots, the ratio of spliced to unspliced RNA was increased by 4-fold in foci transduced with the MGC vector. The relative abundance of spliced transcript per vector copy was calculated in 60 foci generated in 5 separate experiments. The increase in spliced transcript per vector copy in MGC versus G2-transduced spleen colonies ranged from 2-8 fold. Since spliced transcripts are probably translated more efficiently, this increase alone may provide the basis for elevated protein expression in MFG-based vectors. We are currently investigating the relative translation efficiency of the spliced and unspliced transcripts from both vectors. These experiments will address the possibility of effects on translational control mediated by the MFG vector backbone.

C6-428 AAV-ADENOVIRUS HYBRIDS AS POTENTIALLY IMPROVED VECTORS FOR GENE THERAPY

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Adenovirus-based vectors for gene therapy represent a powerful gene delivery system in that they readily infect non-dividing as well dividing cells. They can be produced in very high titers and can be rendered replication deficient. Morever these non-pathogenic human viruses have been proved safe for human use.

Adenoviral vectors do not integrate into the target cell-DNA and remain as episomes in the nucleus- this has both advantages and disadvantages. As the vector does not integrate and does not replicate, in the dividing cells the transgene can be rapidly lost and the expression of the therapeutic gene may be transient .One way to improve stability of the transferred gene is likely to target its stable integration in the genome.

Adeno-associated virus (AAV)is a non pathogenic defective virus. In absence of helper virus, AAV integrates as a provirus preferentially into one region on chromosome 19. AAV linear genome is flanked by two inverted terminal repeats (ITR) of 145 bp; these ITRs appear to be the minimum sequences required for AAV integration.

We are currently testing the capacity of adenovirus vectors containing a reporter gene flanked by the two AAV ITRs to target the stable integration of the transgene into the host genome. As the mechanism of AAV integration is unclear, *in vitro* preliminary studies with selectable and non-selectable gene markers are required to test the functionality of the AAV ITRs in an adenovirus shuttle-vector and to test the feasibility of constructing such vectors.

C6-429 **Episomal MoMLV Expression System which Initiates a Transduction Chain Reaction** Levy, J.P., Dietz, A.B., Link, C.J .Human Gene Therapy Research Institute, Iowa Methodist Medical Center, Suite 218,Des Moines,Iowa 50309.

Retroviral gene transfer is normally accomplished by generating retroviruses from a murine vector producer cell (VPC) such as PA317. Once the retroviral vector (RV) has been transduced and integrated into the target tumor or normal somatic cell it is incapable of being transmitted to any other cell. The structural genes need to be expressed once again in the RV integrated target cell for it to transduce neighboring target cells. This transduction chain reaction (TCR) *can be generated* safely by transiently expressing an MoMLV episomal expression vector (MEEV) in the transduced cells. Initially we demonstrated TCR by liposome transfecting RV transduced cells, in this case LBAGSN A375 cells, in culture with pPAM3 (provided by A.D.Miller), harvesting and transferring supernatants onto NIH3T3 cells and selecting for G418 resistance. We were able to generate low but detectable titers by this method. To increase TCR we have cloned a MEEV by long PCR (Klentaq and PFU enzymes in P.H.9.0 buffer). Using pPAM3 as a template, we amplified a 7.8 kb fragment from the transcription start site extending through to the stop codon of the envelope gene. We initially ligated this fragment into the TA cloning, sequencing vector pCR II (In Vitrogen) and confirmed the correct insertion portion of MoMLV. We have subsequently cloned this fragment into a CMV episomal expression vector, pCEP4 (In Vitrogen). We intend to demonstrate high levels of TCR both *in vitro* and *in vivo*. This MEEV vector may also be useful in making improved versions of both human and murine VPC.

C6-430 INCREASED EFFICIENCY OF IN VITRO AND EX VIVO GENE TRANSFER INTO CANINE BONE MARROW HEMATOPOIETIC STEM CELLS, Kaixuan Liu, Amy E.Mauser,Susan A. Goodman,Tsghe W. Abraha, Clinton D. Lothrop Jr.,Scott-Ritchey Research Center, Auburn University, AL 36849

Efficient gene transfer into hematopoietic stem cells (HSC) of large animals and human beings for high level in vivo gene expression has proven to be difficult. A long term bone marrow culture (LTBMC) system to which 10% neutropenic dog serum (NDS) was added during multiple retroviral transductions increased the efficiency of gene transfer into canine bone marrow HSC. A retroviral vector (LIXSN) containing a human factor IX cDNA and a bacterial neomycin resistance gene (neo^r) was initially used in in vitro experiments. The efficiency of gene transfer into bone marrow HSC was increased twofold with NDS (38.6% versus 19.7%) and also increased after multiple transductions (38.6% versus 13.1%) as determined by CFU-GM assays. The neo^r gene and the factor IX cDNA were detected by polymerase chain reaction in both nonadherent and stromal bone marrow cells. Approximately 40% of factor IX activity in normal human plasma was detected in the culture supernatants almost two months after retroviral transduction. In ex vivo gene transfer experiments, bone marrow cells from four neonatal dogs were transduced in vitro with retroviral vectors containing the neo^r gene using PA317 and PG13 packaging cells and transplanted autologously following sublethal irradiation (1.75 Gy). The efficiency of gene transfer into canine bone marrow HSC ranged from 38.3% to 45.5% and 8.3% to 20.6% before and one month after bone marrow transplantation, respectively, using the CFU-GM assays. Our data imply that the gene transfer protocol established in this study may be applicable for gene therapy of blood diseases.

C6-431 RETROVIRAL VECTORS DEPENDENT ON ENGINEERED tRNA-LIKE PRIMERS FOR REVERSE TRANSCRIPTION: A NOVEL PRINCIPLE FOR BIOLOGICAL CONTAINMENT, A.H. Lund, M. Duch, K.D. Kristensen, J. Lovmand, P. Jørgensen and F.S. Pedersen. Department of Molecular Biology, University of Aarhus, C.F. Møllers Allé 130, DK-8000 Århus, Denmark.

The risk of uncontrolled spread of retroviral vectors or of derived helper viruses may impose limitations on the usage of such vectors. Their wider application in gene therapeutical protocols may therefore depend upon technological advances towards increased vector safety.

The priming of reverse transcription by a specific tRNA annealed to the viral RNA is a key step in retroviral replication. We have studied the specificity of tRNA primer usage and of primer binding site (PBS) interactions as a possible point for control of mobilization of murine leukaemia virus (MLV) derived vectors. These vectors normally use a primer tRNA specific for proline. Our results show that vectors carrying appropriately mutated PBS sequences can replicate efficiently using other tRNA species (acceptors for glutamine, lysine and methionine) as primers for reverse transcription (Lund *et al.*, J. Virol. 67:7125, 1993). Additional mutational studies demonstrate that the replication machinery may tolerate mismatches between tRNA primers and PBS sequences.

Vectors carrying PBS sequences that do not match any naturally occuring tRNA species are severely impaired in their replication capacity (about 10^5 fold reduction in titre). With the aim of restoring the transfer capability of such vectors we have designed artificial tRNA-like primers with 3'ends corresponding to the PBS-mutated vectors. Specialized vector packaging cells with these primers have been developed and found to restore the efficiency of transfer of corresponding PBS-impaired vectors. By sequence marker analysis of transferred vectors proviruses we have provided genetic evidence that these artificial primers are in fact used as primers for reverse transcription.

These results imply that the uncontrolled spread of such vectors beyond the target cells requires a non-natural primer-RNA in addition to viral *trans*-factors. Our studies therefore point to a new safety feature of retroviral vectors of potential interest for some aspects of their use in gene therapy.

C6-432 SELEX VIRAL SELECTION FOR GAINING CELL ENTRY OF NUCLEIC ACIDS, Ian H. Maxwell, University of Colorado Cancer Center, Denver, CO 80262

We hypothesize that a random library of single strand RNA or DNA motifs contains ligands for cell surface receptors that direct internalization of nucleic acids. We are using the genomes of an RNA virus (Sindbis) and a single strand DNA virus (LuIII parvovirus), with insertion of a random oligonucleotide library, to select for such "entry motifs" by viral plaque formation. Sindbis RNA, transcribed from pTR2003 (C. Rice, St. Louis) was infectious for hamster and human cells (65 - 350 pfu per ng by electroporation or lipofection). Unfacilitated background was 2 plaques per µg. pTR2003 contains an ApaI site for library insertion. An ApaI linker in the 3'-untranslated region of a LuIII infectious clone did not compromise infectivity (50 pfu per ng). An oligonucleotide with a 30 nt randomized stretch flanked by fixed sequences (Nexagen, Inc.) was subjected to PCR to attach ApaI restriction sites. Cycle number was minimized to guard against selective amplification and loss of complexity. The 118-mer PCR product (5-7 cycles) was Centricon concentrated and restricted with ApaI to a 102 nt fragment. Ligation with pTR2003/ApaI failed due to inhibitors in the fragment preparation. Initial ligation of oligomers to the randomer improved the PCR template, with increased yields permitting gel purification. We shall report on the effects of exposing cells to single strand nucleic acids derived from the products of ligation with the viral vehicles.

C6-433 PROTECTION OF MICE FROM LETHAL SYSTEMIC METHOTREXATE TOXICITY BY TRANSPLANTATION WITH TRANSGENIC MARROW CELLS EXPRESSING METHOTREXATE-RESISTANT DIHYDROFOLATE REDUCTASE ACTIVITY. Chad May, Roland Gunther, and R. Scott McIvor.
Gene Therapy Program, Institute of Human Genetics, and Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455.

Introduction of variant dihydrofolate reductase (DHFR) gene sequences conferring resistance to methotrexate (MTX) into hematopoietic cells could potentially be applied **(i)** for improved use of MTX as an antitumor agent and **(ii)** as a selective approach to enrich for successfully transduced cell populations. As a model system to study the potential effectiveness of MTX-resistant DHFR gene transfer, marrow cells from previously established lines of transgenic mice expressing either of two different MTX-resistant DHFR's were transplanted into recipient animals to determine the resultant *in vivo* protective effect against toxicity associated with MTX administration. Transplantation of 10^6 transgenic marrow cells expressing either the arg22 (line 04) or trp31 (line 03) DHFR variants rescued normal FVB/N recipient animals from low dose (1.0 mg/kg i.p. daily) MTX administration which was lethal for animals transplanted with 10^6 normal FVB/N marrow cells. Reduced survival of recipients transplanted with transgenic line 04 marrow cells was observed when two-fold or four-fold doses of MTX were administered. However, when 10^7 transgenic line 04 marrow cells were infused, the recipients were found to be resistant to a MTX dose (4.0 mg/kg daily) which was not only lethal for animals transplanted with 10^7 normal FVB/N marrow cells, but also lethal for normal, untransplanted FVB/N mice. These results demonstrate that exclusive expression of MTX-resistant DHFR activity in the marrow can have a substantial, systemic chemoprotective effect in animals which could be applied to improved utilization of MTX for antitumor chemotherapy.

C6-434 MODIFICATION OF THE ADENOVIRUS FIBER PROTEIN FOR TARGETED GENE DELIVERY, Alan McClelland and Susan C. Stevenson, Department of Molecular and Cell Biology, Genetic Therapy, Inc., Gaithersburg, MD 20878

The adenovirus fiber protein is responsible for attachment of the virion to cell surface receptors. The identity of the cellular receptors which mediate binding is unknown, but there is evidence suggesting that there are at least two distinct adenovirus receptors which interact with the group B (Ad5) and the group C (Ad3) adenoviruses. In order to define the determinants of adenovirus receptor specificity, we have carried out a series of competition binding experiments using recombinant native fiber polypeptides from Ad5 and Ad3, and chimeric fiber proteins in which the head domains of Ad5 and Ad3 were exchanged. Specific binding of fiber to HeLa cell receptors was assessed using radiolabeled protein synthesized *in vitro*, and competition analysis using fiber protein expressed in baculovirus. Fiber monomers and trimers were produced *in vitro*, but only the assembled trimers had receptor binding activity. The Ad5 and Ad3 fiber proteins did not compete with each other for binding indicating that Ad5 and Ad3 interact with different cellular receptors. The chimeric fiber containing the Ad5 fiber head domain blocked the binding of Ad5 fiber but not Ad3 fiber. Similarly, the chimeric fiber containing the Ad3 fiber head blocked the binding of labeled Ad3 fiber but not Ad5 fiber. In addition, the isolated Ad3 fiber head domain competed effectively with labeled Ad3 fiber binding to HeLa cell receptors. These results demonstrate that the determinants of receptor binding specificity are in the head domain of the fiber and that the isolated head domain is capable of trimerizing and binding to cellular receptors. Our results also show that it is possible to change the receptor specificity of the fiber protein by altering the head domain. Modification or replacement of the fiber head domain with novel ligands may permit adenovirus vectors with new receptor specificities to be engineered which could be useful for targeted gene delivery *in vivo*.

C6-435 GENE TARGETING IN MOUSE EMBRYONIC STEM CELLS WITH AN ADENOVIRAL VECTOR, Kohnosuke Mitani[1,5], Maki Wakamiya[1], Paul Hasty[3], Frank L. Graham[4], Allan Bradley[1,2] and C. Thomas Caskey[1,2], [1]Department of Molecular and Human Genetics and [2]Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030, [3]Department of Biochemistry and Molecular Biology, University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030, [4]Departments of Biology and Pathology, McMaster University, Ontario L8S 4K1, Canada and [5]Department of Disease-related Gene Regulation Research (Sandoz), Faculty of Medicine, The University of Tokyo, Tokyo 113, Japan

Adenoviral vectors are widely used as highly efficient gene transfer vehicles in a variety of biological research strategies, including human gene therapy. However, because integration of the viral genome into the host chromosome is rare, relatively short-term expression from the vector is obtained. We examined the ability of an E1, E3-defective adenoviral vector to act as a substrate for homologous recombination with chromosomal DNA by including host chromosomal sequence from the mouse *Fgr* locus that also contained a selectable marker. After infection of mouse embryonic stem cells, stable transformants were selected for neomycin resistance and the efficiency of homologous recombination was evaluated. The adenoviral vector was capable of infecting mouse embryonic stem cells efficiently. Between 30 - 50% of the input virus reached the nuclei after 24 hours of infection. Surprisingly, even without negative selection, 25 - 40% of the stable transformants resulted from homologous recombination, although the absolute efficiency of stable transformation was low. This targeting efficiency is five times more efficient than by electroporation of an Fgr vector that does not contain adenoviral sequences. Our results suggest that it is possible to modify the structure of an adenoviral vector to achieve a high gene targeting efficiency, resulting in regulated and long-term expression of an introduced gene.

C6-436 EXPRESSION AND SECRETION OF IL-1β IN TRANSDUCED HUMAN T CELLS, R.A. Monticello, T.P. Quinn, E. Ruhl, M.S. Wing, L.G. Lum, A.S. Goustin, R.H. Rownd and K.T. Trevor, Center for Molecular Medicine and Genetics and Division of Hematology and Oncology, Wayne State University, School of Medicine, Detroit, MI 48201

Interleukin 1-β (IL-1β) has been proposed as a therapeutic mediator for augmenting anti-tumor effects following autologous bone marrow transplant (ABMT). Yet, after ABMT the required concentrations of infused IL-1β can lead to systemic toxicities. Our interest is to enhance the elimination of lymphoma/leukemia cells in ABMT recipients by adoptively transferring human T cells that express and secrete IL-1β, thereby confining the potential benefits to a more local environment. Human T cells derived from peripheral blood lymphocytes (PBLs) were activated with immobilized OKT3+IL-2 for 2 days and transduced with an LXSN-type retrovirus containing a human IL-1β cDNA (MOI=0.1-1 CFU/cell). The vector (kindly provided by Genetic Therapy, Inc.) encodes a fusion protein comprising the rat GH signal sequence preceding the mature 17 kD form of IL-1β for secretion via the Golgi pathway. In retrovirus-transduced cells, RT-PCR indicated the presence of proviral IL-1β transcripts after 3 weeks of growth; no endogenous IL-1β expression was detected. Quantitative DNA PCR indicated that about 3-5% of the initial population was transduced by the recombinant retrovirus. The transduced T cells secreted up to 100-fold the level of IL-1β relative to cultures infected with a control retrovirus (LXSN) or mock-infected cultures. Prior to transduction, the activated PBLs possessed a T cell phenotype consisting of 70% $CD3^+$, 17% $CD8^+$ and 55% $CD4^+$ cells. Following 3 weeks in culture, an expansion of $CD8^+$ cells occurred in both transduced and non-transduced populations: 98% $CD3^+$, 50% $CD8^+$ and 25% $CD4^+$ cells. Our results show that IL-1β-transduced T cells can be obtained in vitro. Further studies will determine whether this T cell population is useful for augmenting anti-tumor effects/immune responses in ABMT.

C6-437 **AAV VECTORS INTEGRATE AND EXPRESS IN THE ABSENCE OF SELECTION,** George Natsoulis, Susan A. McQuinston, Richard O. Snyder, and Gary J. Kurtzman, Avigen, Inc., 1201 Harbor Bay Parkway, Alameda, CA, 94502.

In the absence of infection with a helper virus, wild-type AAV preferentially integrates into a small region on chromosome 19 (19q13.3). Although site-specific integration would be a desireable property for gene therapy vectors derived from AAV, little is known about integration of of recombinant AAV vectors (rAAV). Moreover, since most studies looking at rAAV integration have employed rAAV containing the neo^R gene and G418 selection, the ability of rAAV to integrate via an AAV specific mechanism has been questioned. Indeed, integration of DNA molecules expressing the neo^R gene can be selected for in the presence of G418.

We studied the integration of a *rep*-free rAAV vector containing the ß-galactosidase gene (rAAV-lacZ). 293 cells were infected with a highly purified, adenovirus-free stock of rAAV-lacZ at an m.o.i. of 100, 500, or 1000 (transduction competent particles:cell). One day post-infection, cells were plated at limiting dilution to obtain single cell clones. Between 10 and 25 days post-infection, approximately 10% of the clones were homogeneously $lacZ^+$ while more than 50% of the clones displayed a mixture of $lacZ^+$ and $lacZ^-$ cells. On Southern blot analysis employing a restriction enzyme that does not digest within the rAAV-lacZ genome, the majority of the $lacZ^+$ clones showed one band of variable size indicative of a single integration site. In all but one clone, the size of the band was larger than the vector size, as expected from a full length copy of the vector integrated in the genome of the cell.

These data suggest that stable expression in rAAV transduced cells is the result of integration of the vector into the genome of the cells, but early expression from episomal copies of the vector cannot be ruled out. They also demonstrate that AAV vectors integrate and confer an unselected phenotype to the cell.

C6-438 MAINTENANCE OF HERPES SIMPLEX VIRUS VECTOR DNA IN TARGET CELLS, William J. O'Brien and Jerry L. Taylor, Departments of Ophthalmology and Microbiology, Medical College of Wisconsin, Milwaukee, WI 53226

The use of replication defective herpes simplex viruses (HSV) as vectors for gene delivery may represent a useful approach to the treatment of various diseases. Critical questions exist regarding the input DNA, especially with vectors of viral origin, which have the potential for establishing latent infections. We have infected both replication permissive and nonpermissive monkey kidney cells with a replication deficient mutant of HSV-1 KOS which contains a deletion in the gene for an immediate early class of infected cell protein 27 (ICP27). This mutation makes the virus incapable of replicating in any cell which has not been transfected to express ICP27. We infected cells, embedded them in agarose, and lysed them with proteinase K and detergents. The DNA was analyzed by pulsed field gel electrophoresis to identify the replicative intermediate forms of viral DNA. Cells in which the virus was incapable of replication contained the viral genome as a high molecular weight intermediate as well as reduced amounts of the linear 150-Kb DNA characteristic of replicating virus. The high molecular weight intermediate appeared to be in a concatamenic form which was not integrated into cellular DNA at levels which we could detect. These observations suggest that this virus may offer newly replicated viral DNA for gene expression rather than just input viral DNA.

C6-439 CHARACTERIZATION OF A REPLICATION COMPETENT RETROVIRUS RESULTING FROM RECOMBINATION OF PACKAGING AND VECTOR SEQUENCES E. Otto, A. Jones-Trower, E. F. Vanin, K. Stambaugh, S. Mueller, W. F. Anderson, and G. J. McGarrity, Genetic Therapy Inc., 938 Clopper Rd., Gaithersburg, MD 20878 and Norris Cancer Center, University of Southern California, School of Medicine, Los Angeles, CA 90033

A replication-competent retrovirus (RCR) was detected in three lots of retroviral vector G1Na that were harvested on consecutive days from a single culture of PA317/G1Na producer cells. Using a number of retrovirus-specific primer pairs, it was shown that this RCR was a novel recombinant created by exchanges between G1Na and helper sequence pPAM3 and was not an existing RCR introduced by cross-contamination. Sequencing of clones of DNA amplified in six independent PCR reactions confirmed that the 3' portion of this RCR was composed of retroviral envelope sequences unique to pPAM3 joined to a 3' LTR unique to G1Na. Comparison of pPAM3 and G1Na sequences at the site corresponding to this junction revealed a short segment of patchy nucleotide identity (8 out of 10 bp) suggesting that these helper and vector sequences were joined by homologous recombination. This is the first report of a breakout event occurring in producer cells utilizing this vector/packaging system. The results reported here underscore the importance of screening retroviral vectors destined for human application. Production of 171 lots (855 liters) of various retroviral vectors that were free of RCR, including 42 lots of G1Na, however, indicates that the combination of exchanges required to generate an RCR are infrequent in this system.

C6-440 RETROVIRUS PRODUCTION AND TRANSDUCTION OF PERIPHERAL BLOOD LYMPHOCYTES IN A CLOSED ARTIFICIAL CAPILLARY SYSTEM: AN APPROACH TO LARGE SCALE CELL MANIPULATION FOR CLINICAL GENE THERAPY, Dao Pan, Raji Shankar, David F. Stroncek, Jeffrey McCullough, R. Scott McIvor and Chester B. Whitley, Gene Therapy Program, University of Minnesota Medical School, Minneapolis, MN 55455

The ability to obtain high-titer virus production, and to grow large quantities of primary human peripheral blood lymphocytes (PBL), in a closed system would have profound implications for clinical and experimental gene therapy. Toward a clinical trial of lymphocyte gene therapy for mucopolysaccharidosis type II (ie, Hunter syndrome), we harvested 9.2×10^9 PBL from a patient by conventional apheresis. An aliquot of PBL (5.4×10^7) was stimulated with anti-CD3 and then cultured in an artificial capillary system (ACS; Cellco, #450-011) with AIM-V medium and IL-2. In a separate ACS (Cellco, #480-008), PA317 cells with the retrovirus L2SN—containing the iduronate sulfatase (IDS) and neomycin-resistance genes—were grown in DMEM with 10% newborn calf serum. After 4.5 days growth in the ACS, PBL were transduced with L2SN. In this pilot study, we observed that: 1) Retroviral production in the ACS achieved a titer of $\sim 2 \times 10^7$ cfu/ml, ie, 10-fold higher than titers obtained from concomitant Petri-dish cultures. 2) For PBL transduction, vector supernatant (210 ml) was concentrated (to 11.5 ml) by ultrafiltration into the extracapillary space of the ACS with a 10-fold increase in virus titer. 3) PBL expanded 3-fold over the 2-day period following virus exposure. 4) IDS enzyme activity in non-selected transduced PBL 5 days after transduction was 470 U/mg/hr, a level markedly increased above that of non-transduced cells (<1 U/mg/hr). 5) After 12 days of G418 selection, transduced PBL exhibited higher IDS activity (2,500 U/mg/hr) which was even greater than normal PBL (mean 809, n=10). These data demonstrate that high titers of retroviral vector can be obtained in a closed ACS and that human primary PBL can be cultured and transduced in clinically useful quantities. Supported by Daniel Molinaro Foundation.

C6-441 COMPETITIVE PCR ANALYSIS OF VL30 INTERFERENCE WITH RETROVIRAL VECTOR PACKAGING, D.M. Poetker, A.B. Dietz, J.P. Levy, and C.J. Link., Human Gene Therapy Research Institute, Des Moines IA 50309

VL30 elements are retrotransposons about 5 kb in size found in the genome of many murine species. VL30 sequences occur approx. 200 times per *mus musculus* genome, with many of those sequences being actively transcribed. These elements contain retrovirus-like long terminal repeats at both the 5' and 3' ends of the element and a retroviral packaging sequence. VL30 sequences lack the complete gag, pol, and env genes and are replication incompetent. VL30 sequences may be maintained through concomitant transfer with wild type retroviruses. The retroviral vectors commonly used in gene therapy are produced from murine fibroblasts which contain many VL30 elements. VL30 transcripts are known to compete for virion packaging with retroviral vector transcripts in murine vector producer cells (VPC). We have developed a competitive PCR assay to quantitate the level of VL30 transduced from VPC supernate into target cells. Primers were designed to amplify a 156 bp fragment of VL30 that was verified by sequence analysis. This region can be easily amplified from a human ovarian cancer cell line (IGROV) exposed to supernate from ß-galactosidase VPC, Herpes simplex thymidine kinase VPC, or PA317 packaging cells (without vector). This competitive assay is based on the addition of a competitor DNA fragment having exactly the same primer sequence as the target gene, that flanks a portion of the ampR gene. By this method, variables which affect amplification of the target DNA have the same effect on the competitor. The absolute amount of DNA molecules in the sample is then determined by the ratio between the two amplification products. We are currently measuring more precisely the importance of VL30 interference.

C6-442 THE EXPRESSION OF FUNCTIONAL GENES IN THE RODENT NERVOUS SYSTEM WITH DUAL-EXPRESSING RETROVIRAL VECTORS ENCODING AN *IRES* SEQUENCE. J.Price[1], E.Grove[2], V.Calaora[3], H.Moreau[3], G.Chazal[3], G.Rougon[3], [1]Department of Neuropathology, SmithKline Beecham Pharmaceuticals Ltd., Harlow, UK., [2]Department of Pharmacological and Physiological Sciences, University of Chicago, Ill., USA. [3]Laboratoire de Genetique et Physiologie du Developpement, Université d'Aix-Marseille II, Marseille, France.

We have generated a series of retroviral vectors based on the IRES constructs of Ghattas *et al* (Mol. Cell Biol., 1991, 11:5848). Our constructs encode a bicistronic message comprising a functional cDNA, an IRES sequence, and *lacZ*, all driven from the endogenous MoMLV promoter. The functional cDNAs we have tried include those encoding the NGF propeptide and a GPI-linked protein, P31, expressed transiently by some neuronal precursor cells (Nedelec *et al*, Eur. J. Biochem. 1992, 203:433).

We have used these vectors to address a number of questions: do such constructs express the functional cDNA and *lacZ* simultaneously; is expression stable; can these constructs be used to transduce embryonic neural precursor cells *in situ*, and so alter the pattern of gene expression of these cells and their progeny?

We have generated high titres of ecotropic virus from these constructs in ψ2 cells. We have discovered that in cultured cells, these constructs do indeed deliver stable, high-level expression of both encoded genes. Moreover, these vectors can be used to transduce embryonic rat neural precursor cells *in vivo*. We are currently analysing the clones of cells derived from precursor cells transduced in this way, to see how their phenotype has been altered by viral transduction.

C6-443 PROTECTION OF RETROVIRAL VECTOR PARTICLES VIA COMPLEMENT INHIBITION: A NOVEL STRATEGY FOR IN VIVO GENE THERAPY, Scott A. Rollins, Stephen P. Squinto, James M. Mason and Russell P. Rother, Departments of Molecular Development and Immunobiology Alexion Pharmaceuticals Inc., New Haven, CT 06511

The use of retroviral-mediated gene transfer as an *in vivo* approach for the treatment of human disease has been limited by the inability of retroviral vectors to survive the host humoral immune system. The rapid inactivation of retrovirus that ensues following exposure to human or primate serum is largely attributed to the activity of complement mediated through the classical pathway. In this study, we have further investigated the relationship between the human complement cascade and retrovirus inactivation. Preincubation in normal human serum effectively inactivated LXSN retroviral vector particles, while the vector maintained the ability to transduce cells following incubation in sera deficient in either the C1, C2, C3, C5, C6, C8 or C9 human complement proteins. Preincubation of serum with mAbs that functionally block specific complement components including C5, C6, C8 and C9 successfully protected the LXSN vector from complement-mediated inactivation. Treatment of serum with cobra venom factor, which consumes terminal complement, also effectively protected the vector from inactivation. LXSN vector survival in serum was inversely correllated with the level of complement activity following treatment of serum with anti-C5 mAb as assessed in an erythrocyte hemolytic assay. Additionally, pretreatment of human whole blood with anti-C5 mAb effectively inhibited inactivation of the LXSN vector. Taken together, these data demonstrate that formation of the membrane attack complex (MAC, C5b-9) is required for the inactivation of the murine based LXSN retroviral vector, suggesting that this process is most likely mediated through virolysis. Further, these data suggest that the use of soluble complement inhibitors may enable retroviral vector particles to resist complement-mediated inactivation *in vivo* in humans and other primates.

C6-444 **MURINE MODEL FOR B LYMPHOCYTE SOMATIC CELL GENE THERAPY**, Yacov Ron, Natalie Sutkowski, Ming-Ling Kuo, Alfredo Varela-Echavarria, and Joseph P. Dougherty, Department of Molecular Genetics and Microbiology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854; and the Graduate Program in Microbiology and Molecular Genetics, Rutgers University, Piscataway, NJ 08855

Primary, mature B lymphocytes represent a potentially important cellular target for somatic cell gene therapy, which could prove advantageous for the treatment of certain metabolic and immunologic disorders. Their capacity to serve as antigen presenting cells could be utilized for triggering and/or potentiating immune responses to tumors and viruses. Alternatively, B cells expressing an autoantigen could be manipulated to induce antigen-specific unresponsiveness for treatment of autoimmune diseases. Efficient expression of an exogenous gene product in long-lived B lymphocytes could be particularly useful for providing a corrected gene product into the blood stream. Despite these advantages, efficient gene transfer into mature, primary B cells has not been reported. One reason for this is that current protocols for retroviral-vector-mediated gene transfer into lymphocytes rely on *in vitro* expansion and/or drug selection. This precludes the use of mature, primary B cells as targets, since they cannot be readily cultured for long periods of time. In this report, we describe an efficient and rapid protocol for the introduction of exogenous genes into primary B cells without the need for drug selection. We have used retroviral vectors containing the human *adenosine deaminase* (*ADA*) gene as a marker gene, since the biological activity of this enzyme is easy to measure and is readily distinguishable from the endogenous mouse ADA. Upon adoptive transfer into SCID mice, infected B cells continuously expressing 1-3 copies of the human ADA gene could be found in the spleens of recipient animals for at least 3 months.

C6-445 ***IN VIVO*** **DELETION ANALYSIS OF A MULTIDOMAIN ONCOPROTEIN: HORMONE DEPENDENT SITE SPECIFIC RECOMBINATION WITHIN A RETROVIRAL VECTOR,** Fabio Rossi, Kelly McNagny, Colin Logie, Francis Stewart and Thomas Graf, EMBL, Differentiation Programme, Meyerhofstr. 1, 69117 Heidelberg, Germany.

The analysis of multidomain proteins ideally requires that the function of a specific domain can be abrogated selectively after the protein is expressed in cells. The only approach existing so far that fulfills this requirement is the use of temperature sensitive (ts) mutations in specific locations within the polypeptide. However ts mutations in the correct location are difficult and sometimes impossible to obtain. Furthermore, ts mutations can alter the function of a protein even at the permissive temperature, disturbing the interpretation of the results. We have now developed an alternative approach consisting in the fusion of the yeast FLP recombinase to the hormone binding domain of the human estrogen receptor (Logie and Stewart, submitted). In eukariotic cells expressing this construct the addition of estradiol induces the enzyme to perform a conservative recombination between two specific DNA sequences (FRTs, FLP Recombinase Targets), resulting in precise deletion or inversion of the intervening genomic DNA. Using the E26 avian leukemia virus Gag-Myb-Ets oncogene as a model system, we have been exploring the possibility of inducing specific deletions within this multidomain protein *in vivo* through the hormone dependent recombinase. For this purpose we constructed a retroviral vector expressing the FLP-ER protein and the Gag-Myb-Ets oncoprotein from the same bicistronic transcript, ensuring the coexpression of the two polypeptides in all the infected cells. Minimal FLP Target Sites were also inserted in different locations within the proviral genome in such a way that activation of the recombinase would result in deletion of specific sequences from the integrated virus. We found that treatment of the infected cells with estradiol or estrogen analogs efficiently induced deletions of specific domains of the Gag-Myb-Ets oncoprotein. At the same time, we observed a change in phenotype of the hematopoietic cells transformed by the construct, reflecting their known capacity to differentiate along different lineages This technique therefore allows efficient transduction and subsequent inducible *in vivo* modification of complex proteins, including the creation of in frame deletions and fusions. It should be applicable to a wide variety of systems, particularly those that require tight control of the biological activity of a protein, as well as permitting a detailed analysis of protein domains and their functions in living cells.

C6-446 ANTIBODY-DEPENDENT INACTIVATION OF RETROVIRAL VECTOR PARTICLES BY HUMAN SERUM MEDIATED BY ANTI-αGALACTOSYL NATURAL ANTIBODY, Russell P. Rother, Jeremy P. Springhorn, William L. Fodor, Stephen P. Squinto and Scott A. Rollins, Departments of Molecular Development and Immunobiology, Alexion Pharmaceuticals, New Haven, CT 06511

In vivo retroviral-mediated gene transfer has been restricted in humans by the rapid inactivation of retroviral vector particles by complement. The killing of retrovirus by human or nonhuman primate sera is currently believed to be attributed to an antibody-independent mechanism wherein the surface protein P15E binds the complement component C1 thereby directly activating the classical pathway of complement. In this report, we show that the inactivation of retroviral vector particles is mediated through an antibody-dependent mechanism involving αgalactosyl epitopes present on the viral envelope. The Moloney murine leukemia virus derived LXSN retroviral vector was effectively inactivated in 40% sera from Old World primates (human, baboon and rhesus) but not sera from New World primates (squirrel monkey, owl monkey and tamarin). Retroviral inactivation by human serum was completely inhibited by pretreatment with soluble galactose α1-3 galactose (galα1-3gal) but not by pretreatment with glucose, galactose or fucose. Galα1-3gal was demonstrated on the surface of the LXSN retroviral vector particles by GS-IB4 lectin binding in an enzyme-linked immunosorbent assay. Addition of soluble galα1-3gal to these assays inhibited viral/lectin binding. Treatment of PA317 retroviral vector packaging cells with the glycoprotein processing inhibitor castanospermine rendered vector particles from these cells resistant to human complement-mediated inactivation. Additionally, down regulation of galα1-3gal epitopes in the same packaging cell line through coexpression of the H-transferase enzyme resulted in serum resistant vector particles. These data demonstrate that LXSN retroviral vector particles are inactivated in human serum by an antibody-dependent mechanism and that natural anti-galα1-3gal antibody present in the sera of Old World primates is most likely responsible for the initiation of the complement cascade. Further, these data show that decreased expression of galα1-3gal in retroviral vector packaging cells results in the production of complement resistant retroviral particles.

C6-447 **PROPERTIES OF AN AAV VECTOR PACKAGING CELL LINE,** Richard O. Snyder, Gary Kurtzman, and Susan S. Elliger, Avigen, Inc., 1201 Harbor Bay Parkway, Alameda, CA 94502

The production of recombinant adeno-associated (rAAV) vectors requires four genetic elements: 1) mammalian tissue culture cells (e.g. 293, HeLa, KB), 2) vector sequences containing a heterologous gene flanked on both ends by AAV inverted terminal repeats, 3) helper sequences comprising the AAV genes, and 4) a helper virus (e.g. adenovirus) which acts at the transcriptional and translational levels. A standard method for producing rAAV vectors is by the transient transfection of rAAV vector and helper sequences into tissue culture cells and infection with adenovirus. Transient transfection is inefficient and poorly reproducible, so a stable cell line harboring the vector and AAV helper sequences would be an advantage to avoid transient transfection.

We report the development of a cell line capable of packaging rAAV vectors that stably harbors all of the AAV open reading frames. The AAV non-structural gene encoding for the larger Rep proteins (Rep78 and Rep68) has been placed under the control of a heterologous *lacI/O* inducible promoter. The non-structural gene encoding for the smaller Rep proteins (Rep52 and Rep40) and the structural genes (cap) remain under the control of their endogenous promoters. The Rep78 and Rep68 proteins were difficult to maintain stably even when the promoter was repressed and the cell line obtained appears to have survived a crisis stage. The cell line does not synthesize detectable amounts of the Rep78 and Rep68 proteins until it has been induced, whereupon it makes high levels. The Rep52 and Rep40 proteins are not cytotoxic and are expressed constitutively, but the expression from the P_{40} promoter is undetectable in the absence of adenovirus. This cell line is capable of packaging helper-free transduction-competent rAAV vectors to give titers of up to 1×10^7/ml when vector sequences are delivered by transient transfection and this output has been maintained for nearly one year. In addition, evidence suggests that it may be difficult to create cell lines stably harboring both helper sequences and vector sequences, and means of overcoming this problem are discussed.

C6-448 A NEW SYSTEM PRODUCING RECOMBINANT EPSTEIN-BARR VIRUS, Kenzo Takada, Hironori Yoshiyama and Norio Shimizu, Department of Virology, Yamaguchi University School of Medicine, Ube, Yamaguchi 755, Japan

Epstein-Barr virus (EBV) is ubiquitous and infects most human populations. After primary infection, the virus persists in a latent state for the rest of individual's life although EBV-specific immunity is established in infected person. Reactivations occur frequently and asymptomatically, which is evidenced by the presence of infectious viruses in saliva.

There has been no infection system such as that which produces viruses following EBV infection. Therefore, it has not been possible to produce a large amount of recombinant EBV for human gene therapy. In the present paper we describe a new cell system which allows to propagete recombinant EBV.

The Akata cell line was established from a Japanese patient with an EBV-positive Burkitt's lymphoma. The Akata line is now commonly used to study reactivation of latent EBV, because cells produce EBV rapidly and efficiently after cross-linking of cell surface immunoglobulins (Ig) with anti-Ig antibody. Recently, we noticed that EBV DNA was lost from some of Akata cells during cultivation, and isolated EBV-negative clones. We found that EBV-negative Akata cells are good host for EBV infection and replication.

The procedure for producing recombinant EBV is as follows. EBV-positive Akata cells have about twenty copies of EBV plasmid per cell. After inserting the drug-resistant gene into a EBV plasmid of EBV-positive Akata cells, by using homologous recombination, produced viruses, that were a mixture of wild type and recombinant EBV, were infected into EBV-negative Akata cells. After three weeks of incubation in the selective media, many drug-resistant clones could be isolated very easily, and most of these clones were infected with recombinant EBV alone. By treating cells with anti-Ig, a large amount of virus, more than 10 ug per one litter culture,was produced. Therefore, the system should be very useful for preparing a large amount of recombinant EBV for human gene therapy.

EBV infects B-lymphocytes with a high efficiency. The entire viral genome is stably maintained in cells as plasmids. The genome size is 175 kb and EBV has high capacities for added foreign sequences and is able to transfer and express large, intact genes. When considering to use the EBV vector for application to human, we need to develop the EBV vector deleted of EBV genes responsible for cell transforming activity.

C6-449 PHARMACOLOGIC IMMUNOMODULATION ENHANCES REPEATED *IN VIVO* ADENOVIRUS-MEDIATED GENE TRANSFER, Bruce C. Trapnell, Soonpin Yei, Nanette Mittereder, Ke Tang, Linda Weaver, Chris Mech, and Paul Tolstoshev. Genetic Therapy, Inc., Gaithersburg, MD 20878.

First generation adenovirus (Av1) vectors are efficient *in vivo* gene delivery vehicles currently under evaluation in gene therapy protocols for cystic fibrosis. Despite high efficiency, transient transgene expression has suggested a need for repetitive administration of Av1 vectors. However, recent studies (Yei, et.al. Gene Therapy, 1:192-200;1994) in cotton rats have demonstrated that host responses to these vectors correlate with decreased efficacy of gene transfer and expression after repeated administration. We have evaluated the various components of this host response in cotton rats and non human primates which can be catergorized into 1) direct cellular toxicity; 2) non specific cellular inflammation and cytokine releases; 3) specific anti-adenovirus antibody responses; and 4) specific cytotoxic lymphocyte (CTL) responses. We hypothesized that concurrent brief intermittent immunosuppressive therapy may increase the efficacy of repeated Av1 vector administration. To evaluate this concept, 10^{10} plaque forming units (pfu) of an E1-, E3-region deleted Av1 vector Av1Cf2, expressing the human CFTR cDNA was administered to the lungs of cotton rats (n=3 per group) by intranasal inhalation with or without coadminstration of dexamethasone (2 mg/kg by intraperitoneal injection) daily, beginning one day prior and continuing for ten days after administration of the vector. Six weeks later, all animals received a repeat intranasal pulmonary administration of an Av1 vector that expressed a luciferase reporter enzyme instead of CFTR. The efficacy of transfer and expression of the transgene for the repeat dose was evaluated after 3 days. Repeated Av1 vector transduction was successful and importantly, the efficiency of repeat Av-mediated gene transfer was significantly higher in the group receiving steroids compared to the group without steroids (11,786+3523 light units (lu) vs 622± 192 lu, respectively; $p<0.05$. Various components of the host response were evaluated and changes were correlated with transgene expression.

C6-450 REGULATION OF VIRAL AND THERAPEUTIC GENE EXPRESSION IN ADENOVIRUS VECTORS, SC Wadsworth, D Armentano, C Sookdeo, L Cardoza, P Berthelette, and AE Smith, Genzyme Corporation, Framingham, MA, 01701

Adenovirus gene therapy vectors must function efficiently in two completely different environments. During vector production, efficiency of the viral infection cycle is paramount. In clinical use, efficient expression of the therapeutic gene is desired while viral gene expression must be minimal to eliminate cytotoxicity from viral proteins and to minimize the potential for a cell-mediated immune response. We have been examining both aspects of Ad2-based E1-replacement vector design in parallel to make adenovirus vectors safer and more efficacious while preserving efficient vector production characteristics. With regard to Ad vector safety, it is desirable to delete non-essential sequences. We have deleted sequences from the E4 region not required for vector growth in vitro. Vectors deleted for all but E4 ORF6, resulting in shortening of the genome by approximately 2kb, can be efficiently propagated to high titer. This modification results in a nearly 10-fold reduction in intracellular fiber protein levels although virions contain wild-type amounts of fiber. Fiber expression was subsequently restored by including a more efficient polyadenylation signal. It is also desirable to delete vector sequences that have the potential to recombine with Ad5 sequences in the 293 producer cell line because such a recombination event could generate replication competent adenovirus (RCA). Results of progressive deletion of viral sequences with respect to generation of RCA in 293 cells will be discussed. Regarding the overall efficacy of vectors we have shown that the choice of an efficient polyadenylation sequence as well as a long lasting promoter contributes to the expression level of therapeutic gene sequences. A spectrum of promoters with different strengths has been incorporated into vectors expressing either the cystic fibrosis transmembrane conductance regulator (CFTR) or reporter genes. The strong CMV promoter initially directs high levels of gene expression but reduction of expression occurs rapidly. The E1A and PGK promoters initially direct lower expression levels than that achieved by the CMV promoter but appear to be less prone to inactivation. The use of an efficient polyadenylation sequence was shown to significantly improve CFTR expression levels. In conclusion our studies indicate that useful vector backbone modifications can result in unexpected perturbations in viral gene expression that can in turn hamper vector production. Similarly, insertion of therapeutic gene expression cassettes may not function as expected in the context of the vector genome.

C6-451 DEVELOPMENT OF RETROVIRAL VECTORS FOR GENE THERAPY OF CANINE PYRUVATE KINASE DEFICIENCY. Katharine M. Whitney, Tsghe Abraha and Clinton D. Lothrop Jr., Scott-Ritchey Research Center, College of Veterinary Medicine, Auburn University, Auburn, AL 36849

Inherited hemolytic anemia due to pyruvate kinase (PK) deficiency is caused by an absence or abnormality of the erythroid-specific (R-type) isoform of the glycolytic enzyme. A canine model of the disease exists in the Basenji breed due to a coding sequence point deletion (ΔC^{433}) which causes complete absence of functional R-type PK. We have cloned the normal and mutant canine R-type PK cDNAs, constructed functional chimeric rat/canine cDNAs and developed retroviral vectors to be used in *in vivo* experiments for gene therapy of canine PK deficiency by autologous transplantation of *ex vivo*-transduced hematopoietic stem cells (HSCs). Amphotropic retroviral producer clones derived from PA317 and PG13 packaging cell lines transduced with pLXSN constructs incorporating full-length rat/canine chimeric R-type PK coding sequence (pLχRPKSN) were isolated and characterized by immunoblot, Northern and Southern blot analyses, assay for heat-stable PK activity, and viral titer by transfer of G418 resistance to canine thymic cells (Cf2-Th). Several clones with high titer ($2\text{-}3\text{x}10^5$ colony forming units [cfu]/mL) have heat-stable PK activity (representing transduced gene expression versus heat-labile endogenous M_2-type PK activity) and levels of R-type PK comparable to endogenous fibroblastic M_2-type PK on immunoblots. Supernatants of clones generating high titers are currently being used to transduce canine hematopoietic stem cells from bone marrow and peripheral blood.

C6-452 **Targeted retroviral gene delivery through Stem Cell Factor/c-Kit receptor interaction.** Corinne Wong*, Noriyuki Kasahara#, Andrée Dozy†, Morton J. Cowan* and Y.W. Kan#†, *Department of Pediatric Bone Marrow Transplantation, #Department of Laboratory Medicine and †Howard Hughes Medical Institute, University of California San Francisco, 94143.

A retroviral vector containing the targeting ligand, human stem cell factor (SCF), has been designed to enhance species and tissue-specific infection of SCF receptor (c-kit) positive cells. The virus coat contains a chimeric viral envelope consisting of the extracellular domain of SCF in place of the amino terminus of the wild type ecotropic murine Moloney leukemia virus envelope (SCFenv).

Viruses displaying the chimeric SCFenv were produced in BOSC 23 or GPE86 packaging cell lines. Expression of SCFenv in the packaging cells was confirmed by RT-PCR and Western blot. The presence of SCFenv protein in the virus-containing fractions was also confirmed by Western blot after sucrose density gradient fractionation of the supernatant culture medium from the packaging cells. The viruses were harvested and used to infect NIH3T3, HeLa (SCF receptor negative) and HEL cells (SCF receptor positive). The SCFenv chimeric coat, previously ecotropic, is therefore now capable of mediating tissue-specific, cross-species infection of human cells bearing the SCF receptor (c-kit). These SCF receptor-targeting vectors are now being used to deliver DNA to stem cells derived from fetal liver, peripheral and cord blood.

In summary, the binding interaction of the SCF multi-lineage growth factor and c-kit receptor is being used to alter the natural host range of ecotropic retroviral vectors and thus specifically target infection to hematopoietic stem cells from bone marrow, peripheral blood and fetal liver. Efficient gene transfer to these rare early progenitor cells will have important implications for long term expression of therapeutic genes.

C6-453 EPSTEIN BARR-VIRUS-DERIVED PLASMIDS AS SHUTTLE-VECTORS FOR GENE THERAPY, Reinhard Zeidler, Bettina Kempkes, Dagmar Pich and Wolfgang Hammerschmidt, Institut fuer klinische Molekularbiologie und Tumorgenetik, GSF-Forschungszentrum für Umwelt und Gesundheit GmbH, Marchioninistrasse 25, 81377 München (Germany).

The Epstein Barr-Virus is the only known virus, which is able to infect and immortalize human B cells with high efficiency in vitro. Antibodies against viral proteins can be found in more than 90% of the population. In few individuals, the primary infection causes infectious mononucleosis with up to 10% of infected lymphocytes in the peripheral blood. Due to the hosts immune system the rate of these cells drops rapidely, but for unknown reasons a small population of infected B lymphocytes (1 in 10^{5-6}) escapes elimination. Usually, the first contact with EBV is clinically inapparent but results in a latent infection with EBV for a lifetime.

We have constructed EBV-derived recombinant plasmids ("mini-EBV") which are able to immortalize primary human B-lymphocytes in vitro by transfection of their naked DNA. These plasmids carry all the viral genes necessary for their permanent intracellular maintenance (latency) but lack most of the genes for the productive lytic lifespan. Therefore, no virus is produced and secreted.

Mini-EBVs have been constructed in E.coli using the chromosomal building technique described by O'Connor et al. (Science **244**:1307, 1989). This method is advantageous in that any human gene of interest can be added to mini-EBV plasmids easily. B-lymphocytes of human donors can thus be immortalized by direct transfection of mini-EBV DNA and cultivated in vitro. It is very likely, but remains to be tested, that these cells, after autologous transplantation into the donor, behave like naturally infected cells and will be controlled by the hosts immune system. For these reasons we believe that mini-EBV, carrying one or even more additional human genes of interest is a very good canditate vector for gene therapy.

C6-454 CONTINUOUS PRODUCTION OF HIGH TITER RETROVIRAL VECTORS IN A NOVEL PACKED-BED AIR-LIFT BIOREACTOR Shuyuan Zhang, Gerard McGarrity, Perry Newton, Hitoshi Kotani, Genetic Therapy, Inc., Gaithersburg, MD 20878

A novel packed-bed air-lift bioreactor was designed for long term, large-scale continuous production of high titer retroviral vectors from producer cells. Two different retroviral vector producer cells were cultured successfully in the bioreactor for a period of more than 1 month with continuous harvest of vector supernatant up to 12 L/day. Vector titer in the harvested supernatant maintained in the order of 10^7 CFU/ml for up to 20 - 25 days in culture, with a maximal vector titer of $1x10^8$ CFU/ml. Vector titers decreased in the late stage of cultures to the order of 10^6 CFU/ml. High lactate concentration was found to be one of the inhibitory factors on vector production. Retroviral vectors generated in a production period of 40 days with two different producer cells were all free of replication competent retrovirus (RCR). A total of $4x10^{10}$ producer cells was achieved after 10 days in culture in the pilot scale bioreactor packed with 700 ml of macroporous glass beads as estimated from cell-specific lactate production rates. The total cell number represented a 200-fold increase compared to the inoculation cell number of $2x10^8$. Cell concentration as high as $7x10^7$ cells/ml of beads was achieved in the packed bed. The scaleability and the high cell concentration attained in the bioreactor makes it feasible for continuous production of high titer retroviral vectors in a pharmaceutical level for human gene therapy.

Cancer

C6-500 PATHOBIOLOGY OF THE *IN VITRO* BYSTANDER EFFECT: ROLES OF CYTOKINE RELEASE AND CELL-CELL CONTACTS, Camille N. Abboud*+, Karen E. Frediani*, Jane L. Liesveld*, Scott M. Freeman#, Departments of Medicine* and Medical Oncology*, University of Rochester School of Medicine, Rochester, NY 14642, and Department of Pathology and Laboratory Medicine#, Tulane University School of Medicine, New Orleans, LA 70112

The application of suicide gene therapy to refractory malignancies has relied on the demonstration of a bystander effect *in vivo* and *in vitro* through which tumor cells that express the HSV-TK gene mediate the killing of nearby untransduced HSV-TK negative cells after exposure to ganciclovir (Cancer Res 53:5274, 1993). This report details the behavior of the mesenchymal cell line GCT derived from a fibrous histiocytic tumor, genetically modified to express the HSV-TK gene. GCT-STK cells were killed upon exposure to 50 μM ganciclovir in vitro. Examination of these cells by fluorescence microscopy revealed nuclear fragmentation and apoptotic cell death. This was confirmed by FACS analysis and electron microscopy. Furthermore, unlike other tumors (such as the colon carcinoma HCT-STK) studied in our laboratory, we were able to demonstrate by E.M. the presence of direct cell-cell communications possibly mediating the transfer of toxic phosphorylated GCV. This was underscored by the demonstration of a soluble mediator present in the GCT-STK conditioned media that effectively killed up to 90% of the untransduced GCT cells. This effect was not reproduced with confluent primary human marrow stromal fibroblasts or umbilical cord endothelial cells. In order, to better understand the *in vivo* pathobiology of the bystander effect, we next determined the cytokine(s) released by GCT-STK cells after exposure to ganciclovir. Indeed, these cells increased their release of interleukin-1 α and ß, interleukin-6, TGF-ß. These events underscore the chain of events that are triggered by the *in vivo* bystander phenomenon, and point towards future experimental approaches to elucidate its mechanism(s).

C6-501 **GENE THERAPY OF CANCER USING RECOMBINANT ADENOVIRUS VECTORS EXPRESSING CYTOKINES**

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Many groups have shown that immune responses can be modulated by the delivery of exogenous cytokines, and in tumour bearing animals cytokines can boost anti-tumour activity. We have inserted the human IL-2 cDNA under the control of the human cytomegalovirus IE promoter (HCMV) in an Ad 5 vector. This vector directs the secretion of 1-8 μg of biologically active protein per 10^6 infected human cells over a period of 2 to 4 days, with expression of the gene persisting for at least 7 days post infection.

A transgenic mouse model for breast carcinoma has been used to test the efficacy of these adenoviral vectors in tumour therapy *in vivo*. These mice express the polyoma middle T-Ag under the control of the murine mammary tumour virus LTR (MMTV) and develop carcinomas of all mammary epithelium at 8 to 10 weeks of age (Guy et al, 1992; *Molecular and Cellular Biology*; 12; 954). When tumour cells were infected with AdCAIL-2 *in vitro* and injected s.c. in syngeneic mice, there was a 4-5 week delay in tumour onset compared to controls, but 100% of animals eventually developed tumours. In contrast, if $5x10^8$ pfu of AdCAIL-2 was directly injected intra-tumourally, treated animals showed complete tumour regression while control tumours continued to grow. Mice cured of tumour burden by AdCAIL-2 injection were protected against a subsequent challenge with 10^6 PyMidTAg tumour cells s.c. in the opposite flank, and remained tumour free at least 12 weeks post-challenge. These results suggest that delivery of IL-2 into tumour bearing animals is more efficacious in promoting anti-tumour activity than if co-delivered with the tumour cell load. Direct injection appears to be not only more effective but is more readily applied to therapy of human cancers. Ability to induce protective immunity implies that this approach may be successful in the treatment of metastases.

C6-502 HUMAN MULTIPLE DRUG RESISTANCE GENE TRANSFER INTO HUMAN HEMATOPOIETIC PROGENITORS, Jesusa Arevalo, Jan A. Nolta, and Robertson Parkman, Department of Research Immunology/Bone Marrow Transplantation, Childrens Hospital of Los Angeles, L.A., CA

The human Multiple Drug Resistance gene (MDR) is a drug efflux pump which is expressed at low levels in hematopoietic progenitor cells. The hematopoietic system is therefore extremely sensitive to antineoplastic agents. We are working toward gene therapy for cancer by transferring the MDR gene into $CD34^+$ progenitor cells to allow resistance to chemotherapeutic drugs and to reduce ensuing cytopenia. We have derived high titer clones of a Harvey Sarcoma Virus retroviral vector containing the human MDR cDNA (pHaMDR1/A) in the PG13 cell line, which packages virions with a Gibbon Ape Leukemia Virus *env* protein. We have determined that the PG13 line more efficiently transfers the neo marker gene into $CD34^+$ progenitors from human bone marrow than the standard amphotropic packaging line, PA317 (26% PG13/LN vs. 12% PA317/LN). PG13/HaMDR clones were derived by selection in 60 ng/ml colchicine and by acquisition of single cells expressing increased levels of MDR by FACSVantage. The resulting clones were titered on HeLa cells. Cell-free supernatant from the highest titer clones was used to transduce $CD34^+$ cells from human bone marrow and cord blood. Colony-forming assays were performed with or without 10 ng/ml colchicine or 10ng/ml taxol to quantitate the extent of gene transfer. Cells from each experiment were plated in long-term bone marrow culture to allow analysis of the transduction extent of long-term culture initiating cells (LTCIC). Primers specific for the pHaMDR vector were used to detect the presence of provirus in transduced long-term cultures and single hematopoietic colonies by PCR. Our data will be applicable to gene therapy to reduce supression of hematopoiesis following high dose chemotherapy.

C6-503 VIABLE RECOMBINANT VACCINIA/IL-2 INFECTED MURINE ADENOCARCINOMA CELLS VS. ONCOLYSATE VACCINES,

Jerry A. Bash, Ph.D., Department of Medical Laboratory Sciences, Florida International University, Miami, FL 33199.

Vaccinia virus infection of tumor cells has been shown to augment anti-tumor immune protection in animals previously primed with the virus (helper effect). Our previous work indicated that exogenous IL-2 administered at the time of vaccinia/tumor cell vaccination obviates the need for virus priming to induce protection. Likewise, recombinant vaccinia/IL-2 (vCF13) infected tumor cells were shown to be of enhanced immunogenicity over transfection control vaccinia (vTFCLZ-1) infected tumor cells in a Balb/c adenocarcinoma CT26 protection model. Since the use of lysed vaccinia-infected tumor cell vaccines (oncolysates) has a safety advantage ove viable vaccinia-infected tumor cells in clinical application, the present study was performed to compare the efficacy of these two approaches. Duplicate cultures of CT26 tumor cells were infected with vaccinia/IL-2 recombinant (strain_vCF13) at a 10:1 multiplicity of infection for 24 hr. Infected tumor cells were then harvested and adjusted to 10^6 viable cells/ml and supernatants were assayed for IL-2 production using the CTLL-2 bioassay (^{3}H-thymidine incorporation). Infected cells from one duplicate culture were then disrupted by sonication and freezing and thawing. Balb/c mice (10 mice/group) were given three weekly subcutaneous injections of either 10^5 viable vCF13-infected CT26 or an oncolysate prepared from an equivalent number of vCF13-infected CT26. One week after the third injection all mice in immunized groups as well as in an unimmunized control group were challenged with 10^5 viable CT26 tumor cells. Tumor growth was monitored by palpation and micrometer measurement for three weeks. Tumor-free mice were then sacrificed and splenic lymphocytes were cultured with Mit-C treated CT26 and assayed after 5 days for CTL activity in a standard 4 hr. ^{51}Cr-release assay. The results suggest an important role for cytokine gene expression by viable antigen-presenting tumor cells in the generation of CTL-mediated anti-tumor responses.

Supported by NCI Grant: 1R115CA9975-01

C6-504 RETROVIRAL-MEDIATED TRANSFER OF THE MULTIPLE DRUG RESISTANCE GENE INTO PRIMITIVE PERIPHERAL BLOOD PROGENITOR CELLS (PBPC)

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Drug-induced myelosuppression is a frequent reason for curtailing chemotherapy in cancer patients. We are developing an Mdr1 gene therapy protocol which should render a patient's hemopoietic system more resistant to anticancer drugs. CD34-selection of mobilized peripheral blood resulted in a median CD34+ cell purity of 89%. Hemopoietic cells were either co-cultivated on packaging cells producing the IGmdr1-2 vector or cultured in cell-free virus-containing supernatant (SNT) with a titer of at least $1x10^4$ infectious particles per ml. Subsequently, vincristine titrations were performed to screen for *Mdr1* overexpression. Four-day co-cultivation in the presence of IL-3 resulted in 5.3 (± 1.1) % vincristine-resistant CFU-GM (n=3). The highest transduction efficiency following SNT incubation was observed after 2-day culture in IL-3, which resulted in 3.0 (±2.2) % vincristine-resistant CFU-GM. A 2-day prestimulation in IL-3 prior to a 2-day transduction yielded inferior results of 1.5 (±1.7) % resistant CFU-GM. Aliquots were cultured in a cocktail of SCF, IL-3, IL-6, G-CSF and GM-CSF for 10 days which leads to a terminal differentiation of PBPC into mature myelo-monocytic cells. Rhodamine exclusion, a function of the MDR1 protein, was measured by flow cytometry in these samples. 6% of the 4-day IL-3 co-cultivation group and 1.8 % of the cells incubated for 2-days in SNT stained Rh123-dull. This effect was abolished by incubation with the MDR1 reversal agent cyclosporin A. These results were paralleled by the direct enumeration of week 6 stroma-dependent cobblestone area forming cells (CAFC) in a limiting dilution assay, an *in vitro* model for the most primitive hemopoietic stem cells. Interestingly, in the 4-day IL-3 co-cultivation group chemoresistance could be maintained in 2% of the CAFC for a period of 6 weeks under continuous selection pressure. For the SNT incubation for 2-days in the presence of IL-3 this percentage ranged between 0.1 and 1%. These results suggest that pluripotent hemopoietic progenitor cells were transduced with the Mdr1 gene.

C6-505 FUNCTIONAL EXPRESSION OF A SINGLE CHAIN FV/γ RECEPTOR WITH RENAL CELL CARCINOMA SPECIFICITY IN ACTIVATED HUMAN PBL, Reinder L.H. Bolhuis*, Mo E.M. Weijtens*, Ralph A. Willemsen*, Dinko Valerio**, *Dept. of Clinical and Tumor Immunology, Daniel den Hoed Cancer Center, Rotterdam, The Netherlands. ** Dept. of Medical Biochemistry, University of Leiden, The Netherlands.

We functionally grafted cytotoxic T lymphocytes with a chimeric single chain Fv/γ receptor, specific for renal cell carcinoma. Chimeric single chain antibodies (scFv) consisting of the variable parts of the G250 antibody were constructed and linked to the γ-chain of the FcεRI receptor. The G250 antibody is directed against a renal cell carcinoma specific antigen. Single chain antibodies were constructed by fusing the V-heavy (V_H) chain to the V-kappa (V_K) chain by a flexible linker sequence. The chimeric scFv was fused in frame to the γ chain and cloned into the retroviral vector LXSN.

The chimeric G250 scFv/γ was transfected into the amphotropic packaging cell line PA317. Bulk culture was tested positive for production of amphotropic recombinant retroviral vectors. Anti-CD3 activated human PBMC were transduced by co-cultivation with an irradiated monolayer of the virusproducing cells. Subsequently, transduced PBMC were selected in G418 containing medium for 8 days and expanded in an *in vitro* culture system.

Results: CTL grafted with the chimeric single chain Fv/γ receptor specifically lyse renal cell carcinoma cells. Lysis of the renal cell carcinoma cells is blocked by G250 mAb.

Data on the regulation of signal transduction via the chimeric receptor and lymphokine repertoire will be presented.

C6-506 INDUCING APOPTOSIS INTO TUMOR CELLS VIA RETROVIRAL CONSTRUCTS EXPRESSING VARIOUS P53 ANALOGS AND EFFECTORS. Laurent Bracco, Claude Caron de Fromentel, Emmanuel Conseiller, Laurent Debussche, Michel Janicot, Didier Landais, Isabelle Rey and Bruno Tocque, Cellular and Gene Therapy Division, CRVA, Rhône-Poulenc Rorer, BP14, 13 quai Jules Guesde, 94403 Vitry-sur-Seine, France.

We have studied the effects of expressing various cDNAs able to induce a programmed cell death pathway into tumor cells *in vitro*. Wild-type p53 and several analogs with modified transactivation domains and/or oligomerization domains were compared in addition to one of the known wild-type p53 induced target, i.e, waf-1. Results in several tumor cell lines caracterized for their p53 status will be presented.

C6-507 INTRATUMOR INJECTION OF A REC-ADENOVIRUS EXPRESSING MURINE IL-6 ATTENUATES TUMOR GROWTH IN A TRANSGENIC BREAST CARCINOMA MODEL, Todd Braciak[1], Christina Addison[2], Frank Graham[1,2], William Muller[1,2], Carl Richards[1] and Jack Gauldie[1], Departments of Pathology[1] and Biology[2], McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Two general approaches for a cellular based immunotherapy are currently being developed for cancer. One, adoptive immunotherapy involves expanding tumor reactive lymphocytes in culture with various cytokines. The alternate approach involves immunization with tumor cells themselves, however modified, in hopes of generating enhanced tumor specific immune responses. We are pursuing an active immunotherapeutic approach to cancer. Using our polyoma middle T antigen transgenic mouse model for breast carcinoma, we have begun to evaluate the efficacy of injecting recombinant adenoviruses expressing cytokines directly into tumors. The Ad5E1mIL6A+ vector, a recombinant adenovirus vector containing an E1 substitution of the murine IL-6 gene, produced significant levels of cytokine (up to 10ug/10^6 infected cells) in transgenic breast tumor cells in vitro. These transduced cells were tested for tumorigenicity after subcutaneous injection into syngeneic mice. Tumor cells infected with either Ad5E1mIL6A+ or the control virus AdDl70-3 (an E1 deleted virus) demonstrated no significant difference in their ability to form tumors. In contrast, direct injection into tumor bearing mice appears to modulate tumor progression. Mice were injected subcutaneously in the right hind flank with $1x10^6$ tumor cells and after 21 days had established palpable tumors. At this time, $1x10^9$ pfu of Ad5E1mIL6A+, AdDl70-3 vector or PBS were injected intratumorly and growth was monitored for the subsequent 4 week period. Mice with established subcutaneous tumors showed a marked decrease in tumor growth with the Ad5E1mIL6A+ vector versus controls (up to 3 fold reductions in tumor volume at 4 weeks). Although the mechanism is still to be elucidated, it is interesting to propose tumor infiltrating lymphocytes (TILS) as mediators of this activity since this effect was only observed in the established breast tumor at a time point when TILS are present and IL-6 is a known lymphoproliferative factor with additional CTL inducing activity. Experiments are ongoing to address this issue. (Supported by MRC Canada, NCI Canada and London Life)

C6-508 THE USE OF COMBINATION GENE THERAPIES FOR THE TREATMENT OF CANCER.

Simon Castleden*, J. Arly Nelson,++ Heung Chong*, Ian Hart+ and Richard G. Vile*. *Laboratory of Cancer Gene Therapy, Imperial Cancer Research Fund, and +Richard Dimbleby Department of Cancer Research, Rayne Institute, St Thomas' Hospital, Lambeth Palace Road, London, SE1 7EH, U.K. ++Department of Experimental Paediatrics, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030, USA.

Previously, we have generated expression vectors in which therapeutic genes are driven by the tissue specific tyrosinase promoter. Rather surprisingly, we have now shown multiple intravenous administrations of high titre retroviral supernatant encoding Tyr-HSV*tk*, but not liposome-complexed plasmid DNA, reduced the number of lung metastases of B16 melanoma in C57/BL mice treated with ganciclovir by up to 90% compared to controls. Using PCR analysis, integration of the provirus was observed in metastasis bearing lungs (4 of 6 mice) and in the spleens of some ganciclovir treated animals (2 of 5). However, the retroviral-mediated reduction in the number of lung metastases was not observed in T cell immunodeficient mice, suggesting that the immune system plays a role in mediating these effects. Similarly, data will be presented showing that a partial but significant anti tumour immune response is generated following *in vivo* killing of established tumour deposits with ganciclovir.

To enhance the combination of local cell killing and co-induction of anti tumour immunity to tumour cells killed *in vivo*, we have prepared plasmid and retroviral vectors containing IRES sequences in which the HSVtk gene is co-expressed with a series of immunomodulatory genes (including IL-2 and GM-CSF) As a prelude to *in vivo* delivery studies, tumours were seeded in which a proportion of the cells express either IL-2 alone, HSVtk alone or both genes. Our results show that when as few as 20% of the tumour cells are modified to express both genes together there was a significant reduction in the growth rate of the tumours in ganciclovir-treated mice compared to tumours expressing either gene alone. The immune-mediated mechanisms underlying the action of these vectors will be presented and the potential of their use for direct *in vivo* gene therapy of tumours will be discussed.

C6-509 **TARGETED PRODRUG ACTIVATION IN BREAST CANCER CELLS MEDIATED BY A RECOMBINANT ADENOVIRUS CONTAINING THE DF3 TUMOR-ASSOCIATED ANTIGEN PROMOTER WITH HERPES SIMPLEX VIRUS THYMIDINE KINASE GENE** Ling Chen, Yonghe Dong, Howard Fine and Donald W. Kufe, Division of Cancer Pharmacology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115

Patients with metastatic breast cancer usually succumb to their disease due to the non-selective nature and narrow therapeutic window of the available chemotherapeutic drugs. Recently, adenovirus-mediated transfer of the herpes simplex virus thymidine kinase (HSV-tk) gene has been shown to confer sensitivity to the cytotoxic effect of ganciclovir (GCV) in tumor cells. Since adenovirus infects a broad range of cell types, clinical application of this vector may be limited by non-specific transduction of therapeutic genes into cells other than the target cells. One strategy to circumvent this problem would be to use tumor tissue-selective/specific promoters or enhancers in order to direct gene expression in the desired target cell. A high molecular weight mucin-like glycoprotein, DF3 (MUC1), is a tumor-associated antigen overexpressed in 75-90% of breast cancers. We have constructed E1-deleted recombinant adenovirus containing the DF3 promoter (bps -725 to +31) with *E. coli* β-galactosidase gene (Ad.DF3-βgal) or HSV-tk gene (Ad.DF3-tk). Infection with Ad.DF3-βgal resulted in specific expression of β-galactosidase in DF3-positive breast carcinoma cell lines MCF-7, ZR-75-1 and other DF3+ tumor cell lines, while only a very low to undetectable level of expression was observed in DF3-negative tumor cell lines. Injection of Ad.DF3-βgal into human breast cancer xenografts in nude mice leads to targeted expression of the reporter gene in tumor cells but not in the surrounding normal tissue or injected skeletal muscle. MCF-7 and ZR-75-1 cells infected with Ad.DF3-tk were rendered sensitive to GCV, while Ad.DF3-tk infected DF3-negative Hs578T cells were insensitive. In a breast metastases (peritoneal carcinomatosis) model, our preliminary results demonstrates that injection of Ad.DF3-tk ($1x10^9$ pfu per i.p.) followed by GCV treatment resulted in partial to complete tumor regression. These results demonstrate that the recombinant adenovirus carrying the tumor-tissue selective promoter DF3 with a therapeutic gene is an efficient gene delivery system specifically targeted to DF3-positive cancer cells. These findings provide a potentially novel approach for breast cancer gene therapy.

C6-510 COMBINATION SUICIDE AND CYTOKINE GENE THERAPY FOR METASTATIC COLON CARCINOMA *IN VIVO*

Shu-hsia Chen[1,2], X.H. Li Chen[1], Ken-ichiro Kosai[3], Yibin Wang[2], Milton J. Finegold[3], Susan S. Rich[4] and Savio L.C. Woo[1,2]; 1. Howard Hughes Medical Institute; Departments of 2. Cell Biology, 3. Pathology, and 4. Microbiology and Immunology, Baylor College of Medicine, Houston, Tx 77030.

The efficacy and immune response of combination "suicide gene therapy" and "cytokine gene therapy" were investigated for the treatment of metastatic colon carcinoma in the liver. Tumor in the liver was generated by intrahepatic injection of a colon carcinoma cell line (MCA 26) in syngeneic BALB/c mice. After 7 days of tumor growth, recombinant adenoviral vectors containing various control and therapeutic genes were injected directly into the solid tumors, followed by treatment with ganciclovir. The tumors continued to grow in all animals treated with a control vector or a mouse interleukin 2 vector. Those animals that were treated with a Herpes Simplex Virus thymidine kinase vector, with or without the co-administration of the mouse interleukin 2 vector, exhibited dramatic tumor necrosis and regression. However, only animals treated with both vectors developed an effective systemic anti-tumoral immunity against challenges of tumorigenic doses of parental tumor cells inoculated at distant sites. The anti-tumoral immunity was associated with CD8+ cytolytic T-lymphocytes and were MHC H-$2K^d$ restricted. These cytolytic T-lymphocytes were also cytolytic against a second syngenic colon carcinoma cell line (CC36), suggesting the individual tumor cell lines shared specific tumor antigen(s). The results suggest that combination suicide and cytokine gene therapy *in vivo* can be a powerful approach for treatment of metastatic colon carcinoma in the liver.

C6-511 **RECONSTITUTION OF Fas/APO-1 SENSITIVITY FOLLOWING ADENOVIRUS-MEDIATED TRANSFER OF WILD-TYPE p53,** **James C. Cusack, Wei Wei Zhang, Jack A. Roth, Laurie B. Owen-Schaub, Departments of Surgical Oncology, Thoracic and Cardiovascular Surgery, and Immunology, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030**

Induction of apoptosis following monoclonal-antibody binding of the Fas/APO-1 cell-surface protein, a new member of the NGF/TNF receptor superfamily, has been described in normal and neoplastic cells. The mechanism in the α-Fas/APO-1 resistant cell that expresses the Fas/APO-1 receptor but upon antibody-binding fails to trigger apoptosis has not been identified. To determine the impact of p53 on α-Fas/APO-1 induced apoptosis, we treated an α-Fas/APO-1 resistant colon cancer cell line (KM12L4) that expresses mutant p53 with a recombinant adenovirus expressing wild-type p53, viral vector alone, or sham infection 48 h prior to treatment with α-Fas/APO-1. Fas/APO-1 expression (specific Mean Fluorescence Intensity) and apoptosis (specific DNA Fragmentation) were measured using flow cytometric analysis.

Treatment	Fas Expression (spMFI)	sp DNA Fragmentation
wt p53	6.3	66.2 %
vector	2.0	15.4 %
sham	1.4	1.4 %

Infection with wt p53 increased surface expression of Fas/APO-1 3.2-fold over vector alone group. Transfer of wt p53 similarly increased sensitivity to α-Fas/APO-1 4.3-fold over vector alone. These data demonstrate that transfer of the wt p53 gene 1) increased Fas/APO-1 expression and 2) induced α-Fas/APO-1 sensitivity. Furthermore, these data suggest a possible role of wt p53 in the apoptotic pathway triggered by binding of the Fas/APO-1 surface receptor.

C6-512 ANTISENSE DOWNREGULATION OF THYMIDYLATE SYNTHASE ACTIVITY IN HUMAN TUMOR CELLS: EFFECTS ON DRUG RESISTANCE, Janice M. DeMoor, Mark D. Vincent, Olga M. Collins, James Koropatnick, London Regional Cancer Centre, London, Ontario, CANADA, N6A 4L6

Thymidylate synthase (TS) is a key enzyme in the synthesis of DNA precursors, and an important target for cancer chemotherapeutic agents such as 5-fluorouracil. Treatment with antisense nucleic acids to downregulate TS activity in cancer cells may be useful in increasing the effectiveness of such drugs. We transiently transfected human breast cancer (MCF-7) cells *in vitro* with two vectors expressing antisense TS RNA under the control of a cytomegalovirus promoter (pRC/CMV). Antisense RNAs were targeted to 30 bases of the TS mRNA including part of the stem-loop structure at the translation start site (vector 1) and 30 bases spanning the exon1/exon2 boundary (vector 2). Vectors 1 and 2 were transfected singly, or in combination. On the order of 100 copies of intact transfected vector were present in cells between 24 and 48 h following transfection, as well as low molecular weight degraded vector. Intact and degraded vector were undetectable by 5-7 days. TS enzyme activity in the MCF-7 cells (measured by an [5-^{3}H]dUMP tritium release assay) was decreased by 20-80% within 2 days of transfection. In spite of successful downregulation of TS activity, subsequent resistance to the toxic effect of the antimetabolites 5-fluorouracil (in conjunction with leucovorin) and Tomudex was variable. *(supported by a grant from the LRCC Endowment Fund).*

C6-513 Gene Therapy of Rat Gliomas Using Adenovirus Vector-Mediated Transduction of the Cytosine Deaminase Gene Followed by Treatment with 5-Fluorocytosine. Dong, Y.H., Wen, P., Manome, Y., Hershowitz, A., Chen, L., Kufe, D.W. and Fine, H.A. Division of Pharmacology, Dana-Farber Cancer Institute, Harvard Medical School. 44 Binney Street, Boston, MA 02115

Pro-drug activation following tumor cell transduction with specific cytotoxic "suicide" genes is a potentially useful approach in cancer gene therapy. The nucleoside analogue, 5-fluorocytosine (5-FC), is virtually nontoxic to normal mammalian cells. However, it can be converted by *E. Coli* cytosine deaminase (CD) to the cell cycle specific anti-tumor drug 5-fluorouracil (5-FU). Here we report that a replication-defective adenovirus vector expressing the CD gene can effectively transduce rat glioma cells, resulting in effective anti-tumor activity following 5-FC treatment.

The adenovirus vector, Ad.CMV-CD, was constructed by inserting the CD gene, driven by the human cytomegalovirus (CMV) immediate early gene promoter/enhancer, into the E1 region of adenovirus type 5 genome through homologous recombination. We have previously shown that a 100% transduction of glioma cells can be achieved *in vitro*, in a dose (MOI) dependent manner, using a structurally similar adenovirus vector. Infection of rat 9L glioma cells with the recombinant Ad.CMV-CD led to dramatic increases in CD enzyme activity in the infected cells, again in a dose dependent manner. This increased CD activity was mirrored by increased sensitivity to the cytotoxic effects of 5-FC. For example, the IC50 of 5-FC for adenovirus infected cells was as low as 10μM, while the IC50 for uninfected cells was at least 500-fold higher (>5000μM). More importantly, the culture medium taken from Ad.CMV-CD infected, 5-FC treated cells completely inhibited the growth of uninfected cells in colony-forming assays. Also, co-culturing uninfected cells with varying numbers of infected cells in a dual chamber system resulted in complete inhibition of cell growth in both compartments following 5-FC treatment. These results suggest that the powerful CD-mediated anti-tumor activity following 5-FC treatment may be significantly augmented by a strong "bystander"effect. The combination of highly efficient adenovirus-mediated gene transduction, the large therapeutic window of 5-FC, the cell cycle specific action of 5-FU and the pronounced bystander effect, makes the adenovirus/CD/5-FC system a promising approach to cancer gene therapy. We are currently evaluating the efficacy of the Ad.CMV-CD virus for the treatment of rat gliomas *in vivo*. Data from the animal studies will be presented.

C6-514 *IN VIVO* CYTOKINE GENE THERAPY OF HUMAN LUNG TUMOR XENOGRAFTS IN SCID MICE, Nejat K. Egilmez, Rosa Cuenca and Richard B. Bankert, Department of Molecular Immunology, Roswell Park Cancer Institute, Buffalo, NY 14263

A number of studies have shown that introduction of various cytokine genes into tumor cells can provoke an anti-tumor immune response which can lead to suppression of tumor growth. The majority of these approaches involve *ex vivo* introduction of genes into patient cells which are then re-introduced into patients. The feasibility of this method is limited by the difficulty in successfully cultivating tumor cells *in vitro* and the numerous cumbersome manipulations of patient cells prior to re-delivery. These limitations would be eliminated if genes could be delivered to tumors *in vivo*. To this end, we are currently studying the efficacy of delivering cytokine genes into human lung tumor xenografts in SCID mice by DNA:cationic liposome complexes. We have constructed expression vectors containing a reporter gene (β-galactosidase) and several different human cytokine cDNAs. We have successfully used the cationic liposome mediated delivery to transfect cell-lines *in vitro* with the vector containing the human IL-2 cDNA and have shown that IL-2 production and secretion is stable for at least two weeks. More recently, successfull *in vivo* delivery and expression of the IL-2 gene was also achieved in human lung tumor xenografts. The therapeutic potential of IL-2 is now being tested in SCID mice with established human lung tumors and tumor biopsy tissue orthotopically engrafted into the lung. We also intend to evaluate the tumor-suppressive potential of a number of other cytokines, i.e. GMCSF, TNFα and interleukin-12, alone or in various combinations, using the same approach.

C6-515 Transformation of 10T1/2 cells by the proto-oncogene *snoN* is suppressed by the proto-oncogene *ski*
David J. Eling and Edward Stavnezer, Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0524

The overexpression of either v-*ski*, its proto-oncogene c-*ski* or *snoN* in quail embryo cells transforms these cells, and paradoxically, induces myogenic differentiation. To determine if *ski* and *snoN* could retain these abilities in mammalian cells both dexamethasone-inducible (pMam neo) and constitutive (retroviral, pBabe puro) *ski* - and *snoN*- containing expression vectors were constructed. 10T1/2 cells were used as recipients for gene transfer of each of these constructs. 10T1/2, a mouse mesodermal cell line can differentiate into several lineages, including myocytes. These cells are further characterized by post-confluent growth inhibition and a very low frequency of spontaneous transformation.

Overexpression of *SnoN*, but not *ski*, transformed 10T1/2 cells, as measured by morphology, growth rate, focus formation and growth in soft agarose. Both constitutive and conditional expression of v-*ski*, c-*ski*, human c-*ski* and *snoN*, did not promote the differentiation of these cells. However, co-expression of *ski* and *snoN* resulted in an increase in the number of vacuolated cells cultured in low serum. Furthermore, transformation of 10T1/2 cells by *snoN* was suppressed by the introduction of c-*ski* but not truncated v-*ski*. The carboxy terminus of c-Ski, absent in v-Ski, has been shown to associate directly with SnoN using in vitro cross-linking techniques. These results demonstrate that c-*ski* can suppress the transforming ability of *snoN* in 10T1/2 cells and that this occurs through heterodimerization of SnoN and c-Ski via the carboxy-terminal region of Ski.

C6-516 SUICIDE GENES AS AN ANTICANCER STRATEGY, S.M.Freeman, R.Ramesh, A.J.Marrogi. Department of Pathology and Laboratory Medicine, Tulane Medical Center, New Orleans, LA.

Genetically altered tumor cells expressing the Herpes Simplex Virus thymidine kinase gene (HSV-TK) have recently been shown to have a tumoricidal effect in vivo in mice. These cells demonstrated a potent anti-tumor effect on unmodified "bystander" tumor cells after exposure to ganciclovir (GCV). Further, it was shown that the ability of HSV-TK positive tumor cells to kill unmodified tumor cells was dependent on the mice having an intact immune system. The present study was undertaken to analyze the host anti-tumor response following injection of HSV-TK gene-modified cells into mice. The study was comprised of BALB/c mice in which tumors were established by injecting intraperitoneally (I.P.) syngeneic murine fibrosarcoma cells (kbalb-LNL) on day 0. Ten days later, the mice were inoculated with the PA-1 STK cells, a transduced human ovarian cell line carrying the HSV-TK gene, intraperitoneally. The mice subsequently received GCV twice a day for 5 doses, while control mice did not receive GCV. Mice were sacrificed at regular intervals on one, two, four, and six days post PA-1 STK injection and tumors were isolated from both the treated (n=10) as well as control (n=10) groups. Total cellular RNA was isolated from each of the tumor samples and tested by using reverse transcription (RT) / polymerase chain reaction (PCR) for the expression of cytokines which included TNFα, IL-1, IL-6, IL-10, IL-2, GM-CSF, and IFN-γ. The PCR amplified products were further analyzed by Southern blot hybridization for specificity and sensitivity. Expression of cytokines was not observed among the control group while TNF, IL-1 and IL-6 expression was detected in the treated group within 24 hours of injection of the gene-modified cells. IFN-γ and GM-CSF were identified 48 and 72 hours post-injection, respectively. Immunohistochemical analysis revealed infiltrating mononuclear cells expressing TNF. In addition, there was an increase in T cells, B cells, and macrophages invading the tumor post treatment. The results of this study demonstrate a cytokine cascade after injection of HSV-TK gene-modified cells suggesting that the "bystander effect" is in part mediated by cytokines which are released by tumor infiltrating cells. The use of HSV-TK cells as an anti-tumor agent represents a novel approach to cancer therapy.

C6-517 COMBINED EXPRESSION OF B7.1 AND IL-2: SYNERGY IN TUMOR REJECTION.

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Previous studies have shown that expression of the immune co-stimulatory molecule B7.1 (CD28 and CTLA4 counter receptor) reduces the tumorigenicity of some, but not all malignant cell lines in syngeneic mice. IL-2 expression has also been shown to reduce tumorigenicity. In this study we have examined the effect of expression of B7.1, IL-2 or both B7.1 + IL-2 as a strategy for the induction of immune mediated tumour rejection. Expression of either IL-2 or B7.1 in the NC cell line, originally isolated from a spontaneously arising breast adenocarcinoma in WHT/Ht mice, marginally reduces the rate of tumor formation. However, the combined expression of B7.1 + IL-2 has a synergistic effect on both the rate of tumor formation and disease free survival. Approximately 40% of the animals remaining tumor free for at least 150 days. In order to determine the therapeutic potential of combination B7.1 + IL-2 therapy, tumour bearing animals have been vaccinated with lethally irradiated B7.1 + IL-2 expressing NC cells. Whilst each of the untreated animals developed tumours greater than 20mm in diameter by 21 days, only 20% of the B7.1 + IL-2 treated animals formed slow growing tumours. The remaining 80% appeared to have rejected their tumors and were disease free for at least 60 days?. These studies suggest that vaccination with combination B7.1 + IL-2 modified, lethally irradiated, autologous tumor cells may prove a useful procedure for the induction of immune mediated tumor rejection.

C6-518 EXPRESSION OF CONSENSUS TYPE I INTERFERON GENE DISPLAYS POTENT ANTITUMOR ACTIVITY, INDUCES MHC CLASS I EXPRESSION AND ENHANCES NK CELL ACTIVITY,

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Type I interferons (IFN) are potent antiviral and antiproliferative cytokines. Gene therapy using those cytokines has been hindered due to in part the presence of multiple α-IFN genes. We used a synthetic IFN gene (IFNcon1) corresponding to the consensus sequence of the human type I IFNs to pursue its potential application against malignant diseases. Con1 protein synthesized in *E. coli* has significantly higher antiproliferative activity than other IFN-α's. A series of IFNcon1-containing vectors were constructed, including adeno-associated viral and retroviral vectors. The presence and expression of IFNcon1 in transfected leukemia K562 cells were confirmed by Southern, RNA dot blotting and antiviral assays. Constitutive expression of con1 resulted in significant growth inhibition in culture and reduced colony formation on soft agar in all IFN transfected clones. Furthermore, no tumors developed in nude mice injected with IFN-producing tumor cells whereas rapidly growing tumors were found in 92% of the mice injected with control cells. In animals received a mixture of equal amounts of parental and IFN-producing cells, both the incidence of tumor and the level of tumor growth were significantly retarded. In addition, surface expression of MHC class I molecules was significantly upregulated in IFN transfectants, compared to parental cells. Finally, natural killer cell-mediated lysis against target tumor cells was significantly enhanced when effector cells were pretreated with conditioned media collected from IFN-producer cultures. These results indicated that IFNcon1 exerted not only a direct antiproliferative effect on tumor growth but may also be able to stimulate host immune response against tumor mediated by MHC molecules and/or NK cells.

C6-519 NEUROTOXICITY OF ADENOVIRUS-MEDIATED TRANSFER OF HSV-tk IN PRIMATE BRAIN,

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Adenoviral-mediated transfer of the herpes simplex thymidine kinase (HSV-tk) gene confers sensitivity in glioma cells to ganciclovir (GCV), and *in vivo* leads to tumor destruction and prolonged survival in rodents. The toxicity in normal primate central nervous system of a replication-defective adenovirus vector carrying HSV-tk (ADV-tk) was studied in 6 baboons. A high dose (3×10^{10} particles of ADV-tk) and a low dose (1.5×10^{9} particles of ADV-tk) was injected into the centrum semiovale. The low dose corresponded to the anticipated therapeutic dosage whereas the high dose was expected to be toxic. Two animals (Group I) received a low dose and GCV (10 mg/kg BID IV x 14 d). Two animals (Group 2) received a high dose and GCV, and two animals (Group 3) received a high dose and no GCV. Cerebral MRIs were obtained before treatment, and at 3 and 6 wk after treatment. Necropsies were performed at 3 and 6 weeks, or at death. Both animals in Group 2 became ill during the GCV treatment; one died at 5 d, and the other became moribund and was euthanized at 10 d. Both animals had large areas of coagulative necrosis at the injection sites, cerebral edema, acute inflammation, and mass effect. Group 3 animals did not become ill, but had MRI abnormalities at the injection sites corresponding to 1.5 to 2 cm cystic cavities seen at necropsy. Peri-lesional inflammation was present at 3 wk and had resolved partially by 6 wk. Group 1 animals did not become ill and exhibited small MRI abnormalities at the injection sites. No cavities were seen grossly at necropsy, but microscopic foci of necrosis and resolving inflammation were present at 6 wk.

C6-520 GENE THERAPY OF CERVICAL CANCER BY ADENOVIRUS-MEDIATED p53 GENE TRANSFER,

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Suppression of p53 protein expression is the most frequently detected alteration associated with HPV positive human cervical cancer. HPV oncoprotein E6 can form complexes with p53 and promote p53 degradation. We introduced wild type p53 gene into cervical carcinoma cell lines to determine the effects of a recombinant adenovirus vector, Ad5CMV-p53 on the inhibition of cell growth.

Eight human cervical carcinoma cell lines, C33A, HT-3, CaSki, SiHa, HeLa, C4I, MS751, and ME180 were used, six with HPV16 or 18 present, and two with p53 mutations. These cells were grown in RPMI medium supplemented with 10% heat-inactivated FBS. The Ad5CMV-p53 contains the CMV promoter, wild type p53 cDNA, and SV40 polyadenylation signal in a minigene cassette inserted into the E1-deleted region of modified Ad5. Cells were plated at a density of 5×10^{4}/well in 12 well plates in triplicate, harvested and counted. Cell viability was determined by trypan blue exclusion. Cells were also cultured at 8×10^{3}/well in 96 well plates and pulse treated with [^{3}H]-thymidine. Western blot and immunohistochemical analysis were used to detect p53 protein in the infected cells with mouse anti-human p53 monoclonal antibody(PAb 1801). A recombinant adenovirus β-gal, Ad5CMV-β-gal was used to determine transduction efficiency. Supernatant of the infected cells was collected, centrifuged to remove adenovirus, and added to each cell line to estimate the bystander effect.

The transduction efficiency was 100% in all cells with a dose of 50MOI Ad5CMV-β-gal. The p53 protein was detected in Ad5CMV-p53 infected cells by western blot and immunohistochemical analysis. The growth of the Ad5CMV-p53 infected cells was significantly suppressed. The 50% growth inhibitory effect(IC_{50}) by cell count assay varied between cell lines from doses of 6 to 85 MOI and by [^{3}H]-thymidine incorporation assay between 5 and 95MOI. The supernatant collected from infected cells showed bystander effect with a dose of 100MOI.

These data suggest that Ad5CMV-p53 may be further developed as a potential novel therapeutic agent for cervical carcinoma.

C6-521 THERAPEUTIC CTL FROM MOUSE SPLEEN IMMUNIZED WITH IRRADIATED TNF-α GENE-MODIFIED TUMOR CELLS Eiichi Hara[1], Ken-ichi Inoue[1],Hitoshi Nishimura[1], Kiyoshi Kazumoto[1], Keiichi Itakura[2], John J. Rossi[2], and Donald B. Kohn[3] [1]Saitama Cancer Center, Saitama 362 Japan, [2]Beckman Research Institute of the City of Hope, Duarte, CA 91010, and [3]Children's Hospital of Los Angeles, CA 90027

Many experimental data have suggested that cytokine gene-modified tumor cells augment the host immune responses and are able to induce rejection of a subsequent challenge of the parent cells. But, the vaccination has not been sufficiently potent to effect cure of preestablished tumors. We investigated the therapeutic effect of CTL from mouse spleens immunized with irradiated m-IL-2 or h-TNF-α gene-modified colon 26 tumor cells on mice bearing tumors established in lung, thyroid, or brain. Cytokine genes were transduced via retroviral vectors(LXSN,LNCX) into colon 26. These cytokine secreting clones grew significantly more slowly than controls after sc. and iv. inoculation. Splenocytes from mice which were immunized 3 times sc. and ip. with irradiated unmodified, IL-2 or TNF-α gene-modified colon 26 were cultured in the presence of stimulant for 5 days. CTL from mice immunized with TNF-α gene modified colon 26 ($CTL_{TNF\text{-}C26}$) had the most potent cytotoxicity activity (10-fold) in vitro. Each CTL was adoptively transferred into mice bearing 5 day established parental pulmonary metastases without IL-2 systemic administration. The transfer of $CTL_{TNF\text{-}C26}$ was capable of curing mice (>2 months survival), while those from mice immunized with unmodified or IL-2 gene-modified colon 26 enhanced survival 2-fold, compared with control (mean survival 14 days). The transfer of $CTL_{TNF\text{-}C26}$ also prolonged the survivals of mice bearing tumors established in thyroid and brain. These results suggest that CTL elicited by the immunization with TNF-α gene-modified tumor cells is useful for the adoptive immunotherapy.

C6-522 IN VITRO TRANSDUCTION OF URINARY BLADDER CARCINOMA CELLS WITH MoMLV-DERIVED RETROVIRAL VECTORS IN THE PRESENCE OF URINE. Lisa Hexdall, Mirella Gonzalez-Zulueta, Anne R. Simoneau, Erlinda M. Gordon, W. French Anderson, and Peter A. Jones. Gene Therapy Laboratories, USC/Norris Cancer Center, Los Angeles, CA 90033.

Bladder cancer ranks as the fourth most common malignancy among men in the United States. Up to 80% of patients with superficial tumors will have one or more recurrences after initial standard treatment, and in 10-30% the tumor will progress to invasive disease. As a first step to determine the potential of intravesical gene therapy for bladder cancer using retroviral vectors, four human bladder carcinoma derived cell lines (EJ, HT9, HT1376, J82) were exposed to Moloney Murine Leukemia Virus (MoMLV)-derived vectors in the absence and presence of human urine. EJ cells contain a *H-ras* mutation, and HT9, HT1376 and J82 lines have a mutant p53 gene. At 40-50% confluency cells were infected with the G1BgSvNa vector, which contains a bacterial β-galactosidase gene under the long terminal repeat (LTR) control, in the presence of either polybrene (8 μg/ml) or protamine (10 μg/ml). Gene transfer efficiency in the absence of urine averaged 50-60% in both HT9 and HT1376 lines, and 10-20% in EJ and J82 lines as revealed by X-gal staining three days after transduction. The effect of urine on gene transfer was also tested in each cell line. Comparable transduction efficiencies were obtained in the presence of various concentrations of fresh human urine, and urine did not appear to decrease gene transfer in any of the four cell lines. Subsequently, cells were transduced with the G1Tk1SvNa.7 vector, which contains the herpes simplex thymidine kinase gene. Sensitivity of transduced and untransduced cells to Gancyclovir was determined. The Gancyclovir IC50 decreased from 30 μg/ml in untransduced cells to 0.03 μg/ml in transduced cells. Our results show that retroviral vectors can be used to transfer foreign genes with high efficiency into human bladder carcinoma cells, and that the presence of urine does not hamper retroviral based intravesical gene therapy. Studies in progress include intravesical implantation of tumor cells in nude mice, and surgical implantation of tumor cells in nude rats for future *in vivo* transduction with the G1BgSvNa and G1Tk1SvNa.7 vectors.

C6-523 LOSS OF ONCOGENIC POTENTIAL OF TUMOR CELLS FOLLOWING TRANSDUCTION WITH THE CONSENSUS IFN GENE IN AN AAV VECTOR, C.J. Hu, J.F.Zhang,Y.P.Geng and M.W.Taylor, Department of Biology, Indiana University, Bloomington, IN 47405

An adeno-associated virus plasmid vector was constructed containing the human consensus interferon (Infergen/r-metIFN-con1) downstream of the mouse metallothionein promoter and the neo resistance gene. Virus derived from this plasmid was used to transfect a series of tumor cell lines including K562, Eskol (a hairy cell leukemic cell line),293 and HeLa cells. Transductants were isolated with a high efficiency by selection in G418. All transductants produced Infergen as characterized by immunological specificity. The amount of interferon produced constitutively varied with the cell line, however in most cases, except for 293 cells where amounts were substantial, the levels were low. Infergen could be induced to high levels (8-10 fold) by induction with Zn.

When transformed Eskol, 293 and K562 cells were injected into nude mice no tumors arose, although in all cases parental cells and cells transformed with AAV-neo gave rise to tumor in 1-2 weeks. When equal amounts of transformed and untransformed cell populations were used in the injection, tumors grew much slower(or not all) and by 5 weeks no tumors were detectable.

MCH I and II analysis indicated that changes had occurred in the distribution of such markers on the cell surface. Thus the Infergene appears to act as a tumor suppressor gene in a variety of cell lines.

C6-524 TRANSFER OF HSV-tk GENE INTO MAMMALIAN CELLS : APOPTOSIS AND BYSTANDER EFFECT. Janicot, M., Boiziau, J., Pannier, P., Bonnevaux, H. and Tocqué, B. Rhône-Poulenc Rorer, Division of Cell and Gene Therapy, Molecular Oncology, Vitry/Seine, France.

Thymidine kinase (tk) is a key enzyme in cellular metabolism, catalyzing the transfer of the γ-phosphate from ATP to thymidine to produce dTMP. Various cellular kinases are involved in subsequent phosphorylations of dTMP leading to the production of dTTP which is then incorporated into DNA molecules by DNA polymerase. Taking advantage of the development of antiherpetic drugs such as an acyclic nucleoside analogue of guanine, ganciclovir (GCV), combination of transfer of the Herpes Simplex Virus thymidine kinase (HSV-tk) gene into mammalian cells followed by treatment with GCV gave rise to an effective drug sensitization system. GCV, metabolized by mammalian cells at very low levels, is transformed by HSV-tk into monophosphate form that is subsequently converted to GCV triphosphate by endogenous mammalian kinases. In addition to being a potent inhibitor of viral DNA polymerase, GCV triphosphate competes with normal nucleotides for DNA replication in mammalian cells causing inhibition of cell growth and cell death. Although this system represents an appealing and straightforward strategy for cancer gene therapy, efficacies of HSV-tk gene transfer into tumor cells *in situ* and therefore GCV-induced tumor mass regression represent major issues for such an approach. Using *in vitro* cell model systems, characterization of GCV-induced cell death (apoptosis) in HSV-tk-positive mammalian cells and subsequent transmission of apoptotic signal(s) to HSV-tk-negative cells (bystander effect) will be discussed.

C6-525 **MDR-1 SPECIFIC RIBOZYMES: An "ANTI"-GENE -THERAPY APPROACH TO REVERSE THE DRUG-RESISTANT PHENOTYPE DURING ANTICANCER CHEMO-THERAPY**

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A major obstacle to cancer chemotherapy is the constitutive or acquired resistance of tumor cells to a broad spectrum of cytotoxic drugs. Several mechanisms are thought to confer chemotherapy resistance. The most common form of drug-resistance has been ascribed to overexpression of the MDR-1 gene. MDR-1 encodes a single 175-kd phospho-glycoprotein, designated P-GP. P-GP serves as an energy-dependent drug efflux pump which reduces intracellular drug accumulation and, thereby, cytotoxicity. In order to reverse the multiple drug resistance phenotype, we made use of the ribozyme technique. A hammerhead ribozyme recognizing the GUC sequence at position - 4 close to the translation start site of the MDR-1 mRNA was either prepared by in vitro transcription (MDR-1-RZiv) or was chemically synthesized (MDR-1-RZs). Both MDR-1-RZiv and MDR-1-RZs specifically cleaved the full length MDR-1-mRNA into two parts each of the expected size under physiological conditions in an extracellular system. Site-specific cleavage was dependent upon time, temperature and the concentration of $MgCl_2$ used. In order to examine the in vivo potential of MDR-1RZ, a human pleural mesothelioma cell-line (PXF1118) and two adriamycin or vindesine resistant sublines (termed PXF1118-ADR and PXF1118-VDS, respectively) were established from primary tumor material. Transfer of MDR-1-RZiv and MDR-1-RZs into more than 98% of these cells was accomplished using liposomes. As evidenced by RT-PCR, incorporation of ribozymes resulted in significantly reduced expression of the MDR-1 mRNA. Furthermore, using an anti-MDR-1 specific monoclonal antibody, we were able to demonstrate that MDR-1RZ reduces P-GP overexpression at the protein level and, thereby, abolishes P-GP function as determined by Rhodamine-123 exclusion assays. As a consequence, liposome-mediated transfer of MDR-1-RZiv or MDR-1-RZs reversed the multidrug resistance phenotype in 70 % and 97 % of previously chemoresistant cells, respectively, thereby restoring cellular sensitivity towards chemotherapeutic drugs. Further studies using a multidrug-resistant human colon cancer xenograft animal model are under way to elucidate the potential of MDR-1-RZ to circumvent chemotherapy resistance *in vivo*.

C6-526 REDUCTION IN SEVERITY OF PROLIFERATIVE VITREORETINOPATHY BY SIMULTANEOUS INTRAVITREOUS INJECTION OF FIBROBLASTS BEARING THE HS-Tk GENE AND GANCICLOVIR. Kimura, H., Sakamoto,T., Spee, C., Hinton, D.R., Scuric, Z., Gordon, E.M., Anderson, W.F., Ryan, S.J. Doheny Eye Institute, Departments of Ophthalmology and Gene Therapy Laboratories, USC School Of Medicine, Los Angeles, CA.

Experimental PVR can be induced by intravitreous injection of a critical number of fibroblasts. We tested the potential of gene transfer as treatment of PVR by simultaneous intravitreous injection of fibroblasts transduced with a retroviral vector bearing the Herpes Simplex thymidine kinase (HStk) gene and ganciclovir. The experimental animals were divided into 5 groups as follows: (1) non-transduced fibroblasts and ganciclovir; (2) HStk vector-transduced fibroblasts and ganciclovir; (3) HStk vector-transduced fibroblasts and normal saline solution; (4) 30% HStk vector-transduced-fibroblasts, 70% non-transduced fibroblasts and ganciclovir; (5) 60% HStk vector-transduced-fibroblasts, 40% non-transduced fibroblasts and ganciclovir. 5 x 10^4 cells were administered on Day 0, while ganciclovir (100 ug/eye) was given on Days 0 and 4. Indirect ophthalmoscopy was done on Days 4,7,14 and 28 to look for signs of PVR. PVR was classified into six stages (0 to 5). On Day 14, PVR was most severe in the group that received non-transduced fibroblasts and gancyclovir (mean stage 4.23) or saline (mean stage 3.85). In contrast, PVR was least severe in the group receiving transduced fibroblasts and gancyclovir (mean stage 0.61), as well as in groups that received mixtures of transduced and non-transduced-fibroblasts and ganciclovir (mean stages 1.23 for the group receiving 30% HStk-transduced fibroblasts, and 2.23 for the group receiving 60% transduced fibroblasts). On Day 28, each group showed progression of PVR, but was less extensive in the groups receiving transduced fibroblasts and gancyclovir (mean stage 0.77; $p<0.01$) and a mixture of 30% transduced and 70% non-transduced fibroblasts (mean stage 2.45; $p<0.05$), attesting to a by-stander effect. These data emphasize the potential of gene transfer as treatment of PVR.

C6-527 GENE THERAPY FOR THE TREATMENT OF ANDROGEN INDEPENDENT AND PROSTATE SPECIFIC ANTIGEN PRODUCING HUMAN PROSTATE CANCER, Arthur S.C. Ko, George N Thalmann, Akinobu Gotoh, Chinghai Kao, Wei Wei Zhang, Leland W.K. Chung, Urology Research Laboratory and Thoracic Surgery Research Laboratory, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

The lethal phenotypes of the advanced stage of prostate cancer are androgen independence (AI) and metastasis to the axial skeleton. In our laboratory, we developed the C4-2 cell line, AI mouse model of human prostate cancer/ metastasis. Using this model, we have designed p53, tumor suppressor, gene therapy approach for treating AI and prostate specific antigen (PSA) producing prostate cancer in vivo. As evidenced by their wide-spread overexpression of p53 in response to adriamycin, C4-2 cells have a silent point mutation but functional protein. It has previously been shown that the expression of wild-type p53 protein had no effect on the morphology or growth characteristics of A673 cells which contain normal alleles of p53 but no detectable levels of protein. Since wild-type p53 protein is considered a potent growth inhibitor for a host of malignant human tumor cell lines, we compared the growth inhibitory effect of wild-type p53 protein in AI and PSA producing human C4-2 cell line in vitro and in vivo. We concurrently demonstrated that in vitro Ad-CMV-p53 (wt) has no growth inhibitory effect, despite the high efficiency of infection of C4-2 cells. In contrast, Ad-CMV-p53(wt), when injected intratumorally, significantly depressed the growth of C4-2 tumors maintained in the castrated hosts. In comparison with the control (no treatment), sham (PBS-injected) and control virus (Ad-RSV-Luciferase) treated groups, Ad-CMV-p53 also suppressed serum PSA. Histomorphologic analysis of the p53-treated revealed that more polymorphonuclear cells were attracted to the tumor necrosis sites of p53 injection than luciferase or other controls. All p53 treated mice were tumor free for up to 8 weeks after the cessation of the 10-week treatment regime. Only two of eight p53-treated mice now have small tumors growing at distant sites from the initially treated sites. The rest of them are still tumor free 12 weeks after treatment. The underlying mechanisms for how recombinant adenovirus containing wt p53 can be tumoricidal in vivo and can induce tumor necrosis in seven of eight p53-treated are currently under investigation. One may involve the overexpression of wt p53 as an endogenous potential target of natural killer cells or a few cytotoxic T cells in nude mice. This strategy may be used as a potential immunotherapy of prostate tumor cells.

C6-528 NUCLEAR LOCALIZATION OF RECOMBINANT GLUTATHIONE S-TRANSFERASE TO IMPROVE DRUG DETOXIFICATION, Katharine S. Kramer, T. H. Manoharan, C.Schumacher and W. E. Fahl, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706

The glutathione-S-transferases (GSTs) form a family of detoxifying enzymes which act upon a variety of endogenous and foreign electrophilic compounds, including antineoplastic alkylating agents. An increase in cellular GST activity, whether due to drug selection of cells or due to production of recombinant GST (rGST) correlates with improved protection for cellular DNA and decreased cell kill. To determine whether localization of rGST in the nuclear compartment would improve its ability to compete for electrophilic drug and carcinogen metabolites, we synthesized rGST proteins which included an amino-terminus, constitutive, nuclear localization signal (NLS) from the rat glucocorticoid receptor (28 amino acids) or from the SV40 T antigen (7 amino acids). These constructs (NLS-rGSTs) were stably transfected into Raji cells and transiently transfected into Cos cells. Immunofluorescent staining and Western blot analysis of the cytosolic and nuclear fractions indicated that a portion of the NLS-rGST proteins translocated to the nucleus. Enzyme assays showed that the NLS-rGST molecules retained catalytic activity. NLS-rGSTs that were stably transfected into Raji cells are currently being tested against cytosolic rGSTs in Raji cells to determine their relative abilities to protect cells against the cytotoxic effects of mechlorethamine and melphalan. Suitability of rGSTs and NLS-rGSTs to serve as human somatic transgenes is also being examined. Grant# CA22484.

C6-529 SPECIFICITY OF HUMAN T LYMPHOCYTES IS GENETICALLY REDIRECTED BY CHIMERIC T-BODY RECEPTORS, M. Kruger, A. Bhullar, G. Chiang, R. Goodenow, H. Gregory, P. Harney, A. Kahrs, C. Killion, I. Krapf, C. Lundak, E. McLaughlin-Taylor, J. Reuter, E. Rodriquez, G. Sulya, J. Vernachio, and A. Williams, Gene Therapy Unit, Biotech Group, Baxter Healthcare Corporation, Santa Ana, CA 92705

A T-body is a genetically modified chimeric receptor that consists of the combining site specificity of an antibody with a defined signal transduction element. We are using T-body technology to develop a novel approach toward cancer immunotherapy. Introduction of these chimeric molecules into T cells allows the generation of T lymphocytes with antibody specificity independent of MHC restriction while maintaining T cell effector function. Chimeric genes were constructed using the antigen binding domains of monoclonal antibodies with specificity for either carcinoembryonic antigen or Her2/neu, the predominant tumor associated antigens of colon and breast cancer respectively. Single chain antibody variable regions (V_L/V_H) were linked to different signal transducing subunits (TCRβ, CD3ζ, FcRIIIγ) and cloned into retroviral vectors. We have used these vectors to introduce T-body genes into human peripheral blood T lymphocytes, tumor infiltrating lymphocytes and T cell lines as well as other cell types. Gene integration was confirmed by polymerase chain reaction (PCR) amplification and transduction efficiency was assessed by quantitative southern blot hybridization. T-body gene transcription was detected by reverse transcriptase PCR. Furthermore, these chimeric receptors were expressed on the cell surface and mediated T-cell cytokine secretion through specific recognition of antigen expressed on tumor cells. Parameters are currently being defined to optimize transduction efficiency in human peripheral blood T lymphocytes.

C6-530 ELIMINATION OF INTRAOCULAR TUMORS BY ACTIVATION OF SPECIFIC T CELLS.

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Activation of antigen-specific T cells requires costimulatory signals that can be provided by the interaction of B7-1 and CD28. Recent data from other laboratories indicate that tumor cells that express B7-1 directly activate CD8+ cytotoxic T cells (Tc), bypassing the requirement for CD4+ T helper cells. Our previous results indicate that immunogenic P815 tumor cells grow progressively within the immunologically privileged anterior chamber (AC) of the eye of BALB/c mice. The failure to eliminate AC tumors coincides with the failure of infiltrating pTc to differentiate further into Tc. To determine if the expression of B7-1 initiates differentiation of pTc and rejection of AC tumors, P815 tumor cells were transfected with the episomal vector pBMGNeo containing B7-1 cDNA. Either B7-1 positive, or B7 negative, P815 cells were injected into the AC and tumor growth determined quantitatively by slit lamp examination. As expected B7 negative tumor cells grew progressively. By contrast, B7-1 positive tumors were eliminated completely. Tumor rejection was T cell dependent and SCID mice failed to reject B7-1 positive tumors. Tc were recovered from the tumor-containing eye that lysed specifically P815, but not third-party EL-4 target cells. We conclude that B7-1 positive tumors initiate the differentiation of infiltrating pTc into Tc that eliminate AC tumors. These results imply that the success of immunogenic tumors within immune privileged sites may depend upon preventing APC from up-regulating B7-1 expression and inducing specific Tc. (Supported by NEI-08122).

C6-531 LIPOSOMAL TRANSFECTION OF MURINE BLADDER CANCER CELLS (MBT-2) WITH INTERLEUKIN-2 (IL-2), William A. Larchian[1,3], Warren D. W. Heston[1], William R. Fair[1], Ramila Philip[2], Mohan Philip[2] and Eli Gilboa[3], [1]Division of Urologic Surgery, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, [2]Applied Immune Science, Santa Clara, CA and [3]Department of Experimental Surgery, Duke University Medical Center, Durham, North Carolina 27710

The production of cytokine tumor vaccines has repeatedly been accomplished with transfecting agents that result in the stable and persistent expression of the cytokine. Our previous work with retroviral IL-2 transfection into the MBT-2 murine bladder cancer model, revealed stable expression of IL-2 (2 ng/1 x 10^6 cells/24 hrs) in vitro, and both protection and survival advantages in vivo. With hopes of increasing cytokine expression and eliminating the inherent concerns of using retroviral vectors, especially in the clinical setting, we pursued an alternative transfecting strategy. We studied eight different liposomes, both commercial and investigative, and determined that DMRIE/DOPE (50/50) (Vical Corporation) provided the best transfection results in our MBT-2 model. With the use of an adeno-associated viral (AAV) plasmid containing human IL-2, we generated dose (liposome/DNA 10 ul/1 ug to 80 ul/20 ug) and time (0 to 18 days) response curves. At optimal liposome/DNA ratios (40 ul/10 ug) MBT-2 cells expressed levels of IL-2 (by ELISA) in the range of 180 ng/1 x 10^6 cells/24 hrs, 3 days following transient transfection. All cells were radiated with 7000 rads 24 hours after transfection. Approximately 80% of peak levels of IL-2 expression were maintained for up to 9 days following transfection. In vivo studies analyzing this model's effectiveness are in progress. We believe that liposomal transfection in selected cell lines provides a simple, fast and highly efficient means of transient transfection for cytokine delivery.

C6-532 AAV PLASMID: LIPOSOME COMPLEXES FOR THE GENETIC MODIFICATION OF FRESH PRIMARY TUMOR CELLS: USE IN TUMOR VACCINATION FOR BREAST CANCER. Jane Lebkowski, Mohan Philip, Kim Lyerly*, Brian Clary*, Elisa Brunette, Lydia Kilinski, Deepa Murugesh, Eamonn Coveney*, Thomas B. Okarma and Ramila Philip, Applied Immune Sciences, Inc., Santa Clara, CA 95054, *Duke University, Durham NC 27710.

The use of gene modified tumor cells for the immunization of tumor bearing animals is well established. One complication to its widespread application for human therapy is the inability to efficiently establish lines from many primary tumors. To overcome this problem we have developed procedures to efficiently express transgene in primary tumor homogenates and ascites cells using adeno-associated virus (AAV) based plasmids complexed to cationic liposomes. In these procedures, the primary tumor is homogenized and single cells are collected. T cells are then depleted using an AIS CELLector CD5/8. The T depleted nonadherent fraction is collected and lipofected using cationic liposomes and plasmids containing the inverted terminal repeats of AAV for efficient gene expression. In these studies, primary lung, breast, and ovarian tumors have been transfected with plasmids containing the IL2, chloramphenicol acetyl transferase or nerve growth factor receptor genes. Tumor homogenates transfected in this manner express 500-10,000 pg IL2/10^6 cells/24 hrs after 3 days incubation. Expression of IL2 is maintained in these tumor cells for at least 7-25 days post lethal irradiation at a dose of 10,000 rads. The successful use of these procedures for vaccination protocols has now been verified using a nonimmunogenic mouse breast tumor metastasis model. These procedures will now be implemented in the gene modification of fresh breast tumors for use in immunization of patients with metastatic breast cancer.

C6-533 DEVELOPING A NOVEL GENE THERAPY FOR HEAD AND NECK CANCER

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Abnormalities of the p53 gene constitute the most frequent genetic alteration identified thus far in squamous cell carcinoma of the upper aerodigestive tract (SCCHN). In these studies, we investigated the efficacy of attenuating squamous cell carcinoma growth by the recombinant wild-type p53 adenoviral vector (Ad5CMVp53) both *in vitro* and *in vivo*. Northern and Western analyses showed elevated levels of p53 transcripts as well as protein production. Growth rate was greatly suppressed by p53 adenovirus in all the cell lines tested. Interestingly, this effect is independent of the cell lines' endogenous p53 gene status. *In vivo* studies showed that the Ad5CMVp53 could suppress the growth of established tumors in nude mice. Furthermore, we developed a microscopic residual model which mimics the post surgical environment of head and neck cancer patients with advanced disease. In this model, the p53 adenovirus was able to effectively prevent the establishment of tumors in nude mice. More importantly, a karyotypically normal and non-tumorigenic fibroblast cell line did not respond to the p53 adenovirus treatment. The outcome of these studies suggests that the p53 adenovirus can be further explored as a therapeutic agent for head and neck cancer and potentially other solid malignancies.

C6-534 VCL1102: A PLASMID DNA VECTOR TO EFFECT HIGH LEVELS OF IL-2 EXPRESSION *IN VIVO*. M. Margalith, S. Khatibi, P. Hobart, Vical, Inc. San Diego, CA 92121

IL-2 has shown clinical efficacy for reducing growth of human renal cell carcinomas despite its inherent toxicity when administered systemically. The ability to express therapeutic quantities of IL-2 at a specific tumor site using direct injection of DNA encoding the IL-2 protein would vastly improve the safe, efficacious delivery of this cytokine to effect tumor regression. To achieve therapeutic expression of IL-2, we have compared two strong promoters (Rous Sarcoma Virus [RSV] 3' LTR; and cytomegalovirus [CMV] immediate early [IE] promoter) cloned into a modified pUC18 derived plasmid DNAs designed to express human IL-2. Both the SV40 and the bovine growth hormone transcription terminator/polyadenylation signal sequence were also compared. The level of plasmid DNA based IL-2 expression was initially tested by transfection of cells (baby hamster kidney [BHK], murine B16 melanoma, and murine RENCA cells) grown in *in vitro* cell culture. Our results indicate that a plasmid, desiginated VCL1102, using the CMV IE promoter along with the IE 952 bp 5'UT (comprising its 826 bp intron) coding domain and the bovine growth hormone (BGH) terminator manifested the highest level of IL-2 expression when transfected into B16 cells. Transfected B16 cell secretion reached 3-5 ug (>2 X 10^4 units) of biologically active IL-2 /10^6 cells/24hr period at 72 hr post-transfection. Moreover, expression persisted at 1-2 ug/10^6 cells/24hr for up to eight days in confluent cell culture. The level of expression is more than 60 fold greater than early constructs using the RSV promoter in combination with the SV40 terminator. This IL-2 expression plasmid, designated VCL1102, has been used to effect regression of tumor cell growth when directly injected into subcutaneous murine B16 melanoma tumors *in vivo*.

C6-535 TREATMENT OF ESTABLISHED INTRAHEPATIC SYNGENEIC COLORECTAL TUMORS BY RETROVIRAL HS-Tk GENE TRANSFER IN BALB/c MICE,

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Gene therapy with PA317-based retroviral vector producer cells (VPC) mediating gene transduction of the herpes simplex thymidine kinase gene (HS-Tk) followed by Ganciclovir (GCV) is effective in animal tumor models. Currently, our group is further evaluating safety and efficacy of treatment in human clinical trials for recurrent glioblastoma multiforme. We have also previously shown HS-Tk VPCs followed by GCV can be safely inoculated intrahepatically. VPCs have been shown to survive adequately to allow intrahepatic tumor cell transduction. In this experiment BALB/c mice were injected intrahepatically on day zero with 10^6 CT-26 syngeneic colon adenocarcinoma cells at a single site. On day five 10^7 HS-Tk VPCs were administered by direct injection into the tumor followed on day 12 with GCV 150 mg/kg intraperitoneally b.i.d. for fourteen days in treatment groups. Lac-Z VPCs were injected into some animals as negative controls. Animals were assigned to GCV treatment or nontreatment groups. All animals treated by VPC implantation not followed by GCV had progressive disease except for one animal in the HS-Tk group. Some animals in GCV treated groups were euthanized on day 30 and assessed for tumor status. Among these animals 0/7 (0%) of the Lac-Z VPC treated group and 8/12 (83%) of the HS-Tk group were found tumor free. The remaining animals were followed to day 100 for tumor recurrence. Survival at 100 days was 0/6 (0%) in Lac-Z VPC group and 4/10 (40%) in the HS-Tk treated group. These data suggest that HS-Tk retroviral mediated gene therapy of intrahepatic colon adenocarcinoma tumors can both reduce tumor burden initially and result in the long term survival of some animals.

C6-536 TRANSFECTION OF ACUTE LYMPHOBLASTIC preB LEUKEMIA CELLS CARRYING THE t(4;11) TRANSLOCATION WITH ANTISENSE EXPRESSION VECTORS,

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The preB leukemia cell derived line SEM carries a reciprocal t(4;11)(q21;q23) translocation. The two parental genes interrupted by the translocation are the AF-4 gene on chromosome 4 and the ALL-1 gene on chromosome 11. Reciprocal fusion genes located on the derivative chromosomes der4 and der11, are expressed in this cell line. Using cDNA fragments derived from AF-4 gene transcripts, two antisense-expressing plasmids, ASC1 and ASC2, were constructed. Both carry the *Ecogpt* marker which allows selective growth in medium containing Xanthine, Mycophenolic acid, Aminopterin and Thymidine (XMAT). Antisense mRNA is expressed from both plasmids only after induction with dexamethasone. These antisense mRNA molecules can hybridize either with AF-4- , der11- or der4-derived mRNA molecules. Cells stably transfected with ASC1, ASC2 or both were selected in XMAT containing medium for at least 6 months. The resulting cell populations were analyzed and expression of antisense transcripts derived from the integrated ASC1 or ASC2 plasmids was monitored by RT-PCR. Furthermore, expression of the parental AF-4 gene and both fusion genes was analyzed in the presence and absence of antisense RNA molecules.

C6-537 IN VITRO TRANSDUCTION OF GI TUMOR CELLS BY A RETROVIRAL VECTOR AND SURVIVAL OF VECTOR PRODUCER CELLS IN THE PRESENCE OF HUMAN PERITONEAL FLUID. Lalita Pandit, Don Hegland, Heechung Kwon, Michael Skotzko, Erlinda M. Gordon, W. French Anderson and Dilip Parekh, Department of Surgery and Gene Therapy Laboratories, USC School of Medicine, Los Angeles, CA.

Peritoneal carcinomatosis from GI malignancies remains a target for developing new therapies, because patient survival is poor and present treatment is suboptimal. Retroviral vectors would provide a highly efficient method for gene transfer. Therefore, intraperitoneal infusion of retroviral vectors or producer cells for gene transfer is a potential strategy for treating peritoneal carcinomatosis. In these experiments, we tested the effects of peritoneal fluid on viability of producer cells and gene transfer efficiency. METHODS: In the first experiment, NIH3T3 and vector producer cells were incubated in the presence of 15 heated and non-heated peritoneal fluid samples. In the second experiment, HT29, MIAPACA2 (human colon and pancreas cancer cells) and NIH3T3 cells were exposed to mixtures of retroviral vector supernatant and peritoneal fluid for 4 hrs. Transduction was examined by X-gal staining. RESULTS: All 3 cell lines showed efficient transduction in the presence of 15 samples of peritoneal fluid, attested by + ß gal staining. Interestingly, two-thirds of non-heated peritoneal fluid samples were toxic to producer cells. CONCLUSION: These data indicate that direct infusion of retroviral vector supernatant, and not producer cells, would be a more effective method for gene therapy for peritoneal carcinomatosis.

C6-538 DIRECT PLASMID DNA MEDIATED DELIVERY OF CYTOKINE GENES: A NEW APPROACH FOR CANCER Suezanne E. Parker, Shirin Khatibi, Michal Margalith, Denise Lew, Terrie Latimer, Michelle A. Yankauckas and Magda Marquet. Vical Inc., San Diego, CA 92121

Immunotherapy with cytokines has shown promise for the treatment of malignancy. The administration of high doses of IL-2 protein either alone, or in conjunction with ex-vivo generated lymphokine activated killer cells or tumor infiltrating lymphocytes, has resulted in long lived clinical efficacy for renal cell carcinoma, melanoma and myeloid leukemia. However, systemic administration of IL-2 is limited by the toxicity associated with the *in vivo* infusion of the high doses that are required for efficacy. One way to reduce the toxicity associated with systemic administration of IL-2 would be to introduce recombinant DNA expressing the IL-2 gene directly into tumors *in situ* resulting in a localized, sustained expression of low levels of IL-2. A series of plasmid DNA expression vectors containing the gene for human IL-2 have been constructed and screened for biological activity *in vitro* and for anti-tumor efficacy *in vivo*. *In vitro,* the transfection of tumor cells with the IL-2 containing plasmid VCL-1102, results in the expression of 2-4 µg of biologically active IL-2/10^6 cells in a 24 hour period. *In vivo*, the direct intratumor injection of VCL-1102 into subcutaneous B16 melanoma tumors in mice results in a significant reduction in tumor mass (average tumor volume of 96 mm^3 for the DNA treated mice vs. 3,269 mm^3 for saline treated mice three weeks post tumor inoculation). Intact plasmid DNA was detected in the tumors by Southern analysis following intratumor injection, but the IL-2 protein was not detected in the serum of any of the animals. Detailed pharmacokinetic and GLP safety studies in rodents and cynomolgus monkeys are currently being conducted with the VCL-1102 plasmid.

C6-539 RADIOSENSITIZATION OF TUMOR CELLS USING HALOGENATED DEOXYCYTIDINES IN HStk TRANSDUCED TUMOR CELL LINES. R.M. Paulsen, S.B. Greer, C.J. Link, Human Gene Therapy Research Institute, Des Moines, IA 50309

Radiation therapy is limited by a low therapeutic index between normal and malignant tissues. Radiosensitizing agents such as the halogenated pyrimidines were developed to improve the therapeutic index of radiation therapy. However, the most widely used agents of this class are non-selectively incorporated into the replicating DNA of both tumor and dividing normal cells. The halogenated pyrimidines iododeoxycytidine (IdCyd) and bromo-deoxycytidine (BrdCyd) are preferentially mono-phosphorylated by the Herpes simplex thymidine kinase (HStk). In an attempt to improve the therapeutic index of radiation, we have used a combination of HStk gene delivery with IdCyd and BrdCyd to selectively enhance radiation killing of tumor cells. A375 human melanoma cells were transduced with a retroviral vector (STK provided by F. Moolten) containing the HStk gene. These cells were exposed to either IdCyd or BrdCyd prior to single dose radiation (300 cGy). Clonogenic assays were used to determine the surviving fraction. A375 cells transduced with the HStk gene and treated with IdCyd (1 µg/ml) had a two-fold increase in cell killing after irradiation, above the level of cell killing due to drug toxicity alone. Similar results were obtained with BrdCyd. A375 cells without the HStk gene showed no radiation sensitization. Thus, HStk promoted cell killing of cells pre-incubated in the presence of either IdCyd or BrdCyd. These data suggest that enzyme and pro-drug strategies might be used to selectively enhance radiation-induced tumor cell killing.

C6-540 ADENOVIRUS-MEDIATED GENE THERAPY OF EXPERIMENTAL METASTATIC BRAIN TUMORS, H. D. Shine, A. Colak, J. C. Goodman, S-H. Chen, S.L.C. Woo, R.G. Grossman, Baylor College of Medicine, Houston, TX,

The therapeutic efficacy of adenovirus-mediated gene therapy to treat metastatic brain tumors was investigated in an experimental syngeneic model of mammary brain tumors. Metastatic mammary tumors were modeled by stereotactic injection of cultured mammary adenocarcinoma cells derived from Fischer rats (MAT-B) into the caudate nucleus of Fischer rats. Seven days after MAT-B cell injection, when the tumors were 5.34 ± 1.2 mm^2 in cross sectional area, they were transduced by stereotactic intratumoral injection of a replication-defective adenovirus vector that carried the herpes simplex virus gene for thymidine kinase (ADV-tk) or a control adenovirus vector carrying the β-galactosidase gene (ADV-βgal). After virus injection the animals were treated intraperiotoneally with ganciclovir (GCV) at 50 mg/kg or saline twice daily for 6 days. Sixteen days after tumor injection, the brains of animals were examined and tumors measured using computer-assisted morphometry. Animals in control groups: ADV-βgal + GCV, ADV-βgal + saline, ADV-tk + saline had large tumors that were 15.07 ± 10.1, 16.1 ±. 4.8, and 24.03 ± 5.3 mm^2 in cross sectional area, respectively. In contrast, animals treated with ADV-tk + GCV had no tumors visible. These results reveal that the recombinant adenoviral vector containing HSV-tk gene confers sensitivity to GCV cytotoxicity in mammary tumor cells in the brain in the same manner as it does in experimental glioma cells. The data indicate that this treatment could be used to treat metastatic mammary tumors in the brain.

C6-541 TUMOR SPECIFIC CTLS INDUCED BY HSV-TK GENE TRANSFER AND GANCICLOVIR TREATMENT, Satoru Suzuki, Hiroyuki Shimizu, Arichika Hoshino, Shirou Yamamoto, Taketo Igarashi, and Takashi Shimada, Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo 113, Japan

Transfer of the herpes simplex virus thymidine kinase (HSV-TK) gene into cancer cells followed by treatment of ganciclovir (GCV) is an important strategy of cancer gene therapy. HSV-TK transduced cells were efficiently killed with GCV-triphosphate generated from GCV by the action of HSV-TK. In addition, adjacent non-transduced tumor cells are also killed by the bystander effect, although the mechanism of this effect is not fully understood. We investigated whether the systemic immune response was involved in tumor regression in vivo.

TK(+)RENCA (renal cell carcinoma) and TK(+)CT26 (colon cancer) cell lines were established using a retroviral vector and implanted subcutaneously into BALB/c mice. After complete regression of TK+tumor cells by GCV treatment, the animals were challenged with TK(-)tumor cells. Rejection or significant growth inhibition of challenged tumor cells was observed. Tumor specific $CD8^+$ CTLs were detected in these mice. To study the mechanism of CTL induction, we examined the expression of MHC class I molecules on these tumor cells. Increased levels of class I molecules were detected specifically on HSV-TK/GCV treated cells. These results suggest that HSV-TK/GCV system might be useful not only for short term tumor regression due to the direct cell killing and bystander effect but also for long term tumor regression and prevention of recurrence due to the vaccination effect.

C6-542 USE OF ONCOGENE NEUTRALIZING BINDING DECOYS TO TRIGGER NSCLC APOPTOSIS, Bruno Tocqué, Valérie Housset, Michel Janicot, Marie-Christine Multon, Isabelle Rey, Fabien Schweighoffer, Rhône-Poulenc Rorer, Cell and Gene Therapy, 13 Quai Jules Guesde, B.P. 14, 94403 Vitry sur Seine

There is a number of strategies that are in clinical trials or that can be envision to reduce the tumor burden by gene transfer experiments. We are focusing our attention on identifying Oncogene Neutralizing Binding Decoys (ONBDs) to control tumor development. There is an accumulating body of literature which suggests that the transfection of oncogenic sequences such as activated form of Ha or Ki-ras can increase radiation or chemotherapy resistance. It is also known that overexpression of genes such as c-myc or E1A can confer susceptibility to apoptosis induced by several anticancer agents. Thus resistance of tumor cells to chemotherapeutic regimens may be accentuated by combination of oncogenes. The ability of tumor cells to activate an apoptotic response may determine the ultimate success of cancer therapy. ONBDs will help to reverse the insensitivity of tumor cells to chemotherapy and we have examined the capacity of some of the ONBDs to trigger apoptotic responses in Non Small Cell Lung Carcinomas that express an activated Ki-ras and mutated p53. We have recently identified an isoform of Grb2 that we called Grb3-3 that can trigger apoptosis in a number of cells in vitro. Production of retroviral particles carrying these ONBDs are in progress. Data comparing the efficacy of Grb3-3 to trigger apoptosis and a bystander effect in H460 cells as compared to other ONBDs will be discussed (Science, 264, 971, 1994).

C6-543 MURINE B16F10 MELANOMA CELLS AND THE BENEFICIAL EFFECTS OF IMMUNE MODULATOR GENE TRANSFECTION P. Towner, D. Darling, J. Gäken, M. Kuiper, [1]S. J. Hollingsworth , [1]W. Hirst, F. Farzaneh. Department of Molecular Medicine, [1]Haematological Medicine, King's College School of Medicine & Dentistry, Bessemer Road, London, SE5 9RS, England.

Transfection of tumour cells with cytokines has been a popular method for reducing the tumorigenicity of murine cell lines in syngeneic animals. In B16F10 (B16) murine melanoma cells the expression of either IL-2 or IL-4 results in delayed tumour formation whereas expression of both genes from an individual clone further reduces the tumorigenicity, requiring 51 days to formation compared to 36(IL-4),19(IL-2), and 9(wt) days. Explanted tumours showed reduced levels of cytokine expression, IL-2 by 95% and IL-4 by 98 %, similar reductions were apparent with the explanted combination IL-2/IL-4. In each explant the DNA encoding the cytokine was readily detectable suggesting that tumour formation may result from loss of gene expression rather than deletion of the gene. Transduction of B16 cells with the gene for the immune costimulator B7.1 had no effect on tumour formation. However, preliminary results suggest that the combination of B7.1 with IL-2/IL-4 reduces tumorigenicity and delays tumour formation substantially.

Lethally irradiated transduced cells were used in vaccination, IL-2 or IL-4 transduced cells alone had little or no protective effect against a wild type challenge while IL-2/IL-4 transduced cells were slightly beneficial. IL-2 + IL-4 transduced cells were also used to treat tumour-bearing animals, which led to increased survival times of 30 to 110 days.

C6-544 GENE THERAPY FOR MALIGNANT BRAIN TUMORS

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Transfer of suicide genes into tumor cells is a novel approach which may be included in the treatment of brain tumors (Culver, Science 256:1550,1992). In rat gliomas, we investigated the feasibility of transfer of the Herpes Simplex thymidine kinase (HSV-TK) gene, which renders transduced cells sensitive to Ganciclovir (GCV). For gene transfer, replication defective adenovirus and retrovirus vectors were used.

In our retroviral vector, the HSV-TK is under the transcriptional control of the LTR in which the MoMuLV enhancer is replaced by the enhancer of the mutant polyoma virus (PyF101). Virus producing cell lines were made by transfecting PA317 cells with the viral construct. In our adenovirus vectors, the E1 region is replaced by the HSV-TK gene, which is under the control of the MLP.

Fischer rats were inoculated with rat 9L gliomas in the left frontal lobe. Adeno-and retroviral vectors carrying HSV-TK were inoculated into established tumors. Following GCV, prolonged survival of the rats was seen both following adeno and retrovirus mediated gene transfer. Survival of rats inoculated with Adeno-Tk 3 days after tumor inoculation was more prolonged than after inoculation at 5 days. It was calculated that 2 to 3 log tumorcell kill was achieved by the Adeno-Tk/GCV therapy (Supported by grant KWF/NKB 93-671).

C6-545 DELIVERY OF A DRUG SUSCEPTABILITY GENE INTO HUMAN LYMPHOCYTES FOR *IN VIVO* ELIMINATION OF ALLOREACTIVE T-CELLS AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION.

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Following an allogeneic bone marrow transplantation (BMT) for acute myelocytic or lymphoblastic leukemia, T cells contaminating the graft can cause severe graft versus host disease (GvHD). However, patients who *develop GvHD* have a lower relapse rate compared to patients not showing clinical signs of GvHD. This phenomenon has been attributed to a so called graft versus leukemia reaction (GvLR). The recent development of 'suicide genes' or drug susceptability genes provides a way to *in vivo* deplete donor T cells if GvHD runs out of control. An example of such a gene is the Herpes simplex virus thymidine kinase (HSV-tk) gene, the expression of which renders an eukaryotic cell sensitive to non-toxic levels of ganciclovir. Thus, stable transduction of the HSV-tk gene into primary T-cells should make it possible to abrogate GvHD by selectively killing allogeneic T-cells *in vivo* with ganciclovir. A prerequisite for this approach is that virtually all T-cells that are infused into the patient express the HSV-tk gene and continue to do so a few months later. To achieve this we are developing recombinant viral vectors harbouring two genes (the HSV-tk gene and a selectable marker), either in a bicistronic transcription unit driven by one promoter or as a fusion protein. Efficient translation of both genes in a bicistron can be achieved by either a short intercistron relying on ribosome slipping or by making use of picornavirus-derived internal ribosomal entry sites. Transfection of the various expression constructs into rat fibroblasts and subsequent selection with G418 allows us to compare thymidine kinase activity in these cells. Initial results using the HSV-tk gene and the neo gene as a selectable marker show that after G418 selection cells transfected with bicistronic constructs are more sensitive to ganciclovir than cells expressing a tk-neo fusion protein.

C6-546 A NOVEL DRUG-SENSITIVITY GENE, CYTOCHROME P450 2B1, ACTIVATES CYCLOPHOSPHAMIDE, Ming X. Wei[1], Maureen Chase[1], Yasuhiro Ono[1,2], Fangqin Li[1], David J. Waxman[4], Fred H. Hochberg[3], Xandra O. Breakefield[1] and E. Antonio Chiocca[1,2], [1]Molecular Neurogenetics Unit, [2]Neurosurgery Service, [3]Neurology Service, Massachusetts General Hospital, Harvard Medical School and [4]Department of Biology, Boston University, Massachusetts 02114.

Glioblastoma, the most common malignant primary brain tumor, is considered incurable. Multimodal approaches, such as surgery, radiation therapy, and chemotherapy prolong the survival of patients by only a few months. Cyclophosphamide (CPA) is currently used clinically to treat several types of tumors, but it is not effective for brain tumors. CPA itself is a biologically inactive drug, which must be metabolized by a liver-specific enzyme (cytochrome P450) into its active metabolite (4-hydroxy CPA), which is unstable and spontaneously decomposes into phosphoramide mustard (PM) and acrolein. The former binds covalently to DNA and causes DNA interstrand cross-links which lead to cell death during DNA replication. Our previous study showed that C6 glioma cells engineered to express the P450 2B1 gene (C6-P450) became highly sensitive to CPA both *in vitro* and *in vivo*. Herein, we have examined the cellular mechanism of this novel gene therapy approach for brain tumors. CPA-induced death of C6-P450 cells occurs within three hours after adding 0.5 mM CPA to cultures. This death involvs genomic nucleosomal laddering, which is one of the characteristics of apoptosis. Cytotoxicity appears to be mediated primarily through intracellular generation of toxic metabolites. Cytotoxicity to adjacent tumor cells lacking the cytochrome P450 B1 gene also occurs by transfer of toxic metabolites through cell-cell contacts as well as through the medium (secretory effect). By employing two CPA analogs, which are bioactivated by cytochrome P450 2B1 selectively into either acrolein or PM, we found that the acrolein-generating pathway is primarily responsible for the secretory effect, while the PM-generating pathway is the main component of the cell-to-cell mediated effect. In conclusion, we believe that CPA/cytochrome P450 2B1 gene therapy represents a novel tumor killing strategy which has an increased "bystander" effect through release of diffusible toxic analogs, in addition to transfer of toxic metabolites to adjacent tumor cells.

C6-547 RECOMBINANT ADENO-ASSOCIATED VIRUS (rAAV) ALLOWS STABLE AND FUNCTIONAL EXPRESSION OF B7-1 ANTIGEN (CD80) IN HUMAN PLASMOCYTOMA CELLS.

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The human B cell activation antigen B7-1 delivers a costimulatory signal upon binding to its ligand CD28/CTLA-4, thereby inducing proliferation and lymphokine production of T cells. In several murine tumor models, B7-1 has been shown to induce tumor regression by this T cell dependent mechanism. In order to explore the effect of B7-1 for the treatment of lymphomas, an AAV based vector was constructed in which the human B7-1 gene under control of a CMV promoter and linked to a neomycin resistance (Neo^R) gene cassette was subcloned into a plasmid (psub201) which contained only the AAV ITRs (pAAV/B7). Additionally, rAAV vectors coding either for Neo^R (pAAV/Neo) or β-galactosidase (pAAV/β-gal) were constructed. These vectors were co-transfected with a helper plasmid containing trans-acting AAV genes into human 293 cells that had been infected with adenovirus. rAAV titers of about 10^5 infectious units/ml were obtained. Transduction efficiency was ≈10% of infected cells as determined by β-gal staining 48hr post infection. pAAV/Neo and pAAV/B7 were used to infect the B7-1 negative human plasmocytoma cell line LP-1. After selection in G418 (400 µg/ml), stable expression of the B7-1 antigen (CD80) in LP-1 cells was detected for over 20 weeks determined by FACS analysis. Next, the proliferation of human T cells in response to B7-1 was assessed by [3H]-thymidine incorporation. T cells of healthy donors were stimulated with suboptimal concentrations of concanavalin A (ConA) or PMA and with γ-irradiated (10.000 rad) LP-1 cells, which were transduced with pAAV/Neo (LP1/Neo) or pAAV/B7 (LP1/B7). Stimulation with LP-1/B7 resulted in a twofold higher T cell proliferation than with LP-1/Neo or untransduced LP-1 cells. This effect could be specifically blocked by an α-CD80 MoAb. Taken together, these results suggest that human plasmocytoma cells can be induced to stabily express B7-1 by using a rAAV construct, and that B7-1 expression increases T cell proliferation in an allogeneic model. Future experiments will show whether additional costimulatory molecules and/ or cytokines will improve T cell stimulation and whether tumor regression can be achieved by this strategy in murine lymphoma models.

C6-548 APC TUMOR SUPPRESSOR GENE THERAPY BY RECTAL ENEMA. Carol A. Westbrook, Richard Arenas, Steven Chmura, Glen Otto. Depts. of Medicine and Surgery, University of Chicago, Chicago, Ill. 60637.

APC is a tumor suppressor gene linked to familial Adenomatous Polyposis Coli, which has been implicated in the majority of human colonic neoplasms, and in the MIN mouse (Multiple Intestinal Neoplasm). To investigate the function of normal and mutated APC, and to further examine its potential for gene therapy in cancer, we devised a method to deliver genes to colonic epithelium by rectal instillation of plasmid-containing liposomes. Reporter gene studies in rats and mice, using beta galactosidase (CMV promoter) showed expression of close to 100% efficiency in the treated areas, with no apparent toxicity. Expression is highest on day 1, decreasing gradually to zero on day 4, showing a pattern consistent with the turnover of normal epithelium. APC, also driven by a CMV promoter, was expressed in the rodent colon. Expression from the plasmid was approximately 10% of endogenous APC, confirmed by exon-connection using RT-PCR, and by RNAse protection; it showed a pattern and time course similar to that seen with beta galatosidase. To maintain continuous expression, animals were treated at 72-hour intervals for 3 weeks. There were no differences between animals receiving APC+liposomes or those receiving liposomes alone, confirming that extended expression of APC is not toxic. Studies are underway in MIN and its background strain (C57/Black 6-J) on the ability of wild-type APC to prevent or reverse polyps, and on the role of mutated APC on polyp initiation. Our studies show that liposomal gene therapy to the colon is feasible, and may have utility for the treatment of neoplasia or other human colonic disorders.

C6-549 TREATMENT OF HEPATOCELLULAR CARCINOMA THROUGH ADENOVIRUS MEDIATED GENE TRANSFER OF THE HSV-TK GENE IN COMBINATION WITH THE NUCLEOSIDE ANALOG DRUG GANCICLOVIR. Wills, K.N., Harris, M.P., Huang, W.M., Machemer, T., Maneval, D.C., and Gregory, R.J. CANJI, Inc. San Diego, CA 92121.

Hepatocellular carcinoma (HCC) is one of the most common cancers, causing an estimated 1,250,000 deaths per year worldwide. Currently, surgery is the only treatment offered as a potential cure, although only 20% of patients are considered as candidates for resection. Gene therapy offers an attractive alternative treatment for HCC. Adenoviral vectors are capable of highly efficient *in vivo* gene transfer in a variety of tissue and tumor types. Here we show that recombinant adenoviruses can effectively infect HCC cells and express their transgene products. Through the use of tumor specific promoters and enhancers such as the α-fetoprotein enhancer/promoter, gene expression can be limited to those cells expressing this tumor specific protein. Using the Herpes Simplex Virus type-1 (HSV) thymidine kinase (TK) gene as the transgene in combination with ganciclovir treatment, we have been able to 1) demonstrate specific inhibition of cellular proliferation in TK infected HCC cells using ^{3}H-thymidine incorporation assays, 2) show release of LDH (indicative of cell death) from TK infected cells, and 3) suppression of growth of *in vivo* established HCC tumors in nude mice. TK/ganciclovir treatment does not require that all tumor cells express the TK gene, as a "bystander" killing effect on adjacent replicating tumor cells has been demonstrated. Coupled with the high efficiency of gene transfer by recombinant adenoviruses and their ability to be delivered at high concentrations, a recombinant adenovirus expressing the TK gene is likely to be an effective means of treatment for HCC.

C6-550 ALLOGENEIC MHC CLASS I GENE TRANSFER TO SKIN FOR INDUCTION OF ANTITUMOR IMMUNITY. Ning-Sun Yang*†, Ping Qiu*, Joseph K. Burkholder*, Jerilyn Culp*, Joel Turner*, Jian Sun*. *Cancer Gene Therapy, Agracetus, Middleton, WI. 53562, and †Department of Pathology and Laboratory Medicine, University of Wisconsin Medical School, Madison, WI 53792.

Expression of allogeneic MHC antigens by tumor cells *in vivo* has been shown to result in antitumor responses in humans and mice. We utilized Accell® gene gun technology to introduce the murine H-2K^{s} gene into skin and tumor tissues of H-2K^{d} mice, and measured the cytotoxic T-lymphocyte (CTL) and antitumor responses to transgenic and parent tumor cells. The particle-mediated method for *in vivo* gene transfer has been previously used to induce protective immune responses against influenza, and humoral and CTL responses to a variety of other antigens. In this study, mice were preimmunized twice to the H-2K^{s} alloantigen or a control vector by particle-mediated delivery of plasmid DNA to intact mouse skin. One week after the second immunization, all mice were subcutaneously inoculated with a murine renal carcinoma cell line (Renca-11) that expresses transgenic H-2K^{s}. The Renca-11 line was created by *in vitro* gene gun transfection of the H-2K^{s} and *neo* genes followed by G418 selection and FACS expression analysis. All H-2K^{s} gene treated mice (19 of 19) rejected the Renca-11 cells, compared to 3 of 19 mice treated with control vector, and rejected a subsequent challenge with parent Renca cells. Splenocytes isolated from H-2K^{s} gene treated\Renca-11 challenged mice showed much greater specific lysis of both transgenic and parent Renca cell targets in chromium-release CTL assays, compared to control vector treated mice challenged with Renca-11 or with parent Renca cells. These results demonstrate the potential application of skin immunization with allogeneic MHC class I transgenes for generation of specific antitumor responses. Tests of the antitumor biological mechanisms involved following H-2K^{s} gene delivery to preexisting tumors using this methodology are in progress. Results from this study amplify the findings of Plautz et al. (PNAS 90: 4645, 1993) that showed preimmunization with irradiated H-2K^{s} transgenic tumor cells was required for complete tumor regression in mouse models. Furthermore, this study extends the application of genetic immunization of skin to allogeneic MHC class I gene therapy for cancer.

C6-551 COMBINED THERAPEUTIC EFFECTS OF HSV THYMIDINE KINASE AND CYTOKINE GM-CSF GENE TRANSFER IN A MOUSE TUMOR MODEL, Wen K. Yang, Sung-Jen Wei, Lan-Yang Ch'ang, Den-Mei Yang, Yi-Mei Hung and Jacqueline Whang-Peng, Cooperative Clinical Research Center Laboratory, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, R.O.C.

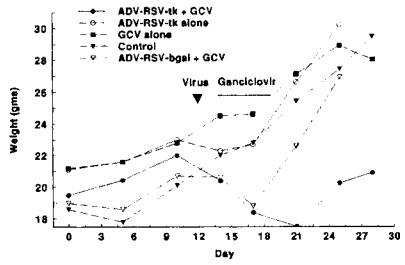
To determine the possible value of combining the thymidine kinase prodrug approach and the cytokine supplement in cancer gene therapy, we have constructed retroviral vectors that contain HSV-TK, mouse GM-CSF and neo-resistance genes and transduced these genes, via amphotropic virion, into highly tumorigenic NG4TL4 cells, a NIH3T3 cell clone initially derived by transfection with murine retroviral integrase gene (regulated by the SV40 origin promoter segment). Individual transduced NG4TL4 cell clones were isolated by selection for G-418 resistance and gancyclovir sensitivity. GM-CSF expression was found to vary considerably among these transduced NG4TL4 cell clones. One high GM-CSF expressing clone, MG412C3, and a nearly non-expressing clone, MG408C7, were selected for the study. The two clones remained highly tumorigenic in FVB/N mice, despite an increase of granulocyte-macrophage infiltration in the MG412C3 tumors. The tumors formed following subcutaneous or intraperitoneal injection of the two clones regressed following gancyclovir administration. The tumor regression, however, occurred faster with the MG412C3 tumors than the MG408C7 tumors. "Bystander" effect of gancyclovir toxicity could also be observed *in vitro* as well as *in vivo*, when non-transduced NG4TL4 cells were mixed with either TK/GM-CSF/neo^{R} transduced clone during the drug treatment. In addition, the gancyclovir-induced tumor regression was faster with the MG412C3 mixed tumors than the MG408C7 mixed tumors, indicating a significant enhancement of the "bystander" effect by GM-CSF expression in the tumor cells. The enhancement was observed only in the animal but not in cultures *in vitro*. Histological examination revealed tumor cell killing and disposal by massive infiltration of granulocytes and macrophages as a possible reason for the enhanced "bystander" effect. (Supported by Department of Health, R.O.C.)

C6-552 DIRECT INTRATUMOR INJECTIONS OF IL-2 PLASMID DNA: AN EFFECTIVE METHOD FOR IMMUNOTHERAPY Michelle A. Yankauckas, Shirin Khatibi, Michal Margalith, Stanislaw H. Gromkowski, Marston Manthorpe, Suezzane E. Parker, VICAL INCORPORATED, San Diego, CA 92121

Intravenous, intralymphatic or intralesional administration of recombinant IL-2 protein has been used clinically as an anti-cancer treatment for renal cell carcinoma and malignant melanoma. However, the therapeutic utility of IL-2 is limited by the toxicity associated with the high systemic doses that are required to achieve efficacy. Here we have attempted to reduce the systemic IL-2 protein load by injecting tumors locally with plasmid DNA coding for the IL-2 protein. A series of plasmid DNA expression vectors containing the gene for human IL-2 have been constructed and screened for biological activity in vitro and for anti-tumor efficacy in vivo. In vitro, the transfection of murine B16 melanoma cells with the IL-2 plasmid resulted in the expression of 2-4 μg of biologically active IL-2 protein per 10^{6} cells per 24 hr. In vivo, the direct intratumor injection of IL-2 plasmid DNA into subcutaneous B16 or Renca tumors results in a significant reduction in tumor mass. For the B16 melanoma model in C57Bl/6 mice, tumor volume measurements at 3 weeks post-injection were 96 mm^{3} for IL-2 DNA treatment vs. 3,269 mm^{3} for saline treatment. For the Renca model in BALB/c mice, average tumor volume measurements at 4 weeks postinjection were 64 mm^{3} for IL-2 treatment vs. 2,243 mm^{3} for saline treatment. Efforts are underway to quantify the amount of IL-2 produced by tumor cells removed following direct intratumor transfection.

C6-553 USE OF ADENOVIRAL MEDIATED GENE TRANSFER OF HSV-TK IN AN ASCITES MODEL OF BREAST CANCER. D Yee*, SE McGuire*, S-H Chen#, SG Hilsenbeck*, N Brünner+, SLC Woo#, *University of Texas Health Science Center, San Antonio, TX, 78284 +Finsenlab, Copenhagen, Denmark, and #Baylor College of Medicine, Houston, TX, 77030.

Malignant effusions often represent a significant clinical problem in the management of breast cancer. We have adapted the MDA-MB-435 human breast cancer cell line to grow in the peritoneal cavity of athymic mice to model malignant effusions. To test the therapeutic efficacy of in vivo gene transduction of HSV-TK, we injected mice (5 per group) intraperitoneally with 10^6 MDA-435 cells. Ten days after inoculation, 10^{10} pfu of an adenoviral vector containing HSV-TK regulated by the RSV promoter (ADV/RSV-tk) or the beta-galactosidase gene (ADV/RSV-bgal) were injected intraperitoneally. 24 hours later, mice were treated with ganciclovir (50mg/kg BID) for six days. Ascites formation was measured by weight and all mice were followed for survival. Mice treated with ADV/RSV-tk and ganciclovir had decreased malignant ascites formation as measured by decreased weight gain (see figure). In addition to decreasing ascites formation, mice treated with ADV/RSV-tk plus ganciclovir had a 39% increase in survival when compared to ADV/RSV-tk mice treated without ganciclovir (32 vs. 23 days, $p<0.005$). These data suggest that in vivo gene transduction of Ad-HSV-TK with ganciclovir treatment may be an effective treatment for malignant effusions due to breast cancer.



C6-554 SAFETY EVALUATION OF Ad5CMV-*p53 IN VITRO* AND *IN VIVO*, W-W Zhang, R Alemany, J Wang, PE Koch, NG Ordonez, JA Roth, Sect Thorac Mol Oncol, Dept Thorac & Cardiovasc Surgery, Depts Path & Tumor Biol, U Texas M D Anderson Cancer Ctr, Houston, TX 77030

In preparation for a clinical trial of the recombinant *p53* adenovirus Ad5CMV-*p53* for the treatment of lung cancer, the potential adverse effects of Ad5CMV-*p53* were assessed *in vitro* and *in vivo*. No infectious replication of Ad5CMV-*p53* was detectable in HeLa cells infected with extracts from HeLa cells previously infected with Ad5CMV-*p53*. No Ad5CMV-*p53* DNA replication was detected by ^{32}P-labeling in lung cancer cells infected with Ad5CMV-*p53* at MOI up to 1000 PFU/cell (total of $5x10^9$ PFU viruses). The infectivity and cytotoxicity of Ad5CMV-*p53* were examined *in vitro* in normal human bronchial epithelial (NHBE) cells. At an MOI of 50 PFU/cell, Ad5CMV-*p53* infection and expression were detectable in 80% of the treated cells. The exogenous p53 protein was first detected by western blotting at 8 hrs and peaked at 48 hrs after infection. Growth of NHBE cells was not affected by Ad5CMV-*p53* infection at an MOI of 100 PFU/cell. The pathogenicity of Ad5CMV-*p53* was assessed in BALB/c mice. The virus was given to four groups of mice by intratracheal injection at dosages from 10^7 to 10^{10} PFU; a fifth group received phosphate-buffered saline alone. None of the viral injections proved to be lethal. Mild to moderate peribronchiolar and perivascular infiltration by mononuclear cells and lymphocytes, with patches of pneumonitis, was the most acute toxic effect detected by histologic analysis in the two high-dose groups. The lung samples of the two lower-dose groups had a histology similar to that of the PBS control. Our results demonstrate that Ad5CMV-*p53* is a replication-defective virus that mediates a relatively low degree of acute pathogenicity in mice; these data document a safety profile encouraging for clinical trials of Ad5CMV-*p53* in the therapy of lung cancer.

Late Abstracts

DEVELOPMENT OF ANIMAL MODELS FOR THE TREATMENT OF HUMAN PAPILLOMA VIRUS INDUCED CARCINOMAS, Thomas Felzmann, Jay Ramsey, R. Michael Blaese, Clinical Gene Therapy Branch, NCHGR, NIH, Bldg. 49, Room 2B07, Bethesda, MD, 20892

Anti tumor immunity requires tumor associated antigens (TAA) presented in the context of MHC molecules as well as costimulation of cytotoxic tumor specific T-lymphocytes. In order to meet those criteria we developed tumor models for human papilloma virus (HPV) induced carcinomas since expression of the human papilloma virus E6 and E7 transforming proteins are predictable TAAs in about 90% of human cervical carcinomas. We developed HPV tumor models by expressing the HPV16 E6 and/or E7 antigens from murine and rat tumor cell lines. Expression of these proteins from cloned tumor cell lines was determined by immunoprecipitation and western blot analysis and found to be in the same range as that seen in human HPV16 induced cervical carcinoma cell lines. Injection of these cells into immunocompetent syngeneic animals caused tumor formation with the same efficiency as injection of the parental tumor cell lines. To provide costimulation from these tumor cells we developed retro- and adenovirus based gene transfer systems to express B7.1 (CD80) molecules. The expression of B7.1 after transduction with these viruses was determined by flow cytometry.

We have established previously, that defects in the expression of Rb and/or p53 in human carcinoma cell lines can partly complement for the DNA replication deficiency of E1a/E1b deleted adenoviral vectors. In HPV induced carcinoma cell lines both Rb and p53 are functionally inactivated by the HPV E6 and E7 proteins, respectively. We transduced rat 9L cells engineered to express HPV16 E6/E7 molecules as well as HPV positive and HPV negative human carcinoma cell lines with E1a/E1b deleted recombinant adenoviruses expressing B7.1. The expression of B7 was greatly enhanced not only in human HPV transformed tumor cells but also in rat 9L cells expressing HPV16 E6/E7 proteins compared to the parental 9L cell line. No such effect could be observed in murine tumor cell lines expressing HPV16 E6/E7 molecules. We are analyzing the mechanism by which this amplification of expression occurs. In vivo experiments will be performed to establish whether the increased transgene expression seen in tumor cells producing E6/E7 TAAs results in increases efficacy of adenovirus based anti-tumor therapies.

FURTHER ANALYSIS OF SMALL FRAGMENT HOMOLOGOUS REPLACEMENT, K.K. Goncz and D.C. Gruenert, Cardiovascular Research Institute, University of California, San Francisco, CA 94143-0911.

The method of small fragment homologous replacement (SFHR) has shown potential as a gene therapy technique for treatment of genetic disorders. In previous work on cystic fibrosis (CF) epithelial cell lines homozygous for the ΔF508 mutation of the cystic fibrosis transmembrane conductance regulator (CFTR), mutant genomic sequences were corrected to wild-type (wt) sequences by the introduction of small fragments of wtDNA (491bp), corresponding to a homologous region encompassing the mutant sequence. Normal genotype and phenotype were restored at a frequency of between 1-10%. In the present study, experiments were performed to 1) further characterize the frequency of SFHR, 2) investigate the occurrence of random integration's of the fragment and, 3) examine different techniques that could improve SFHR efficiency. Human epithelial cells, were transfected with a linearized plasmid vector containing sequences for both mutant neomycin and wt-hydromycin resistant genes. After selection with hydromycin B, resistant cells were transfected by SFHR with a small DNA fragment containing the wt sequence homologous to the mutation. The frequency of homologous replacement was then readily determined from the number of surviving cells after selection with G418. The surviving cells were also tested to determine if it would be possible to enrich for populations of cells that have successfully incorporated the DNA fragment. Epithelial cells derived from cystic fibrosis human subjects homozygous for the ΔF508 mutation were first transfected with the plasmid and then subject to SFHR with the 491bp DNA fragment. The frequency of homologous replacement was then determined by PCR and southern blot hybridization of cellular DNA and RNA. These techniques were also used in the second group of experiments which determined the frequency of random integration of the fragment. In the third set of experiments, CF epithelial cells were transfected by SFHR with the 491bp fragment in addition to various amounts of reagents that might increase the frequency of SFHR. These include promoters of CFTR expression or DNA damaging agents. This work is supported by NIH grants DK46002 and DK47766.

TARGETED GENE REPLACEMENT OF WILD-TYPE AND MUTANT CFTR SEQUENCES IN NORMAL AND CYSTIC FIBROSIS EPITHELIAL CELLS , [1]Dieter C. Gruenert, [1]Kaarin K. Goncz, [1]Zhidong Xu, [1]Linda C. Escobar, and [1,2]Karl Kunzelmann, [1]Gene Therapy Core Center, Cardiovascular Research Institute, Department of Laboratory Medicine, University of California, San Francisco, CA 94143; [2]Institute of Physiology, Albert-Ludwigs University, Hermann Herder Strasse 7, Freiburg, Germany.

A goal of cystic fibrosis (CF) gene therapy is correction of the mutant CF transmembrane conductance regulator (CFTR) with wild-type (wt) DNA sequences to restore the normal CFTR protein and function. Experiments with wtCFTR cDNA expression have shown that the Cl ion transport phenotype can be corrected to resemble ion transport in normal cells. An alternative to cDNA-based vector strategies for correction of function is one that corrects mutant sequences in the endogenous gene with the wild-type homologue. To test whether replacement of specific genomic sequences was feasible for potential application to gene therapy in human epithelial cells, a small fragment homologous replacement (SFHR) strategy was tested in an *in vitro* model system. To this end, ΔF508 or wtCFTR DNA fragments were introduced into transformed airway and pancreatic epithelial cells homozygous for wtCFTR or the ΔF508 mutation, respectively. Fragments with and without a unique Xho I restriction enzyme cleavage site were transfected. Allele-specific polymerase chain reaction (PCR) amplification of mRNA-derived cDNA and genomic DNA and hybridization of PCR products with allele specific probes indicated wtCFTR DNA at the appropriate genomic locus and in expression of wtCFTR mRNA. The amplification of the correct genomic locus was achieved by using PCR primers 5' and 3' to the region of homology. Primers for RNA were such that the PCR products spanned intron-exon boundaries. In addition, to confirmation by allele-specific PCR amplification and hybridization, homologous replacement at the appropriate genomic locus was indicated following Xho I digests of PCR products. Densitometric analysis of Southern hybridization indicated a frequency of approximately 10^{-2} to 10^{-1} in CF nasal polyp cells. Patch clamp analysis showed intact cAMP-dependent Cl transport in about 10^{-2} to 10^{-1} of other transfected CF airway epithelial cells indicating functional correction of the CF Cl transport defect. This method has important implications for gene therapy and the development of transgenic animals. Supported by NIH grants DK46002 and DK47766.

A TUMOUR-SPECIFIC MOLECULAR SWITCH FOR GENE THERAPY OF BREAST AND PANCREATIC CANCER, Jonathan D. Harris, Helen Hurst, Karol Sikora and Nick Lemoine, *ICRF Oncology Unit and Department of Clinical Oncology, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 OHS, United Kingdom*

One of the most promising of the selective genetic strategies against cancer is GPAT (genetic prodrug activation therapy). This involves delivery of a prodrug activating enzyme gene into both tumour and normal cells. By linking the enzyme gene downstream of tumour-specific transcription units, tumour-specific prodrug activation is achieved. Over-expression of the oncogene, *ERBB2*, occurs in many breast, pancreatic and gastric tumours (often due to transcriptional upregulation of single copy *ERBB2*). We have determined that sequences within the first 500bp of the 5' flanking region of this oncogene drive specific transcription within these tumour cell types. We are developing a genetic therapy strategy using GPAT against cancers of the breast and pancreas. We have constructed a chimeric minigene consisting of part of the *ERBB2* promoter linked to cytosine deaminase (converts the prodrug 5-fluorocytosine into the anti-cancer drug 5-fluorouracil) or Herpes Simplex Virus thymidine kinase (activates ganciclovir). We have transduced a panel of *ERBB2* positive and negative pancreatic and breast cell lines using both viral and non-viral delivery methods. In this abstract we describe the tumour-specific expression of both enzyme genes in *ERBB2*-overexpressing cells and the subsequent cell death that only occurs in these cells. We also describe the various advantages and disadvantages of each prodrug activation system and the use of different delivery systems in vitro and in vivo. This tumour cell-specific cytotoxic system is currently being developed for use in a clinical protocol that could be used against tumours that overexpress ERBB2.

LIPOSOME AND ADENOVIRUS-MEDIATED *E1A* GENE TRANSFER AS THERAPIES FOR OVARIAN CANCERS THAT OVEREXPRESS HER-2/*neu*, Mien-Chie Hung, Yujiao Zhang, Weiya Xia, Frank Sorji, Leaf Huang, and Dihua Yu, Department of Tumor Biology, U.T.M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030 and Department of Pharmacology, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15261

The HER-2/*neu* proto-oncogene is frequently amplified or overexpressed in many different types of human cancers, a phenomenon that has been shown to correlate with shorter survival time and lower survival rate in ovarian cancer patients. We previously found that increased HER-2/*neu* expression led to more severe malignancy and increased metastatic potential in animal models, and that the adenovirus 5 *E1A* gene repressed HER-2/*neu* gene expression and was able to suppress tumor dissemination when stably transfected into human ovarian cancer SKOV-3 cells which overexpress HER-2/*neu*. To investigate whether the *E1A* gene may be used as a therapeutic agent for HER-2/*neu*-overexpressing human cancers in living hosts, we first developed tumor-bearing mice by injecting SKOV-3 cells that overexpress HER-2/*neu* intraperitonealy into female nu/nu mice. Five days later, adenocarcinomas that overexpressed HER-2/*neu* developed in the peritoneal cavity and on the mesentery of the mice that received the injection. We then used cationic liposomes to directly deliver the *E1A* gene into these adenocarcinomas. We found that liposome-mediated *E1A* gene transfer significantly inhibited growth and dissemination of ovarian cancer cells that overexpress HER-2/*neu* in the treated mice; about 70% of these mice survived at least 365 days, whereas all the control mice that did not receive the gene therapy developed severe tumor symptoms and died within 160 days. In addition, we also i.p. injected into those ovarian tumor-bearing mice replication-deficient adenoviruses either containing E1A gene (Ad.E1A+), or not containing E1A gene (Ad.E1A-), or PBS buffer for 3 consecutive days, and repeated i.p. injection once a week for 4.5 months. Mice injected with Ad.E1A- or PBS buffer all died of tumor within 4.5 months, whereas 80% of the mice treated with Ad.E1A+ lived more than ten months. These data indicate that liposome-mediated *E1A* gene transfer or Ad.E1A+ virus may be used as safe and effective therapies for human ovarian cancers that overexpress HER-2/*neu* by directly targeting the HER-2/*neu* oncogene.

***In vivo* transfection of foreign DNA into embryonic chick heart**

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We have studied the *in vivo* introduction of recombinant DNAs,i.e., a reporter gene (LacZ) and a functional gene (insulin-like growth factor IGF), into chick embryonic heart. The plasmid vectors, pMiwZ and pMiwHEP, which encode LacZ and IGF gene,respectively, and were driven by RSV LTR and β-actin promoter, were mixed with positively charged liposome. Either construct complex was injected by glass pippete into the paracardiac region of 4-day old shell-less chick embryos. Expressions of foreign genes were analyzed at incubation days 7,10 and 14. In the embryos injected with pMiwZ,the β-galactosidase activity was expressed at all days and limited to the heart and the neighboring region of thoracic cavity. Expression of LacZ did not alter the morphology of the embryo including the heart. In contrast embryos treated with pMiwHEP (IGF) exhibited a significantly greater heart at Days 7 and 14, though the body weight was unaffected. Light microscopic examination revealed a remarkable hypertrophy of cardiomyocytes in pMiwHEP embryos, compared to the control or MiwZ embryos. These results may indicate that; (1) the stable and regional expression of exogeneous DNA could be obtained by liposome-mediated transfection. (2) the promotion of cardiac growth in pMiwHEP embryos could be attributed to the overexpression of IGF. (3)Our *in vivo* gene transfer system may be useful to analyze the developmental role of growth-related genes.

RETROVIRAL-MEDIATED GENE EXPRESSION IN HEMATOPOIETIC CELLS USING MYELOMONOCYTIC CELL SPECIFIC PROMOTERS.

P. Malik, K. A. Pepper, W. J. Krall, D. G. Tenen, A. L. Corbi and D. B. Kohn. Childrens Hospital Los Angeles, Beth Israel Hospital and Hospital de la Princesa.

Gene transfer into hematopoietic stem cells with expression targeted to maturing myelomonocytic progeny has applications for gene therapy of genetic diseases affecting granulocytes and macrophages. Promoters from myeloid specific genes that are upregulated with myeloid differentiation could also upregulate expression of an exogenous gene in a retroviral vector. Based on this hypothesis, we constructed retroviral vectors using promoters from myeloid specific genes CD11b, CD11c, GCSF receptor (GR) MCSF receptor (MR) and PU.1 and compared the activity of these promoters to the MoMuLV LTR using the human glucocerebrosidase cDNA (GC) as the reporter gene. HL-60 cells were transduced with these vectors and vector-derived GC activity was compared in undifferentiated HL-60 cells and the same cells differentiated into granulocytes using DMSO and macrophages using PMA. The MoMuLV LTR, a known strong transcriptional promoter/enhancer, showed the highest vector-derived GC activity in undifferentiated and differentiated HL-60 cells. The vector-derived GC activity transcribed from the CD11b and CD11c promoters was low in undifferentiated HL-60 cells but increased to nearly normal endogenous GC levels with myelomonocytic differentiation. The induction of expression from the CD11b and CD11c promoters correlates with the normal developmental regulation of the CD11b and CD11c genes. The vector-derived GC activity from the GR, MR and PU.1 promoters was not at clinically relevant levels. Myelomonocytic specificity of these promoters and their activity in primary human marrow is currently being tested.

REGULATORY ASPECTS AND SAFETY TESTING OF GENE THERAPY VIRAL VECTORS,

Jeffrey M. Ostrove and Dominick A. Vacante, Microbiological Associates, Inc. and MAGENTA Corporation, Rockville, Maryland 20850.

Approximately 90 gene therapy protocols have been approved by the NIH Recombinant DNA Advisory Committee (RAC) and almost 60 of these have been approved by the FDA. Mammalian cells have been used safely for over a decade as substrates for biopharmaceutical products. Safe and effective viral vaccines have also been produced in mammalian cells for greater than 50 years. Government and industry scientists working together have devised testing programs to assure that these mammalian cell-based products are safe for use in humans and are free of contaminating viruses, nucleic acids, toxins and other micro-organisms. Documents published by the FDA, known as "Points to Consider", are the building blocks for regulatory approval. Microbiological Associates has been involved in the safety testing of biopharmaceuticals since 1980 and the safety testing of gene therapy products since the late 1980's. Approximately 1,000 cell lines have been analyzed for the presence of contaminating viruses, microbes and toxins. This presentation will review our accumulated data on the presence of these adventitious agents as well as the contamination of mammalian cells used for biopharmaceutical and gene therapy products. In addition to adventitious contaminants, genetic rearrangements can occur in retroviral producer cell lines as well as adenoviruses that infect 293 cells. Sensitive assays for the detection of replication competent retroviruses (RCRs) and adenoviruses (RCAs) have been developed. These RCR and RCA viruses have been detected in GMP clinical lots of both retrovirus and adenovirus vectors for gene therapy. Currently the FDA does not allow these replication competent viruses in preparations of viral vectors produced for clinical trials. MAGENTA Corporation is a contract GMP manufacturer that provides viral vectors for gene therapy clinical trials. The concepts of cGMPs as well as building safety into a product will be described.

E2F-1 BLOCKS DIFFERENTIATION AND CAUSES RAPID NEOPLASIA IN TRANSGENIC MICE.

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Gene products involved in cell cycle control may have applications in gene therapy approaches to cancer. The transcription factor E2F-1 has a number of properties that suggest it plays a stimulatory role in the contol of cell division. We wish to understand its role in controlling decisions of proliferation versus differentiation in an *in vivo* developmental system.

In a process common to most hematopoietic lineages, megakaryocyte precursors proliferate and then exit the cell cycle as diploid megakaryoblasts. These cells then mature over several days, finally fragmenting into blood platelets. Previously we generated transgenic mice expressing a megakaryocyte targeted tsA58 SV40 large T antigen. These mice had low platelet counts, exhibiting a block to megakaryocyte differentiation likely due to T antigen's effects on the Retinoblastoma protein (Rb). Because Rb is known to exert at least some of its effects through inhibition of E2F activity, here we addressed the effect of E2F-1 expression on the differentiation of megakaryocytes.

We expressed the human E2F-1 transcription factor in transgenic mice using a megakaryocyte-specific promoter. Several lines of transgenic mice expressing the E2F-1 gene exhibited varying degrees of thrombocytopenia. Examination of megakaryocytes from these animals revealed that most of the megakaryocytes were blocked at an immature stage of development.

Every animal from the most affected line exhibited grossly enlarged spleen and lymph nodes evident from as early as two weeks of age. Histological examination revealed the presence of large numbers abnormal megakaryocytes in these tissues as well as significantly increased numbers of abnormal megakaryocytes in the bone marrow. These animals live less than 3 months, often with infiltration of cells into many tissues.

These mice provide evidence E2F-1 is indeed an oncogene. Because of the complete penetrence and early appearance of proliferating cells, we believe that E2F-1 is able to induce proliferation of the target cells which rapidly progress to the neoplastic state. Currently we are characterizing the alterations and interactions of genes and gene products responsible for the observed effect in the E2F-1 transgenic mice.

GENE THERAPY FOR LYSOSOMAL STORAGE DISEASES: RETROVIRAL TRANSDUCTION OF BONE MARROW CELLS AND THE DEVELOPMENT OF DIRECT SELECTION METHODS. Edward H. Schuchman[1], Patricia Yeyati[1], Cristina Fillat[1], Calogera M. Simonaro[1], Shimon Gatt[2], Mark E. Haskins[3], and Robert J. Desnick[1], [1]Department of Human Genetics, Mount Sinai School of Medicine, New York, NY, 10029, [2]Department of Biochemistry, Hebrew University, Jerusalem, Israel, and [3]Sections of Pathology and Medical Genetics, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, 19104.

Retroviral vectors encoding acid sphingomyelinase (ASM) and arylsulfatase B (ASB), the lysosomal hydrolases deficient in Types A and B Niemann-Pick Disease (NPD) and Mucopolysaccharidosis Type VI (MPS VI), respectively, have been constructed. The full-length ASM/ASB cDNAs were inserted into the pBC140, NAT and MFG retroviral vectors and expression was evaluated in cultured fibroblasts from NPD and MPS VI patients and animals. Each of the vectors expressed high levels of enzymatic activity and resulted in metabolic correction, although MFG vectors consistently expressed higher enzyme levels than pBC140 or NAT. Media obtained from retrovirally transduced cells overexpressing ASM/ASB also corrected non-transduced cells, demonstrating that overexpression of these enzymes led to their secretion. A novel selection method was developed to isolate metabolically corrected NPD cells based on the uptake and catabolism of fluorescently labeled sphingomyelin. NPD fibroblasts were transduced with the MFG vector, grown for 12 hours in media containing lissamine rhodamine labeled sphingomyelin (L12-SPM), and "chased" for 2 days in media devoid of the fluorescent lipid. The labeled cells were then separated using a fluorescence activated cell sorter. The results demonstrated that transduced NPD cells could be efficiently isolated without the need for preselection with G418. Similar studies are underway for MPS VI. Experiments have also been undertaken to evaluate expression of the retroviral vectors in hematopoietic cells. Whole marrow mononuclear cells were obtained from MPS VI cats, transduced with the ASB-MFG vector, and studied in long-term bone marrow cultures (LTBMC). The levels of ASB expression were analyzed in granulocyte/macrophage (GM) colonies and in the non-adherent fraction of the transduced bone marrow. In both cases high levels of ASB activity were detected after retroviral transduction. Since animal models exist for both NPD and MPS VI, these studies lay the foundation for the future development of gene therapy using animal model systems.

DEVELOPMENT OF ADENO-ASSOCIATED VIRUS VECTORS FOR GENE THERAPY OF CHRONIC GRANULOMATOUS DISEASE

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Chronic Granulomatous disease (CGD) is a rare inherited disease characterised by failure of an electron transport chain, the NADPH-oxidase, which is predominantly found in phagocytic cells.
Several groups have now demonstrated that retrovirus-mediated gene transfer can restore NADPH-oxidase activity to immortalised B-cells derived from patients, and to primary cells derived from bone marrow and peripheral blood. However, the inability of current retroviral vectors to transduce quiescent cells greatly limits the efficiency of gene transfer to the desired target cells, pluripotent haematopoietic stem cells (PHSC).
One strategy to overcome this problem is to develop alternative gene delivery systems. Adeno-associated virus (AAV) is a single-stranded DNA parvovirus that is dependent on helper virus, usually adenoviruses or herpes viruses, for replication. In the absence of this function, wtAAV integrates into the host genome at specific chromosomal sites by non-homologous recombination. We have used AAV vector systems to introduce genes to immortalised B-cells derived from patients with CGD, and have shown stable transduction of these cells. In addition, we show expression p47-phox, a protein which is absent in the majority of autosomal recessive forms of disease, and restoration of NADPH-oxidase activity.

HYBRID GENES ENCODING BOTH CYTOSINE DEAMINASE AND URACIL PHOSPHORIBOSYL TRANSFERASE AS NEW SUICIDE GENES.

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Cytosine deaminase (CD) is a microbial enzyme that can convert the antifungal agent 5-fluorocytosine (5-FC) into the antitumor agent, 5-fluorouracil (5-FU).
The *codA* gene of *Escherichia coli* encoding CD is being used as a suicide gene in molecular therapies developped for the selective elimination of unwanted animal cells.
In microorganisms, 5-FU is directly converted to the toxic F-UMP by uracil phosphoribosyltransferase (UPRTase) whereas in animal cells which appear to lack UPRTase, 5-FU is metabolized through the concerted action of uridine phosphorylase and uridine kinase. In order to provide the direct way of 5-FU conversion to F-UMP, we cloned the small *upp* and *cyl* genes encoding UPRTase from *E.coli* and *Saccharomyces cerevisiae* respectively. Then, the genes were fused in frame in a 5' and a 3' configuration to a *Sh::codA* hybrid gene coding for a protein with CD and ability to confer Zeocin resistance. The fused genes *codA::Sh::upp, upp::Sh::codA* with *upp* from *E.coli* or the equivalent with *fcy1* from*S.cerevisiae* driven by a synthetic bacterial promoter were inserted between viral promoter and polyadenylation sequences in vectors for constitutive expression in both *E.coli* and mammalian cell lines. The best association in determining the highest sensitivity of cells to fluorocytosine was established in various *E.coli* mutant strains and animal cells. The fluorinated cytosine was converted to a toxic compound by the cytosine deaminase constructs and this more efficiently when the UPTRase activity was also present in the same entity gene. Furthermore, these experiments emphasized the crucial role of an active cytosine transport in determining the sensitivity of cells to 5-fluorocytosine. The possibility to use these constructions in the gene therapy "suicide" approach is discussed.

HUMAN ARTIFICIAL EPISOMAL CHROMOSOMES (HAECS) FOR GENE THERAPY, Jean-Michel H. Vos[1-2],Subrata Banerjee[2], Tian-Qiang Sun[1], Zachary Kelleher[2], Brian Wendelburg[2] & [1]Dept. of Biochem. & Biophys.; [2]Lineberger Comp. Cancer Center, Univ. of N.C., Chapel Hill, N.C. 27599-7295, USA

We have developed a human artificial episomal chromosome (HAEC) system for the stable establishment and long-term expression of large DNA as circular minichromosomes in human cells [1]. Such system brings the prospects of delivering genes in their genomic configurations including regulatory regions into human cells as engineered extrachromosomal elements [2]. To test the feasibility of the HAEC system for episomal-based gene therapy, we have target human B cells using *ex vivo* protocols [3-4]. HAECs carrying constitutively expressed human HPRT or FACC genes were delivered in B cells from Lesh-Nyhan or Fanconi's anemia patients via Epstein-Barr virus infection [5]. The transferred therapeutic DNAs were established as large 140-160 kb episomes in the stable cell transformants. The cellular HPRT or FACC functions were restored to normal levels with correct HPRT or FACC messenger RNAs expressed from the HAECs. Expression levels remained stable for over a year in cells grown under selection, and for at least two months in the absence of selection. To evaluate the HAEC system in vivo, SCID mice were injected with human B cells carrying various HAEC constructs, and episomal-based gene expression and disease correction were monitored as a function of time. Results of these experiments will be presented, and the prospects of applying the HAEC system to long-term gene therapy of hereditary syndromes will be discussed.

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t-PA Gene Therapy for Preventing the Restenosis after PTCA
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t-PA (Tissue Plasminogen activator) gene therapy may provide a novel therapeutic strategy in preventing the thrombotic diseases. The goal of this study was to detect the expression duration of the t- PA gene transfering into the arterial wall in vivo and whether the gene therapy was able to preventing the restenosis after PTCA. The retroviral vector containing t-PA cDNA were directly delivered into 10 coronary and 12 femoral arterial walls of 12 canines, 6 coronaries of 6 canines in control group were perfused TE buffer , via porous perfusion ballon catheter when canine restenotic model were established. The existence of t-PA cDNA and the expression of active t- PA products were proved by the hybridization in situ , mRNA dot blot and immunohistochemistry in different time of 30, 60, 90 days. Our results showed that the percent area stenosis decreased more than 20% in gene therapy group compared with control one, and further demonstrated that the t-PA gene therapy is able to decrease the neointimal proliferation in the restenotic model.

Keywords : t-PA , Gene therapy , Restenosis , PTCA.

PROGRESS TOWARDS DEVELOPMENT OF AN ADENOVIRAL VECTOR WITH DELETIONS OF E1 AND E2a,
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Most currently available vectors are deleted in the E1 region and are associated with a short duration of transgene expression. There is evidence that the duration of expression may be limited by leaky synthesis of viral proteins followed by host immune responses to eliminate infected cells and that mutations in the E2a gene might lead to reduced expression of viral proteins and improved vectors. The E2a gene encodes a DNA binding protein which functions in the control of replication of viral DNA and in the expression of multiple other genes. We have constructed a plasmid pBH25 suitable for preparation of adenoviral vectors deleted for both E1 and E2a, and have developed an E1-E2a complementing cell line. For construction of the plasmid pBH25, a virus DNA fragment (59.5 to 76.0%) containing the entire E2a open reading frame (ORF) was first cloned. After deletion of 1.3 kb of the total 1.6 kb ORF of E2a, the cloned fragment containing the deleted E2a gene was introduced into an E1 disrupted plasmid pFG140. For development of the E1-E2a complementing cell lines, various plasmids expressing a neomycin resistance gene and the E2a gene, the latter is under the control of the natural viral promoter or of the EF1α promoter, were transfected into 293 cells (an E1 complementing cell line). A virus (Ad5ts125) with a temperature-sensitive mutation in E2a could grow in the cells of several neomycin-resistance clones at the nonpermissive temperature. Of many complementing cell lines, one designated C2 expressing E2a from the natural promoter was tested further and found to support plaque formation of an E1-E2a inactivated adenoviral vector derived from the pBH25 plasmid. The titer of this E1-E2a inactivated virus (AdH25) produced in the C2 cell line is about ten-fold lower than that of the E1 deleted virus produced in the same cell line or in the 293 cell line. The E1-E2a inactivated adenoviral vector system has increased capacity for insertion of transgene sequences, is likely to demonstrate reduced expression of adenoviral proteins, and is less likely to lead to the development of wild-type virus through homologous recombination. Adenoviral vectors expressing reporter genes and carrying both E1 and E2a deletions are being prepared in order to assess whether these vectors are associated with increased duration of expression of transgenes *in vivo*.