TISSUE ENGINEERING

Organizers: Richard Skalak, Randall Swartz and C. Fred Fox

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Poster Sessions

Keynote Address

Q 001 DELIVERY SYSTEMS

by Robert Langer, Massachusetts Institute of Technology, Dept. of Chemical Engineering, Cambridge, MA 02139

Over the past 2 decades, increasing attention has been paid to development of systems to deliver drugs for long time periods at controlled rates. Such systems have been developed for the treatment of eye diseases and birth control. Some of these systems can deliver drugs continuously for over 1 year. However, little attention has been given to developing systems for the controlled release of large molecules (M.W. > 1000) such as polypeptide hormones. In early studies, we discovered that small pellets made of hydrophobic polymers such as ethylene-vinyl acetate copolymer could release many different macromolecules in bioactive form for over 100 days in vitro and in vivo. By combining parameters, such as drug particle size, loading, and coating, release rates for any drug could be changed several thousand fold. Microstructural studies show that the incorporation of powdered macromolecules during polymer matrix casting creates a series of interconnecting channels through which dissolved drug can diffuse. To achieve constant rates, a hemispheric device laminated with an impermeable coating, except for a small cavity in the center face, was developed. Constant release was achieved for over 60 days. Monte-Carlo computer methods are currently being explored to model these systems. By using these techniques, a variety of systems for releasing polypeptides such as insulin, epidermal growth factor, tumor angiogenesis factor, interferon, and protein vaccines have been designed.

Bioerodible polymers, in particular polyanhydrides have recently been explored as vehicles to release both large and small molecules. These polymers are unique in that they show surface erosion and lead to near constant release rates of incorporated drugs. By altering the hydrophobicity of the polymer backbone, release times from 1 week to 6 years can be achieved. They have recently been approved by the FDA for human clinical trials. Twenty-eight medical centers are now testing these polymers in a novel drug delivery application for brain cancer.

Finally, several new biodegradable polymer systems as well as polymer systems that display feedback control will be discussed.

Bone Growth, Development and Repair

Q 002 PURIFICATION, CHARACTERIZATION, AND BIOLOGICAL ACTIVITIES OF A UNIQUE OSTEOINDUCTIVE FACTOR FROM BOVINE BONE

H. Bentz, R. Nathan, R. Armstrong, A. Thompson, P. Segarini, M. Mathews, J. Dasch, K. Piez and D. Rosen, Celtrix Laboratories, Collagen Corporation, 2500 Faber Place, Palo Alto, CA 94303. We have purified and characterized a unique protein that in combination with either TGF-β1 or TGF-β2, promotes osteoinduction in the rat. Osteoinductive factor (OIF) was extracted from the organic matrix of bovine bone with 4M guanidine-HCl and purified employing a combination of Sephacryl S-200 gel filtration, CMC-ion-exchange, concanavalin-A Sepharose and heparin Sepharose affinity chromatographies and reversed phase HPLC. OIF is a glycoprotein containing two asparagine linked glycosylation sites and an apparent molecular mass of 22-28 kDa based on SDS gel electrophoresis. Enzymatic or chemical deglycosylation of OIF reduces its mass to about 12 kDa. In vivo activity of OIF was assayed in rats following subcutaneous administration of OIF in a soluble bovine dermal collagen and calcium phosphate ceramic carrier. In this model OIF activity is dose dependent over a narrow range. Reduction or digestion with trypsin resulted in a complete loss of OIF activity (at a 100 ng/mg implant dose), whereas deglycosylation resulted in a reduction in biological activity. Reduction does not alter its mobility on SDS-PAGE, suggesting that OIF is a monomeric molecule. Amino acid sequence and peptide mapping showed two cysteines at residues 62 and 95 that are intramolecularly linked. The 105 amino acid sequence of bovine OIF is unique and has no homology to other reported proteins. Further utility of OIF has been demonstrated using a rat femoral defect model. In this model, OIF alone or in combination with either TGF-β1 or β2 induced new bone formation. These results suggest that there may be sufficient exogenous TGF-β in bone to synergize with OIF or that the nature of the responding cells are different in the subcutaneum as compared to a bone site. The results demonstrate the ability of a newly identified protein factor to promote bone repair. Once sufficient quantities of OIF are available through recombinant methods, further utility and clinical efficacy can be addressed.

Q 003 BONE RESPONSE TO STRAIN, Stephen C. Cowin, Department of Mechanical Engineering, City College of the City University of New York, New York, NY 10031. The mechanism underlying the functional adaptation of bone tissue (Wolff's Law) has not been established. In particular, the path of the transduction of mechanical information to chemical information has not been determined. The means by which cell populations are subsequently controlled has not been identified. In this contribution the evidence concerning the biological strain transduction mechanisms, such as streaming potentials and ion channels, will be reviewed. The potential mechanisms by which bone cells sense strains and control the modeling or remodeling to adjust to these strains will also be reviewed. Models for the deposition and resorption of bone tissue based on the cellular level concept of a cell activity function will be described. The applications of these models to the prediction of bone modeling and remodeling will be summarized.

Q 004 SAFETY AND EFFICACY OF AN OSTEOINDUCTIVE DEVICE FOR USE IN BONE REPAIR, Thomas F. Parsons* and Ken Beres‡, *XOMA Corporation, Santa Monica, CA 90404, ‡BIOMET Inc., Warsaw, IN 46580. Osteogenic protein preparations, highly purified from bovine bone, have been used in combination with porous hydroxyapatite to formulate osteoinductive devices for the repair of bone defects. A rabbit calvarium trephine defect (8-10 mm diameter) was chosen as a convenient model, allowing placement of osteoinductive devices (discs of 8-10 mm diameter) in close proximity with intramembranous bone and obviating the complications associated with fixation of devices as would be encountered in models for long bone defects. Oxytetracycline hydrochloride was injected at 5 weeks post-implantation to monitor active calcification within the devices at this early time point. Calvaria containing these devices were explanted at 8 weeks or 12 weeks post-implantation. The extent of bone ingrowth into the implants at the time of harvest was histologically evaluated in sections, stained with Basic Fuchsin and quantitated by computer-assisted digitization. The extent of calcification at week 5 was measured by quantitative fluorescence of the incorporated oxytetracycline. These evaluations indicate significant bone ingrowth within these osteoinductive devices (with bone growth observed throughout the device and with the induction of "periosteal" and "endosteal" bone surrounding the device) as compared to the minimal bone ingrowth into untreated hydroxypatite discs (generally resulting in fibrous tissue ingrowth). For the 30 rabbits treated, no distal or systemic osteoinductive effects were noted, and all observations, including clinical chemistries, hemotology, clinical observations, gross/microscopic pathology and antibody responses, indicate no toxicity related to the osteoinductive device.

Cartilage Development and Diseases of Connective Tissue

Q 005 CARTILAGE/BONE: DEVELOPMENT AND REPAIR.
Arnold I. Caplan,
Skeletal Research Center, Department of Biology, Case Western Reserve University, Cleveland, Ohio 44106, USA.

You are alive because you continuously rejuvenate (turnover) the molecules and/or cells of all of the tissues of your body. On the level of cells, the half-life of differentiated cells in the body ranges from a few hours to days, months or even longer. The realization that this turnover exists allows the hypothesis to be put forward that stem cells give rise to these tissue-specific replacement units. Moreover, we anticipate that the pathway of change from stem cell to mature, differentiated cell is a recapitulation of embryonic events. Indeed, recent studies clearly document that such stem cells exist in bone marrow or the periosteum of bone. Such progenitor cells can give rise to either bone or cartilage under the appropriate microenvironmental circumstances. My laboratory has devised the technology for isolating, purifying and mitotically expanding mesenchymal stem cells from animal and human marrow or periosteum. These stem cells can be placed into appropriate delivery vehicles for reimplantation into defect sites to effect repair which the host tissue is not naturally capable of completing on its own. This technology makes it possible to consider autologous grafting to effect self-cell therapy for selected skeletal tissues. The experimental logics and details giving rise to this technology will be presented.

Q 006 BIOMECHANICAL AND BIOCHEMICAL PROPERTIES OF HEALING ARTICULAR CARTILAGE, VC Mow, A Ratcliffe, MP Rosenwasser, SA Shapses, JW Ark, PA Glazer, W Azzo, F Guilak, and TR Gardner, Departments of Orthopaedic Surgery and Mechanical Engineering, Columbia University, New York, NY 10032

Cartilage has a limited capacity to repair and regenerate damaged tissues resulting from joint injuries or diseases. The goal of this project has been to develop methods to enhance cartilage repair in the clinically important high-weight bearing regions of the knee. Histological, bioengineering and biochemical methods have been used to characterize the repair tissues to determine their probable functional capacity.

Materials and Methods: Twenty five New Zealand White rabbits (2.0-2.5 kg) were used. A full-thickness cylindrical defect (4mm diameter) was created in the weight bearing region of the medial femoral condyle. Periosteum was harvested from the ipsilateral tibia and used to form a purse around a disc of either subchondral bone or HTR^R-polymer, with the cambium layer on the outside. This graft was then placed into the femoral defect and recessed 1-2 mm below the articular surface. Postoperatively, the animals were allowed eight weeks of ad lib cage activity and were then sacrificed. The repair tissue and contralateral control cartilage was analysed for histological studies (n=3), and biomechanical (n=3) and biochemical (n=5) properties. Indentation testing and the biphasic indentation theory was used to determine three intrinsic properties of the cartilage (aggregate modulus, Poisson's ratio and permeability). Tissue was analyzed to determine the rate of proteoglycan synthesis, and the collagen and proteoglycan content.

Results and Discussion: Preliminary results indicate that this technique produced abundant amounts of hyaline-like repair tissue within osteochondral defects. Histological studies showed the repair tissue to consist of a large extracellular matrix but was hypercellular compared to the control cartilage. However, this tissue was less stiff and exhibited a significantly greater permanent deformation than control tissue. Surprisingly, no differences in tissue permeability were found. Determination of the biochemical content showed the repair tissue to have a proteoglycan concentration similar to the control tissue, although the collagen content was lower. The repair tissue also had a significantly increased rate of proteoglycan synthesis ($p=0.02$), which was mainly due to increased cellularity. Our ability to generate significant amounts of repair tissue in a high weight-bearing region is encouraging, and we expect that further analysis of the remaining animals will follow the preliminary trends determined for the biomechanical and biochemical properties.

Q 007 FORMATION OF CARTILAGE *in vitro*, Michael Solursh, Department of Biology, University of Iowa, Iowa City, IA 52242

Cartilage as a tissue typically has a limited capacity for repair. Tissue culture methods are now available for the normal growth and differentiation of cartilage. Some of the important variables involved include high cell density or minimal cell-substratum interactions. By manipulating these variables, we are able to culture embryonic mesenchymal stem cells from embryonic chick, mouse or rat sources, so that they differentiate into chondrocytes and subsequently form hypertrophic cartilage. Particularly useful is the culture of cells in agarose gels, a condition which minimizes cell-substratum interaction. The recent application of these same manipulations to human material permits the production of a large mass of cartilage tissue from a small needle biopsy, making it feasible to carry out autologous cartilage grafting. Large numbers of fibroblast-like cells are grown from small explants. Derived cells can be frozen down for storage and will subsequently re-express the cartilage phenotype when cultured in agarose gels. This culture system is ready for application to *in vivo* model systems.

Muscle Replacement and Maintenance

Q 008 INFLUENCE OF IMMUNE RESPONSE AND MIGRATION, ON MYOBLAST CELL THERAPY, Helen M. Blau, Simon M. Hughes, Grace K. Pavlath and Marilyn Travis. Department of Pharmacology, Stanford University Medical Center, Stanford, CA 94305-5332.

Duchenne Muscular Dystrophy (DMD) is a human genetic disease characterized by the destruction of skeletal muscle tissue which typically leads to progressive muscle weakness and death by age 30. Experiments in the mouse suggest that a therapeutic strategy based on the injection of normal human myoblasts may be useful in delaying the progressive muscle weakness typical of 200,000 individuals suffering from human muscle wasting disorders, of which DMD is one example. Two obstacles to cell therapy using human myoblasts have been overcome: quantity and purity. We have developed methods for isolating muscle cells from human biopsy and autopsy material using the fluorescence activated cell sorter. This approach allows an enrichment of human myoblasts to 99%, a purity which may be critical to their acceptance by the donor tissue. In a growth-promoting culture medium, cells from post-natal donors demonstrate a high proliferative capacity, and are capable of yielding kilograms of cells per cell. We have used retroviral constructs encoding β-galactosidase as genetic markers to trace the fate of myoblasts injected into mouse muscle tissue. These experiments have demonstrated that myoblasts can migrate across the basal lamina of extracellular matrix components that surrounds each muscle fiber and gain access to and contribute to the growth of multiple muscle fibers. Studies are underway to determine whether allogenic transplants of myoblasts are rejected, and if so, what protocols for immunosuppression are required to overcome that problem. The extent to which the introduction of new muscle cells interrupts or halts degeneration and promotes regeneration is being investigated. Thus, cell therapy may provide an effective means for introducing missing gene products characteristic of this and other human genetic diseases.

Q 009 PCR METHODS FOR THE ANALYSIS OF DYSTROPHIN EXPRESSION IN *mdx* MICE. Jeffrey S. Chamberlain, Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Michigan. Duchenne muscular dystrophy (DMD) is caused by genetic mutations that lead to defective production of the protein dystrophin. Strategies for a treatment or cure of DMD envision dystrophin replacement which could theoretically be achieved *via* tissue or gene replacement therapies. Such replacement must provide continuous production of functional, exogenous dystrophin at or near physiological levels in the appropriate tissues. Successful development of therapeutic procedures for DMD will require targeted delivery of dystrophin *via* cellular or genetic vectors, methods to assay the production of exogenous dystrophin, and appropriate animal models for testing. Initial testing of DMD therapies will likely utilize the *mdx* mouse. Three independently derived strains of *mdx* mice have been identified. We have been characterizing these mutant mice to develop rapid assays for expression of dystrophin mRNA as well as to develop novel methods for diagnosis of DMD. cDNA clones corresponding to the full-length murine dystrophin mRNA have been isolated and sequenced. These clones have enabled an analysis of the tissue and developmental regulation of dystrophin expression in both wild-type and *mdx* mice. Dystrophin mRNA is detectable *via* PCR at extremely low levels in day 14 embryonic muscle and brain, and increases throughout early development reaching a maximum soon after birth. Both the muscle and brain isoforms are detectable in embryonic muscle, but in the adult the muscle isoform predominates. PCR detects only the brain isoform in adult mouse brain. The developmental and tissue-specific expression of 3'-end alternatively spliced transcripts of dystrophin mRNA in muscle and brain is also being analyzed. All three strains of *mdx* mice express full-length dystrophin mRNAs in both muscle and brain at levels approximately 80% lower than in wild-type mice. Southern analysis reveals a normal pattern in all three strains indicating that the two new mutants, like the originally isolated *mdx* mouse contain point mutations. We are using the murine cDNA sequence to develop rapid scanning methods for the identification of point mutations in the human or mouse dystrophin genes to complement previously described methods for the rapid detection of deletions and duplications. These methods utilize PCR amplification of dystrophin mRNAs followed by chemical mismatch cleavage to localize point mutations prior to automated direct DNA sequencing of PCR amplified transcripts. These methods have identified several polymorphisms between each of the mutant *mdx* mice as well as the wild-type C57/Bl10 strain. In addition these methods confirmed the reported point mutation in the original strain of *mdx* mice and are currently being used to identify the mutation in the new strains. The identification of DNA sequence differences between these various strains of mice enables endogenous and exogenous dystrophin mRNAs to be rapidly analyzed and distinguished *via* PCR. Co-amplification of dystrophin mRNA from mice expressing an artificially introduced gene provides a rapid assay to determine the tissue specificity and relative expression of exogenous dystrophin genes relative to the mutant endogenous gene. PCR can also be used to analyze the ability of implanted myoblasts or gene constructs to produce proper levels of the various alternatively spliced transcripts of the dystrophin gene. Finally, the isolation and sequencing of the 14 kb murine dystrophin cDNA enables the production of full-length cDNA mini-genes with which to examine the feasibility of gene replacement therapy for DMD.

Q 010 TRANSFER OF NORMAL HUMAN MYOBLASTS INTO MUSCLES OF mdx MICE RESULTS IN DYSTROPHIN-POSITIVE MUSCLE FIBERS, George Karpati, Yannick Pouliot, Boaz Weller and Paul Holland, Neuromuscular Research Group, Montreal Neurological Institute, Montreal, Quebec, Canada, H3A 2B4

Skeletal muscle fibers are long multinucleated cylindrical cells, to which mononuclear, potentially myogenic satellite cells are attached. Large numbers of viable satellite cells can be isolated from muscle homogenates and cultured to produce myoblasts. Proliferating myoblasts become fusion-competent and can fuse with either each other, or in certain circumstances, into muscle fibers. Endogenous myoblasts (derived from satellite cells) in mature muscles, can fuse into muscle fibers during natural growth or during regeneration following segmental necrosis.

In mdx muscle fibers, the dystrophin gene suffered a point mutation on exon 25 and as a result, muscle fibers lack dystrophin (dys). Deficiency of dys leads to muscle fiber necrosis followed by regeneration. Large amounts of normal, cloned, human myoblasts were prepared and suspension of viable cells were injected by a 25 gauge needle to multiple sites into quadriceps muscles of 60-day-old mdx mice. 40 days later, large groups of dys-positive muscle fibers were observed in the injected muscles by microscopic immunostaining using a highly specific antidys antibody (Courtesy of Drs. E. Zubrzycka-Gaarn and R. Worton). The dys-positive muscle fibers seemed to escape dystrophic damage. (Contralateral control quadriceps of the same animals injected with saline showed only very rare dys-positive fibers). In a few fibers, dys expression in muscle fibers correlated with the presence of myonuclei derived from donor myoblasts, implying fusion of donor myoblasts into the host fibers.

The following measures have or are expected to increase the number of dys-positive fibers in mdx muscles after normal myoblast transfer (MT): prior crush of muscle, the use of cyclosporin-A for immunosuppression, and prior X-irradiation of the host muscle (presumably by elimination of the endogenous dys-incompetent satellite cell pool).

MT could possibly be used to mitigate muscle damage in certain inherited diseases of skeletal muscles, such as Duchenne muscular dystrophy.

Q 011 APPROACHES TO THE CORRECTION OF MUSCULAR DYSTROPHY BY MYOBLAST IMPLANTATION, Terence A. Partridge*, Jennifer E. Morgan*, Diana J. Watt†, Gary R. Coulton¶,& Eric P. Hoffman§. Departments of Histopathology*, Anatomy† & Biochemistry¶, Charing Cross & Westminster Medical School, London W6, 8RF, U.K. and Genetics§, Childrens Hospital, Boston, MA 02115, USA.

The multinucleated skeletal muscle fibre is formed entirely by fusion of mononucleated muscle precursor cells (mpc), during embryonic development and post-natal growth and also during regeneration following injury. Thus, it is possible to alter the genetic constitution of muscles by grafting genetically distinct mpc, in conditions where they will fuse with host muscle fibres[1]. We applied this idea to the treatment of inherited myopathies, by introducing normal mpc into diseased muscle fibres and looking for for expression of normal genes to ameliorate the inherent defect. As animal models, we have used the ICR/IAn mouse which exhibits phosphorylase kinase-deficiency, a metabolic myopathy, and the mdx mouse, which, lacking dystrophin, is a biochemical homologue of Duchenne muscular dystrophy (DMD) in man. As makers of host and donor tissues, we have used allelic isoenzymes of Glucose-6-Phophate Isomerase (GPI). We find that introduction of normal myonuclei into muscles of phosphorylase kinase-deficient mice, causes only marginal increases in activity of the missing enzyme[2]. By contrast, levels of dystrophin in the muscles of mdx mouse were elevated to >30% of normal levels by injection of normal mpc[3]. Disablement of the mdx host mpc by pre-irradation[4], augments the proportion level of dystrophin expression to >70% of normal. Immunofluorescent studies indicate extensive replacement and repair of host muscle fibres by injected normal mpc, giving largely dystrophin +ve muscle of normal histological appearance. In the latter respect, neonatal mpc seem more efficacious than older mpc. We conclude that, mpc have the required *in vivo* properties for use in therapy of myodegenerative genetic disease, but possibly not for metabolic myopathies, unless they entail muscle fibre degeneration.

1. Watt *et al.*, 1984. *Muscle & Nerve*, 7, 741-750.
2. Morgan *et al.*, 1988, *J. Neurol Sci*,86: 137-147.
3. Partridge *et al.*, 1989, *Nature*,337: 176-179.
4. Wakeford, Watt & Partridge, 1990, *Muscle & Nerve* (in press)

Mechanisms Regulating Endothelial Cell Differentiation (joint)

Q 012 MATRIX REGULATION OF ENDOTHELIAL CELL PHENOTYPE, Joseph A. Madri, Yale University School of Medicine, New Haven, CT 06510 The vessel wall is composed of heterogeneous cell populations residing in and on complex matrices. Each vascular cell type has different functions and morphologies but all of them have roles in maintaining homeostasis and during repair processes. Endothelial cells of the various vascular beds exhibit a broad range of functions and appearances along with their shared features of non-thrombogenicity, polarity and metabolic functions. Endothelial cell response to injury occurs in all vascular beds but the responses vary, depending upon the vascular bed involved, the surrounding and underlying extracellular matrix organization and composition and the soluble factors present in the local environment. Large vessel endothelial cells respond to denudation injury by sheet migration and proliferation. This response is modulated by composition of the underlying matrix as well as by soluble platelet factors and cytokines which affect endothelial cell matrix synthesis. Recent data indicate that selected factors (TGF-β1) affect migration (eliciting an inhibition of migration) by altering matrix expression by the endothelial cells (increasing fibronectin synthesis and deposition). In contrast, microvascular endothelial cells respond to injury by migration into a three-dimensional matrix and ultimate tube formation. As noted with large vessel endothelial cells, microvascular endothelial cell behavior and phenotype is also modulated by matrix composition and organization. Specifically, expression of smooth muscle markers including α-smooth muscle mRNA and protein and PDGF responsiveness, can be induced in microvascular endothelial cell populations by altering matrix composition and organization. Additionally, the responses to selected soluble factors (TGF-β1) are modulated by matrix composition and organization. Specifically, in two-dimensional cultures of microvascular endothelial cells on a type IV collagen substratum, TGF-β1 elicits an eight-fold increase in α-smooth muscle mRNA and dramatic increases in fibronectin accumulation, while in three-dimensional culture in a type I collagen gel, TGF-β1 elicits rapid tube formation with albuminal secretion, deposition and organization of basal lamina components and tight junction formation with assembly of ZO-1 protein.

Q 013 **ROLE OF GROWTH FACTORS IN THE DEVELOPMENT OF THE VASCULAR SYSTEM**, Werner Risau, Max-Planck-Institut für Psychiatrie, Am Klopferspitz 18a, D-8033 Martinsried, FRG

The cardiovascular system is laid down very early during embryonic development. Endothelial cells and blood cell precursor cells differentiate in the mesodermal blood islands. The formation of blood vessels from in situ differentiating endothelial cells (vasculogenesis) and the sprouting from preexisting vessels (angiogenesis) contribute to the development of a functional vascular system. Proliferation of endothelial cells is a central phenomenon of both processes. We have isolated acidic and basic fibroblast growth factors (FGFs) from embryonic stem cells and cystic embryoid bodies (which serve as a model system for vasculogenesis) as well as from embryonic kidney and brain (angiogenic vascular development). The genes that encode these growth factors are expressed during embryonic development. Normal vascular development is dirupted in chimeric embryos which express the polyoma middle T antigen. Multiple hemangiomas appear within the embryo and the yolk sac at mid-gestation when blood islands develop. Endothelioma cell lines established from those hemangiomas rapidly proliferate in vitro and induce hemangiomas in vivo. Since middle T is known to modulate the activity of src-like tyrosine kinases these molecules seem to be important for the regulation of endothelial cell proliferation.

Q 014 **EXTRACELLULAR MATRIX-RESIDENT GROWTH FACTORS AND ENZYMES.** I. Vlodavsky, R. Bar-Shavit, G. Korner, R. Ishai-Michaeli and Z. Fuks, Department of Oncology, Hadassah University Hospital, Jerusalem 91120, Israel.

Cultured bovine aortic and corneal endothelial cells synthesize basic fibroblast growth factor (bFGF) that remains mostly cell associated, but can also be extracted from the subendothelial extracellular matrix (ECM) deposited by these cells (Vlodavsky et al., PNAS 84:2292, 1987). Basic FGF has also been identified in basement membranes of the cornea and blood vessels in vivo (Folkman et al., Am. J. Pathol. 130:393, 1988). Basic FGF binds to heparan sulfate (HS) in ECM as evidenced by lack of binding to ECM pretreated with heparanase, but not with chondroitinase ABC (Bashkin et al., Biochemistry 28:1737, 1989). Exposure of ECM and basement membranes to heparanase but not to chondroitinase resulted in release of bFGF. Likewise, cells incubated with ECM utilized their heparanase activity to release the ECM-resident bFGF. Degradation of the ECM' HS may thus be involved in both cell invasion and neovascularization in normal and pathological situations. Restriction of bFGF bioavailability by binding to ECM and local regulation of its release may prevent a systemic effect of this pluripotent factor. Induction of endothelial cell proliferation and neuronal differentiation by ECM was inhibited by anti-bFGF antibodies. Moreover, PF-HR-9 endodermal cells transfected with the gene for bFGF, but not with the dominant selectable marker SV2-neomycin, were found to express bFGF and to produce ECM which supported proliferation of endothelial cells and neuronal differentiation of PC12 cells (Rogelj et al., J. Cell Biol. 109: 823, 1989). These results indicate that bFGF is an ECM component required for supporting cell proliferation and differentiation. The subendothelial ECM contains also tissue type- and urokinase type- plasminogen activators (PA) which participate in cell invasion and tissue remodeling. Activation of plasminogen by the ECM associated PA was inhibited by the atherogenic lipoprotein(a) particle. Thrombin binds to dermatan sulfate in the subendothelial ECM. The bound enzyme retained its proteolytic and thrombogenic activities, but was protected from interaction with its natural inhibitor, anti-thrombin III (Bar-Shavit et al., J. Clin. Invest. 84:1096, 1989). ECM-immobilized thrombin induced proliferation of vascular smooth muscle cells, independent of its esterolytic activity. These results and similar observations with other ECM immobilized growth factors and enzymes, suggest that the ECM provides a storage depot for biologically active molecules which are thereby stabilized and protected. This may allow a more localized, regulated and persistent mode of action, as compared to the same molecules in a fluid phase.

Wound Healing and Repair

Q 015 TGFβ ACTIONS IN DEEP WOUND REPAIR, Thomas A. Mustoe, Douglas Cromack, Sung Chin Wee, Shashi Kumar, Department of Surgery, Washington University School of Medicine, St. Louis, Missouri 63110

TGFβ is a potent promoter of wound healing as shown in several animal models. We have demonstrated an acceleration of healing in a surgical incision model both in skin and in intestine. In impaired models, TGFβ is able to partially or fully reverse the deficits in healing produced by corticosteroids or total body radiation, or adriamycin. The mechanism of wound enhancement appears to be centrally mediated by induction of collagen synthesis by fibroblasts, independent of its potent chemotactic effects in vitro on macrophages. In vitro TGFβ is inhibitory under multiple conditions. In vivo we saw evidence of a biphasic response with higher doses being less effective in both intestinal and rabbit dermal ulcer models. In the dermal ulcer model that we have described TGFβ is a potent inducer of granulation tissue, but at higher doses inhibits epithelization consistent with its in vitro effects on epithelial cells. In an ischemic ulcer model similar effects are seen.

Insights into the mechanism of wound healing promotion have been gained by a comparison with the effects of PDGF in normal and impaired wounds. Glucocorticoids and total body irradiation induce wound healing deficits characterized by a marked absence of macrophages which TGFβ is able to partially reverse while PDGF is ineffective in these models. The ability to act directly on the fibroblast to induce synthesis of collagen enables TGFβ to be effective in an intestinal model of healing (where PDGF is ineffective) and to see maximal effects on wound breaking strength in the first ten days. PDGF appears to act by attracting and/or activating macrophages including the induction of TGFβ mRNA and directly or indirectly to induce fibroblast proliferation, with secondary effects on collagen/extracellular matrix synthesis with a more delayed and prolonged wound healing enhancement, days 7-49, but peaking at two to three weeks rather than one week. The use of impaired healing models, intestinal healing models, and dermal ulcer models both normal and ischemic, have allowed a clearer understanding of the in vivo mechanisms of TGFβ enhanced wound healing.

Q 016 FIBROINDUCTIVE EFFECTS OF PDGF AND OTHER GROWTH FACTORS IN TISSUE REPAIR, Glenn F. Pierce, Kelly A. Doria, John Tarpley, Ed Shatzen, Arlen Thomason, Departments of Experimental Pathology and Molecular and Cellular Biology, Amgen, Inc., Thousand Oaks, CA 91320. The biologic roles of polypeptide growth factors in normal soft tissue repair are not yet fully understood. However, therapeutic application of growth factors clearly promotes normal and deficient wound healing in animal models, via unique and specific mechanisms of action. The platelet-derived growth factor (PDGF-BB homodimer) and transforming growth factor-B1 (TGF-B1) are potent vulnerary agents which enhance wound strength and induce markedly increased amounts of new extracellular matrix (granulation tissue). Recent functional, morphometric, and ultrastructural studies have shown that PDGF-BB and TGF-B1 each have a unique kinetic profile and duration of activity which may be related directly to their differential abilities to induce matrix constituents such as glycosaminoglycans and collagen. TGF-B1 is a direct inducer of fibroblast procollagen type I mRNA and protein synthesis, and rapidly stimulates increased collagen formation within wounds. PDGF-BB, in contrast, does not stimulate procollagen type I in vitro, and induces markedly increased production of glycosaminoglycans in early full-thickness wounds. PDGF-BB treatment augments influx of macrophages into wounds, which may release increased amounts of endogenous growth factors (such as TGF-B1) which in turn may activate wound fibroblasts to synthesize additional growth factors and matrix constituents. Thus, through direct stimulation of procollagen type I synthesis (i.e., TGF-B1), or direct recruitment of inflammatory cells and autocrine feedback loops (e.g. PDGF-BB) specific growth factors markedly accelerate and augment deposition of extracellular matrix in open wounds. Other growth factors such as basic fibroblast growth factor (bFGF) and perhaps platelet-derived endothelial cell growth factor (PD-ECGF) may have a more direct influence on endothelial cells and neovessel formation, in vivo activities which may be required for the repair of poorly vascularized wounds. bFGF treatment induces a marked endothelial cell response; resultant wound granulation tissue consists almost entirely of activated endothelial cells and neovessels. Epidermal growth factor (EGF), in contrast, has a more specific influence on epithelial cells, and does not augment underlying extracellular matrix formation. Clinical trials will permit further dissection of the cascade of biological activities initiated at the wound site by growth factor therapy.

Q 017 PERSPECTIVES IN WOUND HEALING, Russell Ross, Department of Pathology, School of Medicine, University of Washington, Seattle, WA 98195

The process of wound repair involves acute and chronic inflammatory responses followed by a proliferative and angiogenic response. In each case, the characteristic of the response is dependent upon the tissues involved. Both regeneration (in the instances of epithelial repair) and new connective tissue formation are hallmarks of this process. Small peptides and numerous growth-regulatory molecules can play important roles. These molecules include PDGF, EGF/TGFα, TGFß, FGF, the IGFs, TNFα, IL-1, and numerous others. Some of these factors, such as PDGF, may act directly to induce chemotaxis and mitogenesis. Others, such as TGFß and IL-1, appear to act by chemotactically attracting monocytes, and then induce secondary gene expression in cells such as monocyte/macrophages, fibroblasts, and endothelial cells to express other growth-regulatory molecules such as PDGF. The nature and significance of the cellular interactions and of the growth-regulatory molecules in wound repair will be discussed.

Q 018 EGF AND TGF-ALPHA IN WOUND HEALING AND REPAIR, Gregory Schultz, and D. Scott Rotatori, Departments of Obstetrics Gynecology, and Surgery, University of Florida, Gainesville, FL 32610

Healing of wounds in the skin is a complex process involving the coordinated actions of different cell types. Recent results suggest that several peptide growth factors and their receptors play key roles in the natural healing process by acting as autocrine and paracrine regulators of wound cell function. Two of these growth factors are the structurally related peptides, epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α). In normal, nonwounded human skin, TGF-α mRNA and protein as well as receptors for EGF/TGF-α, are found in the basal cell layers of the epidermis. Since TGF-α or EGF are required for growth of epidermal cells in vitro it is probable that these factors are important for growth of normal epidermis in vivo. Several results also suggest that TGF-α and/or EGF are important in healing wounded skin. Platelets release immunoreactive EGF material that binds to the EGF/TGF-α receptor, and wound macrophages isolated from subcutaneous wound cylinders in mice contain TGF-α mRNA and protein. Wound fluid collected from partial thickness donor sites of patients or from chest drains of women following mastectomy surgery contained immunoreactive TGF-α material and EGF/TGF-α receptor active material. No immunoreactive EGF material was detected in the wound fluids. Concentrations of TGF-α immunoreactive material in the wound fluids decreased during the first 2 days following surgery then increased during the next 3 to 4 days. Total amounts of TGF-α immunoreactive material in wound fluids were highest during the first two days following surgery and then decreased as healing progressed. Treatment of partial thickness burns on pigs or donor sites on patients with topical application of EGF or TGF-α accelerated epidermal regeneration. In addition, topical EGF treatment of chronic wounds in patients may promote healing. EGF also has been reported to promote healing of surgical incisions in normal rats and in doxrubicin-treated rats, and to promote healing of incisions in corneas of rabbits, cats and primates. Additional research is needed to understand the alterations of growth factors and their receptors in chronic wounds and the mechanism by which EGF or TGF-α promote healing of normal and chronic wounds.

Q 019 GROWTH FACTORS AND WOUND REPAIR IN DIABETIC MICE. Katie Sprugel[1], David Greenhalgh[2], Mark Murray[1], and Russell Ross[3]. ZymoGenetics, Inc.[1], Seattle, WA 98105, Shriners Burns Institute[2], Cincinnati, OH 45219, Department of Pathology, University of Washington[3], Seattle, WA 98195.

Chronic nonhealing wounds present a serious clinical problem. The specific defects which underlie the lack of healing in chronic wounds are not understood in most instances. Normal wound healing is a complex and highly orchestrated process involving sequential stimulation of cell migration, proliferation, angiogenesis, and extracellular matrix production. A variety of substances have the potential to stimulate specific aspects of wound repair and may be useful in treating chronic wounds.

We have characterized a model of delayed wound healing in genetically diabetic mice (C57BL/KsJ-db/db). Full thickness skin excisions heal much more slowly in db/db mice than in their non-diabetic littermates. A 250 mm^2 wound heals in 10-14 days in the control mice and in 4-6 weeks in the diabetic mice. Factors contributing to the slower healing are a delayed influx of inflammatory cells, delayed formation of granulation tissue, and decreased contribution of contraction to wound closure.

Several peptide growth factors were evaluated for their ability to enhance wound repair in the db/db mouse. The factors were applied topically for 5-10 days after wounding and the wounds were evaluated 10 or 21 days after wounding. Platelet-derived growth factor (BB and AA isoforms), basic fibroblast growth factor, and transforming growth factor beta-type 1 all improved healing of full thickness skin excisions in the diabetic mice. The improvement in healing was evident as increased amounts of granulation tissue 10 days after wounding compared to placebo treated mice and a decrease in open wound area 21 days after wounding. The granulation tissue in growth factor treated mice at 21 days was also more plentiful and mature than that of the placebo treated animals. Treatment with all of the factors resulted in qualitatively similar responses, including normal, differentiated epidermis covering the wounds. Growth factor treatment is not required throughout the experimental period to induce wound closure, suggesting that the growth factors trigger repair processes which can then proceed without further intervention to healing.

Practical Aspects of Cell Culture and Engineering (joint)

Q 020 CREATION OF MAN-MADE ENDOTHELIAL CELL LININGS, Jarrell, B.E., Williams, S.K., Thomas Jefferson University Hospital, 1025 Walnut Street, Room 605, Philadelphia, PA 19107

Endothelial cells (EC) may be isolated in large number from the microvascular circulation of adipose tissue. These EC demonstrate rapid adherence to PTFE and PET surfaces and is followed in minutes by cell spreading and attachment that is highly resistant to detachment by physiologic shear stresses. After optimization of these processes in vitro, the process of creating an EC monolayer was adapted to an in vivo models. In a canine carotid model, 4 mm diameter, 6 cm. length PTFE grafts were endothelialized with autologous microvessel endothelial cells. The time period from fat procurement to graft implantation was 1 hour. After implantation, grafts were observed for up to 12 weeks and explanted. EC treated grafts demonstrated 1) confluent cell monolayers over the entire graft surface and 2) highly significantly increased patency (9/11) when compared paired control grafts (1/11) (p=0.0.15). The process of creating an EC monolayer was also performed in 5 humans requiring vascular grafts. These grafts were easily endothelialized in an operative setting. Retrieval of two grafts both revealed factor VIII related antigen positive surface cells indicative of EC in the midgraft region. This process can be safely performed in a clinical setting and demonstrates a high potential for significantly improving the thrombogenicity of current grafts.

Q 021 ENDOTHELIAL CELL DYNAMICS AND FLOW. Robert M. Nerem, Peggy R. Girard, Sherry D. Doty, Gabriel Helmlinger, and Thierry Ziegler. Biomechanics Laboratory, Georgia Institute of Technology, Atlanta, GA 30332-0405. In forming the inner lining of a blood vessel, vascular endothelial cells reside in a flow environment. To investigate the influence of this environment, the effect of laminar flow-imposed shear stress has been studied in vitro using a parallel plate flow chamber employing cultured bovine aortic endothelial cells (BAEC). In response to the onset of steady flow, a confluent BAEC monolayer undergoes a change in cell shape and orientation and a reorganization of the F-actin microfilaments into stress fibers aligned with the direction of flow. The latter reflects itself in an enhanced cell stiffness, where a factor of ten increase in the elastic modulus of shear stress-exposed cells as compared to control cells has been measured using the micropipette technique. In addition, immunofluorescent studies show that other cytoskeletal proteins, including the intermediate filament protein vimentin and the focal contact protein vinculin, respond to shear stress by reorganizing their pattern. There also are modifications in cell-associated fibronectin, with the diffuse network of fine fibers characterizing static culture being redistributed into thicker fibers aligned with the direction of flow. Furthermore, with exposure to flow there is an initial decrease in the amount of fibronectin, followed by an increase to levels higher than those of control cultures for longer times. For a subconfluent BAEC monolayer, there is the additional effect of the cell proliferation rate decreasing with increasing level of shear stress. For a shear-stress elongated cell undergoing cell division, the result is two elongated daughter cells. These alterations in cell shape, cytoskeletal structure, and cell proliferation rate by a steady laminar shear stress are accentuated by a simple, 1 Hz sinusoidal, non-reversing pulsatile flow. In contrast, a reversing pulsatile flow produces sharply differing results. It is clear that BAEC not only recognize the presence of flow and transduce that signal into changes in structure and function, but also are able to discriminate between different flow environments. Although the recognition event remains undetermined, many of the second messengers resulting from a chemical stimuli are also present in flow-stimulated BAEC, including increased intracellular calcium, stimulation of phosphoinositol metabolism, and activation of protein kinase C. It is clear that BAEC are responsive not only to their biochemical environment, but also to their mechanical environment. Thus, if one is to engineer the cell culture environment so as to make it more physiologic, it appears that an important consideration for vascular endothelial cells is the simulation of their flow environment.

Skin Replacement and Epithelial Development

Q 022 RECIPES FOR RECONSTITUTING SKIN, Eugene Bell, Paul Kemp, Graham D. Green, N. Muthukumaran, Roger Gay and Mireille Rosenberg, Organogenesis Inc., 83 Rogers St., Cambridge, MA 02142. Reconstituted living skin is made up of a dermal equivalent on which keratinocytes are plated where they give rise to a multi-layered differentiated epidermis. The dermal equivalent (DE) develops through interactions between fibroblasts and collagen fibrils that begin to form after the cell-matrix precursor is cast. The gel that forms as a result of collagen polymerization and fluid trapping is contracted uniformly in all dimensions. By securing it at ends and edges in the mold in which it is cast, the final dimensions, strength and the morphology of the forming tissue are altered. The same phenomena are seen in casting tubular tissues for the fabrication of small caliber blood vessels.

The cells of the dermal equivalent are biosynthetically active and enrich the matrix to different degrees with secretory products, depending on how the cells are stimulated and on the presence or absence of an epidermis. Collagen biosynthesis by dermal cells in the DE is sensitive to growth factors, ascorbate concentrations and amino acid pools. Both ascorbate and TGFβ increase total collagen biosynthesis at least two-fold by one week after tissue formation. No additional enhancement is observed if PDGF complements TGFβ. With TGFβ present, the capacity of cells in the DE to synthesize collagen increases with time, over a two week period. If ascorbate is added just after the tissue is cast and daily thereafter, stable lattice contraction is blocked, and collagen biosynthesis is enhanced relative to contracted controls that had received ascorbate once. The greatest enhancement of collagen biosynthesis by cells in the DE was induced by addition to the medium of glycine, proline and ascorbate. The increase was nearly an order of magnitude over that of controls and was coordinate with a comparable increase in hyaluronate production as shown by TCA-precipitable glucosamine that adds to the intercellular matrix of the DE.

Both the Living Skin Equivalent (LSE) and the Living Dermal Equivalent (LDE) exhibit complex responses to UV radiation and to various chemicals that are greatly different from responses given by monolayered cells. In general, threshold doses are elevated by one or more orders of magnitude for the tissues as compared with cells in monolayer, with the LSE exhibiting higher thresholds than the DE.

The immunogenicity of the human LSE has been tested *in vitro*. Its cells are shown to be unable to stimulate a response in a mixed lymphocyte reaction (MLR) even after Class II antigens are induced by exposure to cytokines. The basis for the immunologic neutrality of the LSE can be referred to the absence of immune cells normally present in skin. The generality of immunologic neutrality is an essential consideration in the fabrication of tissue and organ equivalents for grafting.

Q 023 FETAL SKIN: A MODEL FOR STUDYING TISSUE INTERACTIONS, Karen A. Holbrook, Departments of Biological Structure and Medicine (Dermatology), University of Washington School of Medicine, Seattle, WA 98195

The skin is a complex organ composed of most tissues of the body. Interactions between these tissues during development influence the morphogenesis of each; in the adult they are essential for maintenance of their differentiated states. To understand these tissue interactions, they may be investigated, first, in the more "simple" state in embryonic skin and then in progressively more complex states in fetal skin. Such studies provide parallel information about epithelia, connective tissue, nerves, vessels and cells which may be valuable in devising strategies for "constructing" artificial skin with *in vivo*-like properties.

Data on the morphology and biochemistry of the epidermis, dermal-epidermal junction (DEJ), dermis, hypodermis and epidermal appendages in developing human skin have identified key stages and landmark events. Skin of the embryo (<60d estimated gestational age [EGA]) is a two-layered epidermis (including melanocytes and Langerhans cells) and a watery, hyaluronic acid-rich and cellular dermis separated by a dermal-epidermal junction (DEJ) that includes the "common" basement membrane zone components but lacks the skin-specific antigens and attachment structures. Collagens of adult dermis are identified biochemically although there is little fibrous matrix. Nerves and vessels are organized in single planes.

All regions of the skin change concurrently within a 1-2 wk period surrounding the embryonic-fetal transition (>60d EGA). The epidermis stratifies, adding an intermediate layer of cells which express the differentiation-specific keratins of adult, keratinized epidermis. Coincidentally, cells within the basal layer proliferate and grow into the dermis forming cellular cords or folds which become hair follicles, eccrine sweat glands, or nails. These appendages result from a collaboration between epithelial and mesenchymal cells. The properties of both cell types, the basement membrane separating them, and the extracellular matrix at these sites have been characterized, but their specific interactions on a molecular level are poorly understood. Skin-specific antigens of the DEJ, and the structures that anneal the epidermis to the dermis, are expressed. The now fibrous dermis becomes organized into papillary and reticular regions that are defined by differences in matrix organization, cellular density and orientation, and position relative to vessels of superficial and deep vascular plexes. Elastic fibers are added late in development (~15-20 wks) and remain structurally immature even at birth. Keratinization occurs in the hair follicle around 15 wks; the interfollicular epidermis keratinizes between 22-24 wks, thus marking terminal differentiation of epidermis and completion of cutaneous morphogenesis. The skin of the third trimester fetus is similar to that of the neonate except for a significantly thinner dermis and differences in functional properties.

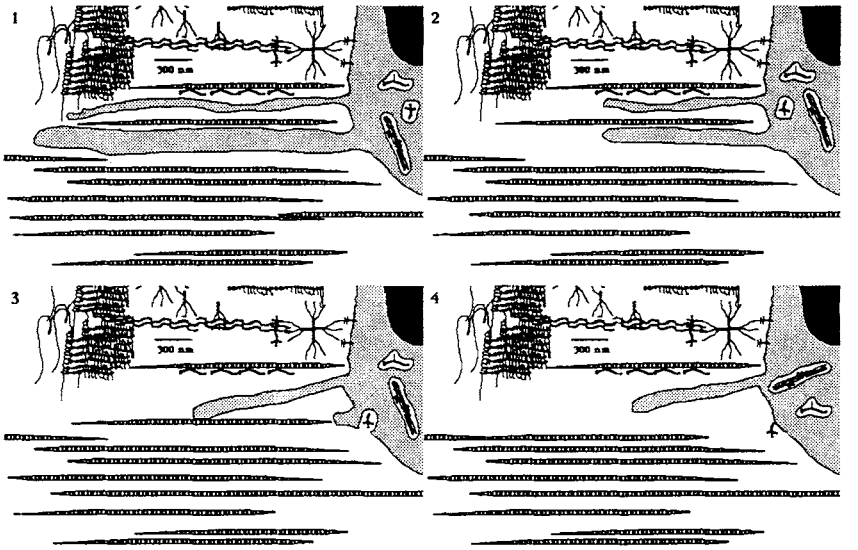
The morphology and biochemistry of the developing skin provide a basis for asking more mechanistic questions about development: How immigrant cells distribute nonrandomly in the epidermis; what signals and/or structures are involved in the induction of epidermal appendages; what messages are transferred in the interplay between epidermal and mesenchymal cells as the follicle grows and differentiates; what cells/soluble factors/matrix factors stimulate angiogenesis and neurogenesis; what growth factors are expressed in the developing skin, by what cells, and what role they play in tissue morphogenesis; and whether any one event occurring at embryonic-fetal transition serves as a cue for coincidental changes in other regions of the skin. These and other questions rely upon the use of fetal skin samples and/or its component cells in a variety of *in vitro* systems; co-cultures of cells (e.g., fibroblasts and keratinocytes of the same or dissimilar ages), raft and suspension organ cultures, and fetal skin grafts to the nude mouse are among the systems used in beginning to explore these problems.

Q 024 HOW WELL DOES THE EPIDERMIS DIFFERENTIATE *IN VITRO*?, Nancy Parenteau, Patrick Bilbo, Cynthia Nolte and Roger Gay, Organogenesis Inc., 83 Rogers Street, Cambridge, MA 02142.

Epidermal cells are able to stratify and undergo squamous differentiation under a variety of *in vitro* conditions. However, it has been found that a culture system incorporating a dermal-like substrate such as in the Living Skin Equivalent (LSE), allows for an increased level of epidermal organization and differentiation. This is in part due to the ability to culture the epidermal sheet at the air-liquid interface, thus providing a more natural environment. The epidermis of an LSE is morphologically similar to that of human skin as seen by light microscopy. It consists of morphologically organized basal, suprabasal, spinous, granular and cornified layers. Thymidine incorporation is limited primarily to the basal layer and the labeling index approximates that of normal skin. The LSE also has a number of ultrastructural similarities with a well-developed stratum corneum with both intra- and extracellular lamellar structures as well as evidence of basal lamina. Immunocytochemistry indicates that a number of differentiation markers as well as basal lamina components are readily produced. However, the ultimate test of epidermal differentiation is function. We have found that morphological organization and the mere presence of differentiation-related proteins are not sufficient indicators of function as determined by rates of percutaneous absorption and resistance to toxic compounds. Poorly functional LSEs exhibited good morphological differentiation but patchy filaggrin distribution (an indicator of granular layer development) and Type I transglutaminase distribution extending throughout the apparently cornified layers. In contrast, LSEs which had abundant, well-distributed filaggrin, exclusion of transglutaminase from uppermost layers, and abundant well-distributed laminin and Type IV collagen possessed a greatly enhanced permeability barrier, as assessed by rates of water penetration, demonstrated a sensitivity to benzalkonium chloride closely approximating that of a normal human response. Keratinocytes are therefore capable of achieving functional differentiation *in vitro*, provided there is proper modulation of synthetic activity.

Q 025 COLLAGEN FIBRIL SEGMENTS: A NEWLY RECOGNIZED INTERMEDIATE IN FIBRILLOGENESIS, Robert L. Trelstad, Emanuel I. Zycband, Donald A. Winkelmann, David E. Birk, Department of Pathology, Robert Wood Johnson Medical School, Piscataway, NJ 08854

Birk, D.E., Zycband, E.I., Winkelmann, D.A. and Trelstad, R.L., PNAS 86:4549, 1989.



Tissue Engineering for Adoptive Immunotherapy and Immune Replacement

Q 026 ISOLATION AND EXPANSION OF FUNCTIONALLY SPECIFIC T CELL POPULATIONS FOR USE IN ADOPTIVE IMMUNOTHERAPY, Allan Haberman, Ana Lages, Don DiMasi, Bruce Jacobson, Gordon Frampton and Randall W. Swartz, Biotechnology Engineering Center, Tufts University, Medford, MA 02155.

Current methods of adoptive immunotherapy involve use of mixed populations of lymphocytes. Such populations must be given in large numbers to be effective, they may contain suppressor cells, and the vast majority of the cells do not reach tumors or regional lymph nodes. Moreover, critical effector cells could be lost due to population shifts during in vitro expansion. One approach to the improvement of adoptive immunotherapy is thus the isolation of specific effector cell subpopulations from peripheral blood lymphocytes or from tumor infiltrating lymphocytes (TIL). We have developed methods for the isolation and expansion of human T cells with affinity for the major basement membrane components collagen IV and laminin. These "extracellular matrix adherent T cells" (EMAT) are highly enriched for T cells bearing γδ T cell receptors. [γδ T cells constitute a distinct lineage which comprises 1-10% of mature T cells; these cells have cytolytic activity and the ability to produce a variety of lymphokines (1).] T cells with specific affinity for basement membrane components may be able to transit the basement membranes of high endothelial venules (blood vessels specialized for lymphocyte homing) and home to epithelial tissues, lymph nodes, inflammatory sites, and tumors; indeed, γδ T cells have been seen associated with high endothelial venules in normal and lymphomatous lymph nodes in humans (2). Activated γδ T cells which home to tumors or to draining lymph nodes could induce anti-tumor immune responses via secretion of lymphokines and activation of other immune cells at these sites. In addition to developing methods for the isolation and activiation of specific T cell populations, we are also developing suspension/perfusion bioreactor systems (3) for the efficient expansion of T cells to numbers required for therapy. The efficacy of our approaches to adoptive immunotherapy will be tested in animal models.

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Q 027 ALTERATION OF PLASMA COMPONENTS FOR IMMUNE SYSTEM ACTIVATION, M. Rigdon Lentz, M.D., G. A. Granger, Ph.D., John Tomich, Ph.D., Ernest Tucker, M.D., Tetsuya Gatanaga, Ph.D., William Hubbard, Ph.D., John F. Kennedy Memorial Hospital, Indio, CA 92201 Human serum ultrafiltrate (UFS) obtained from a variety of cancer patients has been demonstrated to contain material(s) which inhibits cytolytic activity of natural killer cells, Interleukin-II activated lymphocytes and tumor necrosis factor (TNF-alpha) and lymphotoxin (LT-TNF-Beta) in vitro. These factors are found in the ultrafiltrate from patients with different types of cancer and are not detected in the serum of normal donors. Results from molecular sizing studies demonstrate that the molecular weight(s) are greater than 30,000 kD. Inhibition of cell mediated in vitro killing appears to involve both an alteration of cellular recognition as well as specific tumor cytotoxicity. Inhibition of TNF as well as lymphotoxin appears to be through a mechanism of direct competitive inhibition of the effector molecules. It is not clear whether the cell mediated and cytokine blocking factors are the same or different molecules. These factors may have adverse affects on the biologic activities of both natural killer cells, lymphokine activated lymphocytes, as well as those final common pathways to specific cytotoxicity mediated by TNF and LT. The TNF-LT inhibitor is a protein that has been purified, sequenced and cloned. KEY WORDS: Natural killer cells (NK), lymphokine activated killer cells (LAK), tumor necrosis factor (TNF), lymphotoxin (LT), inhibitor, ultrafiltrate of cancer patient's serum.

Q 028 SPECIFIC ACTIVE IMMUNOTHERAPY TO AUGMENT CYTOTOXIC T CELLS AGAINST CANCER, Malcolm S. Mitchell, U.S.C. Cancer Center, Los Angeles, CA 90033. Immunization of the tumor-bearing host with tumor-associated antigens, i.e., active specific immunotherapy (ASI), has been used successfully in rodent tumor models, usually with low tumor burdens. In humans, ASI has delayed recurrence in melanoma, and prolonged survival in at least one trial in colon cancer. Disseminated cancers have been more refractory but some advances have been made in kidney carcinoma and melanoma with immunogens from autologous tumor cells, with regressions in 10-25% of patients. Our treatment with allogeneic lysates from melanoma lines and the adjuvant DETOX caused remissions in approximately 20-25% of patients, with regression of skin, lung, lymph node, and ileal masses and one liver nodule. Median response duration was 17 mo; 4 patients given only ASI are alive $\geq$ 2 yr. The frequency of cytolytic T lymphocytes (CTL) in the blood was generally correlated with response, particularly if objective regressions of 25-50% were included. Antibodies and skin test reactivity to tumor antigens showed no correlation. Immunohistology of rejected skin lesions suggests principal roles of CD4+ lymphocytes and macrophages elicited by them, although CD8+ lymphocytes were also present. T cell clonal analysis revealed both CD4+ and CD8+ CTL, many with ostensibly HLA-unrestricted reactivity against melanomas but not other cancers. Other clones reacted "classically" only against autologous melanoma. Two responders have had brain metastases, but both are alive 8+ and 36+ mo. These results suggest that ASI with allogeneic lysates are effective in advanced melanoma, and should now be studied with microscopic residual tumor in several types of cancer.

Q 029 ANTIBODY/CELLULAR ENGINEERING FOR EFFECTIVE CANCER TREATMENT, Robert K. Oldham, S.K. Liao, Marty Winkler, and John R. Yannelli, Biotherapeutics Inc., Franklin, TN 37064 and Biological Therapy Institute, Franklin, TN 37064
The T cell repertoire is thought to possess specificity in terms of tumor cell antigen recognition. While it is possible to grow large numbers of T cells from peripheral blood, draining lymph nodes, and tumor tissue utilizing current technology, many of the T cells are irrelevant in terms of their tumor cell reactivity. However, it is now possible to broaden the T cell reactivity for tumor cell antigens by biological engineering of antibodies. Antibodies can be used to redirect the recognition event while the T cells deliver the lethal hit. Both chimeric antibodies (human Fc region coupled to murine variable regions) as well as heteroconjugates (anti-CD3 chemically linked to BR1, anti-breast carcinoma antigen) have been studied in our laboratory. The chimeric antibody (Ingene - 1) has been shown to induce cytotoxicity of resting mononuclear cells obtained from normal donors of cancer patients against tumor cell targets to levels as high as 30% at effector to target ratios (E:T) of 100 and 50:1. When LAK cells were generated from the mononuclear cells, enhancement of cytotoxicity was 20% at E:T as low as 10:1. Cytotoxicity was enhanced through the Fc receptor on NK cells. Using heteroconjugates, we have demonstrated cytotoxic potential against tumor cell targets when specific T cell recognition of tumor cells was not available. Boosting of CTL killing was as high as 50% at E:Ts of 3:1 or less. Combining these bioengineered antibodies and either NK cells or CTL offer major possibilities for improving cancer biotherapy.

Replacement in the Hematopoietic System

Q 030 THE STRUCTURE AND FUNCTION OF HUMAN BONE MARROW, Stephen G. Emerson, Departments of Internal Medicine and Pediatrics, University of Michigan, Ann Arbor, MI. 48109 This session is devoted the *in vitro* modeling and engineering of the human hematopoietic system, the bone marrow. The motivations and goals of this project will be enumerated, and the physiologic capabilities of normal human bone marrow which must be modeled will be detailed. The anatomy and physiology of normal *in vivo* hematopoiesis will then be reviewed, focussing on the multicellular nature of the system, the roles of hematopoietic growth factors and the hematopoietic microenvironment, and contrasts between the classical approaches to *in vitro* bone marrow cultures and the status of hematopoiesis *in vivo*. Utilizing these constrasts as a point of departure, possible strategies for experimental intervention to better model *in vivo* hematopoiesis will be explored. This discussion will therefore present the current and near-future state of hematopoietic engineering, and will lead in to the specific presentations by Drs. Quesenberry, Vournakis, Naughton and Palsson in the remainder of the session.

Q 031 HEMATOPOEISIS ON NYLON MESH MICROENVIRONMENTS

Gail K. Naughton, Ph.D.
Brian Naughton, Ph.D.

A physiological three-dimensional long term culture system has been developed for the growth of bone marrow in our laboratory. This system involves the establishment of stromal cells on a suspended nylon mesh, the growth of stromal cells to sub-confluence, and the inoculation of stroma with fresh or cryopreserved bone marrow cells. Stromal elements distribute themselves throughout the meshwork, secrete matrix proteins and establish an intricate microenvironment for the support of hematopoiesis. Active production of myeloid and erythroid cells is seen for at least twelve months in the rodent model and over twelve weeks in the primate systems. Preliminary analysis of this system reveals the adherent zone to be actively proliferating and consisting of multilineage immature cells. This adherent layer supports active proliferation of CFU-GM, CFU-GEMM, and erythroid progenitor cells, as indicated by standard methylcellulose and plasma clot assay systems. As in the in vivo state, the non-adherent zone contains mostly mature cells or late-stage precursors. Multilineage hematologic expression in adherent and non-adherent zones has been confirmed by cytofluorographic analysis and standard differential counts. These findings indicate that the three-dimensional nylon mesh system is a novel in vitro method which supports the growth of several hematologic lineages concurrently, as well as the proliferation of various rat, monkey, and human bone marrow stem cells. It is being utilized effectively as a substrate to study the effect of various purging methodologies and chemotherapeutic effects in vitro and has the potential for use in genetic insertion studies, graft-versus-host studies, blood component therapy, and marrow expansion for transplantation.

Q 032 METABOLIC MODELING, DESIGN AND OPERATION OF CONTINUOUS PERFUSION HEMATOPOIETIC CULTURES, Bernhard Palsson, Department of Chemical Engineering, The University of Michigan, and Ann Arbor Stromal Inc., Ann Arbor, MI 48109-2136.

The bone marrow is the body's primary source of blood cells. This tremendously prolific tissue produces a total of about 400 billion cells daily in humans through a process called hematopoiesis. Study of the hematopoietic process is not only of scientific value in terms of improving our understanding of the differentiation process, but also has significant clinical implications. Reconstructing the hematopoietic process ex vivo would open the possibility of producing blood cells (i.e. red cells, platelets, and the various white cells) on demand, and enable the expansion of bone marrow for transplantation and, possibly, for gene therapy. The construction of functioning ex vivo bone marrow requires the integration of experimental hematology, molecular biology, cell culture/bioreactor technology, and cellular bioengineering.

We will describe the early developmental stages of a perfusion system that is designed to cultivate human bone marrow, and the in vivo condition that it is intended to mimic. Successful operation depends on the introduction of a combination of hematopoietic growth factors, provision of an appropriate microenvironment, and a continuous perfusion system that mimics the dynamics of the in vivo condition. The importance and influence of the media perfusion rate, cell seeding density, hematopoietic growth factors, and extracellular matrix will be described and discussed.

Q 033 LONG-TERM MARROW GROWTH: HUMAN AND MICE SYSTEMS, Peter J. Quesenberry, Daniel S. Temeles, Helen E. McGrath, Rowena Crittenden, and Kotteazeth Srikumar, Department of Medicine, University of Virginia Health Sciences Center, Charlottesville, VA 22908.

Long-term bone marrow growth can be achieved in liquid culture in the presence of adherent cells which appear to form a model for bone marrow stroma. Long-term culture systems for both human and murine marrow have been studied both of which appear to necessitate adherent stromal cell support. Long-term growth has been most effective in murine systems with in general human systems showing a rapid decline in marrow support. Studies in our lab on C57/black, BDF1, or Balb/C long-term murine cultures have shown that the adherent stromal cells are rich sources of growth factors which can be induced by multiple manipulations. In our studies we have evaluated the production of Interleukin-3 (IL-3), Interleukin-6 (IL-6), colony stimulating factor-1 (CSF-1), granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) by adherent stromal cells utilizing bioassay with differential antibody blocking and mRNA analysis. These stromal cells clearly produce other growth factors including Interleukin-1 (IL-1). The stromal system can be isolated in part by exposure to 1100R of donor animals in vivo or alternatively 1100R to 3 week cultures in vitro. These irradiated cultures are capable of producing IL-6, CSF-1, G-CSF, GM-CSF in supporting hemopoiesis. These cultures appear to consist of two basic cell types; a phenotypically typical macrophage and a large alkaline phosphatase positive preadipocyte. The marrow macrophage population can be studied in isolation utilizing CSF-1 dependence as an in vitro selection technique. Studies of this macrophage population have shown it capable of producing growth factors shown to be produced by whole Dexter stroma although differential production at baseline or induced states suggest that the alkaline phosphatase positive preadipocyte is probably the source of CSF-1 and GM-CSF while the sources of IL-6 and G-CSF remain problematical. These systems support a variety of different stem cells and predominantly myeloid differentiation. In toto these results indicate that stromal cells are capable of producing a number of growth factors supporting early hemopoietic stem cells and hemopoietic cell differentiation. This suggests a model of growth factor regulation in which stromal cells produce multiple growth factors, possibly at low concentrations, and acting additively or synergistically to support ongoing hemopoiesis.

Tissue Engineering in the Nervous System

Q 034 RECOMBINANT CELL IMPLANTS IN NEUROLOGICAL DISEASE MODELS. W. Freed, S. Cottingham, M. LaMarca, M. Schultzberg, M. Poltorak, H. Cannon-Spoor, S. Paul, and E. Ginns, NIMH Neurosciences Center at St. Elizabeths, Washington, D.C., 20032, and NIMH Intramural Research Program, Bethesda, Maryland, 20832.

Parkinson's disease is undoubtedly the functional CNS disorder for which the goals of transplantation are most clearly defined. Many of its manifestations are related to a single missing circuit link: the dopaminergic innervation of the striatum. The goal of transplantation has thus been to replace the missing striatal dopaminergic innervation, whether this is accomplished via production of a new dopaminergic innervation, diffuse secretion of dopamine or other catecholamines from grafts, or trophic effects on endogenous host systems. The purpose of this paper is to explore the possibilities for intracerebral grafts of defined and genetically altered cell lines in Parkinson's disease models.

One of the most attractive alternatives is the possibility of a cell line containing tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis. Cottingham et al. (Neurosci. Abstracts,1988) have introduced the cDNA for human tyrosine hydroxylase form 2, into murine NIH-3T3 fibroblasts, in pDOLMP10 retroviral vector. These recombinant TH-3T3 fibroblasts contain TH mRNA as well as TH immunoreactivity by in vitro staining and western blots, using an antibody to rat TH. The TH produced by TH-3T3 cells is catalytically active, with a Km of 22 - 148 μM for tyrosine. When the cells were grown in culture with L-DOPA and cofactor, concentrations of L-DOPA in the medium increased gradually over 24-48 hours.

When transplanted into the brains of Swiss-Webster mice the cells were readily identified; these cells were surrounded by unusual "halos" of TH immunoreactivity, but did not themselves contain TH immunoreactivity. Animals with intrastriatal grafts of TH-3T3 or control cells were treated with reserpine. L-Tyrosine treatment, with or without cofactor, produced locomotor activation of the reserpine-treated mice to a greater degree than the controls. This activation was greatest when the animals were tested three days after reserpine injections. These data suggest that recombinant fibroblasts that are capable of producing L-DOPA from tyrosine can produce functional and behaviorally-significant alterations in the host animal.

Q 035 GRAFTING GENETICALLY MODIFIED CELLS TO THE BRAIN: CONCEPTUAL AND TECHNICAL ISSUES, Fred H. Gage, Lisa F. Fisher, J. Hyder A. Jinnah, Michael B. Rosenberg, Mark Tuszynski and Theodore Friedmann, Departments of Neurosciences and Pediatrics, University of California, San Diego, La Jolla, CA 92093

Neural grafting in the CNS has recently suggested a potential approach to CNS therapy through the selective replacement of cells lost as a result of disease or damage. Independently, studies aimed at direct genetic therapy in model systems have recently begun to suggest new approaches to the treatment of several kinds of human disease. We suggest that a combination of these two approaches, namely the grafting into the CNS of genetically modified cells, may provide a new approach toward the restoration of some functions in the damaged and diseased CNS. We present evidence for the feasibility of this approach, including a description of some current techniques for mammalian cell gene transfer and CNS grafting, and several possible approaches using several different genes including tyrosine hydroxylase, nerve growth factor, and ß-galactosidase.

Q 036 IMPLANTATION OF GENETICALLY ENGINEERED ASTROCYTES INTO THE RAT BRAIN. Herbert M. Geller, Maciej Poltorak* and William J. Freed* Department of Pharmacology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ 08854 USA and *NIMH Neurosciences Center at St. Elizabeth's, Washington, DC 20032 USA.

A7 cells are an immortal astrocyte cell line made by inserting the gene for SV40 large T antigen into primary astrocytes from the rat optic nerve via retroviral-mediated gene transfer (Geller and Dubois-Dalcq, 1988). This cell line has certain astrocytic properties, such as the expression and secretion of PDGF, expression of surface N-CAM, expression of the p185 phosphoprotein product of the *neu* oncogene, and the ability to support the growth of dissociated embryonic neurons in cell culture. We are evaluating the utility of this cell line as an intraparenchymal implant for somatic cell replacement therapy of neurological diseases. A suspension of A7 cells was labelled by bisbenzimide and implanted into the stratum parenchyma of naive Sprague-Dawley host animals using procedures we have developed for implantation of other cell lines (Freed, et al., 1989). Animals were sacrificed after 2-8 weeks of survival and their brains were examined using histological stains as well as indirect immunofluorescence to assess cell survival, cell migration and the effects of A7 cellular implants on the host tissue. Implanted cells were localized by the presence of bisbenzimide labelling as well as with a polyclonal antibody against large T antigen which brightly stains the nuclei of the implanted cells. Whereas only some of the grafted cells survived, those that survived did not produce neoplastic growth. Grafted cells did not induce any evident immunological reactions within the host brain as measured by the presence of OX6$^+$, W3/25$^+$, W3/13$^+$, and OX8$^+$ cell infiltrations. Distinct laminin positive immunoreactivity was closely associated with the implanted cells, which retained their antigenic phenotype, including the expression of N-CAM. In animals examined after 6 and 8 weeks, the Thy 1.1 immunoreactivity within the surrounding host brain seemed to fill the area of grafted tissue. The present data, together with *in vitro* observations that the A7 cells promote neuritic outgrowth and secrete growth factors, suggest that they may be useful in models of restoration of function by intracerebral implantation.

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Q 037 COMBINED USE OF POLYMER TUBES AND BIOLOGICAL SUBSTRATES AS CONDUITS FOR REGENERATING DORSAL ROOT FIBERS IN THE ADULT RAT.
J. Lustgarten, M. Proctor, A. Kader, J. Silver, and M. Kliot, Dept. of Neurosurgery, Neurological Institute, N.Y., N.Y., 10032 and Center for Neuroscience, CWRU Medical School, Cleveland, Ohio, 44106.

Recently, Millipore implants coated with embryonic astrocytes (Kliot et al.,1988) and "peripheral nerve bridges" (Aguayo et al.,1989) have suceeded in stimulating a limited and variable amount of axonal regeneration within the adult mammalian CNS. In order to further define conditions promoting successful axon regeneration, we have begun to employ acrylic polymer tubes as conduits for controlling the microenvironment of regenerating axons. Properties of these tubes shown to influence nerve regeneration include permeability, microgeometry, and charge (Aebischer et al.,1988). We are currently investigating the rate, quantity, and distribution of regenerating dorsal root fibers implanted into specially designed polymer tubes either alone or in combination with various biological substrates. Preliminary findings demonstrate that the tubes are immunologically tolerated and that dorsal root axons will grow within them for a distance of 3mm in 4-6 weeks. We hope this technology will eventually allow us to optimize the delivery of regenenerating dorsal root fibers directly to their target area in the dorsal horn of the spinal cord.

Implantation of Islet and Hepatic Cells

Q 038 BIOENGINEERING ISSUES IN THE DEVELOPMENT OF ISLET IMMUNOISOLATION DEVICES, Clark K. Colton, Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139

The complications of diabetes are thought to arise from poor control of blood glucose concentration because of inadequate pancreatic insulin secretion. One approach to improved therapy is implantation of xenogeneic insulin-secreting pancreatic islets of Langerhans. The islets are immunoisolated to circumvent immunological rejection problems of transplantation by interposing a semipermeable membrane, which passes glucose and insulin but retains white cells and antibodies, between the islets and the host tissue. Several embodiments have been explored: (1) microencapsulation of one or a small number of islets in a spherical configuration, (2) placement of islets within semipermeable hollow fibers or between membrane sheets; and (3) culture of islets on the outside surface of a semipermeable tubular membrane housed in a cartridge which is implanted in the cardiovascular system. Problems common to all of these approaches include: (1) isolation and purification of islets in an efficient and economical manner; (2) development of materials and device designs that maintain islet viability and secretory function in order to provide an insulin secretory response that controls blood glucose concentration at or near physiological levels at all times; and (3) biocompatibility problems associated with thrombosis in intravascular devices and fibrotic capsule formation around devices implanted in tissue. In vivo, each islet is well vascularized and exposed to arterial blood which supplies glucose, other nutrients, and O_2 and rapidly removed secreted insulin. In an immunoisolation device, significant mass transfer resistances exist to the transport of each of these species. These resistances are associated with diffusion within the islet, spaces within the device, the immunoisolation membrane, and the blood or tissue surrounding the device. Perhaps most severe of these transport limitations is that associated with oxygen. Severe oxygen limitations can lead to islet cell death. In addition, recent results demonstrate that exposure of islets to oxygen partial pressures substantially reduced the physiological levels, but well above that which threatens cell viability, can lead to rapid and reversed reduction of glucose-stimulated insulin secretion rate. Thus, conditions can exist wherein islets are maintained viable within an immunoisolation device but are dysfunctional with respect to insulin secretion.

Q 039 SYNTHETIC MEMBRANE-BASED DEVICES FOR PANCREAS AND LIVER ORGAN REPLACEMENTS, Barry A. Solomon, Membrane and Biomedical Research Department, Research Division, W. R. Grace & Co.-Conn., Lexington, MA 02173 The use of synthetic, semi-permeable membranes to culture mammalian cell lines *in vitro* was developed nearly twenty years ago. The broadest application today has been in the growth of hybridoma cell lines in hollow fiber bioreactors for the production principally of high value monoclonal antibodies. The ability to supply a nutrient source to one side of a membrane providing required key nutrients to and removing metabolic waste products from mammalian cells sequestered on the other side of the membrane presents a unique opportunity to create hybrid artificial organs. Membrane devices used as artificial organs must meet performance specifications both focussing on the strict cell culture requirements required for primary cells as well as maintaining biocompatibility between the polymer surfaces of the device and the circulating blood or tissue with which the device comes into contact. Hollow fiber devices for these uses can be categorized depending upon the transport modality of the nutrient supply to the membrane; (a) perfusion devices in which a nutrient stream (blood) is convectively passed through the core of the fibers or (b) diffusion devices in which the nutrients from surrounding tissue bathe the membrane encapsulating the cells. Hollow fibers have been developed and fabricated into these device types which provide appropriate nutrient transport characteristics while maintaining blood and tissue compatibility consistent with the implantation of such devices. Isolated, pancreatic islets have been incorporated into devices of these types and evaluated for use as a long-term, implantable, hybrid artificial pancreas. Extended *in vitro* culture of islets in these devices has been achieved while maintaining insulin secretion and transient glucose responsivity. Similarly, modified hollow fiber culture devices containing primary mammalian hepatocytes, have been evaluated for use as a shorter-term, extracorporeal liver-assist device to provide a broad range of liver functions particularly those related to the P-450 detoxification system. Animal models of diabetes and liver failure have been developed and *in vivo* (pancreas) and *ex vivo* (liver) studies are underway to establish clinical safety and efficacy.

Round Table Discussion and Summary

Q 040 TISSUE ENGINEERING: SUMMARY AND DISCUSSION,
Karl A. Piez, Celtrix Laboratories, Collagen Corporation, 2500 Faber Place, Palo Alto, CA 94303

This conference discusses problems in tissue engineering from the point of view of biological substitution and repair. In my opinion, cartilage, muscle, the immune system, nerves and organs all present problems where only very long term solutions can be expected. Although exciting ideas exist, the paths to success are uncertain. On the other hand, skin replacemen bone repair and wound healing may have short to mid term solutions. In the cases of bone repair and wound healing, this is because specific protein factors have been identified and made available by recombinant DNA technology that are involved in these processes. It is possible to test specific product concepts. The problems to be solved - recombinant protein manufacture, formulation, toxicity evaluation, preclinical efficacy, and clinical testing - are formidable but doable. In the case of skin replacements, advances have been largely empiric in understanding biological matrices and cell culture; here also recombinant protein factors may help produce solutions.

Endothelial Cells; Bone and Cartilage

Q 100 EXPRESSION OF THE CH 21 PROTEIN DURING THE "IN VITRO" DEVELOPMENT OF CHICK EMBRYO CHONDROCYTES. Ranieri Cancedda, Fiorella Descalzi, Beatrice Dozin, Fabio Rossi, Francesco Molina, Armando Negri*, Severino Ronchi*. Istituto Nazionale per la Ricerca sul Cancro, GENOVA-ITALY and *Istituto di Fisiologia Veterinaria e Biochimica, MILANO -ITALY.
When transferred into suspension culture on agarose-coated dishes, dedifferentiated chick embryo chondrocytes resume the chondrocyte phenotype and continue their maturation to the hypertrophic stage. During this process, the synthesis of collagen type I is repressed while the genes encoding collagens II, IX and X are progressively activated. An additional marker expressed and secreted by in vitro differentiating chondrocytes at a late stage of development has been recently evidenced in the laboratory. This low molecular weight protein, named Ch21, is detectable in the cells after a short pulse labelling and is directly secreted into the culture medium. In the embryo tibia, the Ch21 first appears at the boundary of the cone of the hypertrophic cartilage and in the newly formed bone between the 6th and the 10th day of development and thereafter localizes in calcifying hypertrophic cartilage. Based on the aminoacid sequence of isolated clostripain peptides and the nucleotide sequence of an isolated cDNA clone, the complete aminoacid sequence of the protein has been determined. Computer assisted analysis showed significant homology between this sequence and the sequences of proteins that belong to the superfamily of the low molecular weight proteins sharing a basic framework for the binding and transport of small hydrophobic molecules.

Q 101 SECOND MESSENGER LEVELS IN SHEARED OSTEOBLASTS, John A. Frangos, Kathleen M. Reich, and Carol V. Gay‡, Department of Chemical Engineering and ‡Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, PA 16802.
Fluid flow has been shown to stimulate the production of Type I collagen in cultured osteoblasts. The mechanism by which fluid shear is transduced into a biochemical signal, however, is unclear. Since flow is believed to perturb the cell membrane, membrane phospholipid metabolism was analyzed. The levels of inositol trisphosphate (IP_3) were monitored in rat calvarial osteoblasts subjected to 24 dynes/cm^2 of steady shear stress. Inositol trisphosphate levels were increased IP_3 high shear rates. Levels of another second messenger, cyclic adenosine monophosphate, also increase during flow in a time and shear rate dependent manner. This response is shear stress dependent and is also seen in endothelial cells and fibroblasts. The increase in cAMP was found to be mediated by prostaglandin production. The levels at prostaglandin E_2 (PGE_2), which is known to alter bone remodelling and is phospholipid metabolite, were also studied. These studies show that PGE_2 production rises in cells exposed to flow. In summary, cAMP, IP_3, and PGE_2 levels increase in response to fluid shear stimulation of osteoblasts.

Q 102 THE USE OF AN IN VITRO CULTURED CARTILAGE TISSUE ANALOG FOR THE REPAIR OF ARTICULAR CARTILAGE, D. A. Grande, Department of Bioengineering, Hospital for Joint Diseases Orthopaedic Institute, New York NY 10003

It is well documented that articular cartilage has a limited ability for self-repair following injury. A model has been developed for studying cartilage repair in surgically created defects in the knee of the New Zealand White (NZW) rabbit. A composite collagen/chondrocyte tissue analog was prepared in vitro, allowed to grow for a period of 10-14 days, and characterized in vitro prior to allografting into the cartilage defects. Following allotransplantation of the cartilage grafts, animals were sacrificed at four time points postoperatively (2, 4, 6, and 12 weeks) and evaluated with quantitative histology and immunohistochemistry with monoclonal antibodies specific for collagen types I and II as well as specific epitopes on proteoglycan molecules. Initial histological evaluation demonstrated that neochondrogenesis with complete surface reconstitution was present by 6 weeks postoperatively in 62% of samples.

It was concluded that composite graft techniques may be used successfully to treat lesions in articular cartilage, an area where no clinical regimen presently exists.

Q 103 NEW SYNTHETIC BIOMATERIALS FOR BIOSPECIFIC AND SELECTIVE CELL ADHESION BY COVALENT ATTACHMENT OF BIOADHESIVE PEPTIDES, Jeffrey A. Hubbell, Stephen P. Massia and Bradley Lambrecht, Department of Chemical Engineering, University of Texas, Austin, TX 78712.
Animal cell adhesion to biological and nonbiological surfaces is usually mediated by adsorbed proteins; if proteins from the cell adhesion molecule (CAM) family adsorb, then the material will support cell adhesion and spreading. We present here an approach to obtain completely synthetic surfaces which support the adhesion and spreading of cells regardless of protein adsorption. The cell binding domains of the CAM fibronectin is the tripeptide RGD, and that of laminin is the pentapeptide YIGSR. We covalently immobilized on otherwise non-cell-adhesive surfaces these peptides and observed that they were capable of supporting normal cell spreading, focal contact formation, and cytoskeletal organization with and without serum CAMs, indicating a direct interaction between the cell-surface receptors and the peptide ligands. This was observed for primary human umbilical vein endothelial cells (HUVECs) and for primary human foreskin fibroblasts (HFFs). Furthermore, we observed that the rate of HUVEC spreading on the YIGSR-containing surfaces was much faster than HFF spreading, while this was not true for the RGD-containing surfaces, indicating some level of specificity for the HUVECs. Moreover, these surfaces supported the adhesion of human platelets only after preactivation, but were not thrombogenic to flowing nonpreactivated blood. This specificity may be useful in preventing thrombosis and pseudoneointimal thickening in endothelialized vascular grafts. As an effort to develop synthetic models of the heparin binding domains of CAMs, which are usually sequences of peptide rich in lysine and arginine without interposing acidic residues, we have examined cell adhesion and spreading on amine-derivatized surfaces and find them to also support cell adhesion without adsorbed CAMs. We also observe specificity here, in that cell treatment with heparinase abolished spreading on the primary amine surfaces, while treatment with condroitinase abolished spreading on tertiary amine surfaces. This may form a basis for specificity of cell adhesion based upon cell-surface proteoglycan composition.

Q 104 **PATHWAY OF TRANSFORMING GROWTH FACTOR-ß INDUCED MESENCHYMAL CELL DIFFERENTIATION IS DOSE DEPENDANT**, Michael E. Joyce, Ben Kittredge and Mark E. Bolander, Orthopaedic Research Unit, NIAMS, National Institutes of Health, Bethesda, MD 20892.
Transforming growth factor-ß (TGF-ß) regulates the growth, differentiation, and synthesis of extracellular matrix by several cell types found in bone. In fracture healing, repair is initiated when platelets release TGF-ß into the hematoma; TGF-ß is then synthesized by both chondrocytes and osteoblasts as the repair process continues. These findings prompted us to investigate the ability of exogenous TGF-ß to induce osteogenesis and chondrogenesis. Daily injections of TGF-ß1 and TGF-ß2 into the subperiosteal region of newborn rat femurs resulted in localized intramembranous bone formation and chondrogenesis. After cessation of the injections, endochondral ossification occurred, resulting in replacement of cartilage with bone. Control injections with BSA or PBS resulted in only moderate periosteal proliferation. Moreover, injection of TGF-ß2 stimulated synthesis of TGF-ß1 in chondrocytes and osteoblasts within the newly induced bone and cartilage suggesting positive autoregulation of TGF-ß. TGF-ß2 was more active *in vivo* than TGF-ß1, stimulating a mass that was on average 375% larger at a comparable dose ($p < .001$, $n = 87$). With either isoform of TGF-ß, the dose of the growth factor determined which type of tissue formed, such that the ratio of cartilage formation to intramembranous bone formation decreased as the dose was lowered. For TGF-ß1, reducing the daily dose from 200ng to 20ng deceased the cartilage/intramembranous bone formation ratio from 3.57 to zero ($p < 0.001$, $n = 72$); with TGF-ß2, the same dose change decreased the cartilage/intramembranous bone formation ratio from 3.71 to 0.28 ($p < 0.001$, $n = 72$). Taken together the data suggest that TGF-ß2 is not simply more potent than TGF-ß1. Although much larger masses of bone and cartilage were stimulated by TGF-ß2 (at both 200ng and 20ng doses), the ratio of bone to cartilage within those masses was not significantly different than in the smaller masses stimulated by TGF-ß1 at the same dose. These data demonstrate that mesenchymal precursor cells in the periosteum are stimulated by TGF-ß to proliferate and differentiate in a manner that resembles epiphysial bone formation and fracture healing, and suggests that TGF-ß initiates skeletal tissue repair by activating this tissue cascade.

Q 105 USE OF POLYANHYDRIDE POLYMERS AS A DELIVERY VEHICLE FOR SOLUBLE BONE INDUCTIVE PROTEINS, Paul A. Lucas[1], Cato Laurencin, Glenn T. Syftestad, Arnold I. Caplan, and Robert Langer. [1]Department of Surgery, Mercer University School of Medicine, Macon, GA 31207. Protein extracts of demineralized bone matrix (DBM) induce ectopic osteogenesis. These proteins are soluble at body temp. and in isotonic salt solutions, which present problems re-introducing the proteins in vivo. This requires controlled-release of the proteins at the implant site to avoid immediate dispersal. Polyanhydride polymers are biocompatible and biodegradable and have nearly zero order release kinetics. Matrices incorporating lyophilized water-soluble proteins from a 4 M GdmCl extract of bovine DBM were formulated by compression molding with either of two polymers, PCPM or PCPP. Control matrices were formulated with bovine serum albumin. Matrices were implanted into the thigh muscles of CBA mice, harvested 9 and 16 days post-implantation, and examined histologically for the presence of cartilage and/or bone. Lyophilized water-soluble proteins implanted by themselves never induced cartilage or bone. Neither did the matrices incorporating bovine serum albumin. However, PCPP at 60% loading of water-soluble proteins and PCPM at 30% loading of water-soluble proteins both exhibited induction of cartilage and bone. In several implants pieces of polymer were surrounded by induced cartilage or bone. The polyanhydrides can thus serve as a controlled-release delivery vehicle for soluble proteins involved in bone induction and repair.

Q 106 REGULATION OF ENDOTHELIAL CELL FUNCTION BY TGF-ß, Mathew A. Vadas and Jennifer R. Gamble, Division of Human Immunology, Institute of Medical and Veterinary Science, Adelaide, South Australia 5000.

The endothelium presents a non-adhesive anti-thrombotic surface that appears to ensure the unimpeded circulation of blood cells. Pro-inflammatory cytokines such as TNF-α and IL-1 induce endothelial cell (EC) adhesiveness and pro-thrombotic tendencies. We show that the treatment of human umbilical vein EC (HUVE) with the cytokine TGF-ß reduces their adhesiveness for activated or unactivated neutrophils and for T lymphocytes. Furthermore TGF-ß prevents the pro-adhesive state induced by TNF-α on EC for the same cell types. The tonic presence of TGF-ß in blood vessel walls would appear to ensure the maintenance of anti-adhesive states.

The responsiveness to TGF-ß is associated with the age of HUVES, recently explanted (young) cells are routinely responsive whilst the background or TNF-α induced adhesion of >10 day old HUVES for PMN and T lymphocytes is not inhibited by TGF-ß. This loss of responsiveness is selective as the γIFN-induced expression of class II MHC antigen and the proliferation of both types of HUVE is inhibited by TGF-ß. This responsiveness is at least in part dependent on the extracellular matrix (ECM) elaborated by 'young' HUVES as 'old' HUVE acquire responsiveness when cultured on such ECM. Responsiveness to TGF-ß may be a critical determinant in the development of vascular pathology.

Wound Healing; Skin and Epithelia; Muscle

Q 200 THE LIPIDIC PART OF A HEMODIALYSATE CLINICALLY USED FOR THE TREATMENT OF POORLY HEALING WOUNDS STIMULATES S_6-KINASE ACTIVITY, W. Baschong[1], R. Imber[2], K. Huggel[2], A. Bauen[3], H. Isler[3], D. Fabbro[2], 1) MIH, Biocenter Univ. Basel, 2) Dept. Research, Univ. Basel, 3) Pharmacology, Solco Ltd. Basel, Switzerland. The efficacy of a low MW dialysate (cut off 13.5 kDa) from calf blood (Solcoseryl, Solco Ltd., Basel), used for the treatment of poorly healing wounds, is demonstrated in an animal model in a dose-dependent manner. Its effects on poorly healing wounds appears multifactoral suggesting the involvement of mitogenic factors. We tested the hemodialysate for the presence of EGF, αTGF and somatomedin C (SMC) using a radioreceptor assay with the ZR-75 cell line. No competition with neither ^{125}J(EGF) nor ^{125}J(SMC) was observed by the hemodialysate up to 10% on intact ZR-75 cells. However, the hemodialysate and its lipidic part (Folch extract) were able to stimulate in a dose- and time-dependent manner the S_6-kinase activity of ZR-75 cells comparable to the stimulation obtained with 1% FCS. Our data suggest the presence of growth factor-like substances in the lipidic part of the hemodialysate.

Q 201 CHARACTERIZATION OF PORCINE SMOOTH MUSCLE CELLS DERIVED FROM AORTA IN LONGTERM CULTURE, G. B. Boder and T. Burke, The Lilly Research Laboratories, Indianapolis, IN 46285.

Aortic smooth muscle cells were isolated from 10-12 kg swine by two methods: sequential scraping with sterile scalpels or by sequential digestion of the endothelial and smooth muscle layers with type IV collagenase. Cells were plated in RPM-1640 medium supplemented with 10% horse serum and 10% fetal bovine serum (FBS). Scraping resulted in higher proportions of contamination with endothelial and fibroblast-like cells than the sequential digestion method. Cells were frozen in liquid nitrogen at passage four in 1974. In 1988, cells were recovered and weaned into F-12-DME (3:1) medium containing (10% horse serum and 10% FBS) and subsequently weaned to 2% FBS. A combination of PDGF, insulin, human transferrin and fibronectin supported growth at 2% serum levels but not in the absence of serum. Substitution of acidic and basic FGF for PDGF supported growth for short times but quickly resulted in viable but non-dividing cells. Stabilization of FGF with heparin resulted in prolongation to 14-16 transfers and continued differentiation defined by α-actin staining. These results are relevant to definition of cells with potential applications in tissue engineering.

Q 202 BEHAVIOR OF A DESPECIFIED COLLAGEN TISSUE EQUIVALENT GRAFTED ON DESEPIDERMIZED HUMAN SKIN, Annette Chamson, Alain L. Claudy, Jacques Frey, Laboratory of Biochemistry and Department of Dermatology, Medical School, 42023 Saint-Etienne, France.

The aim of our study was to assess the use of a connective tissue prepared "in vitro" from acid-soluble collagen and fibroblast cultures then despecified in the purpose of grafting. The collagen was latticed and contracted by the fibroblasts, treated chemically (formaldehyde or glutaraldehyde) to stabilize the fibrils and the obtained tissue was then despecified by cialit (sodium 2-ethylmercurimercaptobenzoxazol-5-carboxylate) treatment. These connective tissue equivalents were tested on superficial non infected wounds. They took very rapidly a necrotic appearance and were totally lysed after 10 to 12 days without any infection. The histological and ultrastructural studies showed a desorganization of collagen fiber bundles and a tissue invasion by inflammatory cells. But, no circulating antibodies to collagen were observed. These data give informations on the fragility of such a tissue made only with collagen and fibroblasts when these cells have been killed.

Q 203 THE ADVANTAGES OF A DERMAL COMPONENT IN DESIGNING A CULTURED SKIN SUBSTITUTE

Matthew L. Cooper, John F. Hansbrough, and Stanley A. Sakabu, Department of Surgery, University of California, San Diego Medical Center, San Diego, CA 92103.

The use of cultured skin substitutes in the field of wound healing is increasing. Models developed have employed various combinations of dermal substitutes and cultured epidermal cells, or an epithelial layer alone. This laboratory has designed a composite cultured skin substitute using a collagen-GAG dermal membrane inoculated with autologous fibroblasts, with autologous cultured keratinocytes (HK) placed onto a lamination of the collagen-GAG membrane.

When placing the graft onto the wound, its integrity may depend on rapid formation of a basement membrane (BM) between epidermis and dermis. By placing the HK on the dermal component in vitro, this "bond" is already being established prior to wound placement. We have shown this by indirect immunofluorescence in assaying for type IV collagen and laminin deposition on the membrane, and electron microscopy (EM) of the healed wound. An incomplete BM is formed in the culture period. By day 5 post-placement on the athymic mouse the BM is almost complete and by day 10, is continuous. We have seen similar results with EM in clinical studies.

The reformation of dermis in a timely fashion is also important. The dermal framework, with autologous fibroblasts, allows for fibrovascular ingrowth and re-establishment of a dermis with minimal scar formation. We have seen in the athymic mouse that the collagen-GAG membrane is replaced by 4-6 weeks, and elastin fibers have reappeared within six months.

The addition of a dermal component has many potential advantages over epidermal sheets alone. The attachment of the epidermal component in vitro may lead to earlier and more consistent keratinocyte adherence, which is crucial to the success of these grafts.

Q 204 COLLAGEN BUNDLES AND COLLAGEN QUALITY IN BIOMATERIALS, Jacques Frey and Annette Chamson, Laboratory of Biochemistry, Medical School, 42023 Saint-Etienne, France.

According to the works of E. Bell, it is possible to obtain collagen fibers from acid-soluble collagen using the ability of fibroblasts to contract acid-soluble collagen. These fibers do not look like exactly the fiber bundles of normal connective tissue, especially they are less numerous, less organized and the collagen content is less an half than in dermis. It was difficult to increase the initial collagen concentration because the solubility limit of this protein (no more than 3 g/l in acetic acid 0.5 mol/l). So, increasing the fibroblast number which increases the speed of contraction, we obtained a dermal equivalent showing by electron microscopy a high density of collagen bundles. Moreover, we had observed that the quantity of collagen contracted by fibroblasts depended on the quality of collagen solution. In the case of collagen prepared from human placenta by proteolysis, the collagen retained by the fibroblasts was low. In the case of acid-soluble collagen from calf skin or rat tail tendon, it depends on the purification process : the retention was at an upper level (near 80 %) when collagen was prepared in presence of protease inhibitors and at a low level when proteolysis was not avoided or when collagen was prepared in presence of pepsin. The importance of the telopeptides was discussed.

Q 205 LOW CALCIUM STRIPPING OF DIFFERENTIATING CELL LAYERS IN HUMAN EPIDERMAL CULTURES ; AN IN VITRO MODEL OF EPIDERMAL WOUND HEALING. Peter K. A. Jensen, Peter M. Larsen, Steven J. Fey and Lars Bolund, Institute of Human Genetics, University of Aarhus, DK-8000 Aarhus C, Denmark.
Primary human keratinocytes from epidermal explants can be made to form a well differentiated multi-layered tissue in culture. By incubating the tissue culture in low calcium medium the differentiated cell layers can be stripped off leaving a basal cell monolayer. When the basal cell monolayer is re-fed normal calcium medium a reproducible series of cell kinetic, morphologic and biochemical changes take place. It is suggested that these changes represent a general pattern of regeneration after epidermal wounding. After an initial lag phase the regenerative response is principally effected by a recruitment to the proliferating pool of a cohort of cells with a high rate of DNA synthesis. The cells seems to be programmed to rapid differentiation. The regenerative response can be modulated by incubation with the potent growth stimulator, cholera toxin or by incubation with growth regulatory oligopeptides purified from mouse skin. The basal cells remaining after stripping can easily be transfected by calcium phosphate precipitation (efficiency more than 1%); the present in vitro system may thus be suitable for the study of the genetic control of keratinocyte proliferation and differentiation.

Q 206 BASIC FIBROBLAST GROWTH FACTOR ACCELERATES TISSUE REPAIR IN HEALING IMPAIRED DIABETIC AND OBESE MICE, Corine K. Klingbeil and Lucy B. Cesar, California Biotechnology Inc. Mountain View, CA, 94043. The mitogenic and angiogenic properties of basic fibroblast growth factor (bFGF) have been demonstrated using in vitro assays and confirmed in animal models of wound healing. In this study the question of the role of bFGF in the repair of tissue was extended to models where healing is impaired and wounds are subchronic. Genetically diabetic and obese mice provide such models and present some of the symptoms seen in human diabetes mellitus and, as such, may be clinically relevant. In the diabetic mouse, obesity associated with pronounced hyperglycemia and hyperphagia occurs at 3-4 weeks, as well as polyuria, polydipsia, and glycosuria. In the obese mouse, hyperphagia, hyperinsulinemia, and insulin resistance are associated with moderate hyperglycemia. Diabetic mice (db/db) and controls (db/+), and obese mice (ob/ob) and controls (ob/+), were used to determine the effect of recombinant human (rh)bFGF in healing full-thickness excisional ($2cm^2$) wounds. Wounds were covered with an occulsive dressing and the wound area was measured by planimetry every 3 days from day 0 through day 24 (db) or 40 (ob). Additional parameters included body weight, blood glucose, and morphometric analysis of cellular effects based on histology. In controls, healing was complete in about 14 days and treatment with a single dose (day 0) of 1 μg rhbFGF/cm^2 of wound area accelerated closure by 10%. In contrast, the same dose of rhbFGF accelerated the rate of wound closure by $>40\%$ ($p<0.001$) in the healing-impaired db/db and ob/ob mice. Quantitation of fibroblasts, capillaries, and inflammatory cells by histo-morphometry revealed that changes in their levels all correlate with increased rates of wound healing. In addition, the dose response to rhbFGF has also been examined over a range of 0.01-10.0 μg/cm^2 and found to be effective at doses ≥ 0.1 μg in these models.

Q 207 EFFECTS OF EGF, FGF, AND COLLAGEN SHIELDS ON CORNEAL EPITHELIAL WOUND HEALING FOLLOWING LAMELLAR KERATECTOMY. Jamie G. Lopez, Michael Hagenah, and Michael S. Insler, LSU Eye Center, New Orleans, Louisiana. We evaluated the effect of EGF, aFGF, and collagen shields, alone or in combination, on epithelial wound healing in keratectomy wounds in rabbit eyes. Superficial 6 mm keratectomies were performed in 30 eyes of 15 rabbits. The eyes were then treated with i) topically applied EGF (100 μl/ml in PBS, 4x/day), ii) topically applied heparin-stabilized aFGF (100 μl/ml in PBS, 4x/day), iii) collagen shields hydrated in PBS every 12 hours, iv) collagen shields hydrated in 100 μl/ml EGF, or v) collagen shields hydrated in 100 μl/ml aFGF, or vi) were left untreated. The wound healing process was determined from photographs of the fluorescein stained wound area every 12 hours. Analysis of wound area was performed using computerized planimetry to calculate the wound radius. Considering the epithelial wound healing as a biphasic process involving a latent stage after injury followed by a linear wound healing phase starting approximately 8 hours after wounding, we determined the wound healing rate by calculating the wound radius measurement beginning 12 hours after keratectomy to the last measurement (72 hours). We found significantly higher wound healing rates for the groups treated with either the collagen shield alone (0.04 ± 0.005mm/h, $p=0.011$) or the combination of collagen shield with aFGF (0.045 ± 0.005 mm/h, $p=0.001$) compared to the untreated control group (0.031 ± 0.004 mm/h). The other groups did not show significantly higher wound healing rates compared to the control. The projected time to wound closure calculated by regression analysis showed the highest wound closure rate for the eyes treated with collagen shields hydrated in aFGF. These results suggest that collagen shields hydrated in aFGF may further speed wound healing, compared to shields hydrated in PBS, whereas EGF had no apparent effect. We conclude that collagen shields containing aFGF have a beneficial effect on corneal epithelial wound healing following keratectomy.

Q 208 PRODUCTION AND PURIFICATION OF RECOMBINANT IGF BINDING PROTEIN BP3 IN E. COLI, Christopher A. Maack, Rino Y. Lee, Eric T. Rhodes, Desmond Mascarenhas, and Andreas Sommer. BioGrowth Inc., 3065 Atlas Rd. Suite 117, Richmond, CA. 94806.
Insulin-like growth factor (IGF) is involved in a number of growth processes, including wound healing and bone growth. In the circulation, essentially all of the IGF is found bound to its binding protein (BP3). Recent results indicate that this IGF/BP3 complex is more efficient than IGF alone in stimulating fibroblast growth and in promoting wound healing. The gene for BP3 has been isolated, and expressed as an intracellular inclusion body in E. coli using a T7 RNA polymerase based promoter system. BP3 protein has been fully reduced and solubilized by urea from these inclusion bodies. The fully reduced, inactive IGF-BP has then been refolded in the presence of cysteamine into an active form. This active protein has been purified to homogeneity by a combination of S-Sepharose cation exchange, C_4 reversed phase, and Phenyl-Superose hydrophobic interaction chromatography.

Q 209 A PHYSIOLOGICAL SUBSTRATE FOR WOUND HEALING, Gail K. Naughton, Leslie Jacob, Brian A. Naughton, Ron Cohen, Marrow-Tech, Inc., LaJolla, CA, 92037, and Hunter College School of Health Sciences, New York, NY, 10021.
A physiological skin substitute has been developed in our laboratory and is being studied in expanded preclinical trials. This substrate is a universal dermal equivalent consisting of human neonatal fibroblasts on a biodegradable mesh made of polyglycolic acid. The fibroblasts stretch across mesh openings, secrete collagens Type I and III, release growth factors and remain mitotically and metabolically active. Sheets of dermal equivalent are sealed in Teflon bags and stored in liquid Nitrogen. Cells remain 90-95% viable after thawing. Preclinical trials to date have been performed on Charles River micropigs. Full-thickness wounds 4x4 cm in size were treated by application of dermal equivalent on mesh, mesh combined with growth factors, or mesh alone. A sheet of cultured autologous keratinocytes was applied to the dermal equivalent in vivo 10 days post dermal implantation. Areas treated by the universal dermis/autologous keratinocyte method showed rapid healing with minimal contraction and no evidence of rejection. Mesh gradually hydrolyzed in vivo over 3-4 weeks. Wound areas which received mesh alone or a combination of mesh and growth factors showed substantial scarring. This methodology shows promise for providing a living wound treatment which can be stored frozen and used immediately in burn victims and patients with chronic decubitus ulcers.

Q 210 EFFECT OF PLATELET DERIVED WOUND HEALING FACTORS (PDWHF) ON FIBROBLAST MITOGENIC ASSAY. Dawn D. Newman. CuraTech, Inc., 14 Research Way, Setauket, NY 11733
PDWHF is released from platelet alpha granules following thrombin induced platelet aggregation. This growth factor extract has been shown to potentiate chronic wound healing in several clinical trials. One method used to quantify growth factor activity in PDWHF is the Fibroblast Mitogenic Assay (FMA). In this assay confluent BALB/c-3T3 cells are stimulated with serial dilutions of PDWHF and the level of ^3H thymidine incorporation determined. PDWHF stimulated ^3H thymidine uptake 185% over maximal stimulation induced by Platelet Derived Growth Factor (PDGF). A similar enhancement in total cell number was observed when PDWHF stimulated cells were compared to PDGF stimulated cells. A level of stimulation comparable to PDWHF was obtained with a combination of TGFß, PDGF and EGF (TGFα was as effective as EGF). No other combination or single growth factor tested was as active as PDWHF. Other growth factors tested include: ILGF1, acidic and basic FGF, and thrombin. These results show optimal in vitro growth of fibroblasts requires multiple growth factors. It is likely that other growth and chemotactic activities associated with wound healing also require multiple growth factors for maximal efficiency.

Q 211 **FIBROBLAST MEDIATED GENE THERAPY: TRANSPLANTED SKIN FIBROBLASTS PERSIST LONG TERM BUT PRODUCE THERAPEUTIC PRODUCTS ONLY TRANSIENTLY** **T.D. Palmer°*, G. Rosman°, W.R.A. Osborne*, A.R. Thompson#, and A.D. Miller°, °University of Washington, #Puget Sound Blood Center, and °Fred Hutchinson Cancer Research Center, Seattle, Washington, 98104.** It may be possible to treat many genetic diseases by introducing therapeutic genes into somatic cells of affected individuals. Toward this end, gene transfer into dermal fibroblasts was examined with retroviral vectors containing a selectable marker (neo) and a potentially therapeutic gene, clotting factor IX or adenosine deaminase (ADA). With a single exposure to virus, up to 80% of the cultured fibroblasts expressed neo and produced high levels of active therapeutic protein. The amounts of protein produced in culture suggested that fibroblasts could provide therapeutic levels of protein *in vivo*. This was confirmed by transplanting factor IX producing fibroblasts into rats or mice where circulating human factor IX was easily detectable, but only for one month. Antibodies to human factor IX in some animals suggested that immune mediated graft failure or immune clearing was responsible for decreased levels of protein. To examine this possibility, the transplantations were repeated using an ADA gene. ADA is an intracellular enzyme and would be less likely to elicit an immune response. In addition, ADA could be detected at levels as low as 1% of endogenous ADA. Rat fibroblasts expressing twenty-fold more human ADA than endogenous rat ADA were transplanted into syngeneic rats. The transplants were assayed at various times for ADA activity and survival of transplanted cells. Approximately 50% of the cells in the transplants contained neo sequences at 8.5 months. However, human ADA activity decreased to less than 1% of rat ADA within one month. These results suggested that gene inactivation, rather than immune intervention, was responsible for decreased therapeutic protein production. We conclude that genetically modified fibroblasts survive long term *in vivo*, but that if such therapy is to be effective, new strategies that guarantee continued gene expression must be found. In this regard, we are developing vectors which use normal cellular promoters that are constitutively expressed in fibroblasts *in vivo*.

Q 212 SELECTIVE INDUCTION OF ANGIOGENESIS AND MATRIX SYNTHESIS IN HEALING WOUNDS BY bFGF AND PDGF ISOFORMS, K. Doria, E. Shatzen, D. Yanagihara, A. Thomason, G.M. Fox and G.F. Pierce, Amgen Inc., Thousand Oaks, CA 91320.

A full thickness lapine ear excisional model was utilized to identify in vivo vulnerary activities of bFGF and PDGF, and to correlate those activities to known in vitro biologic functions. Growth factors were delivered once at the time of surgery in an aqueous vehicle; wounds were harvested 1-2 weeks later and new tissue formation was quantified. bFGF [both native and a serine (70,88) analog] at doses of 2-10 μg per wound induced nearly exclusively by an angiogenic response, as assessed using lectin immunohistochemistry. aFGF with or without heparin, at similar doses was without effect. bFGF induced significantly more neovessels than observed in controls ($p<0.001$) or PDGF-BB-treated ($p<0.03$) wounds. PDGF-BB induced supportive neovessel formation, but was a more potent inducer of new extracellular matrix (i.e., granulation tissue) than bFGF. PDGF-BB functioned via augmenting inflammatory cell influx into wounds (primarily macrophages), likely triggering a cascade of cellular and growth factor activities, leading to a 4-day acceleration of healing during the first 2 weeks. PDGF-AB and PDGF-AA dimers had in vivo biologic activities similar to those found for PDGF-BB; in contrast PDGF-B monomer was without effect. In vitro all 3 dimeric isoforms induced peak monocyte chemotaxis at 5-10 ng/ml, consistent with their observed in vivo effects on the inflammatory phase of tissue repair. Thus, PDGF isoforms and bFGF are potent vulnerary agents having specific cellular targets and unique biologic activities in vivo.

Q 213 TOPICAL APPLICATION OF A RECOMBINANT VACCINIA GROWTH FACTOR ENHANCES HEALING IN PARTIAL THICKNESS BURNS, Timothy M. Rose, A. Gregory Bruce, Jennifer J. Lee, Janet A. Marvin, Baiba J. Grube, David M. Heimbach and George J. Todaro, Oncogen, 3005 First Ave., Seattle, WA 98121.

Vaccinia virus infection results in the production of an EGF-like growth factor, called Vaccinia Growth Factor (VGF) which is 38% homologous to human EGF and 30% to human TGFα. Bacterial expression of a synthetic gene for a truncated VGF-like molecule resulted in a 56 amino acid polypeptide, VGFa, which was renatured, purified and shown to be active in the EGF receptor competition assay. To determine the effectiveness of VGFa in epidermal regeneration, we conducted a randomized, double-blind clinical trial where 8 patients with partial thickness dermal burns were treated with VGFa in Thermazene, a cream vehicle containing silver sulfadiazene, or with Thermazene alone. VGFa treatment at 0.1 μg/ml in Thermazene lead to a mean decrease of 19% in the time required for 50% healing of mid-dermal burns with a P value < 0.04. No adverse reactions to the growth factor treatment were observed. Similar results were obtained in a study of skin-graft donor sites treated with hEGF (Brown et al., N. Engl. J. Med 1989;321:76-79). However, the concentration of EGF in this study was 10 μg/ml, 100 fold higher than that used with VGFa. Further studies are needed to determine if increased dosage of VGFa could increase the rate of healing to make this treatment clinically useful.

Q 214 CONCENTRATION AND GENE-EXPRESSION OF IGF-I IN DIFFERENT PHASES OF WOUND HEALING IN THE RAT, Henrik H. Steenfos, John-Olov Jansson, Departments of Plastic Surgery and Physiology, University of Göteborg, Sweden.

In wound granulation tissue the IGF-I gene is expressed, and IGF-I is present in wound fluid and fibroblasts. Moreover, IGF-I stimulates fibroblast growth and wound healing in combination with other growth factors. In the present study we measured the time course of changes in the levels of IGF-I and IGF-I mRNA during wound healing.

Method: Four stainless steel wire mesh cylinders were implanted subcutaneously in 60 rats. Ingrown granulation tissue and wound fluid were sampled at different time points (week 1-18) after implantation. Wet weight of granulation tissue were determined, and levels of IGF-I mRNA were measured by solution hybridization/RNAse protection assay. The IGF-I concentration was measured in wound fluid by RIA after acid ethanol extraction to remove binding proteins.

Results: The wet weight of the granulation tissue increased significantly between week 1.5 and week 4-5, whereafter it decreased again confirming previous studies. The IGF-I mRNA concentration displayed a similar pattern as wet weigth with a significant 3-fold increase between week 1.5 and week 3-5, and a decrease between week 5 and week 7. In contrast, IGF-I concentration in wound fluid remained constant untill week 8 and then increased to a higher level during week 11-18.

Conclusion: The present study shows that the gene expression and concentration of IGF-I change during wound healing.

Q 215 KINETICALLY ENGINEERED FUNCTIONAL SKELETAL MUSCLES IN VITRO
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Brown University and The Miriam Hospital, Providence, RI 02906.

Mononucleated avian skeletal myoblasts grown in monolayer cultures on an elastic substratum were subjected to some of the mechanical forces found during embryogenesis in vivo. These included slow continuous stretching of the substratum in one direction, simulating bone growth, as well as repetitive stretch/relaxations, simulating muscle movement. A new horizontal mechanical cell stimulator was used (IN VITRO 25, 607; 1989). Three dimensional artificial muscle organs formed after 3 to 4 weeks of stimulation. These muscles, approximately 25 mm long, 1 mm in cross section, and attached only at their ends, contained parallel arrays of long unbranched myofibers organized into fascicle-like structures. The muscle organs stained uniformly with a monoclonal antibody for fibronectin, but staining for the myotendinous antigen tenascin was concentrated at one end. The tissue had a mean resting longitudinal axial tension of 149 $\pm$ 47 mdynes/muscle which rapidly increased to 285 $\pm$ 53 mdynes/muscle ($P<.001$) when extracellular potassium was increased. The organs thus performed functional work. Applying mechanical forces to tissue cultured cells is another step toward simulating the in vivo environment for more complete tissue growth and development.

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Q 216 INSULIN-LIKE GROWTH FACTOR-1 STIMULATES PROLIFERATION IN MYOGENIC LINEAGE-COMMITTED AND UNCOMMITTED PLURIPOTENT STEM CELLS, Henry E. Young, Donna C. Morrison, and Will Sealy, Basic Medical Science/Surgery, Mercer University School of Medicine, Macon, GA 31207. Insulin-like growth factor-1 (IGF-1) has been postulated to effect the repair of damaged skeletal muscle by stimulating myogenesis. It has been suggested that this activity resides in the direct stimulation of mononucleated stem cells to differentiate into multinucleated myotubes. A 0.1 ng to 500 ng dose range of recombinant IGF-1 was incubated in defined medium with two culture models, myogenic lineage-committed stem cells and uncommitted pluripotent stem cells, to test this hypothesis. After 8 days in culture (1) an increase in proliferation of mononucleated cells occurred in both stem cell models, (2) an increase in myosin content occurred in the lineage-committed stem cells, while (3) no increase in myosin content was detected in the uncommitted pluripotent stem cells. These results suggest that the IGF-1-stimulated increase in myosin content probably involves a mitogenic influence on mononucleated precursor cells prior to fusion rather than affecting their overt cytodifferentiation into fused myotubes. Further analysis of endogenous growth factor influence on stem cell differentiation using these two model systems is currently underway. Supported by Rubye Ryle Smith Charitable Trust.

Immunotherapy; Hematopoiesis; Nervous System; Islet and Hepatic Cells

Q 300 CONTACT GUIDANCE AND MONITORING OF CULTURED CELLS BY MICROENGINEERED SUBSTRATES Julian A.T. Dow, Lorna J. Breckenridge, Peter Clark, *Patricia Connolly, Adam S.G. Curtis, *Robert Lind, *Chris D.W. Wilkinson & Richard J.A. Wilson, Departments of Cell Biology and *Electronic Engineering, University of Glasgow, Glasgow G12 8QQ, Scotland, UK.

The behaviour of animal cells can be modified by surface topography and adhesiveness, interactions believed to be important in normal development. These processes have been modelled *in vitro*, using cultured fibroblast, epithelial, leukocyte, muscle and neuronal cells. Synthetic topographies are produced in planar substrates by the photolithographic techniques normally employed in microchip fabrication. Cells are strongly aligned by patterns of parallel grooves on the micron and sub-micron scales. Additionally, the direction of neuronal regeneration can be modified by such substrates (1,2).

Using arrays of up to 64 planar extracellular microelectrodes underlying patterns of grooves in a silicon nitride/polyimide substrate (3), we have succeeded in establishing, recording from and stimulating small neural networks of cultured invertebrate neurons. This allows the developing network properties of these networks to be monitored, or modified by external signal application, over much longer time periods than is possible in conventional neurobiology. This technology has clear applications to the manufacture of those biocompatible prosthetic devices where the interaction of cells with electrodes must be controlled.

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Q 301 DETECTION OF CLONOGENIC LEUKEMIC CELLS (CFU-L) IN A LIQUID "DOUBLE LAYER" METHOD, Salamon J., Hirsch J., Weber E., Majdic O., Lutz D. Ludwig Boltzmann Institute for Leukemia Research and Hematology, Hanusch Hospital, A-1140 Vienna, Austria.

Growing clonogenic blasts from acute leukemias either in liquid phase on top of a soft agar feeder or a in methylcellulose,is tedious and replating is not possible in all cases. We have developed a culture method using the Transwell^{R} chambers with a membrane of 0.4µm pore size between feeder cells (irradiated normal PBMC) and effector cells (CFU-L), both of which are kept in liquid phase. In all AML patients studied so far (n=12) we were able to grow and replate the samples at least twice, without any difference between blood (n=9) or bone marrow (n=8) derived blasts in replating capacity and cloning numbers. The median number of colonies was $22/10^5$ in the 1st, $135/10^5$ in the second and $37/10^5$ in the 3rd plating. Biphenotypic leukemias (n=2) showed a similar growth pattern ($21/10^5$ in the 1st; $120/10^5$ in the 2nd and $113/10^5$ in the 3rd plating). Morphologic and cytogenetic examination and immunophenotyping proved propagation of the malignant clone. In contrast, ALL derived blasts (n=5), although showing clonogenic growth in the 1st plating ($63/10^5$), could only be replated once in 2/5 cases. This method thus seems to be a convenient and reproducible method for growing AML blasts in preference to ALL blasts.

Q 302 MOLECULAR CLONING AND EXPRESSION OF cDNA FOR HUMAN HEPATOCYTE GROWTH FACTOR, Kazuhiro Takahashi[1], Kenji Miyazawa[2], Hirohito Tsubouchi[2], Daiji Naka[1], Yasushi Daikuhara[4] and Naomi Kitamura[2], 1 ; Mitsubishi Kasei Corporation, 1000, Kamoshida-cho, Midori-ku, Yokohama 227, Japan, 2 ; Institute for Liver Research, Kansai Medical University, Moriguchi, Osaka 570, 3 ; Second Department of Internal Medicine, Faculty of Medicine, Kagoshima University, Kagoshima 890, 4 ; Department of Biochemistry, Kagoshima University Dental School, Kagoshima 890. Human hepatocyte growth factor (hHGF), which stimulates the growth of adult rat hepatocytes in primary culture, have purified from a plasma of patients with fulminant hepatic failure. The purified hHGF shows multiple forms with molecular weights between 76,000 and 92,000, and consists of two chains, heavy and light, with molecular weights of 54,000-65,000 and 31,500-34,500, respectively. And these chains are linked together by disulfied bonds. Four peptide fragments of hHGF, purifeid from patients plasma, were sequenced. Based on the amino acid sequence of one of the four fragments, two oligodeoxyribonucleotide mixtures were synthesized and used to screen a human placenta cDNA library. On the screening, two overlapping cDNA clones, hybridized to the mixture probes, were isolated and the nucleotide sequence of the cDNA was determined. Deduced nucleotide and amino acid sequence from hHGF cDNA clone revealed that heavy and light chains are encoded by the same mRNA and are produced from a common translation product, consists of 728 amino acid residues, including possible signal peptide at the N-terminus. The expressed recombinant hHGF, constructed with expression vector and transfected into cultured cells, showed same activity as purified hHGF.