TISSUE ENGINEERING

Organizers: Robert Nerem and Donald Ingber

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Tissue Engineering

Keynote Addresses (Joint)

PZ 001 PHYSICOCHEMISTRY OF RECEPTOR-MEDIATED CELL FUNCTIONS, Douglas A. Lauffenburger, Departments of Chemical Engineering and Cell & Structural Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801.

Understanding cell functions such as proliferation, adhesion, and migration will require, in addition to identification of molecular components involved, elucidation of the physicochemical mechanisms by which these components operate as an integrated, dynamic system. Regulation of such functions by receptor/ligand interactions depends on parameters characterizing physical and chemical properties of these interactions, and resulting cell behavior can be strongly influenced by quantitative parameter values. Despite the current emphasis on receptor/ligand binding affinity as a central property governing signalling for cell function, a number of additional parameters are often important in determining the response. Among these parameters are included kinetic rate constants for receptor/ligand binding, coupling, and trafficking processes along with mechanical strengths and compliances of receptor/ligand bonds and receptor/cytoskeleton linkages, as well as molecular concentrations. This talk will present a series of examples demonstrating how physicochemical properties of receptor/ligand interactions beyond simple binding affinities are crucial in a variety of cell functions, and how quantitative analysis of these properties can lead to increased mechanistic insights. Since the quantitative parameter values can be altered by genetic or pharmacologic means, an ability to predict consequent effects on cell behavior may be of biotechnological use.

Cell Growth and Stem Cell Isolation

PZ 002 HB-EGF STRUCTURE AND FUNCTION; BIOACTIVITY OF RELEASED AND MEMBRANE-ASSOCIATED GROWTH FACTOR FORMS, Michael Klagsbrun[1], Minoru Ono[1], Shigeki Higashiyama[2], Michael Gagnon[1], Judy Abraham and Gerhard Raab[1], [1]Childrens Hospital, Harvard Medical School, Boston, MA 02115, [2] Osaka University Medical School, Osaka, 565, Japan.

Growth factors have long been known to act as soluble factors that are released from cells to stimulate cell growth. There is mounting evidence that growth factors are also active when insoluble, for example when bound to extracellular matrix (ECM) or when bound to the cell membrane. The ability of cell-associated growth factors to signal neighboring cells via cell to cell contact has been termed juxtacrine regulation. Heparin-binding EGF-like growth factor (HB-EGF) is bioactive both as a secreted soluble growth factor and a cell-associated one. HB-EGF was originally identified in the conditioned medium of human macrophages. It was named HB-EGF because of its EGF-like activities and its strong affinity for immobilized heparin (elution with 1.0-1.2 M NaCl), and cell surface heparan sulfate proteoglycan (HSPG), a property not shared by EGF or TGF-alpha. HB-EGF is synthesized as a precursor of 208 amino acids including signal peptide, mature protein, trans-membrane and cytoplasmic domains. The mature secreted form of HB-EGF is a single chain, cationic, 20-22 kDa glycoprotein 86 amino acids in length. Mature HB-EGF consists of two functional domains. The C-terminal portion is an EGF-like domain with a homology to EGF and to TGF-α of 42-46%. Consistent with its EGF-like structure, HB-EGF competes with EGF for binding to A-431 cells and phosphorylates the EGF receptor (EGFR). Within the N-terminal domain of HB-EGF, there is a stretch of 21 amino acids that constitutes a putative heparin-binding domain responsible for HSPG binding. HB-EGF is a potent mitogen for smooth muscle cells (SMC), much more potent than EGF or TGF-α even though all 3 bind to the same receptor. The incubation of SMC with heparitinase, with chlorate (an inhibitor of sulfation) and with a 21 amino acid synthetic peptide corresponding to the heparin-binding domain of HB-EGF, all inhibit HB-EGF activity suggesting that SMC HSPG modulate HB-EGF activity. Besides being processed to the secreted mature form, the HB-EGF precursor also exists in a membrane-associated form that is tethered to the cell via its transmembrane domain. Purified HB-EGF precursor is mitogenic in culture. In co-culture, transfected cells expressing the HB-EGF precursor, but not control cells, stimulate phosphorylation of EGFR in A431 cells. Thus HB-EGF can act in a juxtacrine manner to signal neighboring cells expressing EGFR. The HB-EGF precursor is also the receptor for diphtheria toxin (DT). Cells transfected with and expressing HB-EGF precursor are DT-sensitive unlike parental cells which are DT-resistant. Phorbol esters promote the processing of the HB-EGF precursor, release HB-EGF into the medium and cells become DT-resistant. Taken together, it appears that the structure of HB-EGF enables it to be multifunctional, as a released mitogen, as a cell-associated factor that mediates cell-cell interaction, and as a receptor for a non-growth factor ligand.

PZ 003 MYOGENIC CELLS WITH STEM-CELL PROPERTIES. Terence Partridge[1], Jennifer Morgan[1], Charles Pagel[1], Jonathan Beauchamp[1], Michelle Peckham[2], Mark Noble[3] Parmjit Jat[3], [1]Charing Cross & Westminster Medical School, [2]King's College London, [3]The Ludwig Institute, London, U.K.

Skeletal muscle consists of large syncytial non-proliferative "end cells". They are formed by fusion of mononucleate myogenic cells which diminish in number during postnatal life but persist in small numbers in mature muscle as 'satellite cells'. These are activated to proliferate and to repair or replace muscle fibres damaged during post-natal life. With a view to therapy of primary genetic diseases such as Duchenne muscular dystrophy, we and others have attempted to make use of this process to alter the genetic constitution of skeletal muscle by grafting normal myogenic cells into the muscles of dystrophic mice and boys. It is also of interest to genetically alter the grafted myogenic cells prior to grafting them, both as a treatment for primary muscle disease and in non-muscle diseases where skeletal muscle could be used to synthesize a variety of systemically active peptides such as hormones and clotting factors. For research purposes this approach is of interest for investigation developmental and disease processes by grafting cells bearing genetic markers of genes implicated in developmental or pathogenic processes. Such techniques entail the ability to clone genetically modified cells extensive for many cell doublings while retaining normal differentiation. Most committed tissue cells either become senescent immortalize as cell lines when cultured for many generations. We have been investigating whether cells can be isolated from mature skeletal muscle with the properties of myogenic stem cells, i.e. which are capable of self replacement and of generating committed myogenic cells throughout the life span of the animal. Long-term studies of grafted myogenic cells and serial passage studies indicate that such stem cells probably exist. We have also isolated myogenic cells from a mouse transgenic for an inducible construct which immortalizes cells while the construct is expressed and allows them to differentiate under conditions in which the transgene is not expressed. These cells, have most of the useful properties of stem cells: they can be clonally expanded and can be transplanted into suitable sites in host animals to produce large amounts of genetically modified normal muscle These cells do not become immortalized or lose ploidy as rodent cells normally do after extensive proliferation in tissue culture. Since they also retain the ability to differentiate into normal muscle they will be a useful tool for the investigation of muscle cell biology and for preliminary evaluation of strategies for gene therapy.

Basic Principles: Control of Tissue Development (Joint)

PZ 004 SIGNAL TRANSDUCTION BY EXTRACELLULAR MATRIX UTILIZES BOTH CELL SHAPE-DEPENDENT AND CELL SHAPE-INDEPENDENT PROCESSES, Calvin Roskelley and Mina J. Bissell, Lawrence Berkeley Laboratory, Berkeley CA, 94720.

The differentiated state of the mammary epithelium is exquisitely sensitive to changes that occur within the glandular microenvironment. A critical component of this microenvironment is the extracellular matrix (ECM). In three-dimensional culture, mouse mammary epithelial cells undergo complex morphogenetic re-arrangements and they begin to differentiate over a period of days in the presence of ECM and lactogenic hormones. On the other hand, when cell monolayers were first primed with ECM and then treated with prolactin, synthesis of the milk protein ß-casein was initiated within hours. Thus, the latter experimental protocol allowed us to examine the functionally important aspects of cell-ECM interactions in isolation. ECM-priming was associated with a concomitant cellular rounding and clustering which led us to ask two specific questions: 1) Are changes in cell shape necessary for differentiation? 2) Are additional signals initiated by cell-ECM contact required? Agents that prevent cell rounding or force cell spreading, such as phorbol esters and fibronectin, strongly inhibited differentiation when added during ECM-priming. However, when the cells were forced to round and cluster independently, using the non-adhesive substratum polyhema, this inhibition was abrogated. Conversely, genestein treatment during priming had no effect on morphology but differentiation was inhibited, and this inhibition could not be reversed by polyhema. These data suggest that both cell shape-dependent changes, which rely on an intact protein kinase C pathway, and cell shape-independent changes, which are sensitive to alterations in tyrosine phosphorylation, are critical for transducing signals emanating from the ECM. As we have already shown that $ß_1$-containing integrin receptors are involved, the challenge ahead will be to identify the precise molecular nature of these pathways. (Supported by the Office of Health and Environmental Research of the Department of Energy and NCI Canada).

PZ 005 ROLE OF ADHESIVE RECEPTORS IN REGULATING KERATINOCYTE DIFFERENTIATION AND STRATIFICATION, Fiona M. Watt, Keratinocyte Laboratory, Imperial Cancer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London. WC2A 3PX, UK.

Within the epidermis proliferation takes place in the basal cell layer that is attached to the basement membrane. Basal cells that become committed to differentiate withdraw from the cell cycle, detach from the basement membrane and move into the first suprabasal layer. Thereafter cells undergo terminal differentiation as they move through the suprabasal layers until finally they are shed from the surface of the skin.

Adhesion of basal keratinocytes to the basement membrane is mediated by receptors of the integrin family. Studies with cultured keratinocytes have shown that the $ß_1$ integrins are subject to both transcriptional and post-translational regulation (1,2) and that different subpopulations of basal cells can be distinguished on the basis of integrin expression and function. Keratinocytes with characteristics of stem cells express the highest levels of $ß_1$ integrins, while transit amplifying cells (which have a lower self-renewal capacity and higher probability of differentiating) express lower integrin levels (3). The $ß_1$ integrins on the surface of basal cells that are committed to differentiate become inactive and are no longer capable of binding to basement membrane proteins; this helps ensure that committed cells are selectively expelled from the basal layer. On initiation of terminal differentiation, transcription of integrin genes is inhibited, N-linked glycosylation and transport of immature integrin subunits is inhibited and receptors on the cell surface are endocytosed.

Integrins not only mediate keratinocyte adhesion and stratification; they also regulate the onset of terminal differentiation. Plating keratinocytes on a substrate that inhibits spreading acts as a stimulus for differentiation. Differentiation is regulated by the proportion of $ß_1$ integrins on the cell surface that have bound ligand rather than by cell shape per se (4, 5). The differentiation-regulatory function of integrins is independent of receptor clustering and cytoskeleton assembly (6). The aim of current experiments is to identify the intracellular signalling pathways by which integrin-ligand binding regulates keratinocyte behaviour.

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From Basic Research to Clinical Experience: The Musculoskeletal System

PZ 006 CELL-POLYMER CARTILAGE IMPLANTS, Lisa E. Freed[1], Gordana Vunjak-Novakovic[1], Daniel A. Grande[2], Janson Emmanual[3], and Robert Langer[1]. [1]Massachusetts Institute of Technology, Cambridge, MA. 02139. [2]North Shore University Hospital, Manhasset, [3]W.L. Gore, Flagstaff.

Cartilage implants for potential use in reconstructive or orthopedic surgery were created by growing isolated cartilage cells (chondrocytes) on fibrous polyglycolic acid (PGA). The polymer provides a three dimensional cell scaffold which biodegrades in a controlled fashion during tissue regeneration. The cell-polymer constructs closely resembled normal cartilage in parallel *in vitro* and *in vivo* studies. *In vitro,* implant composition depended on the culture conditions[1]. In static cultures, cell proliferation and the production of glycosaminoglycan (GAG) and collagen were limited by diffusional constraints. In mixed cultures (i.e. bioreactors), a more spatially uniform distribution of cells was achieved, and the rate of collagen production was increased. High implant GAG content was associated with high intial cell density, presumably due to cooperative cell-to-cell interactions. *In vivo,* chondrocyte-PGA allografts were successfully used to repair full-thickness articular cartilage defects in rabbit knees, as assessed histologically based on cell alignment, extracellular matrix composition and distribution, reconstitution of the subchondral plate, and bonding of the repair tissue to the underlying bone. These tissue engineering studies show that regeneration of implants with appropriate form and function for *in vivo* use relies on optimization of the *in vitro* culture environment.

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PZ 007 TISSUE ENGINEERING APPROACHES FOR REPAIR OF KNEE INJURIES, Daniel Grande, Robert Schwartz,Lisa E. Freed[1] and William Ertl , Dept. of Surgery/North Shore Univ. Hosp., Manhasset NY 11030, [1]Div. of Health Sciences & Technology, Mass. Inst. of Tech., Cambridge,MA 02139.

Injury to the knee joint occurs more frequently then diagnosed by clinical or radiographic examination. Such injuries usually involve one or more of the cartilages present, articular and/or meniscal. Available treatment at present is inadequate in repairing these injuries ultimately resulting in early degeneration of the native tissue and any repair tissue present. The optimal strategy for cartilage repair requires integration, by grafting, of tissue engineered cartilage to replace damaged or lost cartilage. Several candidate materials were evaluated in vitro for their ability to support chondrocyte growth and synthesis of the matrix products collagen and aggrecan. Rates of collagen and aggrecan synthesis as well as total accumulation of matrix were assayed. A similar protocol was implemented using a cell line derived from menisci. Meniscal cells were cultured under various conditions which are known to be stimulatory to these cells, such as incubation with platelet derived growth factor, fibroblast growth factor, endothelial cell growth supplement, fibronectin and RGD peptide. After invitro characterization, tissue constructs were assembled using the two best materials, collagen or polyglycolic acid (PGA) as scaffold materials for in vivo animal testing. Surgical defects were created bilaterally in the distal femora in the knees of skeletally mature New Zealand White rabbits and implanted with either tissue constructs, material alone (minus cells), or left empty. Animals were sacrificed in groups for up to one year post-operatively. Assessment of repair was made macroscopically, histologically and biomechanically. Results showed that the repair tissue resulting from cell-based constructs had properties which more closely resembled native cartilage over time as compared to defects alone. The use of tissue constructs yielded more functionally viable cartilage repair compared to the mixture of fibrocartilage and fibrous tissue consistently observed in samples undergoing a native repair response or implanted with materials alone.

PZ 008 GENETICALLY MODIFIED SKELETAL MUSCLE STEM CELLS: A NOVEL SYSTEM FOR DELIVERING RECOMBINANT PROTEINS TO THE SYSTEMIC CIRCULATION *IN VIVO*, Sandeep Tripathy[1], Eliav Barr[1], Christopher Sullivan[1], Marion Verp[1], Eugene Goldwasser[1], and Jeffrey M. Leiden[1], [1]University of Chicago, Chicago, IL 60637.

A number of diseases are currently treated by the repeated subcutaneous or intravenous infusion of purified or recombinant proteins. These include pituitary dwarfism treated with repeated intravenous injections of recombinant growth hormone, Hemophilias A & B treated with intravenous infusions of purified Factors VIII & IX, Diabetes Mellitus treated with subcutaneous or intravenous infusions of insulin, and the erythropoietin-responsive anemias which are treated by the subcutaneous or intravenous infusion of recombinant erythropoietin (Epo). The ability to develop a cell-based system which could produce stable and physiological levels of recombinant proteins in the systemic circulation would represent a significant step forward in our ability to treat these diseases. A number of cellular systems have been tried previously without success. Skeletal muscle stem cells or myoblasts are attractive candidates for such a serum protein delivery system for several reasons: (i) skeletal myoblasts can be readily obtained from routine skeletal muscle biopsies which are relatively non-invasive procedures, (ii) such cells have a large proliferative capacity *in vitro*, (iii) these cells can be transduced with a variety of viral and plasmid-based vectors, (iv) cultured skeletal myoblasts can be implanted into host muscle by simple intramuscular injection--such implanted myoblasts have been shown previously to fuse with the endogenous myocytes to become stably incorporated into the host muscle, (v) such implanted myoblasts do not interfere with host muscle function, and, (vi) such implanted myoblasts remain localized at the site of injection and can therefore be removed if it is necessary to terminate therapy. We have reported previously that murine C2C12 myoblasts transfected with a human growth hormone expression vector secrete large amounts of growth hormone *in vitro* (12 ng/10^6 cells/hour). Following implantation into syngeneic C3H mice, such genetically modified myoblasts produce stable and physiological levels of growth hormone in the systemic circulation for prolonged periods of time. Prior to utilizing this system for human therapy, it is necessary to demonstrate that primary human myoblasts can produce and secrete high levels of recombinant proteins *in vitro* and, more importantly, that such cells can be used to produce physiological levels of recombinant proteins and appropriate biological effects in animal models *in vivo*. Accordingly, we have recently used a lipofectamine-mediated transfection to produce primary human myoblast clones that secrete high levels of recombinant human erythropoietin (hEpo) *in vitro* (14-16 u/10^6 cells/day). We have shown that the human erythropoietin secreted by these myoblasts is appropriately glycosylated and possesses full biological activity *in vitro*. Implantation of these hEpo producing primary human myoblasts into scid mice by intramuscular injection, resulted in a significant elevation in hematocrit (47±1.4 vs. 59±1;$p \leq 0.005$). Thus, genetically modified primary human myoblasts can be used to stably deliver physiological levels of therapeutic proteins to the systemic circulation *in vivo*. This approach will be useful for the therapy of erythropoietin-responsive anemias, a disease which affects more than 100,000 patients in the U.S.

From Basic Research to Clinical Experiences: The Gastrointestinal System

PZ 009 HOLLOW FIBER ENTRAPMENT BIOREACTOR AS XENOGENEIC BIOARTIFICIAL LIVER Madhusudan V. Peshwa[1], Timothy D. Sielaff[2], Florence J. Wu[1], Scott L. Nyberg[1,2], Russell A. Shatford[2], Bruce Amiot[6], Michael Y. Hu[2], Rory P. Remmel[3], Henry J. Mann[5], William D. Payne[2], Joseph R. Bloomer[4], Frank B. Cerra[2], and Wei-Shou Hu[1], Departments of [1]Chemical Engineering and Materials Science, [2]Surgery, [3]Medicinal Chemistry, [4]Medicine, [5]Pharmacy Practice, University of Minnesota, and [6]Cellex Biosciences Inc., Minneapolis, Minnesota.

A hollow fiber bioreactor has been developed as an extracorporeal bioartificial liver (BAL) to provide interim liver support to patients in hepatic failure. A suspension of xenogeneic hepatocytes is entrapped in a three-dimensional collagen matrix within the intraluminal space. While the patients' blood circulates on the extracapillary side, a hepatocyte-supporting medium is perfused through the intraluminal comparment. We have demonstrated long term preservation of hepatocyte-specific differentiated functions in the BAL. Entrapped rat hepatocytes for *in vivo* treatment in an anhepatic rabbit model provides good liver-specific biosynthetic and metabolic biochemical functions. Treated animals, in contrast to untreated control animals, showed improved hemodynamics and stable blood chemistry. However, in order to demonstrate efficacy of treatment and develop protocols for human therapy, a 50-100 fold scale up in activity is required.

An engineering analysis was performed and the BAL was scaled up 10-fold over the rabbit prototype to accommodate approximately 10g of entrapped rat hepatocytes. For evaluation of the scaled-up system, a clinically relevant large animal model of fulminant hepatic failure was developed using D-galactosamine (Gal-N) induced liver failure in dogs. BAL treated dogs showed transient recovery from coma and improvement in aromatic amino acid levels; immunohistochemical staining revealed immunoprotection for the xenogeneic hepatocytes from dog IgG and IgM. Functional activity of entrapped porcine hepatocytes in the large scale BAL is currently being assessed. Evaluation of the Gal-N canine model is under progress in randomized animal trials as a survival model and will be used for acute phase studies to demonstrate treatment efficacy for human clinical trials.

Further scale up in performance of the BAL is currently being evaluated in terms of effect of growth factors, addition of extracellular matrix components, and enhancement of specific hepatocyte funtion. Cultivation of hepatocytes as 'spheroids' or multicellular aggregates has been observed to lead to enhanced liver specific function. Our results indicate that hepatocytes in spheroids mimic the *in vivo* cytoarchitechture of the liver and can be maintained viable and differentiated over prolonged culture periods. The mechanistics of spheroid formation have been evaluated quantitatively with an objective of engineering the spheroid formation process to yield increased rates and efficiencies of spheroid formation. It is predicted that use of entrapped spheroids instead of a suspension of freshly harvested hepatocytes within the BAL can lead to significant improvement in device performance. Liver specific function within spheroid entrapment BAL is presently being evaluated *in vitro*.

PZ 010 TISSUE ENGINEERING A BIOARTIFICIAL ENDOCRINE PANCREAS, Thanassis Sambanis[1], and I. Constantinidis[2], [1]Georgia Institute of Technology, Atlanta GA 30332-0100 and [2]Emory University, Atlanta GA 30322.

A most promising method for the long-term treatment of diabetes is the implantation of functional artificial tissues based on insulin-secreting cells. Due to the relatively low molecular weight of insulin, implanted cells can be isolated from the immune system of the host by encapsulation in a synthetic membrane imposing a molecular weight cutoff of approximately 60,000. The effectiveness of such implants in restoring normoglycemia has been repeatedly demonstrated in both small and large diabetic animal models. The method is generic and, with proper cells, may also be applied to the treatment of other endocrine disorders [1,2]. Nevertheless, before any routine clinical application, several obstacles need to be overcome, most important of which is the growth or procurement of a sufficient number of cells.
We are investigating the use of transformed endocrine cells in the fabrication of a functional bioartificial pancreas. Transformed cells can be grown to large homogeneous populations, so artificial tissues can be manufactured reproducibly at a medically relevant scale. We are studying the secretory function and long-term stability of insulin-producing mouse insulinoma ßTC3 and recombinant mouse pituitary AtT-20 cells entrapped in calcium alginate/ poly-L-lysine/ calcium alginate microbeads. Using models based on measured diffusivities through the polymeric matrix and on intrinsic insulin secretion kinetics, beads are designed so as to be rapidly responsive to changes in the bulk glucose concentration. We have developed a perfusion bioreactor and radiofrequency coil which permit the non-invasive assessment of cellular energy metabolism by nuclear magnetic resonance spectroscopy. The probe has excellent radiofrequency homogeneity, while the bioreactor operates differentially and is macroscopically homogeneous. Immunoisolated cells do not grow but maintain constant metabolism, bioenergetics and basal secretion for periods in excess of a month [3]. This type of behavior is most promising for the development of long-term functional artificial tissues based on transformed endocrine cells.

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PZ 011 CONSTRUCTION OF REPLACEMENT LIVER AND INTESTINE USING CELL-POLYMER CONSTRUCTS, Joseph P. Vacanti, M.D., Children's Hospital, Boston, MA02115

The major problem in all areas of reconstructive surgery including transplantation is insufficient tissue. This problem has led to the topic of this symposium, tissue engineering. Our approach to tissue engineering has been the development of synthetic, degradable polymer templates onto which tissue-specific cells can be added to construct a device for implantation. At intervals differing from tissue from tissue, this device can then be implanted where remodeling, regeneration, and angiogenesis take place to form a new tissue equivalent that is stable and hopefully can provide differentiated function. Over the past eight years, several tissues have been studied in the laboratory. A major model for investigation has been the liver. We have found that to provide clinically relevant replacement function, approximately ten percent of liver mass must be successfully implanted. We have also discovered that this tissue mass must be exposed to hepatotrophic stimulation to produce stable cell numbers for long term function. We have studied this phenomena in several different animal models and currently are studying small animal metabolic defects including the Gunn rat of hyperbilirubinemia and the vitamin C deficient rat. Likewise, we have studied replacement function in the Dalmation dog model of hyperuricosurea. Providing hepatotrophic stimulation through portosystemic shunting and implanting large numbers of cells mostly in the mesenteric bed, we have been able to replace the needed function to six weeks post transplantation.

In more preliminary work, tubular scaffolds have been constructed of synthetic, degradable materials and have been laden with intestinal epithelial cells from crypt enriched fractions of rat intestine. We have documented neoepithelial tube formation reminiscent of early rat fetal development. Current and future studies will include composite structures made of smooth muscle-epithelial cells and studies to measure absorptive capacity of these epithelial lined tubes. In summary, tissue engineering using cell-synthetic, degradable polymer templates continues to show experimental promise and may lead to clinical application.

Biomaterials in Tissue Engineering: From Natural to Synthetic (Joint)

PZ 012 POLYMERS FOR PROMOTING AND RESISTING CELL ADHESION IN CARDIOVASCULAR TISSUE ENGINEERING, Jeffrey A. Hubbell[1,2], Jennifer L. Hill-West[2], Sanghamitra M. Chowdhury[1], Paul D. Drumheller[1], and Marvin J. Slepian[3], [1] Department of Chemical Engineering and [2] Department of Biomedical Engineering, University of Texas, Austin, TX 78712, [3] University Heart Center, University of Arizona - VA Medical Center, Tucson, AZ 85723.

Cell adhesion plays an important role in healing in injured vessels and in artificial vascular grafts. Adhesion proteins, which are constituents of the vessel wall or are adsorbed to the surface of a vascular graft, determine the adhesive events occurring during exposure to blood. Two approaches in the control of cell adhesion are presented, one involving the promotion of cell-type specific adhesion to polymeric vascular graft materials, and the other involving the blockade of platelet adhesion and aggregation upon the surfaces of injured blood vessels. Adhesion peptides that have been chemically or physicochemically incorporated into a polymeric material have been used to promote adhesion of endothelial cells to vascular graft materials. Many examples exist on the incorporation of RGD peptides into polymeric surfaces. Work has also been done with the synthetic laminin peptide YIGSR, for which a platelet receptor has not been identified. This would provide the opportunity to promote the adhesion of blood vessel wall cells to a graft material while avoiding blood platelet adhesion. However, for this to occur, adhesion proteins must not adsorb to the graft surface, which would result in signals for cell adhesion other than the incorporated peptide. Toward this end, copolymers of acrylic acid and monoacrylyl-poly(ethylene glycol 1900) (poly(AA-co-PEG)) were synthesized, with various amounts of acrylic acid and mono-acrylyl-PEG. These polymers were further incorporated into a substrate of crosslinked trimethylolpropane triacrylate (TMPTA) as an interpenetrating polymer network (IPN). A poly(AA-co-PEG) with 85% AA incorporated into an IPN with linear, nonacrylated PEG 4000 (0.2 g poly(AA-co-PEG), 0.2 g PEG, 1 g TMPTA) was found to be highly resistant to fibroblast adhesion with incorporated control RDG peptide, but highly adhesive with immobilized RGD peptide. Materials such as this provide a path to highly specific cell adhesion in the vasculature, where tissue responses may be controlled by the incorporated peptides without complication by potentially adsorbing proteins. Controlling cell adhesion is also important in tissue engineering of the native blood vessels. During the therapeutic process of balloon angioplasty, the vessel wall is damaged and becomes thrombogenic. Thrombosis upon the vessel wall is thought to play a causational role in restenosis, the thickening of the intima by smooth muscle cell migration and matrix secretion. Adherent platelets generate thrombin and release platelet-derived growth factor, which may be involved as stimulatory signals. Direct contact of angioplasty-injured vessels with blood was blocked in rabbits by a degradable hydrogel barrier upon the arterial surface. A prepolymer of PEG 10000 with oligomeric lactic acid extensions at both ends and with acrylate end-caps was interfacially photopolymerized by illumination with green light after staining the vessel lumen surface with a solution of eosin Y. The mean ratio of the cross-sectional area of the intima to media was reduced from 1.7 in untreated animals to 0.3 in treated animals ($p<0.003$) 14 d after injury and treatment, more than 10 days after the disappearance of the hydrogel barrier.

PZ 013 SYNTHESIS OF POLYMERIC SUBSTRATES AND CHARACTERIZATION OF CELL-POLYMER INTERACTIONS, W.M. Saltzman, J. Belt, W. Dai, C.E. Krewson, T. Livingston, M.R. Parkhurst, and M. Shin, The Johns Hopkins University, Department of Chemical Engineering, 3401 N. Charles Street, Baltimore, MD 21218.

For many decades, polymeric materials have been used as structural supports for cultured cells. Although most contemporary cell culture studies utilize a substrate composed of a synthetic polymer (typically surface-modified polystyrene), no general principles regarding the interactions of vertebrate cells with synthetic polymers have emerged. This situation limits the engineering of tissue replacements, so there is considerable interest in developing new polymers and characterizing their interaction with cells.

The interaction of cells with polymers has been studied at three levels of complexity: i) adhesion and spreading of cells on the surface of a polymer; ii) growth, motility, and orientation of cells on the surface of a polymer ; iii) motility, aggregation, and organization of cells within a polymer gel or matrix. Cell adhesion to polymers has been quantified by using flat, homogeneous polymer films that are purely synthetic [1, 2] or modified by covalent coupling of proteins or peptides [3], carbohydrates [4, 5], or glycolipids [6]. In short term studies, cell adhesion and spreading usually vary predictably with surface chemistry. Changes in cell growth or motility are more subtle and less predictable [2, 7], probably because cells modify the polymer surface during longer periods of observation. Rates of cell motility on a surface can be altered by changing the density of adsorbed extracellular matrix (ECM) molecules [8]. Similarly, rates of cell migration through gels of ECM molecules depend on concentration [9, 10]. Regulation of the composition or isotropy of the gel can result in changes in cell aggregation, process outgrowth [9], or cell orientation. These results have been obtained using gels composed primarily of naturally occuring polymers; the development of synthetic polymer gels that produce this same behavior would be a significant advance.

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PZ 014 THE INTERACTION OF MAN-MADE SURFACES WITH BIOLOGICAL SYSTEMS. George M. Whitesides, Amit Kumar, Gabriel Lopez, Hans Biebuyck, Paul DiMilla, Ralph Haerter, and Kevin Prime, Department of Chemistry, Harvard University, Cambridge MA 02138.

Self-assembled monolayers (SAMs) of alkanethiolates on gold and silver provide organic surfaces with well-defined structures. The functional groups presented at the solid-liquid interface by these SAMs can be readily controlled and modified by organic synthesis; they can be patterned in two dimensions; they can be made optically opaque or transparent, depending on technique of fabrication. This talk will outline the techniques used to make SAMs, and illustrate the application of appropriately functionalized SAMs to the study of the adsorption of proteins on surfaces, and to the study of the adhesion and control of cell growth by attachment to surfaces.

From Basic Research to Clinical Experiences: The Hematopoietic and Nervous Systems

PZ 015 HEMATOPOIETIC PERFUSION BIOREACTORS: SCIENTIFIC AND CLINICAL UTILITY, Bernhard O. Palsson, The University of Michigan, Ann Arbor.

There are many compelling reasons for reconstituting human tissues *ex vivo*: 1) successful *ex vivo* tissue models enable the study of the dynamics and mechanics of cell differentiation and organ development; 2) functioning *ex vivo* organs are useful in a clinical setting for transplantation therapy and for gene therapy, and 3) further, meaningful pharmacological studies can be carried out in such systems. Extensive research has been performed in recent years to obtain prolific human bone marrow cultures from adult donors [1]. In this talk we show how the approach of *in vivo* mimicry has led to prolific *ex vivo* bone marrow perfusion cultures [2,3] and their development to the stage of clinical utility. The key to this accomplishment is bioreactor design and implementation of the dynamic *in vivo* environment [4,5].

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PZ 016 *EX VIVO* EXPANSION OF PRIMITIVE HEMATOPOIETIC CELLS UNDER PERFUSION CONDITIONS, Eleftherios Terry Papoutsakis[1], Manfred R. Koller[2], Craig E. Sandstrom[1,3], William M. Miller[1], and James G. Bender[3], [1]Department of Chemical Engineering, Northwestern University, Evanston, IL 60208-3120, [2]Aastrom Biosciences, Inc., PO Box 376, Ann Arbor, MI 48106, and [3]Baxter Healthcare, Round Lake, IL 60073.

Sources of hematopoietic cells for bone marrow transplantation are limited by the supply of compatible donors, the possibility of viral infection, and autologous (patient) marrow that is depleted from prior chemo- or radiotherapy or has leukemic involvement. An *in vitro* system to amplify hematopoietic progenitor cells could increase the number of patients eligible for autologous transplant, allow use of cord blood hematopoietic cells for repopulating adults, reduce the amount of bone marrow required for transplantation, and allow collection of mobilized peripheral blood stem and progenitor cells to replace the bone marrow harvest process. Present methods for hematopoietic cultures on stromal (i.e., accessory cells that support hematopoiesis) layers in flasks lack a well-controlled growth environment. The prospects for use of hematopoietic cultures in bone marrow transplant are very good if an efficient culturing system can be developed. Our research suggests that perfusion conditions improve significantly the performance of hematopoietic reactors. Several questions arise in the design of such reactors pertaining to the choice of design and operational parameters which affect the nutritional and physichochemical environment of the culture. These culture parameters affect cell proliferation and differentiation, two cellular aspects that frequently appear antagonistic in hematopoietic cultures. In any case, there are several objectives that must be met by these cultures depending on the desired use of the cultures cells. As such, there are potentially several "optimal" designs and operational protocols. I will discuss the design and operational criteria that such reactors must meet, and our research on identifying conditions that allow for rapid expansion of hematopoietic progenitors in a perfusion bioreactor without the need for stromal cells and serum in the culture medium. Eliminating the stromal layer and serum in the medium, eliminates the need for a stromal cell donor, reduces culture time, and simplifies the culture system.

Basic Principles: Mechanical Forces as Regulators of Biological Activity (Joint)

PZ 017 ENDOTHELIAL GENE REGULATION BY BIOMECHANICAL FORCES, Nitzan Resnick[1], Bauer E. Sumpio[2], and Michael A. Gimbrone Jr.[1], Vascular Research Division, Dept. of Pathology, Brigham and Women Hospital Boston, MA, and Vascular Surgery division, Yale University, New Haven,CT.

Vascular endothelial cells are exposed to hemodynamic forces generated by flowing blood. These forces, which include wall shear stress and cyclic strain, have been shown both in-vitro and in-vivo to modulate the morphology and function of the endothelium. Recently several groups have demonstrated that, in well defined in-vitro systems both shear stress and cyclic strain can modulate endothelial gene expression. In particular,our group has demonstrated that PDGF-B chain gene transcription is induced by physiologic levels (10 dynes/cm^2)of laminar shear stress. We have defined a region within the PDGF-B chain promoter that is responsible for the induction of the gene by shear stress, and called this the "Shear Stress Responsive Element" (SSRE)[1]. This promoter element binds shear-stress inducible nuclear proteins from endothelial cells, as demonstrated by gel shift analysis. Computer analysis of the promoters of several other endothelial genes that are responsive to shear stress revealed a core sequence within the SSRE that exists in all of them. In recent set of experiments, using gel shift analysis and mutated SSRE probes we have better defined the SSRE showing that indeed the core sequence (GAGACC) is a functional binding site. Moreover, hybrid promoters containing the SV40 promoter (which is not responsive to shear stress) coupled to the SSRE sequence (as taken from the PDGF-B chain promoter), were inducible by shear stress in bovine aortic endothelial cells, thus verifying the fact that the SSRE is both necessary and sufficient for gene induction by laminar shear stress. SSRE probes taken from other shear stress responsive genes formed similar DNA-nuclear protein complexes as observed with the PDGF-B chain probe, suggesting that the SSRE is functional in these genes as well. Since cyclic strain has also been found to modulate endothelial gene expression, we tested the presence of SSRE-binding-proteins in nuclear extracts made from endothelial cells exposed to this hemodynamic force. We have found that the SSRE does bind to nuclear proteins from endothelial cells exposed to cyclic strain (24% strain, 60 cycles/min), that this binding peaks at 30 min. after the exposure of the cells to cyclic strain, is dependent upon the level of strain and on the cell type (endothelial cells vs. smooth muscle cells). These results thus suggest that biomechanical forces act on the endothelium through a common-cell-type-specific mechanism to activate gene transcription. (supported in part by PO1-HL36028.)

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PZ 018 THE REGULATION OF VASCULAR ENDOTHELIAL BIOLOGY BY FLOW, Gabriel Helmlinger, Olivier Thoumine, Theodore F. Wiesner, and Robert M. Nerem, Bioengineering Center, Georgia Institute of Technology, Atlanta, GA 30332-0405

The biology of vascular endothelial cells (EC) is regulated by a variety of factors including the flow environment in which the endothelium resides. Over the past decade, much has been learned about the influence of flow through cell culture studies. In these a cultured EC monolayer is exposed to a sudden onset of a steady, laminar flow. Although this is not truly a physiologic model of flow, considerable insight has been gained. What we now know is that flow, and the associated shear stress, influences EC shape, its cytoskeletal structure and the resulting mechanical properties of the cell, and the extracellular matrix, including the distribution of focal contact proteins. The ability to proliferate also is affected, with cell proliferation being decreased by flow, and it has been shown that the synthesis and secretion of certain substances by EC is influenced by flow, with this effect extending to the gene expression level. More recent results from our laboratory indicate that EC are influenced differently by the sudden onset of pulsatile flow as compared to steady flow. In these experiments a non-reversing pulsatile flow, a reversing pulsatile flow, and a purely oscillatory flow with a zero mean component have been studied. These all employ a sinusoidal waveform with a 1 Hz frequency. From these studies it is clear that such properties as cell shape, the localization of F-actin, and the extracellular matrix differ depending on the specific pulsatile flow environment. Although the exact mechanisms involved in the recognition of the onset of flow and the associated shear stress are unclear, the elevation of intracellular calcium appears to be an important second messenger in the transduction of the signal. There are slow oscillations present in the flow-induced calcium signal, the frequency of which depends on the composition of the media. Particularly noteworthy is the increase in the frequency of these calcium oscillations for pulsatile flow as compared to steady flow. This alteration in the frequency content of the calcium signal suggests that the temporal nature of this signal may be essential to the information content provided the cell and to the ability of EC to discriminate between different flow environments. The ability to tissue engineer a blood vessel may be dependent on understanding the influence of flow and the mechanical environment imposed, as well as the signaling mechanisms involved.

From Basic Research to Clinical Experiences: The Vascular System

PZ 019 A NON-CELLULAR BIOLOGICAL SUBSTRATE MATERIAL FOR USE AS A VASCULAR GRAFT, Stephen F. Badylak[1], Arthur C. Coffey[2], Gary C. Lantz[1], and Leslie A. Geddes[1], [1]Purdue University, 1293 Potter #204, West Lafayette, IN 47907-1293, [2]Center for Surgery Education Department, 1633 N. Capitol #650, Indianapolis, IN 46202.

Xenogeneic small intestinal submucosa (SIS) has been used as a template around which recipient animals develop new blood vessels. The type of new blood vessel formed, that is, an artery or a vein, depends upon the location in the body in which the SIS material is placed. Microenvironmental stimuli affect the type of tissue remodeling which occurs around this resorbable biomaterial. For example, the presence of a circumferential stress (hoop stress) can cause the formation of organized smooth muscle cells in the wall of the neovessel. Likewise, the intraluminal pressure within an SIS vascular graft plays a role in the morphology of the new blood vessel. When placed in the arterial circulation, SIS grafts transform to a wall thickness of approximately 600 μM. When placed in the venous circulation, the wall thickness transforms to approximately 70 μM. The principal that microenvironmental stimuli affect tissue remodeling can be extended to other connective tissues for which SIS can serve as a graft material. SIS has been used to replace injured ligaments, tendons, bone, urinary bladders and other body tissues in animal models. It appears that not only the structure of SIS but also the composition of SIS are important in its function as a biological substrate for tissue engineering.

PZ 020 GENE THERAPY AND THE BIOLOGY OF ARTIFICIAL BLOOD VESSELS, Author: Alexander W. Clowes, M. D., Professor of Surgery, University of Washington Medical School, Department of Surgery, RF-25, Seattle Washington, 98195. Vascular smooth muscle cells (SMCs) are a potential vector for introducing genes of therapeutic importance. We have been able to introduce human adenosine deaminase (hADA) and alkaline phosphatase in a replication-defective retrovirus into rat SMC and seed these cells into the intima of rat carotid artery. These cells form an intimal thickening identical to the intimal thickening induced by injury of the vessel alone. These cells continue to express the human genes as late as twelve months after seeding. Furthermore, they do not demonstrate any evidence of neoplastic transformation. This system for introducing genes in vivo through the construction of an artificial intima is now being extended and potentially could be used to develop models of human disease as well as to explore gene regulation.

PZ 021 REGULATION OF ANGIOGENESIS BY COPPER/SPARC AND BY TRACTION-GENERATED TEMPLATES OF COLLAGEN I, E. Helene Sage[1], R. B. Vernon[1], L. Iruela-Arispe[1], and T. F. Lane [2], [1]University of Washington, Seattle and [2]HHMI, Harvard University, Boston.

We envision angiogenesis as a continuum of activities that endothelial cells must exhibit during the formation of new capillary networks. Many of these functional states are manifested as changes in cellular phenotype, e.g., in morphology, extracellular matrix, or degree of proliferation, that are concomitant with the altered levels of protease activity, cytokines, and accessory protein synthesis known to occur in the immediate tissue environment. More subtle changes, such as those which facilitate directional migration, intercellular adhesion, and temporal or spatial control of proliferation, are not yet understood.

We have proposed two regulatory pathways of angiogenesis: proliferation and morphogenesis, and have characterized two proteins secreted by endothelial cells that appear to act at different stages of the angiogenic process. SPARC, as a soluble mediator, is thought to intervene at control points that regulate cell number: initially, more endothelial cells are required to establish leading cords, whereas ultimately, cell division must abate or cease to avoid disorganized, florid angiogenesis. SPARC itself is an inhibitor of G1 → S in the endothelial cell cycle. Its role as a stimulator of early phases of angiogenesis is thought to be mediated by the highly-conserved sequence KGHK, a copper-binding peptide that is released from SPARC by plasmin and/or proteases. Conversely, type I collagen, as an insoluble mediator of angiogenesis, promotes the formation of endothelial networks by the provision of traction centers upon which the cells exert directional tension. Moreover, fibrillar type I collagen secreted by endothelial cells during this process provides the growing cord with a mandril or template that might be eventually degraded to form a patent lumen.

Requisite participants in the transition from a quiescent state to tube formation and subsequent remodeling are several morphogens/cytokines, and their cognate receptors, that affect endothelial cells at specific points during angiogenesis. We will present data confirming the contextual sensitivity of cells to several cytokines and the selective expression of cytokine receptors at discrete stages of angiogenesis *in vitro*. Understanding how proliferation and morphogenesis are controlled during angiogenesis is likely to reconcile several models with respect to the pathways and the participants that appear to regulate this dynamic process.

From Basic Research to Clinical Experiences: Artificial Skin

PZ 022 **PARACRINE REGULATION OF KERATINOCYTE GROWTH AND DIFFERENTIATION BY EPITHELIAL-MESENCHYMAL INTERACTIONS**, N.E. FUSENIG, H. Smola, G. Thiekötter, H.-J. Stark and D. Breitkreutz, Div. Carcinogenesis and Differentiation, DKFZ, 69120 Heidelberg, Germany

Epithelial-mesenchymal interactions are essential for the establishment of organ architecture and the maintenance of tissue homeostasis, but the regulating mechanisms are largely unknown. Organotypic coculture models have been developed to study the reconstitution of a structural and functional satisfying equivalent of the skin including epithelial and mesenchymal cells (1,2). In feederlayer cultures, the simplest coculture system, keratinocyte proliferation is drastically stimulated by postmitotic fibroblasts and capillary endothelial cells. This could not be replaced by fibroblast-conditioned medium, and it was suggested that the "feeder" effect was not mediated by secreted stable factors. However, mRNA analysis of the separated keratinocyte and fibroblast compartments demonstrated that growth factor expression (KGF, Il-6, GMCSF) being constitutively low in proliferating and postmitotic fibroblast monocultures was substantially stimulated by cocultured keratinocytes (3). As a possible inducer signal, Il-1α was expressed by keratinocytes, leading to upregulation of Il-1α receptor in fibroblasts and thus to a sensibilization of the feeder cells. Similar mutually induced regulatory circuits were observed in organotypic cocultures of keratinocytes growing on lifted, fibroblast- or endothelial-cell-populated collagen gels. Proliferation in both compartments (determined by histone 3 mRNA, BrdU incorporation, and cell counting) was stimulated in cocultures. In addition, TGF-α expression was grossly induced in keratinocytes growing on lifted gels, but was independent of the presence of dermal cells. In such a reconstituted in vitro epidermis, proliferation was hyperstimulated with delayed expression of differentiation markers and aberrant coexpression of vimentin. Both alterations were normalized in surface transplants of such cultures on nude mice. Thus, such overstimulated organotypic keratinocyte cocultures, resembling wound situations or hyperproliferative diseased states in skin, are probably caused by overstimulated and unbalanced proliferative signals of mesenchymal cells. For the improvement of skin equivalent models the regulation circuits between epithelial and mesenchymal cells have to be studied more in detail and the derived knowledge applied to elaborate stable in vivo-like tissue recontructions under defined conditions. In serum-free, defined culture medium reconstitution of a regularly structured epidermis occurs in such organotypic cocultures with fibroblast-containing collagen gels as well, however with improved tissue architecture and stratum corneum formation. Such cultures, under completely defined growth conditions, are better suited for further identification of mediators and mechanisms regulating tissue homeostasis in reconstituted skin equivalent structures in vitro.

1. Boukamp et al., Differentiation 44:150-161 (1990)
2. Mackenzie et al., Epithelial Cell Biology 2:107-119 (1993)
3. Smola et al., Journal of Cell Biology 122:417-429 (1993)

PZ 023 DEVELOPMENT OF A BILAYERED 'SKIN EQUIVALENT': FROM BASIC SCIENCE TO CLINICAL USE, Nancy L. Parenteau, Michael L. Sabolinski, Leon M. Wilkins and David T. Rovee, Organogenesis Inc., Canton, MA.

The problems of cutaneous wound healing and skin replacement have been actively studied for many years. The wound healing focus of tissue engineering has been on the possibility of using a biological skin substitute formed *in vitro* from extracellular matrix and/or cultured cells. Basic components of skin which likely play an interactive role in healing are the extracellular matrix, dermal connective tissue cells, the epidermis and the stratum corneum. Formation of a bilayered skin construct allows us to provide these components in a normal morphological and physiological relationship. Our model consists of a condensed bovine collagen matrix containing dermal fibroblasts overlaid with an epidermis. The construct is cultured at the air-liquid interface to allow the epidermis to form a protective stratum corneum on its outer surface. Studies of grafting onto nude mice indicate that the skin construct can "take" and persist as a split-thickness graft. The grafted material assimilates well with host tissue. There is gradual remodeling of the dermis over time which appears to be dependent on wound depth and is modulated by the presence of epidermis. The dermal fibroblasts in turn support the maintenance of the epidermis. In a clinical setting, the variety of wound conditions, treatment modalities, and patient medical history provide a more challenging environment for graft function. To date, 234 patients have been studied using the construct (Graftskin) in three separate indications. Interim results for venous ulcer show that the rate of wound healing, time to 50% wound closure, and the incidence of complete wound healing are improved with Graftskin when compared to standard treatments. Differences between groups are most pronounced in the most advanced, recalcitrant ulcers. When the construct has been applied to cleanly excised wounds resulting from dermatologic surgery procedures, improved rates of healing and cosmetic outcomes have been reported. In burns where the proteolytic environment is different from chronic or other acute wounds, rapid dissolution of the dermal matrix has been observed. The matrix therefore does not survive long enough to direct remodeling and control formation of new dermal tissue. However, preliminary observations using meshed Graftskin over meshed autograft indicate that if graft survival is prolonged by the "protection" afforded by the autografted tissue, the construct is able to improve the quality of the healed tissue. In all three clinical indications tested, Graftskin has been shown to enhance the healing process. Our experimental and clinical experiences indicate that the bilayered construct can establish new epidermal and dermal tissue and take as a graft in an optimum environment. One of our remaining goals is to improve the durability and function of the extracellular matrix so the construct's full clinical potential can be realized under a broader spectrum of conditions.

Control of Tissue Development, Growth and Repair

PZ 100 A TYPE I COLLAGEN ALPHA 1 HOMOTRIMER-PRODUCING BONE MARROW CELL TRANSFECTED WITH ALPHA 2 EXPRESSES TYPE I HETEROTRIMER. G. Balian, P.C. Anderson, A. Lim* and B.D. Smith*. Depts. of Orthopaedics and Biochemistry, University of Virginia School of Medicine, Charlottesville, VA 22908 and *Dept. of Biochemistry, Boston University School of Medicine, Boston, MA 02118.

A cell which was cloned from mouse bone marrow cultures synthesizes a collagen homotrimer consisting of three α1 type I chains. The homotrimer producing cells do not express collagen type I α2 polypeptides or α2 mRNA. To determine if α2 can be expressed and incorporated into heterotrimers, the cells were transfected with a construct that expresses the full length mouse α2 type I chain and neomycin resistance gene. The transfected cells were selected with G418. Alpha 2 mRNA was detected by hybridization to Northern blots of total RNA prepared from the transfected cells. The cells were incubated with ^{14}C-proline and the proteins in the cell layers and in the media were quantitated by treatment with bacterial collagenase, digested with pepsin, and analyzed by SDS-PAGE. Transfection with α2 increased total collagen synthesis from 19% in the original cells to 30% in the transfected cells. A collagen heterotrimer, $(\alpha 1)_2\alpha 2$, could be distinguished from the homotrimer, $(\alpha 1)_3$, based on differences in melting temperature after limited digestion with pepsin. The appearance of a heterotrimer in the transfected cells demonstrated that the α2 polypeptide is incorporated into triple helical molecules together with the endogenous α1 polypeptides. These experiments highlight the possibilities for treatment of cells in vitro to express stable triple helical collagen heterotrimers.

PZ 101 SYNTHETIC EXTRACELLULAR MATRIX ACCELERATES HEALING OF MUCO-GINGIVAL SURGERY DONOR SITES. Marie C Béné, Gérard R Martin, Nathalie Godard, Jacques Penaud, Neil Miller, Gilbert C Faure. Periodontology Department, School of Dentistry and Lab. Immunology, Faculty of Medicine. Université Nancy I. France.

Palatal donor sites are commonly used in periodontal surgery. Their fast healing, expected for the patient's comfort, can be enhanced by the use of collagen and/or extracellular matrix dressings. We used such a compound, containing chondroitin IV sulfate, heparan sulfate and fibronectin (Glycagen®), in order to accelerate donor sites healing in 10 periodontal patients. Immunohistological techniques were used on frozen-cut biopsy samples from the wound's margin to appreciate tissue reconstruction around the synthetic material, and on a control biopsy where a classical dressing had been used alone. Oriented serial sections were performed, spanning both the healthy wound margin and the healing area. Extracellular matrix components were studied using antibodies directed to human laminin, fibronectin and types I, II and IV human collagen. Factor VIII labelling was used to further assess the vascularization and angiogenesis. Epithelial growth characteristics were explored using monoclonal antibodies to four different human keratins, CD9, and Ki67. Reactive cells in the mucosa were immunophenotyped using monoclonal antibodies to CD3, CD4, CD8 and MHC-Class II antigens. Newly-formed extracellular matrix components appeared rapidly within the synthetic sponge. A thin growing epithelium, with selective keratin expression, was developing over the synthetic material, supported by newly-formed basement membrane or a thin layer of fibrinogen. Cytoplasmic and intranuclear expression of Ki67 was evidenced in different epithelial segments. The deepest areas of the underlying mucosa, beneath the synthetic dressing, showed clear signs of angiogenesis, with small capillaries perpendicular to the epithelial plane. Selective inflammatory reactions, including polymorphonuclears and mononuclear cells, could be seen at specific sites in all samples, respectively in the vicinity of the synthetic material and, nearer to the healthy tissue, around the wound margin. These data indicate that synthetic collagen sponges supplemented with extracellular matrix components are liable to accelerate wound healing by initiating fast epithelial growth, neovascularization, and spatial organization of new matrix.

PZ 102 GROWTH OF EMBRYONIC AND ADULT RAT CNS NEURONS IN DEFINED MEDIUM,
Gregory J. Brewer, Southern Illinois University School of Medicine, Springfield, IL 62794-9230

Present efforts to repair neural tissue are limited by the ability to maintain and differentiate donor tissue. We have developed a defined serum-free supplemented medium combination which was optimized for 4 days of growth of rat embryonic hippocampal neurons (Brewer et al., J. Neurosci. Res. 35:567(1993)). This B27/Neurobasal™ medium supports embryonic neuron growth without glia for weeks at densities as low as 80 cells/mm^2. For cells plated at 640/mm^2, survival after 4 weeks was greater than 90%. Now we report the density dependent growth of neurons from embryonic striatum, substantia nigra, septum, cerebral cortex and postnatal cerebellum and dentate gyrus. Development of the dopaminergic phenotype was studied in striatal neurons by quantitative immunofluorescence. Immunoreactivity of tyrosine hydroxylase was very high after one day in culture when process sprouting was just beginning. Over a fourteen day period, immunoreactivity first was diluted evenly throughout all processes and later showed some discrete focal concentrations. Hippocampal neurons were virtually unlabeled. Striatal neurons also showed dopamine uptake detected with anti-dopamine antiserum. These neurons also showed punctate monoamine fluorescence after reaction with glyoxylate. In other experiments that start with adult rat hippocampus, we have enzymatically isolated viable neurons with sheared axons and dendrites. In B27/Neurobasal, these isolated cells regrow axons and dendrites and remain viable for weeks. This defined medium may be useful for in vitro growth and repair of damaged adult brain.

PZ 103 DEVELOPMENT OF ARTIFICIAL THYMUS, LYMPHOCYTES, AND OTHER TISSUES FROM WILD TYPE AND TRANSGENIC MOUSE EMBRYONIC STEM (ES) CELLS IN A COMBINED *IN VITRO* AND *IN VIVO* DIFFERENTIATION SYSTEM Una Chen, Basel Institute for Immunology, Grenzacherstrasse 487, CH 4005 Basel, Switzerland

Mouse embryonic stem (ES) cells can differentiate in culture into late stages of many lineage-committed precursor cells. We have found organ culture conditions that are favorable for the differentiation of ES cells into hematopoietic cells at a stage equivalent to days 11 to 14 of fetal liver. We have determined growth conditions that allow ES cells to differentiate efficiently into cystic embryoid bodies (ES fetuses) that contains precursors of most hematopoietic lineages including lymphoid cells. We have followed the development of tissues from ES fetuses *in vivo* in appropriate hosts and characterized both mature T and B-lymphocytess as well as myeloid populations. Since the mature untransformed ES-derived lymphocytes are functional, this seems to be a quick (a total of 7 weeks) and efficient system for purifying large quantities of both precursors and mature lymphocytes for multiple purposes including somatic gene therapy. Also, with this combined *in vitro* and *in vivo* system, we can study the differentiation of a variety of tissues: thymic stroma, endothelial venules, cartilage, bone with bone marrow, skin, neuron, *etc.* We have studied the differentiation of mutated ES cells into hematopoietic lineage-committed precursor cells by using several transgenic/knock out ES cells. Selected transgenic/knock out ES cell clones can differentiate in an orderly fashion to ES fetuses after electroporation and drug selection. Cellular analysis has shown that transgenic ES cells which contain minigenes of recombinase substrates can differentiate into mature lymphocytes. Molecular data of ES-derived lymphocytes and their hybridomas have indicated that they have both correct and aberrant rearrangements. We reason that the natural selection force for perfect fetuses exercised in normal embryogenesis does not exist in the culture system, thus it allows for every possible event of recombination to appear in ES-fetuses. The advantage of this system is the open window of the first tissue culture step that allows the study of the influence of growth factors; and the second *in vivo* environment that completes the maturation step of lineage/tissue committed stem cells. Indeed, we have shown that there are factors preferentially regulating the maturation and migration of lymphoid subpopulations.

PZ 104 CULTURED DERMAL CELLS INHIBIT WOUND CONTRACTION IN VIVO, IN CONTRAST TO NON-CULTURED DERMAL CELLS, Henry J.C. de Vries, Esther Middelkoop, Richard P. Dutrieux, Charles H.R. Wildevuur and Wiete Westerhof. Department of Dermatology, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands.

Scarring and wound contraction of full thickness skin defects can be prevented with dermal templates seeded with cultured autologous fibroblasts. The time required to cultivate these fibroblasts influences the quality of regeneration adversely. The use of non-cultured cells could eliminate the three week period required for cell cultivation. We evaluated the effect of a collagen/elastin dermal substitute seeded with cultured cells (CC, n=11), with non-cultured cells (NC, n=8) and without cells (CO, n=11) in our porcine full thickness wound model. Dermal sponges were seeded in a concentration of 10^5/cm^2. Per pig, 14 3x3cm full thickness wounds were created. The wounds were covered with the test materials and split skin mesh grafts. A semi permeable wound dressing (Exkin®) protected the wounds from infection and excessive fluid loss. Evaluation parameters were myofibroblast accumulation (using a anti-α smooth muscle actin monoclonal) and wound contraction.

CC lowered the percentage of α-smooth muscle actin positive cells in the first week (CO 78.6$\pm$1.3%, CC 50.9$\pm$7.4%, $P<0.02$) and inhibited wound contraction after 8 weeks (CO 37.9$\pm$8.5%, CC 29.5$\pm$6.6%, $P<0.02$). NC did not lower the α-smooth muscle actin positive cell ratio (NC 72.3$\pm$6.2%) significantly and had no reducing effect on wound contraction (NC 36.8$\pm$1.3%). NC fibroblasts were four times smaller than cultured fibroblasts which could indicate a lower metabolic function. The NC suspensions also contained capillary wall fragments. These fragments contained endothelial cells, pericytes and smooth muscle cells. The endothelial cells could have stimulated wound contraction through endothelin-1 production. Pericytes and smooth muscle cells possess contractile capacities which might have contributed to wound contraction.

PZ 105 HOLLOW FIBER BIOREACTORS ENGINEERED FOR EXPANSION OF THERAPEUTIC CELLS, Randal A. Goffe, PhD, Unisyn Technologies, Inc., 14272 Franklin Avenue, Tustin, California, 92680

Hollow fiber bioreactors were first used to culture mammalian cells over two decades ago. Since that time they have been employed extensively for growing hybridomas in the production of monoclonal antibodies. While other biomolecules and viruses have also been produced successfully in these "cell entrapment" devices, they have only recently been considered for the production and harvesting of therapeutic cells.

A hollow fiber-based bioreactor is described which has been engineered specifically for the cultivation and harvesting of viable therapeutic cells. Endothelial cells were selected as a primary cell line model for two reasons: 1) they are a key component in stroma, which appears to be critical to successful expansion of progenitor cells; 2) these cells are useful in studying adhesion molecules. Micro-environmental factors that enhance the growth and prolong the propagation time for endothelial cells were investigated and incorporated into a novel bioreactor design.

The latest findings are presented and discussed with a view to the clinical and research impact of such a system.

PZ 106 NEPHROGENESIS IN VITRO: GLOMERULAR DEVELOPMENT WITH TARGETED ANGIOGENESIS, H.D. Humes, D.A. Cieslinski, A. Funke, and S. Liu, VAMC and Univ. of Mich., Ann Arbor, MI.

Embryonic morphogenesis of the kidney is dependent upon a timely orchestrated inductive interaction between mesenchyme and epithelium. After the initial stages of tubulogenesis, the development of the glomerulus proceeds due to the release of angiogenic factors by the epithelial cells in the cleft of the S-shaped tubules. The secretion of these factors promotes targeted migratory angiogenesis to form the glomerular capillary network within the developing tubule. To recapitulate these events in vitro, experiments were devised using an amphotrophic, replication defective, recombinant retrovirus containing a full length coding sequence for fibroblastic growth factor (FGF)-5, a known angiogenic factor containing a signal peptide for processing along the classic secretory pathway. Using this vector, rabbit renal proximal tubule progenitor cells were transduced and seeded into 3-dimensional collagen gels and allowed to grow into tubules in defined non-serum containing media for 7 days. After this time interval, collagen gels were transplanted on chorioallontoic membranes (CAM) of 7-9 day old chick embryos for 7 days to assess targeted angiogenesis into the gels. The transduced cells promoted an intense capillary ingrowth into the gels containing transduced cells but not in gels containing nontransduced cells. Morphologic assessment of the gels demonstrated endothelial-epithelial cell interactions characteristic of early glomerular development. These data suggest that recapitulation of embryonic kidney development may be developed in in vitro systems as a first step towards a tissue engineering construct for an implantable bioartificial kidney.

PZ 107 GROWTH OF HUMAN COLORECTAL CARCINOMA CELLS IN NASA ROTATING WALL VESSEL (RWV) PRODUCES HIGH CELL YIELDS WITH DIFFERENTIATION AND REDUCED GLUCOSE CONSUMPTION. J.M. Jessup, R.D. Ford, T.J. Goodwin, D.A. Wolf, G. Spaulding. Deaconess Hospital, Boston, MA 02215 and NASA-Johnson Space Center, Houston, TX 77058.

Successful batch culture of mammalian cells requires efficient mass transfer of nutrients and wastes in an environment that attains high cell concentrations. The NASA-JSC RWV Bioreactor has several ideal characteristics for such batch cultures since it is a zero headspace reactor that rotates around the horizontal axis and produces low shear stress (<3 dynes/cm^2) for microcarrier bead-based cultures. Cultures are not exposed to a gas-liquid interface with attendant surface denaturation effects because gas exchanges through a central silicone-covered spindle. A poorly differentiated human colon carcinoma cell line, HT-29, and a subline, HT-29KM, that differentiates when grown in nude mice were cultured in the RWV and in 5 ml T25 conventional monolayer cultures for periods of up to 30 days. Maximum concentrations of both lines in conventional cultures were 3 x 10^5 cells/ml in T25 flasks, 3 x 10^6 cells/ml in the RWV, and 9 x 10^6 cells/ml in the RWV when co-cultured with human colonic fibroblasts. Both lines were poorly differentiated in T25 and RWV monocultures and in T25 co-cultures with human colon fibroblasts. Co-cultures of HT-29 with human colon fibroblasts did not differentiate in the RWV but co-cultures of HT-29KM formed glands and mucin-filled signet ring cells. HT-29 and HT-29KM co-cultures produced cell cycle lengthening associated with a decrease in glucose consumption from 47 x 10^{-14} M/hr/cell at 9 days of culture to 9 x10^{-14} M/hr/cell at 25 days of culture with viabilities of > 95% without lactate dehydrogenase released into medium. Monocultures of fibroblasts did not decrease glucose consumption in the RWV vs T25 flasks but HT-29KM and HT-29 in the RWV were 16 - 30% of T25 flask glucose consumption. These results demonstrate that 1) zero head space RWV bioreactors, but not conventional *in vitro* systems, provide an environment in which cells may recreate architectures that are similar to *in vivo* tissue, 2) high density, viable epithelial RWV cell cultures reduce their glucose consumption, possibly by altering their energy utilization in the RWV.

PZ 108 DNA SYNTHESIS AND MULTINUCLEATION IN CARDIOMYOCYTES DERIVED FROM MURINE EMBRYONIC STEM CELLS, Michael G. Klug and Loren J. Field, Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, IN 46202

The temporal aspects of DNA synthesis and nucleation in cardiomyocytes derived from murine embryonic stem cells (ES) was examined. Cardiomyocytes, as well as other cell types, develop when pluripotent ES cells are grown in suspension culture to form embryoid bodies (EBs). These cardiomyocytes form regions of rhythmic, spontaneous contractile activity; additionally, the mRNA and protein levels of several markers have been shown to parallel that of embryonic development. When allowed to attach, EBs spread out and contain sheets of beating cells. Areas with concentrated regions of cardiomyocytes were isolated by dissection, dissociated and grown on coated slides. On separate days the cells were given a short (2 hour) tritiated thymidine pulse, washed, then fixed. Cardiomyocytes were identified on autoradiograms by immunostaining with an antibody to sarcomeric myosin (MF-20) and/or the use of a clone of ES cells containing a construct which directs cardiac specific expression of ß-galactosidase to the nucleus. Analyses indicate that initially about 10% of the cardiomyocytes synthesize DNA and about 95% of the cells are mononucleated. At later time points, the percentage of DNA synthetic cells decreases and the percentage of multinucleated cells increases. From in vivo studies, it is known that the embryonic heart grows by hyperplasia. In contrast, the adult mammalian heart grows only by hypertrophy, accompanied by increases in nuclear content. Although proliferation was not directly addressed by these experiments, the presence of multinucleation furthers the tenet that the development of ES derived cardiomyocytes parallels, at least in some aspects, in vivo development. Therefore this system should be useful in study of terminal differentiation of cardiomyocytes with the intent of establishing a molecular platform for the treatment of myocardial infarction.

PZ 109 LARGE-SCALE CULTIVATION OF MURINE BONE MARROW: THE EFFECTS OF INOCULUM DENSITY AND CELL TYPE ON THE PRODUCTIVITY OF CELLS GROWN IN AN AIRLIFT PACKED BED BIOREACTOR, Dhinakar S. Kompala and John G. Highfill, Department of Chemical Engineering, University of Colorado-Boulder, Boulder, CO 80309-0424

The *ex vivo* expansion of bone marrow cells has many important applications for treatment of various types of cancer including breast cancer, leukemia, and bone marrow cancer. However, large-scale production of hematopoietic precursor cells has not been accomplished. This work utilizes a fiberglass fiber matrix for the attachment and growth of stromal cells. The presence of stromal cells in sufficient concentration is required for the initiation and maintenance of hematopoiesis in the absence of exogenously added growth factors and cytokines. This work demonstrates the effects of inoculum density and cell type on the productivity of cells grown in large-scale culture operations. Glucose uptake and analysis of suspended cells are used to determine reactor productivity.

PZ 110 BIOHYBRID ISLET-GLAND EQUIVALENT FOR TRANSPLANTATION, Chao-Ying Kuo, George A. Burghen, and Henry G. Herrod, Department of Pediatrics, College of Medicine, The University of Tennessee, Memphis, Memphis, TN 38163.

A three-dimensional (3-D) culture system was constructed for development of islet gland-like tissue for transplantation. Islets of Langerhan were isolated from Wistar Furth rats by the established collagenase digestion procedure followed by Ficoll gradient separation. Rat small intestinal submucosa (SIS) was prepared in accordance with the method of Sandusky et al (American J. Pathology 140:317-324, 1992). Expanded polytetrafluoethylene fiber and Matrigel were added to support the islets inside the SIS-pocket. The 3-D islet gland-like tissue was cultured in a medium containing equal parts of CMRL-1066 and dMEM with 10% FCS and 1% Antibiotics-Antimycotics (Gibco, Grand Island, NY). A week later, the 3-D biohybrid islet gland equivalent were; (1) fixed for histological studies, (2) tested, in vitro, for insulin secretion and (3) transplanted into STZ-induced diabetic mice.

The islets that grew inside the SIS-pocket preserved their 3-D morphology as confirmed by histological section studies. The islet cells fused with the SIS and there was no evidence of cell death. Very few fibroblasts were present in the area of SIS-islet contact. The amount of insulin released/secreted by the islets into culture medium was fairly constant in a given biohybrid between 24h - 96 h of culture. 214, 250, 53 and 10 ng/hr of insulin were released from 4 separate islet-SIS grafts. In transplantation studies, one islet-SIS graft reversed serum glucose in a mouse from 600 to 35 mg/dl blood glucose within 24 hours. In two other transplants, one produced complete normalization of blood glucose and the other achieved partial normalization. These islet-SIS grafts remained functional for a week with cyclosporin A (CsA) treatment of recipient mice. The hyperglycemia returned within a week after CsA was withdrawn.

In conclusion, SIS appears to be a suitable extracellular matrix for in vitro engineering of islets into a gland-like tissue. This islet-gland equivalent may be a viable model for preparation of islet implants for future transplantation. (This study is supported in part by the Crippled Children's Hospital Foundation of Memphis).

PZ 111 GRANULATION TISSUE CONTAINS CELLS CAPABLE OF DIFFERENTIATING INTO MULTIPLE MESENCHYMAL PHENOTYPES, Paul A. Lucas, Andrew F. Calcutt, Paul Ossi, Henry E. Young, and Sheila S. Southerland, Department of Surgery, Mercer University School of Medicine at the Medical Center of Central Georgia, Macon, GA 31201

We have isolated cells from adult rat skeletal muscle capable of differentiating into a number of mesenchymal phenotypes when treated with a non-specific differentiating agent such as dexamethasone. We have have postulated they may be present in granulation tissue. Stainless steel wound chambers were implanted subcutaneously into 7 week old male rats and removed 7, 14, or 28 days post-implantation and scraped of adhering tissue. The cells were isolated by enzymatic digestion, cultured in media with pre-selected horse serum until confluent, then trypsinized and frozen in 7.5% DMSO at -80°C . The cells were thawed and cultured in the same media supplemented with 10^{-6} to 10^{-10}M dexamethasone. Cells from all time points behaved similarly in culture. Control cultures contained cells with a stellate morphology with no definite phenotype. Upon treatment with dexamethasone, the following phenotypes were observed: skeletal myotubes (long multinucelated cells that stain with an antibody to myosin), cartilage (nodules of cells stained with Alcian blue, pH 1.0), bone (round cells stained with Von Kossa's stain for mineral), adipocytes (Sudan black B staining), smooth muscle (mononucleated cells stained with an antibody to smooth muscle α-actin), endothelial cells (uptake of di-acyl LDL) and fibroblasts (spindle-shaped fibrillar cells). These results point to the presence of a population of pluripotent mesenchymal stem cells in granulation tissue not limited to differentiation into fibroblasts. If they can be appropriately manipulated, actual tissue regeneration could be achieved as opposed to the formation of scar tissue. Supported by funds from the Medcen Foundation

PZ 112 THE USE OF HUMAN EPIDERMAL MODELS AS TOOLS TO UNDERSTAND THE ACTION OF SULFUR MUSTARD, Robert G. Van Buskirk, Jeffery R. Cook, and Laura S. Rhoads, Department of Biological Sciences, State University of New York, Binghamton, NY 13902-6000.

Synthetic human epidermal models are being used as in vitro alternatives for identifying topical skin protectants which might protect human skin when exposed to sulfur mustard. Normal Human Epidermal Keratinocytes were grown on collagen-coated microporous membranes and stratified for two weeks. This epidermal model exhibited ultrastructural and biochemical hallmarks of epidermal differentiation including desmosomes, laminin synthesis and a basal lamina with associated hemidesmosomes. Some of these differentiation markers were differentially expressed depending on the matrix form of collagen (i.e gel, dry film, etc.) used as the substrate. EpiDerm (MatTek Corporation, Ashland, MA), a commercially available synthetic human epidermis resembling this model, was exposed to a mustard simulant, 2-chloroethylethylsulfide (CEES) for 2hrs and its viability assayed at multiple times using the reduction/oxidation indicator dye, Alamar Blue. Data indicate that 2 days were necessary for 8.0 and 0.8 mM CEES to compromise viability, whereas only 2 hrs was necessary for 80 mM CEES to cause a cytotoxic effect of similar magnitude. Separation of the EpiDerm from its underlying substrate, a process resembling microvesication which occurs in human skin exposed to mustard, occurred only with 8.0 and 0.8 mM CEES exposure. We conclude that EpiDerm responds to CEES in a manner consistent with in vivo epidermis. Furthermore, the fact that EpiDerm lacks a thick collagen dermal equivalent allows the possibility of interceding with protective agents at the epidermis/dermis interface, the major site of CEES cytotoxicity, so as to determine the mechanisms underlying mustard toxicity. This human epidermal model, coupled with fluorescent indicator dyes, are valuable tools for identifying topical skin protectants.

PZ 113 KINETICS OF CHONDROCYTE GROWTH IN CELL-POLYMER CARTILAGE IMPLANTS, Gordana Vunjak-Novakovic, Lisa E. Freed, John C. Marquis and Robert Langer, Whitaker College of Health Sciences and Technology and Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139.

Cartilage implants based on isolated cells and polymer scaffolds need to have high cell densities for differentiated cell function (e.g., glycosaminoglycan, GAG, production), and high GAG contents for biomechanical function. The kinetics of chondocyte growth within polymer scaffolds and alginate beads was studied in order to optimize implant culture conditions. In static cultures, cell growth rates decreased as either implant thickness or implant cell density was increased; cell growth stopped at 3-4 weeks, and implant cell density remained low. In well mixed cultures, high cell growth rates were maintained over 7 weeks, and implant cell density reached that of normal cartilage. The permeability of cell-polymer implants to glucose was only 3% that of the plain polymer scaffold after 4 weeks of *in vitro* cell culture. These findings imply that: 1) implant growth is diffusionally limited, due to the combined effects of high nutrient requirements and low effective porosity, and 2) optimization of the tissue culture environment is thus essential in order to cultivate clinically useful cartilage implants *in vitro*.

L.E. Freed, G. Vunjak-Novakovic, J. C. Marquis, and R. Langer: Kinetics of Chondrocyte Growth in Cell-Polymer Implants, *Biotechnol. & Bioeng.* 1994, Vol. 43 (in press).

PZ 114 EMBRYONIC DEVELOPMENT OF THE PANCREAS
Lisa-Anne Whittemore, Prajna Panda, Jane Aghajanian, and Gordon Wong

We have been studying embryonic development of the pancreas with the hope that a better understanding of the regulatory factors and cell lineages involved in pancreas development will lead to novel approaches for the treatment of diabetes. We have been using an *in vitro* system modelled after that of Rutter et al.*, in which pancreatic epithelial buds from E12 rat embryos are cultured in the absence or presence of mesenchyme. Morphogenesis of the pancreatic epithelium, as well as cytodifferentiation of exocrine cells, depends on the presence of mesenchyme.

We have now generated monoclonal antibodies to developing (E12) rat pancreas and hope to identify antigens which will serve as markers for specific cell lineages in the developing pancreas. One of the antibodies specifically labels the apical surface of epithelium. We have seen labelling of epithelium from pancreas, intestine, trachea, and nasal pharynx in E14 rat embryos. Interestingly, this antibody also labels the floor plate of neural tube, notochord, and nerve fibers in E14 rat embryos.

In addition to using these monoclonal antibodies as markers, we are investigating their ability to block pancreas development *in vitro*. Such an approach may lead to the identification of factors involved in pancreas development.

* Rutter, W. J., Wessels, N. K. and Grobstein, C. 1964. Control of specific synthesis in the developing pancreas, *J. Natl. Cancer Inst. Monogr.* 13: 51-70.

Musculoskeletal System

PZ 200 ALGINATE AS AN ARTIFICIAL MATRIX FOR ARTICULAR CHONDROCYTES, Hilary P. Benton and Grace A. Loredo, Department of VM Anatomy, Physiology and Cell Biology, Haring Hall, University of California, Davis, Ca 95616.

Alginate, a derivative of seaweed, has been successfully used as a matrix to entrap differentiated chondrocytes and allow longterm culture of cells capable of producing cartilage matrix molecules (1,2). Culture in alginate gels has several advantages, most importantly temperature independence, longterm stability and reversibility. Alginate polymerizes to form a gel in the presence of multivalent cations, the final consistency of the gel being determined by the ratio of guluronic acid to mannuronic acid. Alginates with a high guluronic acid content produce strong brittle gels that are more heat stable, while high mannuronic acid content produces gels that are weaker and more elastic and show less heat stability. By addition of a chelating agent, such as sodium citrate, the gelation reaction is fully reversible, allowing easy recovery of cells from the matrix. We have compared various alginate preparations with different guluronic/mannuronic acid ratios for their suitability as matrices for articular chondrocytes. We found Keltone HV, an alginate with longer polymer chains, to have advantages over Keltone LV, the alginate used in previously published studies. Alginate has potential for artificial cartilage engineering, not only as a means of immobilizing chondrocytes but also as a potential slow-release delivery system for chondrogenic or matrix stimulatory factors. 1) Guo et al, Conn. Tiss. Res. 19: 277-297, 1989. 2) Hauselmann et al, Matrix 12: 116-129, 1992

PZ 201 TISSUE ENGINEERING OF THE ANTERIOR CRUCIATE LIGAMENT (ACL): COMPARATIVE CONTRACTILE PROPERTIES OF FIBROBLASTS, Charlotte Caron, Lucie Germain, Francine Goulet, François A. Auger, Laboratoire d'organogénèse expérimentale (LOEX), Université Laval et Hôpital Saint-Sacrement, 1050 chemin Ste-Foy, Québec, Canada, G1S 4L8.

The ACL is undoubtedly a very important ligament and its rupture has dire consequences. Inert biomaterial is an effective immediate method of surgical repair but implant fatigue and rupture are well described. Transposition of ligament surgery is also a well known therapeutic option but secondary degenerative knee disease frequently ensues. Thus we have devised a tissue engineering method for the reconstruction of the ACL. Fibroblasts were isolated from ligaments cut into 0,1 cm^2 pieces and digested according to a sequential collagenase (0,125%)-trypsin (0,1%) method. The dermal fibroblasts were obtained in a similar protocol for skin (1). Two human partially demineralized bones were used as anchorage while a collagen type I solution (2 mg/ml) with 20×10^6 fibroblasts was poured in between. These ACL equivalents were cultured with or without slight tensile charges (1-10 g). Contraction kinetics of various floating ACL equivalents were recorded. Histological and indirect immunofluorescence analysis (type I and III collagens, elastin) were performed. Contraction curves of ACL fibroblasts had a more gentle and regular slope than dermal fibroblasts. Histological analysis revealed a better organisation of collagen fibers and fibroblasts for the ACL equivalent with tensile charges. The junction of the ACL equivalent with spongious bone showed excellent collagenous /cell material penetration. The immunofluorescence analysis showed presence of high amounts of elastin all along the ACL equivalent but type III collagen was noted mostly at the anchorage level. In conclusion the tissue engineered ACL may eventually be a therapeutic option but it can already serve as a model for pharmacological or physiological studies.

1. Germain L. et al, *Burns* (1993) **19**(2), 99-104

PZ 202 RECOMBINANT HUMAN BONE MORPHOGENETIC PROTEIN-2 (rhBMP-2) INDUCES SEVERAL MESENCHYMAL PHENOTYPES IN CULTURE, Martin L. Dalton, Karen Dixon, and Paul A. Lucas, Depatment of Surgery, Mercer University School of Medicine at the Medical Center of Central Georgia, Macon, GA 31201

rhBMP-2 has been repeatedly shown to induce the de novo formation of cartilage and bone in vivo in a cascade reminiscent of endochondral bone formation. Recently, we have discovered a resident pluripotent population of cells in adult rats capable of differentiating into multiple mesenchymal phenotypes and have shown that Swiss 3T3 cells are also pluripotent in culture. We have termed these cells mesenchymal stem cells (MSCs) and have tested rhBMP-2 for its inductive activty on these cells. Rat mesechymal stem cells (rMSCs) and Swiss 3T3 cells were plated at 5 or 10,000 cells/16 mm culture well and cultured in EMEM + 10% pre-selected horse serum and rhBMP-2 in a dosage range of 0 - 100 ng/ml for up to 4 weeks. Control cultures maintained their mesenchymal phenotype and did not exhibit discernible phenotypes. However, cultures treated with rhBMP-2 at doses of 5 - 100 ng/ml exhibited adipocytes (Sudan black B), cartilage nodules (Alcian blue, pH 1.0), mineralized tissue (Von Kossa's), and skeletal muscle myotubes (multinucleated, spontaneously contracting cells that stain with an antibody to skeletal muscle myosin). While cartilage was predominant, it was not overwhelmingly so. It appears that either rhBMP-2 has a broad spectrum of inductive activiy or that it is cross-reacting with receptors for more specific inductive factors for phenotypes other than cartilage.

Supported by grants from the Medcen Foundation; rhBMP-2 was a generous gift of Genetics Institute

PZ 203 IN VIVO METHOD FOR MEASURING STRAIN IN TENDON GRAFTS, Jack C. Debes, Michael C. Hiles, Klod Kokini, Stephen F. Badylak, Hillenbrand Biomedical Engineering Center, Purdue University, West Lafayette, IN 47907-1293

Rehabilitation techniques serve to control and regulate the motion of a joint, which in turn affects the *level of strain in the tendons and/or ligaments*. A hypothesis is made that the levels of strain in a tendon or ligament graft during healing influence the growth and remodeling of the graft. In order to test this hypothesis, a technique has been developed for the *in vivo* measurement of strain in an Achilles tendon graft. The method presented here makes use of the ultrasound transit-time (USTT) technique. We performed the USTT on a unique xenogeneic Achilles tendon graft in dogs using the commercially available Sonomicrometer® (Triton Technology, San Diego, CA.). The Sonomicrometer measures the distance between two points in soft tissue by measuring the time it takes for a sound burst to travel from one point to another. The output is an analog voltage proportional to the distance between the two piezo electric crystals - one is a transmitter and the other is a receiver. The calibration is developed from a stable quartz crystal oscillator with a period of oscillation equal to the time it takes sound to travel 1 mm in myocardium. The grafts tested in this study were composed of porcine small intestinal submucosa (SIS). This unique xenogeneic graft material has been studied extensively in our laboratory for vascular, orthopedic, and other connective tissue applications. In order to correct for any possible difference in the speed of sound in myocardium and that in our SIS Achilles tendon grafts, we calibrated the Sonomicrometer against radiographic images of the spacing between the piezo electric crystals. Two dogs were implanted with SIS Achilles tendon grafts containing Sonomicrometer crystals for periods of up to 15 days. Radiographic calibration data of the Sonomicrometer output signal was recorded immediately after implant and then periodically prior to each *in vivo* recording session. The radiographic calibration plots compared with the internal calibration of the device based on the speed of sound in myocardium indicate a small but significant difference. The Sonomicrometer output signal was recorded periodically in anesthetized animals during passive motion of the joint. The data appears relatively noise free and tracks with the flexion of the joint during both cyclic and step change motions. *In vitro* tests of the load vs. elongation relationship performed prior to implant and post-mortem indicate a dramatic decrease in effective compliance of the graft during the first 17 days of remodeling. This technique serves as a valuable method for measurement of *in vivo* strain in various graft applications in order to determine the relationship between strain and remodeling.

PZ 204 FORMATION OF CARTILAGENOUS TISSUE IN VITRO, Kandel R, Cruz T, Boyle J, Speagle M, Luan B, Connective Tissue Research Group, Mt. Sinai Hospital, Toronto, CAN M5G 1X5

A cell culture method has been optimized in which chondrocytes accumulate extracellular matrix. In this study, we compare the cartilagenous tissue formed in culture to bovine articular cartilage. Chondrocytes were obtained from bovine articular cartilage by sequential enzyme digestion and the cells plated as a monolayer at a density of at least 1 x $10^6/cm^2$ on Millicell CM^R filters precoated with collagen. The cultures were maintained in Ham's F12 with up to 20% fetal bovine serum. After one week, ascorbic acid (50 or 100 µg/ml) was added with each media change. The cultures were harvested at various times up to 35 days. The cultures and representative pieces of bovine cartilage were paraffin embedded for histological and morphometric assessment using the Bioquant Image Analysis system. The glycosaminoglycan content of the matrix was measured following papain digestion using the dimethylmethylene blue dye binding assay and spectrophotometry. To examine collagen synthesis, the cultures were incubated with [^{14}C]proline for 24 hrs followed by pepsin digestion. The extract was separated by SDS-PAGE and processed for autoradiography or transferred for Western blot analysis using antibodies reactive with Type II or Type I collagen (Southern BioTech Associates,AL). Proteoglycan synthesis was analyzed by incubating the cultures with [$^{35}SO_4$] for 24hrs followed by extraction with 4M guanidine HCl. The proteoglycans were examined by horizontal agarose gel electrophoresis. Bovine cartilage, incubated with [$^{35}SO_4$] for 24hrs, was extracted similarly to the cultures.

The cells plated as a monolayer became multilayered and accumulated extracellular matrix which appeared histologically similar to articular cartilage. By 21 days, the superficial cells were flattened and the deeper cells were spherical. The distribution of collagen fibers, as demonstrated by picrosirius staining showed that the collagen in the superficial zone was organized parallel to the surface whereas collagen surrounded the chondrocytes in the deeper zone. The thickness of the cultures was dependant on the cell density at plating and ranged from 34±2 to 186 ±2 um (X±SD). The chondrocytes were synthesizing and incorporating large proteoglycans and type II collagen, the major macromolecules of cartilage. No Type I collagen was identified. When compared to articular cartilage, the in vitro tissue at 35 days was more cellular (26% vs 8%). The GAG content/unit area when corrected for cellularity was similar between the two tissues. In conclusion, chondrocytes grown on filters in culture generate cartilage tissue which could potentially be transplanted into damaged joints.

PZ 205 CYTOSKELETAL CONTROL OF ACTIVE TENSION AND DYNAMIC STIFFNESS OF AN *IN VITRO* CONNECTIVE TISSUE EQUIVALENT, Michael S. Kolodney and Elliot L. Elson, Dept. of Biochm. & Mol. Biophys., Washington U. Schl. of Med., St. Louis, MO 63110

Forces generated by fibroblasts are believed to contribute to the remodeling and morphogenesis of connective tissue. The fibroblast populated collagen lattice (FPCL), formed by contractile cells embedded in a collagen gel, provides an *in vitro* model for connective tissue morphogenesis. We have developed a technique to investigate determinants of the active and passive mechanical properties of the FPCL. One edge of the FPCL is connected to an isometric force transducer; the opposite edge is fixed to a feedback controlled piezoelectric motor, which provides a small (<0.5%) lengthening strain at a prescribed frequency. Active force generated by the cells populating the collagen lattice, the tissue's dynamic stiffness, and length-tension relationship were measured. At lengths less than about 110-120% of the tissue's original length, most tension was mechanically in series with the cells. Stimulation with fetal bovine serum (FBS) resulted in myosin activation through phosphorylation of the myosin regulatory light chain as well as 2-3-fold increases both in isometric force and tissue stiffness. Disruption of cytoskeletal actin by cytochalasin D reduced force to nearly zero and caused a 5-10-fold decrease in tissue stiffness, which correlated with the decline in force. Tissue stiffness was linearly proportional to cell genereated force during treatment with FBS or cytochalasin D. Tissues stimulated to contract with FBS were 2-3-fold stiffer at 1 Hz than at 0.01 Hz. The tissue's resistance to deformation had a more viscous character at lower frequencies. These results indicate that the cytoskeletal tension generated by cellular actin and myosin can dominate the mechanical properties of a FPCL.

PZ 206 TOTAL REPAIR OF RAT NASAL BONE DEFECTS USING TYPE I COLLAGEN GELS, Thomas M. Sweeney, William H. Lindsey and Roy C. Ogle, Departments of Orthopaedics and Otolaryngology, Univ. of VA Medical Center, Charlottesville, VA 22908

The goal of this research is to improve management of extensive bone defects by developing a broadly applicable, osteogenic, graft for use as an alternative to current bone grafting methods. Inadequate quantity and potential for morbidity frequently limits the utility of autografts. Allogenic bone is widely available but, carries an increased incidence of non-unions, fatigue fracture, rejection, and risk of infection when compared to autogenous bone. Previously, native, neutral pH, isotonic gels of type I collagen were shown to mediate total repair of critical size rat calvarial defects through intramembranous ossification. A rat nasal bone defect model was developed to test whether type I collagen gel implants will be broadly applicable to sites which develop through endochondral ossification. Sixteen, 6 month old, Sprague-Dawley rats were randomly assigned to two groups of eight animals. The nasal bones were entirely excised using a drill. The mean defect size was 0.49cm^2 (±0.04SD) in controls which received no implants. The others were implanted with 200µl gels of type I collagen in 0.52cm^2 (±0.05) defects. The rats were sacrificed after one month and the defects were photographed and measured by planimetry. The defects were then excised, fixed, decalcified, embedded, sectioned, and stained with H and E. Total repair of all defects was observed in animals treated with type I collagen gels. Control animals showed 5.7% (±3.7) healing by area. Histology revealed restoration of the anatomy with a thin plate of immature bone spanning the defect in continuity with the cartilage of the nasal septum. Native, isotonic, neutral pH, space filling collagen gels apparently provide a favorable environment for osteoconduction in bone defects. Recent observations that collagen binds growth factors of the transforming growth factor family and bone morphogenetic proteins, suggests that type I collagen gels may facilitate osteoinduction as well. Type I collagen gel may bind peptide growth factors released at the wound site which subsequently stimulate migration of osteoprogenitors and osteogenic differentiation. Collagen gels may be an excellent vehicle for combination with growth factors or transplanted osteoblasts to further enhance bone formation in facial, calvarial and long bone defects.

PZ 207 TYPE X COLLAGEN EXPRESSION BY PERIOSTEUM. R.E. Topping, P.C. Anderson and G. Balian, Univ. of Virginia School of Medicine, Department of Orthopaedics, Charlottesville, VA 22908.

Periosteum has been shown to have osteogenic as well as chondrogenic potential both in vivo and in vitro. Periosteal grafts have been used to repair defects in articular cartilage and in cortical bone. To better understand the repair process in cartilage and bone, we incubated periosteum in vitro and studied the expression of type X collagen which is synthesized by hypertrophic chondrocytes during endochondral ossification. Periosteal explants (2 x 3mm) were dissected from the tibias and femurs of Sprague Dawley rats, suspended in agarose and collected at weekly intervals for histological examination and immunolocalization of type X collagen. Prior to incubation, the tissue was fibrous, with random areas of cells that stained intracellularly for type X. The incubated tissue consisted of enlarged cells which were surrounded by cartilaginous matrix. At 2 weeks, clusters of several cells stained positively for type X intracellularly. By 5 weeks the chondrocytes appeared more hypertrophic and their matrix was intensely positive for type X. Periosteum explants both prior to incubation and after 5 weeks were incubated in organ culture with ^{14}C-proline; proteins were extracted, treated with pepsin and analyzed by SDS-PAGE and fluorography. Type X collagen synthesis was detected at both time points. Type X collagen expression precedes the process of mineralization of cartilage during its transition to bone. Chondrocytes in articular cartilage adjacent to the tidemark with subchondral bone and the hypertrophic chondrocytes in soft fracture callus synthesize type X collagen. The finding of type X expression by periosteum and its extracellular localization after incubation in culture suggests that this may be a model that could be used to improve cartilage surfacing or fracture healing with periosteal grafts.

PZ 208 LOADING TENDON EXPLANTS STIMULATES FIBROBLAST PRODUCTION OF CYCLIC ADENOSINE MONOPHOSPHATE,
R. Vanderby Jr, M.E. Cooke, R.J. Thielke, and B.K. Graf, Orthopedic Surgery, University of Wisconsin, Madison, WI 53792

Unloading tendons can reduce their strength and stiffness. Conversely, exercise can cause increases. Since these mechano-adaptive processes are critical for treating injuries, they have been studied with cultured fibroblasts. These methods remove cells from their matrix, making any mechanical perturbations hard to interpret. The purpose of this study was to investigate the effect of tissue loading on fibroblasts that are still in their extracellular matrix, using cyclic adenosine monophosphate (c-AMP) as a measure of cellular stimulation.

Twentyone specimens, harvested from the patellar tendons of 2, 6 month old rabbits, were dissected into subunits of approximately 0.3 mm^2 in cross section by 10.0 mm in length. Subunits were gripped and loaded in specially designed culture wells filled with McCoy's media. The specimens were divided into 4 groups; 5 subunits were subjected to 100 cycles of sinusoidal tensile strain with amplitude of 1.5% (mid-substance surface) strain at 5 Hz, 6 subunits received 100 cycles at 6% strain, 5 subunits were not loaded and 5 were treated with forskolin and not loaded. The forskolin released all c-AMP, therefore serving as a standard. Five minutes prior to loading, 3-isobutyl-1-methylxanthine was added to the media as an inhibitor of c-AMP phosphodiesterase. Levels of c-AMP were measured using a radio-immunoassay kit and expressed per wet weight of each specimen, normalized by forskolin controls. Groups were compared using an ANOVA with Tukey's post hoc t-test.

The loading at the highest strain level (6%) produced a greater amount of c-AMP/mg than the low (1.5%) level ($p=0.06$) and than the unloaded controls ($p=0.06$). The c-AMP of the low level strain was not different from the unloaded specimens. The forskolin specimens exhibited a level of c-AMP much higher than any of the other groups.

Although the number of specimens are small (n=5 or 6 per group), we observed a strong trend ($p=0.06$) suggesting that a strain of 6% stimulates a cellular response. Also, these preliminary data suggest that there may be a threshhold for cellular stimulation, since no response was observed for a cyclic strain of 1.5%.

Vascular System; Biomaterials

PZ 300 ON THE CONDITIONS WHICH PROMOTE ENDOTHELIALIZATION OF DERIVATIZED SUBSTRATES
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In addition to adhesion, design of novel biomaterials for vascular implantation can and should promote subsequent cellular functions (such as motility and proliferation) for enhanced endothelialization of prostheses.

The present *in vitro* study was an investigation of bovine pulmonary artery (BPAE) and human umbilical vein (HUVEC) endothelial cell adhesion, proliferation, and motility on model surfaces (borosilicate glass) modified with the covalently-bound [1,2], bioactive adhesive peptide sequence Tyrosine - Isoleucine - Glycine - Serine - Arginine - Glycine (YIGSRG) from laminin [3], in the presence and absence of soluble basic fibroblast growth factor (FGF) and soluble transforming growth factor-beta (TGF-β).

There were no significant differences in BPAE or HUVEC adhesion on plain (reference material) glass and on glass modified with YIGSRG. In contrast, BPAE and HUVEC cell proliferation and motility over a 72 hour period were both affected by the presence of FGF. The highest BPAE cell densities were observed in the presence of 1 ng/ml FGF on substrates modified with YIGSRG; this result was statistically significant ($p < 0.05$) compared to all other proliferation and motility conditions tested. BPAE and HUVEC proliferation and motility were not significantly affected by the presence of 0.1 ng/ml TGF-β.

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PZ 301 MECHANICAL PROPERTIES OF XENOGENEIC SMALL-INTESTINAL SUBMUCOSA WHEN USED AS AN AORTIC GRAFT IN THE DOG,
Michael C. Hiles, Stephen F. Badylak, Gary C. Lantz, Klod Kokini, Leslie A. Geddes, and Robert J. Morff, Hillenbrand Biomedical Engineering Center, Purdue University, West Lafayette, IN 47907

Small-intestinal submucosa (SIS) has been shown to induce tissue remodeling *in vivo* when used as a vascular graft. The present study investigated the physical and mechanical properties of remodelled aortic grafts derived from xenogeneic SIS material. Eight infrarenal aortic grafts were implanted in mongrel dogs. The grafts were explanted at 1 or 2 months and tested for compliance and hoop mechanical properties. The morphologic changes within the grafts were also characterized. The remodelling produced graft structures which were significantly stronger than both the normal artery ($P=0.012$) and the original SIS graft ($P=0.0001$), and the compliance of these structures was one third that of normal artery and more like that of the SIS grafts. The remodelled grafts were greater than 10 times the thickness of the implanted SIS. Immunohistochemical analysis of remodelled tissues suggest that the SIS material is degraded and resorbed over time. The remodeling process transformed a material which was physically and mechanically quite different from normal aorta into a blood conduit which was physically and mechanically similar to artery.

PZ 302 ELECTROMAGNETIC INTERACTION OF ENDOTHELIAL CELLS, Jongwon Kim, Jin H. Kim, Jun K. Chang, Dong-han Yoon*, and Byoung G. Min, Dept. of Biomedical Eng., Seoul National University, Seoul, 110-744 and *Dept. of Electronic Eng., Kum Oh National University of Technology, Gumi, Kyoungbok, KOREA

The growth of the cells is strongly dependent on its environmental conditions such as substrate, media fluid dynamics and other source of stresses. Pulsed electromagnetic field (PEMF) is also considered as one of the interactive source. In order to investigate the effect of the PEMFs on the human umbilical vein endothelial cell (HUVEC) growth, we have performed the HUVEC culture on the PEMF generating device which was developed especially to give rectangular shape and adjustable frequency and magnitude. We seeded HUVECs on two 24-well culture plates (Falcon®) and placed one of them on the PEMF generating device. Both of the culture plates were located inside CO_2 incubator. The complete medium composed of M 199 medium, 20 % fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l glutamine was changed every two days. The number of HUVECs was counted by Coulter counter and synthesized DNA amount was monitored every day until fourth day after seeding. Two levels of PEMF (3 and 10 gauss) were applied at 50 Hz. The shape change of cultured HUVECs was also investigated by an image processing system. The PEMF energy was calculated from the frequency and magnitude of the PEMFs in order to analyze their effects on the growth rate and DNA synthesis of the HUVECs. The higher growth rate was observed at 3 gauss. The growth rate of the 10 gauss PEMF exposed cells was rapidly decrease at the fourth day after seeding. The shapes of the HUVECs exposed the PEMFs were different from those of the unexposed cells. We are trying to develop a new growth model of the HUVECs exposed on the PEMF energy using the Arrhenius type reaction of the activation energy.

PZ 303 STRUCTURAL ANALYSIS OF A TISSUE ENGINEERED VASCULAR EQUIVALENT: PHOTONIC AND ELECTRONIC MICROSCOPY STUDIES, Nicolas L'Heureux, Lucie Germain, Raymond Labbé, François A. Auger, Laboratoire d'Angiogénèse Expérimentale, Québec, Canada.

INTRODUCTION and METHODES:There is a definite clinical need for small vascular grafts of less than 4 mm in diameter and with good post-implantation patency. Thus, we constructed a three layer (adventitia, media and endothelium) vascular equivalent through tissue engineering. Endothelial (HUVEC) and smooth muscle (HVSMC) cells were obtained from human umbilical cords by collagenase digestion. The fibroblasts (HSF) were isolated with a collagenase-thermolysin®-trypsin method. Cell purity was established through morphological and indirect immunofluorescence methods. The construction was initiated by incorporating HVSMC ($5x10^5$ ¢/ml) into a tubular human collagen gel (3 mg/ml). After 3 to 4 days of maturation, the adventitia was added in a similar fashion: HSF were embedded in a human collagen gel to form the outer layer. A central glass cylinder insured a lumen of constant diameter throughout the following maturation process. Finally, this bi-layered construction was cannulated and seeded with HUVEC ($1x10^6$ ¢/ml).

RESULTS:The arrangement and orientation of HVSMC was quite interesting: after 48h, a complex tridimensional network of interconnecting cells was noted. Furthermore, a circular cell orientation (to the axis of the vascular equivalent) was observed, particularly at the extremities. The HUVEC showed a slightly elongated morphology with a longitudinal axis parallel to the axis of the equivalent This confluent endothelium was kept in culture at least 50 days. Ultrastructurally, HUVEC could be seen as a continuous string of well linked cell containing characteristic Weibel-Palade bodies. These cells showed a typical high nucleus/cytoplasm ratio and small projections into the collagen gel. No basement membrane like structures was deposited.

CONCLUSION:These results indicate that this vascular equivalent model may be a invaluable tool to elucidate the mechanical and intercellular phenomenons that generate vascular architecture. We are presently involved in such studies.

PZ 304 ENDOTHELIAL CELL ACTIVATION ON BIOMATERIALS. Ann E. Schmierer and Buddy D. Ratner, Center for Bioengineering, University of Washington, Seattle, Washington 98195

Cell adhesion molecules (CAMs) are responsible for many cell-cell and cell-extracellular matrix interactions. Our group is interested in the CAM expression on human endothelial cells seeded on biomaterial surfaces, particularly endothelial-leukocyte adhesion molecule-1 (ELAM-1). CAMs are responsible for the recruitment of many cell types involved in acute and chronic inflammation, and may be a key to understanding how biomaterials influence cell behavior *in vivo*. Investigators are currently trying to produce "biocompatible" vascular prostheses by lining them with endothelial cells. Unfortunately, most studies report the association of inflammatory cells, such as neutrophils, with the seeded endothelial cells. This unwanted recruitment of neutrophils could ultimately affect the number of endothelial cells retained on the vascular prostheses through endothelial cell death or detachment from the graft surface. We have utilized an anti-ELAM antibody for fluorescence cell surface staining to determine the effect of the substrate biomaterial on ELAM expression. Preliminary results show higher levels of ELAM cell surface markers for cells grown on expanded (porous) polytetrafluoroethylene (PTFE) versus the chemically identical smooth (nonporous) PTFE. This indicates some influence of substrate morphology on ELAM expression. Increased CAM expression may be the mechanism by which endothelial cells are lost from the luminal surface of seeded vascular grafts through interactions with circulating white blood cells.

PZ 305A COMPARISON OF THE HEALING CHARACTERISTICS OF A NOVEL SYNTHETIC COLLAGEN GRAFT AND E-PTFE. Paul L.Termin , Robert M. Carr, Kim D. O'Neil. Organogenesis, Canton, MA, 02021

A novel two layer small diameter vascular graft (GraftArtery) composed of bovine Type I collagen cast on a sleeve of processed porcine intestinal collagen has been fabricated. Grafts were treated with benzalkonium-Cl heparin complex. Neither of the two collagen layers were cross-linked. The grafts were implanted in the rabbit infra-renal aorta and canine femoral arteries. As part of the canine femoral artery study, a similarly configured contra-lateral reference material, e-PTFE, was implanted. Grafts were explanted from 30 to 180 days.

Histological evaluation of the synthetic collagen graft demonstrated cellular ingrowth into the graft at 30 days, with more than 90 percent of the graft collagen remodeled by 90 days; and a mature 'neo-artery' at 180 days. Host tissue bridged the anastomosis by 60 days with the anastomosis only demarcated by the non-resorbable sutures. The predominant cell type in the neo-artery was a positive g-Actin staining smooth muscle-like cell. The surface of the remodeled graft was lined by endothelial cells as demonstrated by SEM, TEM and factor VIII staining.

In contrast, at times to 180 days, no ingrowth into the e-PTFE artery was observed either across the anastomosis or along the body of the graft. Only a thin smooth muscle cell hyperplastic response was demonstrated extending from the adjacent artery a short distance on the graft's luminal surface. The graft was encapsulated by mature fibrous tissue with no evidence of cellular or tissue extension into the graft.

Conclusion: Remodeling of a novel non-crosslinked vascular graft has been demonstrated in two species. In comparison, there is no evidence of remodeling in the e-PTFE reference material.

PZ 306 SPONTANEOUS ANGIOGENESIS IN VITRO IS MEDIATED BY TYPE I COLLAGEN THAT IS SYNTHESIZED IN SITU AND REMODELED BY THE TRACTION OF ENDOTHELIAL CELLS, Robert B. Vernon, Stephanie L. Lara, M. Luisa Iruela-Arispe, John C. Angello, Thomas N. Wight and E. Helene Sage, Department of Biological Structure, University of Washington, Seattle, WA 98195

Selected populations of vascular endothelial cells, when cultured as *confluent* monolayers on tissue-culture plastic, generate planar networks of cellular cords that resemble beds of capillaries. We studied in detail (by time-lapse videomicroscopy, immunocytochemistry, and ultrastructural images) the spontaneous morphogenesis of networks by a population (Clone A) of bovine aortic endothelial cells (BAEC) to determine the mechanism by which the cells formed capillary-like patterns. We found that Clone A BAEC organized into networks in association with narrow cables that were comprised of highly-aligned fibrils of type I collagen. The collagenous cables, which originated as networks beneath the cellular monolayer, induced BAEC to organize into a corresponding network of cellular cords by a process of cellular elongation, alignment, and elevation above the monolayer. It was apparent that cellular traction was important in the formation of the collagenous networks: concentrations of cytochalasin D that inhibited the contraction of collagen gels by Clone A BAEC *in vitro* also prevented the BAEC from forming networks of cells and collagen spontaneously *in vitro*. Moreover, we observed that monolayers of BAEC cultured on sheets of highly-malleable, unorganized type I collagen generated forces of tension that reorganized the collagen fibers into networks *via* the radial expansion of small, circular defects in the collagen layer.

Collagen gel contraction assays were employed to identify factors that influenced the reorganization of collagen by BAEC traction *in vitro*. Contraction of collagen gels by BAEC was stimulated strongly by fetal calf serum, bovine serum albumin (BSA), and bovine serum fetuin, but was not stimulated by bovine serum fibronectin, ovalbumin, lactalbumin hydrolysate, or the protein analog polyvinylpyrrolidone. Contraction of gels by BAEC that was induced by BSA required protein synthesis and the activity of BAEC-derived metalloproteases.

Our observations lead us to propose that the reorganization of type I collagen into ordered structures by forces of cellular traction (under the regulation of serum-associated factors) might facilitate the migration and spatial organization of endothelial cells during angiogenesis and vasculogenesis *in vivo*.

PZ 307 CO-CULTURE OF ENDOTHELIAL CELLS WITH SMOOTH MUSCLE CELLS IN A MATRIX OF COLLAGEN: EFFECT OF FLOW ON CELL MORPHOLOGY. Thierry Ziegler and Robert M. Nerem, Biomechanics Laboratory, Georgia Institute of Technology, Atlanta, GA, USA, 30332

In vitro flow experiments on cultured endothelial cells (EC) showed less elongation and growth inhibition than what is observed *in vivo*. To evaluate the role the neighboring smooth muscle cells (SMC) play in the response of EC to flow, porcine aortic EC were cultured on top of gels of collagen type I seeded with porcine aortic SMC and then subjected to a steady, laminar shear stress. Morphology was assessed by phase-contrast and electron microscopy. Cell cytoskeleton was observed by fluorescence. The morphology of EC grown on top of a gel of collagen without SMC depended on the shape of the gel (i.e. circular or rectangular) and whether or not its borders were restrained to prevent retraction. An EC monolayer seeded on a circular gel had the typical cobblestone morphology whereas an EC monolayer on a rectangular gel showed a progressive elongation starting from the borders. Cells were oriented parallel to the borders of the gel with the cells at the center being aligned with the major axis. EC in a static co-culture, with SMC residing inside a rectangular collagen gel, were also elongated and showed a random orientation. The degree of elongation was even greater and occurred more rapidly in bound collagen gels. When EC on top of the collagen gel were subjected to shear stresses of 10 and 30 dynes/cm^2 for 24 to 48 hours, no particular re-orientation was observed. The F-actin cytoskeleton of EC in static co-cultures was composed of stress fibers aligned with the major axis of the cells, i.e. in a random manner. After exposure to shear stress, the same distribution was observed. No difference in SMC morphology and cytoskeleton was observed after exposure to shear stress. We believe that elongation of EC on top of collagen gels is induced because of the formation of stress lines inside the gel. The stress lines are created by both SMC and EC through their attachment to the collagen fibers and subsequent traction. Assuming that collagen fibers align with the direction of maximum stress, the stress lines usually occurred in a random manner in floating gels seeded with SMC which explain the observed random EC orientation. However, they were aligned with the major axis in rectangular, attached gels, and strongest when induced by SMC. Tension also induced the formation of actin stress fibers in EC in static gel cultures. Shear stress also has an observable effect on the cells by inducing a flattening of the EC. Studies are in progress to measure the effect of both co-culture and shear stress on the growth rate of EC and SMC.

PZ 308 PROTEIN ADSORPTION ON GLYCERYLPROPYLSILYL GLASS, WITH AND WITHOUT AN IMMOBILIZED LIGAND FOR $\alpha4\beta1^+$ HEMATOPOIETIC CELLS. J. R. Bain, A. S. Hoffman, and T. A. Horbett, Bioengineering, University of Washington, Seattle, WA 98195

Tissue engineers try to create materials to attract or repel specific proteins and cells. Silanized glasses have become popular model systems. Glycerylpropylsilyl glass (GPSG) is generally thought to resist protein adsorption and cell spreading, and is used as a negative control in cell-spreading experiments, as well as an immobilization support for cell-adhesion ligands. Spreading of A-375 melanoma cells on GPSG in the presence of serum led us to examine protein adsorption on this diol-rich surface. We also evaluated albumin adsorption on GPSG covalently linked to the peptide, Gly-Ile-Asp-Ala-Pro-Ser-Tyr (GIDAPSY, 10 picomoles/cm^2). GIDAPSY is based on IDAPS, a fibronectin ligand of the integrin $\alpha4\beta1$. $\alpha4\beta1$ is widespread on CD34$^+$ hematopoietic progenitors. Albumin was chosen because it is the most abundant protein in most cell-culture systems. ^{125}I-albumin was adsorbed at 0.1 mg/mL, 37 °C, pH 7.4, for 2 hrs. Contrary to expectations, GPSG adsorbed *more* albumin than glass (27.6 $\pm$ 3.1 and 17.7 $\pm$ 3.9 ng/cm^2, respectively). GIDAPSY-linked GPSG adsorbed fully half the albumin of glass, and a third as much as GPSG. Desorption studies in a pH series showed that albumin was most tightly bound to all surfaces near its isoelectric point (~4.9). Above the pI, all surfaces showed decreasing retention with increasing pH, but oligopeptide-linked GPSG retained more albumin at pH 7.4 than either GPSG or glass. Cell spreading on GPSG may result from adsorption of serum proteins. Protein adsorption on peptide-grafted surfaces needs further investigation, since adsorbed albumin can affect the binding efficiency of cell-adhesion ligands such as fibronectin.

PZ 309 PRODUCTION OF KNITTED COLLAGEN FABRICS FOR USE AS BIOLOGICAL SCAFFOLDS John F. Cavallaro and Paul D. Kemp. Organogenesis Inc., Canton, MA 02021

Tissue engineered grafts are designed not only to perform an immediate physical function, but also to guide and encourage appropriate tissue formation within the graft. Materials used to produce such remodelable grafts are the focus of a great deal of study. Biologically derived materials contain information that facilitates cell attachment and function, whereas synthetics may not interact with cells in the desired manner. By utilizing textile technology, it may be possible to mimic the natural organization of collagen fibers using reconstituted collagen threads.

Although the production of reconstituted collagen threads has been previously reported, knitting collagen fabrics requires long lengths of flexible, uniform thread. One advantage of knitting over weaving is that it incorporates knots, reducing unraveling and enhancing suture retention.

Continuous collagen threads were formed at 240 cm/min by extruding a solution of 5 mg/ml of bovine Type I tropocollagen in 8.8 mM acetic acid into a flowing coagulation bath of 20% (w/v) polyethylene glycol 8000 in phosphate buffer at pH 7.6. Residence time in this bath was 4 min. The material was dried by passing it under a stream of warm air either immediately after coagulation, or after drawing through a second bath containing distilled water. Although both threads had similar loads at break, only the rinsed thread was flexible enough to be knitted on either Raschel or Tricot knitting machines.

The fabric can be crosslinked and sterilized by traditional methods. We have produced threads with wet UTS over 100 MPa. Collagen fabric crosslinked with 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide and implanted into full thickness abdominal wounds in the rat demonstrated rapid tissue in-growth and vascularization.

PZ 310 ECM PROTEINS COUPLED TO DEVICE SURFACES IMPROVE *IN VIVO* TISSUE INTEGRATION

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For several years, it has been hypothesized that the tissue compatibility of implant devices could be improved by coating their surfaces with extracellular matrix (ECM) proteins that: 1) are less likely to be recognized as foreign (than the original device surface) and 2) promote the attachment and overgrowth of specific desirable cell types. Attempts to test this hypothesis with adsorbed ECM proteins have produced only marginal improvements. In contrast, recent studies have used photochemistry to covalently couple ECM proteins at $\geq$ monolayer levels onto three implant devices which were then implanted, evaluated histologically, and found to promote desirable tissue responses. First, a combination of fibronectin (FN) plus type IV collagen (COL IV) produced 3-fold increases in lumenal endothelial cell coverage of 4 mm ePTFE and polyurethane vascular grafts that were implanted 30 days in dog femoral arteries. Each protein immobilized individually was less effective than when immobilized in combination. Second, a coating of type I collagen was applied to a PMMA intracorneal lens and implanted in rabbit corneas for 15 months. As compared to uncoated controls, the coated lenses promoted bonding of stromal tissue, reduced inflammation adjacent to the device, and greatly reduced necrosis of corneal tissue over the device. Finally, a coating of COL IV was applied to 100 ml model silicone rubber breast implants and implanted subcutaneously in pigs for 4 months. As compared to uncoated controls, the coated model breast implants showed greater bonding of tissue to the device surface and a 48% thinner fibrous capsule. In contrast to the thinner capsules observed with COL IV immobilized alone, a combination of FN plus COL IV produced thicker capsules than uncoated controls. These implant studies show that: 1) the tissue response to implant devices can be significantly improved by covalently bonding ECM proteins to their surfaces and 2) different proteins or combinations of proteins are required for different devices.

PZ 311 **EVALUATION OF A NOVEL POLYMERIC INORGANIC MATRIX FOR USE IN TISSUE ENGINEERING APPLICATIONS**, **Mark B. Lyles, David L. Carnes, W. Elaine Hardman, Stephen B. Milam, and Ivan L. Cameron, Departments of Cellular and Structural Biology, Endodontics, and Oral and Maxillofacial Surgery, University of Texas Health Science Center, San Antonio, TX 78284**

We are investigating the development *of in vitro* tissue models for wound repair and for the construction of autologous cultured graft material for transplant. Recent investigations have tested a variety of synthetic materials for use in tissue engineering applications. We have tested these materials in an *in vitro* model utilizing several different primary and established cell lines. Human cell types including; osteoblasts, adipocytes, fibroblasts, chondrocytes, and keratinocytes, have been cultured from primary tissues taken via biopsy. Early data from cell culture indicates that cell growth and differentiation manifests itself differently depending on the material utilized. Current literature supports the hypothesis that cell orientation and adhesion may permit normal phenotypical expression of primary cells in culture. We intend to show that surface chemistry as well as cellular orientation may have a primary role in cell growth and differentiation. For example, the transformed cell line MG-63, a human osteosarcoma cell line, under certain conditions, has shown nodule formation when cultured using a specific substrate. These results support developing theories as to the optimum conditions for the construction of future biomaterials for tissue engineering applications, both *in vitro* and *in vivo*. **(Supported by NIH grant DE00152)**

PZ 312 DESIGN AND ASSEMBLY OF ARTIFICIAL PROTEIN-BASED MATERIALS, Alyssa Panitch, Maurille J. Fournier, Thomas L. Mason and David A. Tirrell, University of Massachusetts, Amherst, MA

This poster will describe the design and construction of artificial proteins of potential interest in tissue engineering applications. The design of such materials draws on ideas taken from polymer chemistry, physics and structural biology, and exploits the sequence control and chain-length uniformity provided by genetic engineering.

Synthetic Strategies. The overall synthetic scheme has been described previously, and consists of the following steps: i). synthesis, cloning, and amplification of a DNA "monomer" encoding the repeating unit of the target polymer, ii). multimerization of the monomers, iii). cloning and isolation of the individual multimers, iv). transfer of each coding sequence to an expression plasmid, and v). protein expression in *E.coli*..

Design and Synthesis of Macromolecular Crystals. Crystal growth is a process dependent both on the details of local structure and the homogeneity of the molecular population. Genetic engineering allows control of both of these aspects of macromolecular architecture. We have prepared the series of periodic copolypeptides represented by $[(AlaGly)_x L_1L_2]_n$ where x=3-6. The design relies on the known propensity of alanylglycine-rich sequences to adopt extended β-sheet-like arrangements in the solid state. The periodic residues L_1 and L_2 were chosen, by virtue of their size and/or polarity, to terminate the β-sheet structure and initiate (or accommodate) reversal of chain direction. The objective is the formation of folded-chain lamellar crystals of controlled thickness and surface functionality.

Incorporation of Non-natural Amino Acids. The 20 natural amino acids provide a broad range of chemical functionality; nevertheless, many functional groups of interest to materials science are not represented. To address this limitation, we have initiated a broadly based exploration of the prospects for *in vivo* incorporation of non-natural amino acids into artificial proteins. The approach uses a bacterial host strain that is auxotrophic for one of the 20 natural amino acids. Growth of the host on a medium supplemented with an analogue of the limiting amino acid will in some cases result in high levels of incorporation of the analogue.

Monodisperse Helical Rods. We have recently completed a synthesis of several monodisperse derivatives of poly(α,L-glutamic acid) (PLGA). The acidic side chains of PLGA can be benzylated quantitatively to produce poly(γ-benzyl glutamate) (PBLG), a widely studied rod-like polymer that forms liquid crystalline solutions and ordered monolayer films. The prospects for materials design based on monodisperse PBLG derivatives will be discussed.

PZ 313 ISOLATION & CHARACTERIZATION OF PURE POLY-N-ACETYLGLUCOSAMINE: CONTROLLED ENZYMATIC DEACETYLATION AND FORMULATION FOR TISSUE ENGINEERING APPLICATIONS, John N. Vournakis, Ernst R. Pariser, S. Finkielsztein, Steve E. Parker and Mike E. Helton, Marine Polymer Technologies, 7 Washington St., Beverly, MA 01915

A novel polysaccharide biomaterial consisting of pure poly-N-acetyl glucosamine (p-GlcNAc) fibers has been isolated and characterized. The polymer is produced under sterile and strictly controlled processing conditions; this newly developed proprietary manufacturing procedure yields a product having consistent properties, free of proteins, amino acids and inorganic impurities. The material can be formulated into many formats including membranes and sponges. Data from elemental and carbohydrate analyses indicate that the material is fully-acetylated. This finding is supported by solid-state NMR. Infra-red and NMR spectrscopy also indicate that the material has a highly ordered structure. The pure p-GlcNAc biomaterial is enzymatically deacetylated using a purified proprietary deacetylase to generate surfaces having reactive primary amines, sites at which protein and other factors can be covalently attached. Circular dichroism spectroscopy has been used to quantitate the degree of deacetylation of p-GlcNAc membranes. Both acetylated and partially deacetylated membranes and sponges are excellent support matrices for mammalian cell growth. Partially deacetylated p-GlcNAc materials having controllable rates of degradability in-vitro have been prepared. Acetylated and partially deacetylated membranes and sponges are reabsorbed in-vivo, are non-inflammatory, and have other properties making them potentially ideal delivery matrices for implantable therapeutic devices and other tissue engineering applications

PZ 314 DESIGN AND SYNTHESIS OF SEMIPERMEABLE, NON-POROUS, POLYURETHANE MEMBRANES FOR TISSUE ENGINEERING

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Traditionally, synthetic membranes developed or used in supporting cell growth have been microporous to allow for permeability of nutrients, salts, gases, and products. We have synthesized a variety of semipermeable polyurethane membranes that allow passage of nutrients, salts, and gases but are impermeable to larger molecular weight species. However, the membranes are non-porous and achieve permeability by different modes of transport than that of porous membranes. These membranes exhibit high tensile strengths. Yet unlike most microporous membranes they are elastomeric, optically transparent, heat sealable, and hydrophilic. Molecular weight cutoffs are controlled at the synthesis step instead of the polymer processing steps as in the case of microporous membranes.

We have supported growth *in vitro* of several cell lines macro-encapsulated within the nonporous membranes. These include serum-dependent cell lines where serum was only available in the external media. Pancreatic islet cells have been encapsulated in the membranes and have survived up to six months of *in vitro* culture. In animal implantation the membranes have promoted neovascularization with or without cells. In addition, explants of devices exhibited little fibrotic tissue surrounding the devices.

PZ 315 THE EFFECT OF OXIDATION STATE OF ELECTRICALLY CONDUCTING POLYMERS ON CELL SHAPE, Joyce Y. Wong[1], Robert Langer[2], and Donald E. Ingber[3], [1]Department of Materials Science and Engineering, [2]Department of Chemical Engineering, MIT, Cambridge, MA 02139, and [3]Departments of Surgery and Pathology, The Children's Hospital, Harvard Medical School, Boston, MA 02115

The study of interactions of cells and proteins with surfaces is important since most mammalian cells are anchorage-dependent and must attach to a surface in order to grow and proliferate. Electrically conducting polymers are novel in that their surface properties, including charge-density and wettability, can be reversibly changed with an applied electrical potential. Such properties might render conducting polymers unique for biological applications.

We synthesized optically-transparent thin films of polypyrrole via the electrochemical method and characterized these films in environments suitable for protein adsorption and cell culture. Polypyrrole synthesized via this method is in its oxidized state, and the neutral form of the polymer was attained via electrochemical reduction at -0.5V (vs. Ag/AgCl) in cell culture medium. Cyclic voltammetry and UV/VIS spectroelectrochemistry measurements were used to confirm the oxidation state of the polymer.

Bovine aortic endothelial cells attach and spread on fibronectin-coated oxidized polypyrrole but remain rounded on fibronectin-coated neutral polypyrrole. Since cell function has been shown to closely correlate to cell shape, electrically conducting polymers could potentially be used as a substrate material to control the function of a cell via its oxidation state.