TISSUE ENGINEERING

Organizers: Eugene Bell

April 3-10, 1992

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Keynote Address

CE 001 HOW DOES EXTRACELLULAR MATRIX REGULATE GENE EXPRESSION?: VIA INTEGRINS AND TRANSCRIPTIONALLY,
Mina J. Bissell, Lawrence Berkeley Laboratory, Berkeley, 94720

Along with the spectacular advances in our understanding of the structure of genes and their regulatory sequences, the pains-taking work of developmental and cellular biologists has determined that the microenvironment in which a cell finds itself decisively and specifically regulates the expression of tissue-specific genes. In the last decade, my laboratory has used two versatile model systems to define the important regulatory elements of such microenvironments: 1)the interaction of the extracellular matrix (ECM) with the mouse mammary epithelial cells in culture and 2)the interaction of Rous sarcoma virus with the embryonic limb *in ovo*.

While I will concentrate on the first system, the conclusions from both systems are that the microenvironment is dominant in allowing the expression of both normal and malignant phenotypes, and that extracellular matrix and the three dimensional structure of the tissues are crucial determinants of such regulations.

Our evidence indicates that the basement membrane in general, and laminin in particular, regulate the expression of β-casein gene, that the regulation is via interaction with integrins, that such regulation is transcriptional and that there is a unique ECM and prolactin response element (an enhancer) in the 5' region of the β-casein gene that requires both ECM and hormones for induction of gene expression. I will present a working model for how such elements may play a role in expression of tissue-specific genes in general.

Furthermore, the ECM selectively suppresses the expression of a number of other genes including growth factors and the ECM molecules themselves. I will discuss the importance of ECM *in vivo* and the possible relevance of these findings to malignancy and metastasis.

These studies were made possible by funding from the Office of Health and Environmental Research of the Department of Energy and by a gift for research from the Monsanto Company.

Organization of Cells into Higher Ordered Structures in Vitro

CE 002 TISSUE ENGINEERING, AN OVERVIEW: RECONSTITUTING TISSUE AND ORGAN EQUIVALENTS FOR GRAFTING AND FOR USE AS MODEL SYSTEMS, Eugene Bell, Massachusetts Institute of Technology, Cambridge, Massachusetts, 02139.

In the broadest sense tissue engineering has two goals: the first is to provide replacements for damaged, diseased or worn out parts of the body; the second is to provide histiotypic and organotypic model systems that help to enlarge understanding of tissue and organ function in health and disease. The models can be used to develop therapeutic strategies for disease management, they can also be used for diagnostic applications, for screening and for toxicity testing. The first goal can be approached by reconstituting body parts in vitro as faithfully as possible, including specialized cells, with certain restrictions governed by immunologic ground rules. But however faithful to nature the imitation may be, after implantation, vascularization and some degree of remodeling is expected to occur. Another approach is to develop acellular matrix replacements which when implanted mobilize cells from adjacent tissues and circulating cells that are induced to remodel the implant, ideally, into a functional replacement part. The development of materials designed to replace a segment of blood vessel, or of bone are examples of the foregoing strtategy. The limitation of the latter approach is that treatment of certain diseases, diabetes for example, requires restoration of a lost or impaired biosynthetic capacity and of the glucose sensing apparatus on which the fine tuning of on demand insulin secretion depends. The need here is for the right kind of cells. Thus as part of its program, tissue engineering is expected to join in the search for stem cells, to provide the banks of specialized cells required for tissue fabrication and to find ways of promoting cell survival or replacement, when the functions the cells are expected to carry out when inplanted, are the disease targets. The tissue and organ products of the second goal, model building for research, therapy, diagnosis, differentiating systems in which to grow virus and general testing, need not be subject to the same immunologic restrictions imposed on tissues and organs for grafting, since they are used only in vitro. In vitro they can be enriched with subsets of immune cells added to the parenchymal cells needed for organ specific function. Improving their resemblence to actual counterparts in nature would greatly enhance their value for most applications and certainly for studying tissue remodeling and wound healing. In discussing the goals of tissue engineering outlined above I will draw on our own work with skin, blood vessels, glands, and various other tissues as well as the work of others to illustrtate my principal points.

CE 003 Synthetic Polymers for Cell Transplantation and for Controlled Release of Drugs
by Robert Langer, Massachusetts Institute of Technology, Department of Chemical Engineering
Cambridge, MA 02139

Polymers represent the broadest class of biomaterials. By designing polymers in different ways they can be extremely useful in a variety of ways with respect to tissue engineering. One of these ways involves their use as controlled release vehicles for different growth factors, such as fibroblast growth factor or epidermal growth factor. Furthermore, polymers can be used to microencapsulate cells or to provide scaffolds to create new cellular structures. In this respect, we have focused, in our laboratory, on the use of polymer scaffolds for cartilage transplantation. The results of studies in these and related areas will be discussed.

CE 004 ENGINEERING THE ANATOMY OF ARTIFICIAL ORGANS, Malcolm S. Steinberg, Department of Molecular Biology, Princeton University, Princeton, NJ 08544.

During organogenesis, cells and tissues engage in "morphogenetic movements" by which they arrange themselves into organotypic configurations. What tells the cells how to assemble correctly? The first clues came from experiments which showed that isolated bits of early embryonic tissues, combined in vitro, rearranged to form organ-like structures and that similar structures could also be assembled by the sorting-out of the corresponding cells intermixed within a common aggregate. On the basis of experiments designed to discriminate among alternative explanations of cell sorting behavior, I formulated the "differential adhesion hypothesis" (DAH), which explained the approach toward the same anatomical configuration through either cell sorting or mutual tissue spreading as analogous to the approach toward a minimal free energy configuration by a spreading oil/water pair. If confronted as a pair of droplets, the oil (of lower surface tension) spreads over the surface of the water, while if codispersed in a single droplet, the dispersion "breaks" or sorts out, the oil again coming to occupy the external surface. For a system to show such behavior it must (1) be composed of many subunits which (2) cohere while (3) being mobile. In ordinary liquids the subunits are molecules and the mobility is thermal; in rearranging cell populations the subunits are living cells and the mobility is "amoeboid." The differences must be borne in mind but the shared properties cause tissues whose cells are mobile to behave as liquids. Many details of cell population behavior during cell sorting and tissue spreading mimic liquid behavior. According to the DAH, the equilibrium or most-stable configuration of any population of mobile, mutually adhesive cells should be specified by the volumes of the member cell populations and the intensities of the adhesions formed at the various possible cell-cell interfaces. A cell population approaches this configuration as its cells increase their areas of mutual adhesion and exchange weaker adhesions for stronger ones, and achieves it when the sum of all intercellular binding energies is maximized. Direct measurements of aggregate cohesiveness earlier confirmed that less cohesive aggregates envelop more cohesive ones but the adhesiveness between unlike cell aggregates could not be measured. Since that time, genetic engineering has made it possible to generate cell lines with predetermined adhesive properties. We have therefore set out to evaluate the DAH empirically by creating a number of such cell lines, measuring their adhesive intensities, combining their cells in known proportions, allowing the resulting masses to reach conformational equilibrium and comparing the resulting configurations with those predicted by the DAH. Our initial experiment investigates the configuration adopted when two cell populations are combined, differing only in the level of cell surface expression of a single kind of homophilic (self-recognizing) adhesion molecule. We have utilized cells of very low original mutual adhesiveness (L cells), stably transfected with cDNA inserts containing the entire coding region of P-cadherin downstream from either the ß-actin promotor, yielding higher P-cadherin expression ("PLß2 cells"), or the SV40-thymidine kinase promotor, yielding lower P-cadherin expression ("PLs5" cells), provided by M. Takeichi (Kyoto). We earlier predicted that a mere quantitative difference in adhesion site density between two cell populations should cause them to be immiscible, the cells with fewer adhesion molecules completely enveloping those with the greater number[1]. This has proved to be the case. In aggregates containing mixed PLß2 and PLs5 cells, distinguished by di-I fluorescent labeling, the two cell populations sorted out, the more cohesive PLß2 cells forming internal islands which coalesced to form a spheroid totally surrounded by the less cohesive PLs5 cells. When pure PLß2 and PLs5 cell aggregates were fused, the latter spread over and completely enveloped the former. Two important conclusions from these results are that not only can two cell populations be rendered immiscible by a merely quantitative difference in the expression of a single kind of adhesion system, but that a specific anatomical configuration -- a more cohesive medulla surrounded by a less cohesive cortex -- is thereby also determined. Supported by NIH grant CA13605.

1. Steinberg, M.S. (1964) The problem of adhesive selectivity in cellular interactions. In "Cellular Membranes in Development" (M. Locke, ed.), Academic Press, New York pp. 321-366.

Cell Matrix Interactions in Development (Joint)

CE 005 SYNDECAN, THE PROTOTYPE OF A FAMILY OF INTEGRAL MEMBRANE PROTEOGLYCANS, ACTS AS A HIGHLY REGULATED "RECEPTOR PARTNER", Merton Bernfield, Harvard Medical School, Boston, MA 02115

All adherent vertebrate cells have heparan sulfate, a heparin-like glycosaminoglycan (GAG), at their surfaces. These GAG can bind a wide variety of components in a cell's microenvironment, including extracellular matrix, growth factors, degradative enzymes and proteinase inhibitors. These components change during embryonic development and tumor invasion and can influence the adhesion, shape, growth, and differentiation of cells. Indeed, adding heparin to model systems developing in culture modifies their morphogenesis. Therefore, we hypothesized that changes in the amount and type of cell surface heparan sulfate could control the influence of these components during morphogenesis and tumorigenesis. A major source of cell surface heparan sulfate is an integral membrane heparan sulfate-containing proteoglycan, syndecan (from the Greek, *syndein*, to bind together), that is the prototype of a proteoglycan gene family. The expression of syndecan in embryos and neoplastically transformed cells is highly regulated. Syndecan appears soon after fertilization and localizes to the cells that will form the embryo. It is lost from the anterior mesenchyme following gastrulation and its subsequent expression follows morphogenetic rather than histologic patterns and is dictated by epithelial-mesenchymal interactions. It also shows cell-specific expression: syndecan is predominantly at the cell surface on epithelia, where it has smaller heparan sulfate chains, but is predominantly intracellular in mesenchymal cells, where its heparan sulfate chains are larger. Indeed, these heparan sulfate chains from epithelial and mesenchymal cells differ in the number and size of N-sulfated, iduronic acid-rich domains. Syndecan on epithelia associates with the actin cytoskeleton via its cytoplasmic domain and inducing syndecan deficiency in cultured epithelia alters their shape, ability to migrate within matrix and response to growth factors. Syndecan expression is reduced upon neoplastic transformation. Thus, because syndecan may interact with a variety of extracellular effectors, we propose that syndecan is a "receptor partner", acting in combination with more highly specific receptors, which mediates the actions of matrix components and growth factors on cells. Thus, change in expression of the syndecan family of proteoglycans at distinct times or sites may regulate the effects of the microenvironment on cells.

Bernfield M and Sanderson RD. Syndecan, a developmentally regulated cell surface proteoglycan that binds extracellular matrix and growth factors. Phil Trans R Soc Lond Ser B. 1990; 327:171-186.

Bernfield M and Hooper KC. Possible regulation of FGF activity by syndecan, an integral membrane heparan sulfate proteoglycan. Ann NY Acad Sci. 1991;638:182-194.

CE 006 GENETIC ANALYSES OF CELL ADHESION IN FLIES AND MICE, Richard O. Hynes,[1,2] Elizabeth L. George[1,2], Elisabeth N. Georges,[2] Yevgenya Grinblat,[3] Fotis Kafatos,[3] Stephenie Paine-Saunders,[1,2] Helen B. Rayburn[3], Joy T. Yang,[1,2] Gene Yee[2] and Susan Zusman,[1,2]
[1]Howard Hughes Medical Institute and [2]Center for Cancer Research, Department of Biology, M.I.T., Cambridge, MA and [3]Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA.

Cell-matrix adhesion plays crucial roles in embryonic development, in normal physiological processes such as hemostasis and wound healing and in pathological processes such as thrombosis, inflammation and cancer. The molecules involved in these processes have been studied extensively *in vitro* but the functions *in vivo* have been much less analyzed.

In order to analyze the functions of the adhesive extracellular matrix protein, fibronectin, and of cell surface integrin adhesion receptors, we are using genetic methods to investigate their roles in intact organisms, namely flies and mice.

Fibronectin is being analyzed in mice using techniques of homologous recombination in embryonic stem (ES) cells to derive strains of mice which contain defective fibronectin genes. The fibronectin transcript is alternatively spliced to produce multiple different fibronectin isotypes. These alternatively spliced forms are expressed in tissue and developmentally specific fashion but their differences in function are not yet understood. We have generated both null mutants of fibronectin and alleles altered in their pattern of alternative splicing. The results so far demonstrate that FN-null is a recessive early embryonic lethal mutation. Mice heterozygous for fibronectin are viable but express reduced levels of the protein in their blood. They will be examined for more subtle defects in functions thought to involve fibronectin. The mutations altered in pattern of splicing will provide information on the functions of the alternatively spliced segments of fibronectin. Work in progress to generate transgenic mice expressing different alternatively spliced forms of fibronectin should provide complementary information. We are also analyzing the functions of certain integrin subunits which participate in fibronectin receptor function by similar approaches in mice.

We have been unable to detect fibronectin in *Drosophila* but integrins are present. Using preexisting mutations in the gene encoding the β subunit of the position-specific (PS) integrins we have been analyzing the functions of this family of integrins in the development of *Drosophila* embryos, muscles, wings and eyes. Using various genetic combinations and somatic clones, we have shown the involvement of these receptors in all four situations. Using P-element transposons, we have reintroduced wild type and mutant integrin subunits under the control of either their own promoter or a heat-inducible promoter. This allows definition of the times of requirement for integrins in the various processes and the requirement for specific structural elements within the protein.

We have also detected alternative splicing in the extracellular domain of the PS integrin β subunit and again using P-element rescue, have shown that some systems can use either spliced form but that proper embryonic development requires both forms. Further work should reveal the significance of the alternative splice for the structure and functions of this family of integrins.

Many processes involving cell adhesion and movement and axonal outgrowth proceed almost normally in flies in the complete absence of these integrins, suggesting that alternative adhesion molecules can suffice. Accordingly we have used PCR to search for other integrins in *Drosophila* and have discovered a novel β subunit which shows a highly restricted pattern of expression in the midgut of the developing embryo. The gene for this subunit has been mapped and small deficiencies obtained. These deficiencies should allow isolation of mutants in the gene encoding this novel β subunit.

Matrix Molecules and their Ligands

CE 007 STUDIES OF THE IN VITRO DEVELOPMENT OF THE ANCHORING COMPLEX AT THE DERMAL-EPIDERMAL JUNCTION. M. Peter Marinkovich[1], Gregory P. Lunstrum[2], Douglas R. Keene[2], and Robert E. Burgeson[3]. [1]Department of Dermatology, Oregon Health Sciences University, Portland, OR 97201, [2]Shriners Hospital, Portland, Oregon, 97201, [3]Departments of Dermatology, Anatomy and Cellular Biology, Cutaneous Biology Research Center, Harvard University Medical School, Massachusetts General Hospital - East, Charlestown, MA, 02129.

In addition to the ubiquitous basement membrane constituents, laminin, nidogen, type IV collagen, and heparan sulfate proteoglycan, the basement membrane at the dermal-epidermal junction underlying ezternal epithelia contain specialized proteins that contribute to the structures of the hemidesmosomes, anchoring filaments and anchoring fibrils. In the past, we have shown that the anchoring fibrils are derived form type VII collagen. Recently we have identified one component of the anchoring filaments as the protein kalinin. Antibodies to kalinin localize to the anchoring filaments and when added to the medium of cultured keratinocytes, the antibody causes rounding and detachment of the cells. Purified kalinin promotes the rapid attachment and spreading of keratinocytes but not of fibroblasts upon bacterial plastic. Kalinin contains three disulfide bonded subunits, two of which become proteolytically processed following secretion. The final processed form seen in cell culture is electrophoretically identical to the molecule extracted from tissue. Structural determinations of the kalinin chains show homologies to laminin chains. Further, the rotary shadowed image of kalinin resembles the long arm and G-domain of laminin, but lacks the three short arms.

We have recently described a laminin variant, K-laminin, that co-purifies with kalinin and is covalently bonded to kalinin solubilized from intact tissue. The tissue distribution of K-laminin is restricted largely to the basement membrane zone underlying external epithelia, identical to that of kalinin. K-laminin contains the B1 and B2 laminin chains, but lacks an A chain. Instead, a novel chains of 200kD substitutes for the A chain. Rotary shadowed images of K-laminin show a Y shaped structure, indicating the the 200kD alternate A chain does not contribute a short arm.

We have successfully synthesized in vitro an apparently intact basement membrane resembling the dermal-epidermal junction. Ultrastructural analysis of this product indicates the presence of hemidesmosomes, anchoring filaments, and anchoring fibrils. Using heterotypic constructs of bovine and human keratinocytes or mesenchymal cells, together with species specific monoclonal antibodies, we have observed that with the exception of kalinin (and possibly K-laminin), the mesenchyme synthesizes the heparan sulfate proteoglycan, laminin, and type VI collagen, and type VII collagen, and these molecules become incorporated into the basement membrane. Therefore, at least in vitro, the basement membrane zone of the dermal-epidermal junction is a product of both the epithelium and the mesenchyme.

CE 008 LAMININ-MEDIATED NEURITE OUTGROWTH: ACTIVE SITES, RECEPTORS AND INTRACELLULAR EVENTS, Hynda K. Kleinman, Peter D. Burbelo, Maura C. Kibbey, Mattias Jucker* and Benjamin S. Weeks, National Institute of Dental Research, NIH, Bethesda, MD 20892. *National Institute of Aging, NIH, Baltimore, MD 21225.

Laminin promotes neurite outgrowth in both peripheral and central neural cells and lines. We have identified a 13 amino acid domain on the A chain containing an active site of 6 amino acids (SIKVAV) which mimics the effects of whole laminin with many but not all neuronal cells. The receptor for this site was isolated from brain extracts by affinity chromatography and found to be a novel non-integrin protein. This receptor has both cellular (140 KD) and cell surface forms (110 KD) and is increased at sites of mechanical injury and in an ischemia model in the brain. Laminin signal transduction does not involve protein kinase C, cyclic nucleotides, or calcium fluxes but does involve protein dephosphorylation and the induction of novel genes. One gene identified by subtractive hybridization is a unique leucine zipper protein. Thus, the neuronal cell response to laminin is rapid, involves specific receptor ligand interactions and results in protein dephosphorylation and the induction of various genes. These responses are unique to neural cells as endothelial cells and Sertoli cells utilize other mechanisms.

CE 009 THE ROLE OF NON-FIBRILLAR COLLAGENS IN MATRIX ASSEMBLIES, Bjorn Reino Olsen, Department of Anatomy and Cellular Biology, Harvard Medical School, Boston.

The mechanical properties of tissues and organs depend to a large extent on tissue-specific three-dimensional patterns of collagen fiber scaffolds. The precise molecular interactions that lead to the establishment of these patterns are not yet known; however interactions between collagen fibril surfaces and other matrix components and cells are likely to be important. Members of the recently discovered FACIT class of extracellular matrix molecules are attractive candidates as molecules involved in such important interactions because they are associated with collagen fibrils, are expressed in tissue-specific patterns, and show tissue-dependent variations in domain structure. Three members of the family have been identified: Collagens IX, XII, and XIV. Type IX collagen molecules are localized along the surface of type II-containing fibrils in cartilage and in the vitreous of the eye, while types XII and XIV collagens are expressed in type I-containing tissues such as skin, tendon, ligaments, perichondrium and periosteum. The three types of collagen contain homologous carboxy-terminal triple-helical domains of about 100 amino acid residues (COL1) which are believed to mediate interaction with specific sites along the surface of collagen fibrils. Their amino-terminal domains differ substantially in that type IX collagen in cartilage contains one globular domain of about 250 amino acid residues while types XII and XIV collagen molecules have three very large (> 1,500 amino acid residues) non-triple-helical finger-like domains attached to a central globule. The finger regions contain a number of fibronectin type III repeats interspersed with sequences that are homologous to von Willebrand factor A domains. The amino-terminal domains of all three types are attached to the COL1 domains by a triple-helical connecting stalk. This suggests that the amino-terminal domains of type IX, XII, and XIV molecules provide potential binding sites for matrix components or cells along collagen fibrils. Type IX molecules may be important for the binding of proteoglycan aggregates to collagen fibrils in cartilage, while types XII and XIV collagen may contribute to the formation of fibril bundles. Further diversity in the amino-terminal domains of FACIT molecules is generated by tissue-specific alternative transcription start sites and/or exon splice patterns in their genes. For type IX collagen two forms that differ in their amino-terminal globular domains are generated in cartilage and the vitreous by the use of alternative exons in the $\alpha 1(IX)$ gene. For type XII collagen two forms that differ in the number of fibronectin type III repeats and von Willebrand factor A-like domains are expressed at different ratios in different tissues. To test hypotheses about the functional significance of such variations and the general function of FACIT molecules in matrix assemblies, we have cloned portions of the mouse $\alpha 1(IX)$ and $\alpha 1(XII)$ collagen genes, and have used these clones to make constructs for altering the expression of the genes in mouse embryonic stem cells and transgenic mice.

CE 010 PROTEOGLYCANS IN VASCULAR BIOLOGY, Thomas N. Wight, Michael Kinsella, Elke Schönherr, Hannu Järveläinen, Susan Potter-Perigo and Alan Snow, Department of Pathology, University of Washington, Seattle, WA 98195.

Although proteoglycans (PGs) consitute a minor component of vascular tissue, these molecules influence a number of arterial properties such as viscoelasticity, permeability, lipid metabolism, hemostasis and thrombosis by virtue of their ability to interact with component molecules involved in these processes. The major classes of PGs synthesized by both vascular endothelial and smooth muscle cells are those that contain chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS). The proportions of these PGs change during arterial development and disease. Endothelial cells modulate their PG synthesis when stimulated to migrate and when induced to form capillary tubes in vitro. Some of these changes can be induced by the addition of basic fibroblast growth factor (bFGF) to confluent cultures of endothelial cells. Arterial smooth muscle cells modulate their PG synthesis when either stimulated to proliferate by platelet derived growth factor (PDGF) or inhibited from proliferating by transforming growth factor beta (TGF-ß1). In all cases, modulation of PG synthesis involves changes in the transcription/translation and post translational processing of specific classes of PGs. Specific vascular PGs interact with a number of ligands involved in vascular development and disease. Small interstitial PGs that contain DS interact with PDGF and TGF-ß1. Large interstitial CSPGs interact with low density lipoproteins in specific and defined ways. Proteoglycans that contain HS bind amyloid in various forms of amyloidoses as well as participate in the coagulation cascade by interacting with antithrombin III. Some forms of vascular disease are characterized by abnormalities in PG metabolism. In some patients with the Marfan syndrome, an autosomal dominantly inherited disease characterized by aortic dilation and dissection, the synthesis of DSPGs (decorin and biglycan) by the patients cells is greatly reduced. These studies emphasize the importance of PGs in the normal and pathophysiology of blood vessels.

Co-Cultures and Other in Vitro Systems for Promoting Differentiation and Tissue Formation

CE 011 **MODULATION OF CARDIAC GROWTH AND CONTRACTILE FUNCTION BY SYMPATHETIC INNERVATION,** Dianne L. Atkins and Steven H. Green, Departments of Pediatrics and Biology, University of Iowa, Iowa City, IA.

We have developed a co-culture model of sympathetic neurons and neonatal rat cardiac myocytes to study the neurotrophic influence of innervation on the developing myocyte. Explants of thoracolumbar sympathetic chain ganglia are added to newborn rat myocytes harvested prior to the onset of *in vivo* innervation. Neuromuscular junctions are observed and neuroeffector transmission is present by 48 hours in culture. Using stereologic techniques adapted for single cell examination, we assessed sympathetic influences on myocyte growth. Innervation did not affect the shape or height of the cell but did increase cell volume by 44%. All cytoplasmic elements increased proportionately. Complete adrenoceptor blockade did not prevent this effect of innervation and cardiac myocytes incubated within the co-cultures, but not anatomically innervated, showed a similar increase in cell size. This indicated that the neurotrophic effect was independent of neuroeffector transmission or anatomic contact.

Contractile function of individual myocytes was measured by video motion analysis of spontaneously contracting cells. Sympathetic innervation decreased spontaneous contraction frequency and increased contraction amplitude, and peak velocities of contraction and relaxation. There was no relationship between the decrease in contraction frequency and the increase in contractile function. This response was not blocked by complete adrenoceptor blockade but was present in non-innervated myocytes distant to neuronal explants.

We hypothesized that the effects of innervation on myocyte growth and contractile function were mediated by a soluble factor produced by the neurons but different from the neurotransmitter. Since identification of such a factor is complicated by the cellular heterogeneity of the primary cultures, we examined the myocyte response to conditioned medium from PC12 cells, a pheochromocytoma cell line which assumes a neuronal phenotype when exposed to nerve growth factor. PC12 conditioned media induced size and contractile changes which were qualitatively similar to those induced by innervation. Moreover, adrenoceptor or muscarinic antagonists did not alter the response to the conditioned medium. These cells share with sympathetic neurons the ability to release a non-adrenergic factor which regulates contractility and can serve as a source for purification of the factor.

CE 012 *Abstract Withdrawn*

CE 013 STRATEGIES FOR *IN VITRO* MODELING OF HUMAN CYTOTROPHOBLAST INVASION,

Susan J. Fisher, Clifford Librach, Marilyn Fitzgerald, Yang Zhou, Dee Dao, Ken Kosten and Caroline H. Damsky, University of California San Francisco, San Francisco, CA 94143-0512.

Successful development of the human embryo is critically dependent on the rapid differentiation of placental trophoblasts. During this process chorionic villus cytotrophoblasts, originally a polarized epithelial monolayer anchored to a basement membrane, aggregate into multilayered columns of nonpolarized cells that penetrate the uterine wall and associated arterioles. The transient tumor-like behavior of cytotrophoblasts, which peaks early in pregnancy, is developmentally regulated. Since the dynamic aspects of human cytotrophoblast invasion are not accessible for study *in vivo*, we have developed *in vitro* models that mimic this process and can be used to test the function of relevant molecules. In culture, human cytotrophoblasts plated as a monolayer aggregate on certain basement membrane-like substrates, including Matrigel and laminin gels. Then, early gestation, but not term, cytotrophoblasts rapidly invade these substrates. To determine the degree of fidelity of this model to actual cytotrophoblast invasion of the uterus, we used immunohistochemical techniques to compare the expression of key molecules *in vivo* (using placental bed biopsies which contain cytotrophoblasts that have invaded the uterus) and *in vitro*. Using this experimental strategy we have shown that cytotrophoblast differentiation and invasion are accompanied by dramatic changes in the expression of matrix degrading metalloproteinases, extracellular matrix ligands and their integrin receptors, as well as the trophoblast-specific class I molecule, HLA-G. With regard to the role of proteinases, cytotrophoblasts synthesize both metalloproteinases and urokinase-type plasminogen activator. Metalloproteinase inhibitors and a function-perturbing antibody specific for the 92 kd type IV collagen-degrading metalloproteinase completely inhibited cytotrophoblast invasion *in vitro*, whereas inhibitors of the plasminogen activator system had only a partial (20-40%) inhibitory effect. We conclude that the 92 kd type IV collagenase is critical for cytotrophoblast invasion. We also studied the expression of adhesion receptors and their extracellular matrix ligands during human trophoblast invasion *in vivo*. Then the function of molecules that were modulated was investigated using an *in vitro* model of the invasion process. Cytotrophoblast differentiation to the invasive phenotype *in vivo* was characterized by a switch from the expression of integrin $\alpha 6/\beta 4$ and multiple forms of laminin (Ln) to the synthesis of the $\alpha 5/\beta 1$ Fn receptor, the $\alpha 1/\beta 1$ Ln/collagen receptor and a fibronectin (Fn)-rich extracellular matrix. Function-perturbing antibodies against collagen IV (Col IV), Ln and integrin $\beta 1$, but not Fn, blocked cytotrophoblast invasion *in vitro*. Antibodies against individual $\alpha/\beta 1$ receptors had distinctive effects. Anti-$\alpha 5/\beta 1$ enhanced cytotrophoblast invasion 250%, and addition of exogenous Fn to Matrigel blocked invasion by 80%. In contrast, antibodies against the $\alpha 1/\beta 1$ Col IV/Ln receptor inhibited invasion by 60%. Antibody against $\alpha 6$ ($\alpha 6/\beta 1$ is a Ln receptor) had no effect on invasion when added alone, but complemented anti-$\alpha 1/\beta 1$. These results suggest that interactions between $\alpha 5/\beta 1$ and Fn may restrict invasion, whereas interactions of $\alpha 1/\beta 1$ and $\alpha 6/\beta 1$ with Ln and Col IV may promote invasion. Finally, the expression of HLA-G, a trophoblast-specific nonpolymorphic class I molecule, is also upregulated during trophoblast invasion, both *in vitro* and *in vivo*. Taken together, these results suggest that cytotrophoblast invasion is a complex process that requires differential expression of metalloproteinases, adhesion molecules and HLA-G. Experiments in progress are directed towards using the invasion models and their *in vivo* correlates to determine the autocrine and/or paracrine role of cytokines and growth factors in regulating this complex differentiation process. (supported by HD 26732, HD 24180, HD 22210 and contract NICHD-N01-8-2903)

CE 014 MECHANOCHEMICAL SWITCHING BETWEEN GROWTH AND DIFFERENTIATION BY EXTRACELLULAR MATRIX: POSSIBLE USE OF A CELLULAR TENSEGRITY MECHANISM,

Donald E. Ingber, The Children's Hospital, Brigham and Women's Hospital, and Harvard Medical School, Boston, MA 02115.

To engineer artificial tissues with desired functions it is first necessary to identify the critical determinants that cells require for control of cell growth and differentiation. Over the past ten years, we have found that the growth and development of functional tissues, such as branching capillary networks, requires both soluble growth factors and insoluble extracellular matrix (ECM) molecules. However, ECM molecules were found to be the dominant regulators since they dictate whether individual cells will either grow or differentiate in response to soluble stimuli. For example, we have shown that a variety of cells including capillary endothelial cells, vascular smooth muscle cells, and hepatocytes, increase their growth rates in direct proportion as the ECM molecular coating density is raised (e.g. increasing the number of fibronectin molecules adsorbed/cm^2) on otherwise non-adhesive bacteriologic plastic dishes. All of these studies were carried out in serum-free, defined medium containing saturating amounts of soluble mitogens (FGF, PDGF, and EGF in these three cell types, respectively). Furthermore, as growth was turned on, both capillary differentiation (tube formation) and expression of liver-specific genes in hepatocytes were switched off. Analysis of the molecular basis of these effects has revealed that ECM molecules alter cell growth via two integrated mechanisms: 1) by clustering specific transmembrane integrin receptors and thereby activating chemical signaling pathways (e.g., Na^+/H^+ antiporter, inositol lipid turnover, tyrosine phosphorylation) inside the cell, and 2) by promoting changes of cell shape (i.e., a biomechanical alteration). Release of chemical second messengers by integrin binding is sufficient to induce quiescent cells to pass through the G_0/G_1 transition and enter the cell cycle. However, further progression through G_1 and entry into S phase appears to require large-scale changes of cell and nuclear shape (i.e., cell and nuclear spreading).

To address the question of how ECM might alter cell shape and thereby provide regulatory information to the cell, modeling studies were carried out using three dimensional "tensegrity" (tensional integrity) models. These "stick and string" models were built by interconnecting multiple compression-resistant struts (analogous to microtubules, stress fibers) with a continuous series of tension elements (contractile microfilaments) that can vary in length in response to force. These cell models also contained a nucleus that was constructed out of another tensegrity sphere which was linked to the "cell" surface via additional tensile elements (e.g., analogues of intermediate filaments). Analysis of the mechanism by which these models change their form suggest that both cell and nuclear shape alterations may be determined through the action of tensile forces that are generated by intracellular microfilaments and resisted by ECM attachment points. Both tensegrity models and living cells spread and flatten when allowed to adhere to a highly adhesive substratum whereas they spontaneously round when their ECM attachments are dislodged. Recent studies with both living and membrane-permeabilized cells (whole cytoskeletal preparations) also confirm that cell and nuclear shape are determined through this type of mechanical force balance. These findings suggest that ECM molecules may regulate cell function by both binding integrins and physically resisting cell-generated forces that are applied to these receptors. These results also suggest that future design of artificial tissues and ECMs may need to take into account the structure's biophysical properties as well as its chemical specificity.

CE 015 RAPID AMPLIFICATION OF HUMAN KERATINOCYTE STEM CELLS IN CULTURE AND APPLICATIONS TO SKIN GRAFTING AND RECONSTRUCTIVE SURGERY,

James G. Rheinwald, Therese O'Connell-Willstaedt, Kristina Lindberg, and Ben Bronstein, BioSurface Technology, Inc., Cambridge, MA 02139.

The epidermis is a stratified squamous epithelium that continually renews, turning over every 2-4 weeks by the multiplication of basal keratinocytes followed by their terminal differentiation and ultimate sloughing as squames from the skin surface. Keratinocyte growth regulation is complex, involving multiple mechanisms controlling cell division and the decision to commit irreversibly to terminal differentiation. A culture system that employs a 3T3 fibroblast feeder layer and a cocktail of medium supplements and mitogens promotes rapid growth and serial subcultivability of normal keratinocytes from donors of all ages and retains stem cells in the cultured population. The bacterial protease Dispase releases a confluent keratinocyte culture as a coherent sheet that will attach, stratify, and differentiate when placed on a wound bed, thus enabling the clinical use of cultured autologous keratinocytes as permanent skin grafts.

Small skin biopsies can be expanded >1000-fold within three weeks of culture into a large epidermal tissue surface which can be grafted to provide permanent skin coverage for major burn victims. BioSurface Technology, Inc. (BTI) has prepared cultured grafts for about 300 burn and other skin surgery patients at over 80 hospitals in the *U.S.* and *Europe* during the past three years. Skilled clinicians using optimal protocols typically achieve >70% take, but results have varied widely, depending largely upon clinical variables such as graft bed preparation, infection, and post-surgical care. Analysis of data we have compiled from >100 of these patients has disclosed the value of using a grafting method pioneered by Dr. Charles Cuono at Yale. In this two-stage grafting procedure, cultured epithelium is applied to the allogeneic dermal remnant ("allodermis") resulting from partial excision of temporarily engrafted allogeneic donor skin. Fourteen patients from eight U.S. burn centers grafted this way with cultures grown at BTI have experienced a consistently excellent result (mean take = 90%, median take = 95%).

Cultured epidermal cells also are of potential value for smaller burns and other full-thickness wounds, such as those resulting from the excision of giant congenital nevi, because their use avoids the creation of donor sites and the meshed patterns of conventional skin grafts. Other epithelia have immediate potential for grafting from cultures, as well. There are at least eight specialized keratinocyte subtypes, including interfollicular and palmar/plantar epidermal cells and the cells that form the epithelia of the oral cavity, esophagus, cornea, urethra, and vagina. All exhibit the same growth characteristics in culture as the epidermal cell and several subtypes have already been used as cultured grafts in humans for internal tissue reconstruction.

Evidence has now accumulated to establish convincingly that allogeneic epidermal keratinocyte grafts do not take permanently in humans. Thus, they will not provide permanent skin coverage of large *full-thickness* wounds. However, cultured epidermal allografts have been demonstrated to function as "biological bandages", markedly stimulating healing of acute *partial-thickness* wounds such as conventional skin graft donor sites and second-degree burns, and chronic skin lesions such as venous and decubitus ulcers.

CE 016 HEPATOCYTE DIFFERENTIATION INDUCED BY EXTRACELLULAR MATRIX CONFIGURATION, Martin L. Yarmush[1,2], James C.Y. Dunn[2], Ronald G. Tompkins[2], [1]Department of Chemical and Biochemical Engineering, Rutgers University, Piscataway, NJ 08854, [2]Department of Surgery, Massachusetts General Hospital, Boston, MA 02114.

The influence of extracellular matrix configuration on the tissue-specific function of cultured hepatocytes was investigated. Adult rat hepatocytes sandwiched between two layers of collagen gel were compared to cells cultured on a single layer of collagen gel for differences in cytoskeletal organization, the level of albumin-specific mRNA, the rate of albumin gene transcription, and the rate of albumin mRNA translation. When hepatocytes were placed on a single collagen gel, actin stress fibers were observed near the collagen attachment sites. This abnormal actin distribution could be converted to a pattern almost identical to the normal liver when the second layer of collagen was overlaid. Adult hepatocytes in the sandwich system were shown to maintain a level of albumin mRNA similar to that found in the normal liver for at least six weeks, whereas the level of albumin mRNA declined rapidly in the single gel system. The level of mRNA in the sandwiched hepatocytes was shown to correlate with the level of albumin gene transcription. In addition to transcriptional control, the overall rate of albumin production was shown to depend on the rate of translation, which increased with culture time and reached a plateau in one-two weeks. This increase in translational activity over time in culture was observed in both the sandwich and the single gel systems and, thus, appeared to be independent of the configuration of extracellular matrix.

Induction Phenomena-I (Joint)

CE 017 REGULATORY FACTORS IN ADIPOCYTE DEVELOPMENT, Bruce M. Spiegelman, Reed A. Graves, Peter Tontonoz, and Lisa Choy, Dana-Farber Cancer Institute, Division of Cellular and Molecular Biology and Harvard Medical School, Department of Biological Chemistry and Molecular Pharmacology, Boston, MA

We have been studying the regulation of adipocyte gene expression during cell differentiation and tissue development. The intracellular factors that activate adipocyte-specific gene regulation are not well understood. Toward this end, we have recently discovered an adipocyte-specific enhancer, and are performing a molecular dissection to identify key transcription factors binding to this DNA. This enhancer, from the adipocyte P2 gene, functions in cultured cells and transgenic animals. It binds several nuclear proteins including a member of the NF-1 family and mutational analysis indicates that binding at 5 separate sites are necessary for full activity. One factor, termed ARF6, appears to be developmentally regulated and may be the switch that activates this enhancer only in fat cells. The ARF6 factor itself is observed only in nuclear extracts from adipocytes. Multimers of the ARF6 binding site are sufficient to activate gene expression from a minimal promoter in adipose cells. C/EBP, a transcription factor that is induced during adipocyte differentiation, does not bind to this enhancer and an expression vector for C/EBP cannot transactivate this enhancer in preadipocytes. Current experiments address the general importance of the ARF6 factor in the activation program of differentiation in adipocytes. We have also been examining adipocytes for production of extracellular factors that may influence systemic energy balance. Our recent data indicates that adipocytes make and secrete several complement factors including factor D/adipsin. They also activate part of the alternative pathway of complement and generate several complement peptides with known biological effects. The role of this pathway in systemic energy metabolism is being investigated.

CE 018 GROWTH FACTOR-MEDIATED INDUCTIVE SIGNALLING DURING *C. elegans* DEVELOPMENT, Paul W. Sternberg, Russell Hill, Phoebe Tzou, Jing Liu, Helen Chamberlin, Gregg Jongeward, Junho Lee, Linda Huang, and Raffi Aroian, Howard Hughes Medical Institute, Division of Biology 156-29, California Institute of Technology, Pasadena, CA.

An intercellular signalling pathway consisting of a growth factor, transmembrane receptor tyrosine kinase and a ras protein acts in several defined inductive events during nematode development. During vulval induction in the hermaphrodite, a single cell in the gonad, the anchor cell, induces three of six multipotent vulval precursor cells [VPCs] to undergo three rounds of mitosis and generate vulval cells. The three uninduced VPCs undergo a single mitosis and generate nonspecialized epidermis. Among genes necesary for this induction are *lin-3*, *let-23* and *let-60*. Defects in any of these three genes leads to a failure of vulval induction. Transgenic nematodes carrying high copy *lin-3* or *let-60* transgenes have excessive vulval differentiation. Using these dominant transgenes, we have ordered the action of these three genes: *lin-3* acts via *let-23*, which acts via *let-60*. *let-60* encodes a ras protein. *let-23* encodes a *C. elegans* homolog of the human EGF receptor and related receptor tyrosine kinases. *lin-3* encodes a putative growth factor precursor with the architecture of TGFα. Predicted *lin-3* proteins have an N-terminal leader sequence, a single EGF growth factor repeat, a membrane-spanning domain. Two alternatively spliced forms of *lin-3* differ in the presence of 15 amino acids between the EGF repeat and the transmembrane domain. *lin-3* is expressed in the anchor cell at the time of vulval induction. We propose that *lin-3* encodes the signal that induces vulval development. During male spicule development, we have shown that the F and U cells use this *lin-3-let-23-let-60* pathway to induce particular neurectoblasts fates including Bα and Bγ, which generate particular subsets of spicule cells. Ablation of F and U has the same result as mutation of *lin-3*, *let-23* or *let-60*: defects in the Bα and Bγ cell lineages. We are examining the negative regulation of this pathway by examining mutations that result in excessive vulval differentiation. Some of these mutations are in the *let-23* gene, others are in unlinked loci, *lin-15*, *unc-101*, and *rok-1*.

CE 019 ADHESION AND MOTILITY OF EMBRYONIC AND CANCER CELLS.

Jean Paul Thiery, Jean Claude Boucaut, Brigitte Boyer, Florence Broders, Thierry Darribere, Annie Delouvée, Sylvie Dufour, Jeanne Marie Girault, Jacqueline Jouanneau, Victor Koteliansky, Giovanni Levi, Ginette Moens, Jean Pierre Saint-Jeannet, Pierre Savagner, Lionel Simonneau and Ana Maria Valles. Laboratoire de Physiopathologie du Développement, CNRS-Ecole Normale Superieure 46, rue d'Ulm, 75230 Paris Cedex 05 FRANCE.

We have analysed in detail the program of expression of several adhesion molecules during morphogenesis focusing primarily on epithelial-mesenchymal cell interconversions and on migratory events. The neural crest, a transient embryonic structure of the neural epithelium undergoes a conversion to a mesenchymal state; these cells subsequently migrate thoughout the embryo to give rise to many derivatives including most of the peripheral nervous system and melanocytes. The pattern of expression and modulations of the cell adhesion molecules (CAMs) and the substrate adhesion molecules (SAMs) correlate with the different morphogenetic steps in the neural crest. During migration crest cells do not express functional CAMs but interact specifically with fibronectins in the extracellular matrix. Several distinct cell binding domains on the fibronectin molecules have been mapped and their relative contribution to adhesion, spreading and motility will be described. The role of cell adhesion is also investigated in amphibian embryos particularly during gastrulation.

A rat bladder carcinoma has been used as a model system to study the conversion of an epithelial to a migratory fibroblast-like state. This morphological transformation is triggered by collagens but not by fibronectins or laminin. A similar conversion is induced by acidic Fibroblast Growth Factor (aFGF) in subconfluent cultures while this multifunctional growth factor acts as a mitogen on high density cultures. In low density cultures, aFGF and several other growth factors acting through tyrosine kinase receptors induce a rapid internalization of desmosomes, a major adhesive structure of epithelia. The newly formed fibroblasts progressively lose their cytokeratins which are replaced by vimentin intermediate filaments. The transformation is fully reversible upon removal of the growth factor. Acidic FGF also triggers cell motility and production of gelatinases. On collagen substrates, the speed of locomotion is enhanced in the presence of aFGF and under these conditions the bladder carcinoma cells readily invade 3D collagen gels. The bladder carcinoma line can also become fibroblastic after transfection with an expression vector coding for aFGF, most likely through an autocrine mechanism. Thus, this model system may offer a unique opportunity to evaluate the role of the different adhesion modes and signalling factors in morphogenetic processes.

Physical Forces as Requirements for Gene Expression, Growth, Morphogenesis and Differentiation

CE 020 PHYSICAL FORCES AS A FACTOR IN TISSUE GROWTH AND REMODELING,

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The effect of physical stress is illustrated by changes in rat pulmonary artery when the circumferential stress is suddenly increased stepwise by putting the rat in a hypoxic chamber (10% O_2, 90% N_2). Three changes were measured: structural histological change, residual stress change revealed by opening angle at zero-stress state, and mechanical properties changes revealed by constitutive equations. Figure 1 *center* shows the main pulmonary artery of rat. *Right* side shows the structure of vessel wall. Clear changes can be seen at two hours, and progressively later. *Left* side shows the zero-stress state of artery, obtained by cutting a short segment, then cutting the ring which springs open into a sector. The angle subtended by radii from the midpoint of the endothelium to the tips of the sector is the *opening angle*, which varies with the location, can be larger than 360° at the main trunk, and can change more than 100° due to tissue remodeling in hypoxia. Further, the arterial wall obeys a nonlinear stress-strain relation, whose material constants vary with time in the course of hypertension. A unified interpretation of these processes is that a certain stress-growth law exists. I offer a stress-growth law for discussion.

Ref: Fung, Y.C. and Liu, S.Q.: *J. Appl. Physiol.* **70:** 2455-2470, and *Circ. Res.* **65:** 1340-1349, 1989.

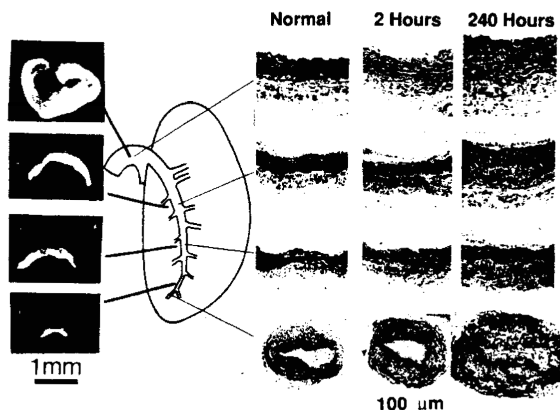

Figure 1. Left: Zero-stress state of normal rat pul. artery. Endothelium downward. **Right:** Histology of normal and hypertensive rats.

CE 021 MECHANICL STRESS EFFECTS ON VASCULAR CELL GROWTH,

Robert M Nerem,[1] Bradford C. Berk,[2] Masako Mitsumata,[1] Thierry Ziegler,[1] and R. Wayne Alexander,[2] [1]Georgia Institute of Technology, Atlanta, GA 30332-0405 and [2]Emory University, Atlanta.

In the application of tissue engineering to the reconstitution of a blood vessel in culture, an important issue is the regulation of vascular cell growth. Our studies have focused on the vascular endothelial cell and the influence of its mechanical environment on both its intrinsic and extrinsic growth programs. In vivo, the endothelial cell 'sees' pressure, a normal stress, and a shear stress which acts tangentially and is directly related to the blood flow pattern; it also 'rides' on a basement membrane which is being cyclically stretched by the pulsatile pressure. There is now considerable evidence indicating an influence of this hemodynamic or mechanical environment on endothelial structure and function, including endothelial cell turnover rate. In spite of such studies, most cell culture investigations of vascular endothelial biology have been carried out in static culture under no-flow conditions where endothelial cells exhibit a polygonal shape. However, when subjected to flow and the associated shear stress environment, it is known that bovine aortic endothelial cells (BAEC) elongate in shape and align their major axis with the direction of flow. Concommitant with this, there is an alteration in F-action localization, an increase in cell stiffness as measured using the micropipet technique, and a variety of other changes including alterations in the synthesis of certain vasoactive substances. Recent studies indicate that, associated with this response of the endothelial cell to flow, there is an influence on its intrinsic growth program. For subconfluent monolayers this effect is represented by a decreased rate of proliferation in the presence of a laminar, steady-state flow. The higher the level of shear stress, theslower the rate of growth, and with 1Hz sinisoidal, non-pulsatile flow, this inhibiting effect of shear stress is accentuated. This influence of flow is confirmed by a decrease in DNA synthesis as measured by 3H-thymidine incorporation. Flow cytometry measurements indicate that this is due to an inhibition of entry into S-phase. Similar results have been obtained for confluent BAEC monolayers. In addition, using time-lapse videos of the process of cell division, we have demonstrated that, whereas in static culture BAEC round up to undergo cytokinesis, elongated BAEC in the presence of flow remain flattened in shape through the entire process of cell division, dividing into two elongated daughter cells. The influence of flow also is at the gene expression level, and this has been demonstrated through alterations in mRNA expression for the proto-oncogene c-myc and for platelet-derived growth factor (B chain). The latter indicates that the influence of flow is not only on the endothelial cell's intrinsic growth program, but also on its growth program. An important unanswered question is how does an endothelial cell recognize its flow environment and discriminate between different flow conditions, and having done so, how does it transduce this into changes in structure and function. Current studies include investigations of the role of the phosphoinositide system, intracellular calcium, and protein kinase C as second messengers.

CE 022 **EVIDENCE FOR THE ROLE OF PHYSICAL FORCES IN GROWTH, MORPHOGENESIS AND DIFFERENTIATION**
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Historically, evidence for the role of physical forces and internal stresses on growth, morphogenesis and differentiation has been observed and utilized for a long time. Both soft and hard tissues need stress for their proper development, maintenance, and repair. However, the detailed mechanisms of cellular response to physical forces are not known at a molecular level. The sensing, transduction, and monitoring mechanisms associated with stress effects on cells are just now being elaborated.

Selection and Design of Molecules and Marked Cells For Tissue Engineering

CE 023 EXPRESSION AND MAINTENANCE OF SYNTHETIC GENES ENCODING ANALOGS OF NATURAL PROTEINS IN *E. COLI*,
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A new field of Polymer Chemistry is developing to exploit the use of repetitive polypeptides as performance polymers. Such polypeptides offer several advantages over conventional hydrocarbon-derived polymers in that precise control can be exerted over sequence, composition, stereochemistry, and molecular weight. A wide range of applications is being envisioned for peptide polymers including use as piezoelectric materials, molecular wires, in non-linear optics, in ceramics fabrication, industrial coatings, industrial and apparel fibers, and in the medical arena. Medical applications include use in various types of grafts, prostheses, wound dressings, sutures, etc. The utility of polypeptides for such applications lies in the possibility of designing sequences that reduce or eliminate inflammatory, necrotic, and immunologic reactions, as well as in the potential to program tissue regeneration by incorporating appropriate cellular attachment factors and response modifiers. Biotechnology promises economic large-scale production of tailor-made polypeptides. Economic production of peptide biopolymers may ultimately require the use of microbial secretion systems or transgenic plants and/or animals, particularly for high-volume commodity industrial markets. However, current production systems generally utilize *E. coli*. Requirements for a suitable *E. coli*-based expression system include: (1) proteolytic stability of the peptide biopolymer, and (2) genetic stability of the corresponding repetitive gene.

Efforts in our laboratory have focused on developing *E. coli* as a generic production system for repetitious proteins. These studies have used as models analogs to structural proteins such as a precursor to the bioadhesive protein from *M. edulis* {poly(A-K-P-S-T-P-P-T-Y-K)}, collagen [poly(G-P-P)], and elastin [poly(V-P-G-V-G)]. The tandem repeat DNA of repetitious polypeptide genes is particularly prone to deletion of repeat units in *E. coli*, occurring at rates on the order of 10^{-2}-10^{-4}/plasmid division. This is roughly 10^{3}-10^{5} fold higher than similar events occurring on the bacterial chromosome. Rates in the lower end of this range provide acceptable stability for at least moderate-scale fermentation. Although RecA protein may play a role in this phenomenon in certain circumstances, the major mechanism responsible for this process is independent of RecA function. The process appears to be highly dependent on the base composition and sequence of the repeat. The frequency of deletion also increases with increasing number of the repeat units. The length of the repeat units (complexity of the DNA) has a relatively small effect on the deletion frequency within the range of 9-120 bp. The polypeptides produced from these genes can be highly labile in *E. coli*. Such polypeptides can often be stabilized by using protease-deficient hosts and/or various expression systems. For example, the T7 expression system could be used to stably produce bioadhesive precursor at levels up to 60% of total cell protein, even in hosts containing proteases known to degrade the protein. The ability of the T7 expression system to partition bioadhesive protein into inclusion bodies may be responsible for the observed stability. (Supported in part by ONR contract#N00014-89-c-0293)

CE 024 BIOELASTIC MATERIALS AS MATRICES FOR TISSUE RECONSTRUCTION. Alastair Nicol and Dan. W. Urry, Laboratory of Molecular Biophysics, School of Medicine, University of Alabama at Birmingham, UAB Station, P. O. Box 300, Birmingham, AL 35294-0019.

Perhaps the principal properties required for tissue reconstruction matrices are biocompatibility, the capacity to support cell adhesion and tissue modelling and the capacity to adopt appropriate physical forms having suitable mechanical properties. The bioelastic materials based on the repeating amino acid sequences of elastin and selected analogs have properties which suggest their use as foundation materials for tissue reconstruction matrices. The remarkable biocompatibility of the primary bioelastic material has recently been described (1). They have been formed into sheets, rods and tubes by γ-irradiation cross-linking in molds, and it is clear that other shapes could be readily produced. The cross-linked materials have adjustable physical properties (e.g. elastic moduli ranging from 10^5 to 10^8 dynes/cm^2) which appear appropriate for tissue reconstruction. A major area of development required of these materials is in the realm of cellular interaction. We have shown in cell adhesion studies (2) that X^{20}-poly(GVGVP), the elastomeric matrix material based on the repeating pentamer GVGVP of mammalian elastin, is a very poor support for cell adhesion. The synthetic incorporation of RGDS, the cell attachment site of fibronectin, as GRGDSP into the matrix results in a material with cell attachment properties equivalent to those of fibronectin coated tissue culture plastic. Cellular infiltration of these matrices remains to be addressed. The specific requirements of cells for tissue regeneration are unknown but the demonstration that the 'biologically neutral' X^{20}-poly(GVGVP) can be transformed into a good support for cell adhesion gives strength to the idea that other more specific signals when identified may be similarly incorporated.

Thus the biocompatibility and appropriate mechanical properties of these bioelastic materials combined with the capacity for incorporation of bioactive peptide sequences indicate their potential for development as matrices for tissue reconstruction.

1. D. W. Urry, T. M. Parker, M. C. Reid, D. C. Gowda (1991) J. Bioactive and Compatible Poly. **6**, 263-282.
2. A. Nicol, D. C. Gowda and D. W. Urry (1992) J. Biomed. Mater. Res., **26** (In press).

Approaches to Allografting Engineered Cells and Tissues

CE 025 TOLERANCE INDUCTION IN ADULT ANIMALS, Kevin J. Lafferty, and Ronald G. Gill, Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, 4200 E. 9th Ave., Box B140, Denver, Colorado 80262.

Although the allograft reaction to MHC incompatibilites is a most violent response, MHC antigens are intrinsically weak immunogens within the species. This paradox is explained by the two signal model for T cell induction which is based on the proposal that two signals, engagement of the T cell receptor and the provision of a separate signal by the APC (co-stimulator activity), causes T cell induction when these signals are provided in conjunction. The model leads to the notion that tissue APC's are a major source of tissue immunogenicity and that in the absence of these cells tissues can be grafted without recipient immune suppression. Such grafts eventually lead to the development of donor-specific tolerance in the adult recipient animal. Donor-specific tolerance also can be induced to untreated allografts transplanted under the cover of short-term recipient immunosuppression by agents such as RS-61443 or Cyclosporine A. An attractive hypothesis is that the delivery of donor antigens (signal 1) by APC-depleted grafts or by tissues presented to the recipient in the presence of immunosuppression leads to the deletion or anergy of donor-reactive T cells. However, donor reactivity in tolerant animals does not support this hypothesis. Rather, animals tolerant of allografted islet tissue appear to have potential anti-donor reactivity that is indistinguishable from age-matched control animals as determined by proliferative responses, cytotoxic T cell precursor frequency, and lymphokine production. Such results suggest that tolerance induced in adult animals is due to the regulation of anti-donor responses rather than the elimination/inactivation of donor-reactive T cells.

CE 026 "NEUTRAL ALLOGRAFTS" CULTURED ALLOGENEIC CELLS AS BUILDING BLOCKS OF ENGINEERED ORGAN TRANSPLANTED ACROSS MHC BARRIERS.

Mireille Rosenberg, Valerie A.Theobald, Jeffrey D. Lauer, Kate B. Baker. Organogenesis Inc., 83 Rogers Street, Cambridge MA., 02142

The immunogenetic elements of an allograft have generally been tought to be cells of hematopoietic origin or small vessel endothelium rather than the parenchymal and mesenchymal cells. Tissue engineering techniques have been applied to the fabrication of organs using cultured cells of non hematopoietic origin and extra cellular matrix. If cells can be selected which are not recognized and rejected as foreign, it should be possible to engineer so called "neutral allografts" that would be accepted across major histocompatibility barrier.

Pure populations of passaged cultured cells are now easily available due to progress in cell culture and availability of defined culture media. Human dermal fibroblasts, epidermal cells as well as vascular cells such as smooth muscle cells and endothelium cells have been isolated and maintained as pure populations. When used as stimulators in an in vitro test of alloreactivity such as MLR, only endothelial cells were able to initiate a reaction. Although all cells contitutively express MHC class I antigens only,interferon gamma can induce expression of MHC class II antigens as well as ICAM. Expression of MHC class I, class II antigens and ICAM although necessary for alloreaction is not in itself sufficient to initiate an alloreaction by fibroblasts, smooth muscle cells and epidermal cells.In contrast endothelial cells are potent stimulators. Necessarry additional signals are generally provided by cytokines, however addition of cytokines during allostimulation does not restore an alloreaction. Some cells so called "neutral" are missing some as yet unknown costimulatory factor(s) that are necessary to initiate an allogeneic response and a rejection if grafted across MHC barriers. Although the mechanism by which those cells elude the immune system is unknown, may be partial induction, may be clonal anergy or indeed failure to induce a specific response, selected cultured allogeneic cells can be used for production of engineered organs and tissue grafts to be transplanted across MHC barriers.

Stimulating Tissue Remodeling in Vivo: The Plasticity of the Organism

CE 027 DIRECTED CONNECTIVE TISSUE REMODELLING UPON A BIOLOGIC COLLAGEN SUBSTRATE, Stephen F. Badylak[1], George Boder [2], Robert Morff [2], Gary C. Lantz [1], [1]Purdue University, W. Lafayette, IN 47907, [2] Eli Lilly, inc., Indianapolis, IN 46285.

Regeneration of specialized and differentiated tissues is poorly understood in mammalian systems. Repair via scar formation or complete loss of structure is often the result of injured organs or organ parts. A heretofore obscure collagen based connective tissue derived from the small intestinal submucosa (SIS) has been shown to stimulate the growth of certain body parts such as arteries, veins, ligaments, and tendons in xenogeneic recipients. SIS stimulates an aggressive proliferation of vascularized, undifferentiated connective tissue that subsequently assumes the shape and function of certain replaced body parts. Long term animal studies have shown that SIS, when placed in an arterial location, is completely replaced by endothelialized intima which in turn is surrounded by organized circumferential layers of smooth muscle cells. Venous grafts with SIS form thin vascular structures devoid of smooth muscle but composed of thromboresistant endothelial lined collagen conduits. Likewise, SIS placed either within synovial joints or as extraarticular support structures becomes replaced by appropriate connective tissues which function effectively as infection resistant, strong ligament and tendon like structures. Cell culture work shows that SIS supports a variety of cell cultures and may enhance growth of cells in surrounding media.

CE 028 MANIPULATION OF MOLECULAR AND CELLULAR PARAMETERS OF SKELETAL MUSCLE FOR CARDIAC ASSIST AND NEOSPHINCTER MYOPLASTY AND DURING SURGICAL LIMB LENGTHENING PROCEDURES.

Geoffrey Goldspink[1], Pamela Williams[2], Hamish Simpson[3], John Kenwright[3], Peter Kyberd[3] and David Goldspink[4] [1] Molecular and Cellular Biology, The Royal Veterinary College London University, [2] Hull University, [3] The Nuffield Orthopaedic Centre, Oxford University, [4] The University of Leeds, UK.

Adaptability of Skeletal Muscle. Muscle has a remarkable ability to adapt to the type of activity to which it is habitually subjected. This inherent adaptability is the result of quantitative and qualitative changes in gene expression. Our work has been focused on the myosin heavy chain (MyHC) isoform genes as these encode the myosin crossbridge; the force generator for contraction. We have also studied the way skeletal muscles elongate during growth and have shown that new sarcomeres are added serially to the ends of existing myofibrils and are thus capable of adapting to a new functional length The stretch effect and the adaptation to an increased functional length is known to be associated with increased protein synthesis. More recently they have studied the way gene expression in muscle is influenced by electrical stimulation and/or stretch. This therefore offers the prospects of engineering the tissue to meet a changed activity regime as might be required for certain surgical and other procedures including those described below.

Cardiac assist. Surgical procedures for "cardiac assist" myoplasty have now been established by several teams in the States and Europe. The Latissimus Dorsi (LD) muscle is used as a contractile wrap which is activated by a specially designed pacemaker. The muscle fibres adapt surprisingly well to their changed activity pattern and become considerably more fatigue resistant. The main problem is that when the LD flap is translocated to form a ventricle or aortic wrap there is a marked decline in contractile power output. This is attributable to muscle fibre atrophy and also the slowing of the contractile apparatus.

Neosphincter. N.S. William's Group at the Royal London Hospital surgical Unit have pioneered the construction of anal sphincters using the gracilis muscle. The muscle is stimulated with a specially designed pacemaker to remain contracted and generate enough force to close the anal canal. The pacemaker can be switched off during defecation using a small magnetic switch, the problem is the length of time it takes the gracilis muscle to adapt to its new role by becoming fatigue resistant.

Surgical limb lengthening. Bone distraction is used in orthopaedic centres to correct major deformities and limb length discrepancy. The procedure involves osteotomy followed by distraction of the two parts of the bone using an external frame. New bone forms between the cut ends and if carried out at the appropriate rate the muscle fibres also adapt by producing more sarcomeres in series. The remodelling of the collagen framework of the muscle was somewhat slower. Nevertheless the results indicate good adaption to the imposed length.

Supported by The Wellcome Trust and Action Research UK.

CE 029 MATRIX ENGINEERING: DESIGN AND FABRICATION OF A COLLAGEN VASCULAR GRAFT FOR REMODELING IN VIVO.

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Autogenous veins remain the "gold standard" for arterial grafting. They do not function as normal blood vessels immediately after surgery; rather, many of the cells die and the grafts serve as scaffolds for extensive remodelling. This results in an artery-like structure having an endothelial lining and smooth muscle cell media within weeks to months (1). Other biological materials, such as the small intestine submucosa, can also serve as scaffolds for remodeling and arterialization (2). Our approach is to use biological molecules such as collagen to fabricate *in vitro* a construct designed to function as a vascular graft that will be remodeled in an orderly manner *in vivo.*

By concentrating collagen around a mandrel prior to fibril formation, a collagen matrix has been produced containing long, native-banded collagen fibrils at densities approaching those of normal tissues. By controlling the pH at which this dense fibrillar collagen (DFC) matrix is laid down, a smooth, relatively non-thrombogenic surface can be produced (3). Implantation experiments show that the DFC matrix can be rapidly remodeled *in vivo.* In order to provide sufficient strength for suturing and to maintain the structure during the remodeling process, the graft is integrated with an open knit mesh. Most experiments to date have utilized a Dacron™ polyester mesh, but recently we have produced DFC threads (4) and knit them into a mesh which can meet the physical requirements for vascular grafting when integrated with DFC matrices. Crosslinking the threads slows their remodeling so that the knit collagen threads should be able to maintain the graft's mechanical strength during the remodeling process.

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Designs and use of Cellular Implants for Replacement Therapies

CE 030 **IMPLANTATION OF CULTURED SCHWANN CELLS TO FOSTER REPAIR IN DAMAGED MAMMALIAN SPINAL CORD,**

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Tissue cultured Schwann cells (SCs) were grafted into lesioned adult rat spinal cord to determine if they elicit CNS axonal regeneration. Cystic cord cavities were formed by a photochemical lesioning technique. Highly purified SC populations were obtained from E15 rat dorsal root ganglia, rolled in polymerized collagen to form an implant 4-6 mm long, and grafted into the lesion cavity. No immunosuppression was used. Implants containing only the collagen roll served as controls. At 14 and 28 days and 3 and 6 months after grafting, animals were perfused and analyzed histologically with silver and toluidine blue stains and electron microscopy (EM). The grafts integrated well with host tissue, fusing with it in many places with only limited astrogliosis. By 14 days bundles of unmyelinated and occasional thinly myelinated axons populated SC implants. By 28 days and thereafter, numerous unmyelinated and myelinated axons were present in all grafts. Photographs were taken of plastic-embedded, toluidine blue-stained cross-sections and assembled into montages to count the number of myelinated axons at those levels; in such montages the number of myelinated axons varied from 647-3214 (mean, 1549). EM of implants at 28 days and 3 and 6 months showed that unmyelinated axons were more numerous than myelinated axons, and that myelin thickness was greater by 3 months. Random EM sampling in one SC graft showed that there were 7.6X more unmyelinated than myelinated axons. This suggests that the total number of axons in a cross-section of a graft could be as high as 24,000. Additional grafts are being evaluated. Silver staining revealed branching of axons at the implant border at 28 days and long bundles of axons within the implant at 90 days. Acellular collagen grafts, on the other hand, did not contain axons. In sum, grafts can be prepared by rolling SCs on a layer of supporting collagen, they fill a lesion cavity, and they are well tolerated by the host. There is rapid and substantial axonal growth into grafts containing SCs; the SCs ensheathe and myelinate these axons in the normal manner. *Supported by NIH Grants NS28059, NS09923; The Miami Project; and the Spanish Ministry of Education.*

CE 031 MESENCHYMAL STEM CELLS AND THEIR USE IN REGENERATING ADULT TISSUES, Arnold I. Caplan, Ph.D., Skeletal Research Center, Department of Biology, Case Western Reserve University, Cleveland, Ohio.

A number of adult mesenchymal tissues exhibit relatively high turnover rates and a correlated ability to repair/regenerate damaged tissue. Bone, for example, repairs damage by the migration and proliferation of primitive mesenchymal cells which differentiate into extracellular matrix-producing cells with the eventual regeneration and reformation of bone at the fracture site. These reparative cells at the bone fracture site are capable of differentiating into either chondrocytes or osteoblasts depending upon a number of locally controlled factors and physical conditions. Such a bipotential cell is referred to as an osteochondral progenitor cell, but may actually be a pluripotent progenitor cell capable of developing into tendon, ligament, adipose tissue, marrow stroma, muscle or dermis which can be referred to as a MESENCHYMAL STEM CELL. Our laboratory has developed the technology to isolate Mesenchymal Stem Cells from the bone marrow or periosteum of adult animals and humans; these cells multiply in culture through a number of passages so that millions of such cells can be obtained.

With the availability of large quantities of autologous or syngeneic Mesenchymal Stem Cells, the re pair/regeneration of a number of mesenchymal tissues can be explored. Experiments involving the repair of massive bone, ligament, tendon or cartilage defects and marrow stroma involve the successful reimplantation and delivery of Mesenchymal Stem Cells to discrete tissue locations. The use of tissue-specific cell delivery vehicles and specific surgical implantation motifs defines a new era of cell-based therapies to engineer tissue regeneration. Data will be presented which focuses on the isolation, mitotic expansion and use of Mesenchymal Stem Cells to effect various skeletal repair.

Supported by grants from the National Institutes of Health.

CE 032 PREVENTION OF REJECTION OF ISLET ALLOGRAFTS AND XENOGRAFTS BY IMMUNOALTERATION OR ENCAPSULATION OF DONOR ISLETS, Paul E. Lacy, Washington University School of Medicine.

Passenger leucocytes in donor islets have been shown to be responsible for initiation of rejection of islet allografts in rodents. Some methods for alteration or destruction of donor leucocytes in the islets prior to transplantation are in vitro culture at low temperature or in 95% O_2, treatment with specific antibodies against lymphoid cells, ultraviolet irradiation, use of multiple donors, and purification of islet cells. These pretreatment regimens prevent rejection of islet allografts in mice and in conjunction with temporary immunosuppression also prevent rejection of islet allografts in rats. The procedures induce specific immune tolerance in the recipients.

These immunoalteration techniques for the donor islets and temporary immunosuppression of the recipients also prevent rejection of islet xenografts transplanted across a closely related (rat to mouse) and wide species barrier (human to mouse). Treatment of donor rat islets with transforming growth factor beta and temporary treatment of recipient mice with a monoclonal antibody to either interferon-γ or tumor necrosis factor α prevents rejection of rat islet xenografts. An unexpected finding was that daily injections of the recipients with tumor necrosis factor α prevented rat islet xenograft rejection in mice until the injections were stopped. This finding indicates that alteration of the level of a lymphokine affects initiation of the rejection process. Immune tolerance to islets from the donor strain is present in mice with established rat islet xenografts.

Immunoisolatory devices including microencapsulation, intravascular devices and acrylic copolymer hollow fibers have been used for prevention of rejection of islet allografts and xenografts. Recently, we have shown that rat islets immobilized in a matrix within acrylic copolymer hollow fibers will maintain normoglycemia and prevent rejection of islets in the fibers, transplanted intraperitoneally into mice. An amazing finding was that the encapsulated islets would also maintain normoglycemia when implanted subcutaneously. Neither free nor encapsulated islets have been shown previously to function for long periods of time in the subcutaneous site.

The goal of the studies on prevention of rejection of islet allografts and xenografts is to be able to utilize these approaches for the prevention of rejection of human or animal islets transplanted into diabetic patients without the need for immunosuppressive therapy. The first objective was to demonstrate that transplants of human islets into diabetic patients already receiving immunosuppressive therapy for maintenance of their kidney transplants would produce normoglycemia in the recipients without insulin therapy. Recently, methods have been developed for the mass isolation of human islets which permitted the initiation of these studies. In the past two years, it has been shown that human islet allografts in immunosuppressed diabetic patients will achieve and maintain normoglycemia in the recipients. The next phase is to determine whether immunoalteration of donor human islets will prevent rejection without immunosuppression in diabetic patients. With appropriate scaling up of immunoisolatory devices, clinical trials with encapsulated human and/or animal islets can be initiated in the future.

CE 033 IMMUNOISOLATION OF CELLS AND TISSUE: RATIONALE, TECHNIQUES, & RESULTS, [1]Michael Lysaght, [1]Orie Hegre, [1]Frank Gentile, [1]Seth Rudnick, [2]Patrick Aebischer. [1]CytoTherapeutics Incorporated, Providence, [2]Brown University, Providence.

Biohybrid organs were first described in the late 70's for the treatment of diabetes in rodent models and were largely studied for that purpose during the following decade[1]. To be effective they require that therapeutically useful dispersions of cells survive and function after transplantation into a discordant host when surrounded by a semipermeable membrane with pores suitably sized to screen out immunoglobulins and immunologically active cells while allowing passage of nutrients, electrolytes, oxygen, and therapeutically useful cell secretions. The scope of technology applied to this approach has recently broadened, most significantly including: 1. *Therapeutic Targets* - Models of CNS diseases have been successfully ameliorated by implants containing cells which constitutively release neurotransmitters or neurotrophic factors; treatment of experimental Parkinsons is most advanced but early results with other neurodegenerative disorders and chronic pain are also convincing and encouraging[2] 2. *Cell Sourcing* - Techniques have been developed to prepare immunoisolated implants from dividing cells, both genetically engineered and established lines, which are easier to procure than primary cells and which produce a wide variety of therapeutically useful products. Appropriately selected cells do not outgrow capsules and will survive in a xenogeneic host only when encapsulated 3. *Extracellular Matrices* - Surrounding encapsulated cells with a synthetic or naturally occurring hydrogel has been shown to prolong cell survival as well as to influence morphology and orientation 4. *Design and Fabrication* - Implants have advanced from relatively crude handmade laboratory preparations to well controlled, GMP-level preclinical prototypes. These can be retrieved after implantation or, in some cases, recharged *in vivo* with cells. As a cumulative result of these advances, investigators working with canine models of diabetes[3] and subhuman primate models of Parkinsons[4] have routinely achieved efficacy for periods of three months to one year in the absence of pharmacological immunosuppression and with cell viability documented both by reversal of symptoms during implantation and by histology obtained at the conclusion of the study. In combination with rodent experiments[5,6], such studies persuasively demonstrate that currently achievable levels of biocompatibility, immunoprotection, and transport kinetics are now adequate to allow long term therapeutic efficacy of immunoprotected well established animal models. Investigative groups are currently acquiring the extensive preclinical data needed to begin human trials.

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Late Abstract

CELLS THAT ARE THOUGHT TO BE DIFFICULT OR IMPOSSIBLE TO GROW: NORMAL HUMAN NEUROBLASTS. Hayden G. Coon, Lab of Genetics, NCI, NIH, Bethesda, MD 20892.

Fetal neuroblasts have not received the necessary public acceptance for use in human grafting. Another potential source of human neuroblasts exists: the adult olfactory epithelium (OE). We have worked first with rats and then with human OE and succeeded in growing cloned cell strains that develop neuron markers in culture and that can respond (cAMP accumulation and/or Ca^{2+} release) to submicromolar quantities of chemical odorants (1,2). Among the neuron markers identified both by immunohistochemistry and immunoblot techniques in the human cells are: neuron specific enolase (NSE), three size classes of neurofilaments, olfactory marker protein (OMP), growth associated protein (GAP-43), and peptides: calcitonin gene related peptide (CGRP) and carnosine (carnosine synthetase activity) both associated with the OE nerve tracts. At the same time that these more differentiated products occur in the cultures, cloned strains also show glial fibrillary acidic protein (GFAP) and keratin which suggest the presence of more primitive, stem cells in the population. We and others have described conventional bipolar neuron morphologies in cultures that do not resemble the unique knobbed dendrite with cilia characteristic of the sensory neuron in the OE. When OE tissue was transplanted to the brain, streams of neurons not resembling sensory neurons were observed around the graft (3). These observations suggest the possibility of obtaining a pluripotent neuroblast from normal mammalian OE; perhaps a kind of 'generic' neuron. Adult human OE cells can be obtained from biopsies or from organ donors (6 - 10 hours post mortem). They have been cultivated for at least 30 population doublings *in vitro* and are, therefore, suitable for banking and for engineering as pseudotissue grafts. We are undertaking to study these cultured human neuroblasts as potential material for grafts in immune compromised animals and ultimately in humans.

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Tissue and Organ Equivalents; 3D Constructs

CE 100 ***IN VIVO* EXPRESSION OF hGH FROM GENETICALLY AUGMENTED HUMAN MICROVASCULAR ENDOTHELIAL CELLS IN THREE-DIMENSIONAL MATRIGEL**, Paula N. Belloni, Lori Ross, Melinda Van Roey, Peggy Ho-Faix, Shirley Clift, Lawrence K. Cohen, and Miriam E.C. Hancock, Somatix Therapy Corporation, Alameda, California 94501
Human microvascular endothelial cells MVEC have been investigated for cell-based drug delivery *in vivo*. MVEC were isolated from human adipose tissue and characterized for expression of Ac-LDL and UEA-I receptors and cytoplasmic vWF. A retroviral vector (α-gl-hGH.b27) containing the cDNA for human growth hormone (hGH) was constructed based on MMLV. HMVEC were transduced at high efficiency with a single exposure of α-gl-hGH.b27 particles. hGH expression was determined qualitatively by immunohistochemical staining followed by FACS (90% + for hGH) and secreted proteins quantitated using a human specific RIA. Transduced HMVEC cultured in either 2 or 3 dimensional matrigel secreted hGH into the culture media at a rate of 500 ng/10^6 cells/hr. *In vivo* expression of hGH was achieved by implanting 2 x 10^6 transduced HMVEC with matrigel containing FGF and heparin at subcutaneous sites in nude mice. Plasma hGH was initially detected at significant levels (25ng/ml), but decreased 100 fold by 10 days. HMVEC rescued from the implant after 10 days secreted ~500 ng/10^6 cells/hr. Histochemical analysis of the matrigel implants demonstrated that the HMVEC were retained in the graft. Neovascularization of the matrigel implants was observed within 24 hrs and persisted beyond two weeks. The matrigel implants also contained fibroblasts and a modest monocytic infiltrate. These data suggest that matrigel is a suitable implantation material for assessing the properties of genetically augmented endothelial cells *in vivo* and *in vitro*. Currently, we are using this model system to assess the potential effects of immune elicited cytokines on protein expression from genetically-modified HMVEC.

CE 101 REGULATION OF COLLAGEN SYNTHESIS IN DERMAL EQUIVALENTS BY ASCORBIC ACID, FIBROBLAST GROWTH FACTOR AND TGFβ, Richard A. Berg, Jeffrey C. Geesin, Laura J. Brown and Joel S. Gordon, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, NJ 08854 and Johnson and Johnson Consumer Products, Inc., Skillman, NJ 08558.
Ascorbic acid has been shown to stimulate collagen synthesis in monolayer cultures of human dermal fibroblasts. In the present studies, we compared the response of fibroblasts contained in monolayer cultures and in dermal equivalents to regulation by ascorbic acid, transforming growth factor β, and fibroblast growth factor. Dermal equivalents were prepared by combining fibroblasts with soluble type I collagen and incubated for 5 days to form a gel prior to determining the concentration and time dependence for ascorbic acid's effects on collagen synthesis. The concentration dependence of ascorbic acid was measured in cultures incubated for 24 hours with ascorbic acid. The results indicated that in both monolayer cultures and in dermal equivalents ascorbic acid stimulated collagen synthesis at concentrations of 10 μM and above. The degree of response to ascorbic acid at 50 μM was 5-fold in monolayers and 2- to 4- fold in dermal equivalents. At a concentration of 200 μM, the time-course for the effect of ascorbic acid was measured and found to be maximal at 2 days. The magnitude of stimulation by ascorbic acid in monolayer cultures was 8-fold, while the magnitude of stimulation in dermal equivalents was 3-fold. Transforming growth factor β (TGFβ) stimulated collagen synthesis and fibroblast growth factor (FGF) inhibited collagen synthesis in ascorbic acid treated human dermal fibroblasts. TGFβ stimulated collagen synthesis in both tissue culture models; however, the effect was greater in monolayer cultures (11-fold) compared with dermal equivalents (2.5-fold). The inhibition of ascorbic acid stimulated collagen synthesis by FGF was also greater in monolayer cultures than in dermal equivalents. These results indicate that the presence of an extracellular matrix alters the response of dermal fibroblasts to modulation of collagen synthesis. Supported in part by NIH AM31839.

CE 102 **THE EFFECTS OF HUMAN PAPILLOMAVIRUS ONCOGENES IN ORGANOTYPIC CELL CULTURES** **Rebecca A. Blanton[1], Marc D. Coltrera[2], George J. Todaro[1,3], and James K. McDougall[1,4], [1]Fred Hutchinson Cancer Research Center, 1124 Columbia St., Seattle WA 98104, and Depts of [2]Otorhinolaryngology, [3]Pathobiology, and [4]Pathology, University of Washington, Seattle WA 98195.**

We have modified procedures for the organotypic culture of epidermal keratinocytes in order to culture a variety of lining epithelial cell types. With these modifications it has been possible to culture epithelial cells from the endo and ectocervical regions of the human uterine cervix such that they specifically reform the fully differentiated tissue from which they were derived.

We have utilized this organotypic culture system to assess the role of Human Papillomavirus (HPV) oncogenes in malignant progression. In these experiments, human foreskin and cervical epithelial cells were infected with recombinant retroviruses which express the HPV16 E7 or E6 genes, selected for neomycin resistance, and cultured organotypically prior to senescence of the cells infected with the retrovirus vector alone. The major conclusions from these studies are: (1) E7 expression alone results in continued proliferation in the suprabasal epithelial layers of stratified squamous tissues, (2) suprabasal layers of cultures expressing HPV16 E7 retain the ability to terminally differentiate to an extent comparable to that seen in normal or vector-containing cultures, and (3) coexpression of HPV16 E6 and E7 did not alter the neoplastic appearance of stratified cultures compared to cultures expressing E7 alone. However, as previously reported, E6 and E7 together are able to immortalize a variety of epithelial cells.

CE 103 PROTECTION OF ALLOGRAFT TISSUES FROM REJECTION BY IMPLANTATION WITHIN AN IMMUNOISOLATION DEVICE. James H. Brauker, Laura A. Martinson, Victoria E. Carr-Brendel, and Robert C. Johnson, Applied Sciences, Baxter Healthcare Corp. Round Lake, IL, 60073. Tissues derived from a donor of the same species, but different genotype (allografts) are rejected in 7-14 days when implanted into a host. It is currently thought that the allograft cells exhibit endogenous peptides on their cell surface in association with MHC class I molecules, and that the class I-peptide complexes are recognized by cytotoxic T-cells, which kill the allograft cells. We implanted embryonic lung tissues within membrane-enclosed devices to protect the tissues from this cellular immune response (immunoisolation). Allograft tissues (Sprague-Dawley rat into Lewis rat) in membranes that allowed entry of host cells (Millipore brand Biopore™ membranes in which we made 70-110 μm holes) were killed within 15 days when implanted in epididymal fat pads. In contrast, allograft tissues implanted within membranes that prevented entry of host cells (intact Biopore™ membranes) were protected from rejection for at least 9 months. These tissues were histologically indistinguishable from control tissues derived from a donor with the same genotype (isograft). The isograft controls were implanted in the same animals as the allograft tissues on the opposite fat pad. The Biopore™ membrane has a nominal pore size of 0.45 μm, and therefore allows entry of humoral immune factors. Tissues from a donor of a different species (xenograft), for example ICR mice, were destroyed within 21 days when implanted in membranes that prevent the entry of host cells (intact Biopore™). The death of the tissues was accompanied by a substantial inflammatory response, including a heavy, localized plasma cell response, not seen around allograft implants under the same conditions. These data indicate that this membrane allows transit of a rejection signal from implanted xenograft tissues, but not from allograft tissues. The data show a specific response of the host to xenograft tissues, indicating the likelihood that the xenograft tissues are rejected by a humoral immune response. In contrast, the allograft tissues, which are clearly rejected when cell contact is allowed (holes poked in the membrane) are protected from rejection for at least 9 months by an intact membrane. Allograft immunoisolation chambers have advantages over autologous cell transplants. Some examples of the advantages are; (i) genetically altered cells are isolated from the tissues of the patient, (ii) cells are retrievable, (iii) the same well characterized cells can be used for all patients, and (iv) the cells are implanted in a well-vascularized ("organoid") milieu.

CE 104 ORGANIZATION OF AN OSTEOID TISSUE "IN VITRO" : ROLE OF TENSILE FORCES, Annette M. Chamson, Nathalie H. Vergely, Jacques M. Frey, Department of Biochemistry, Medical School, 42023 Saint-Etienne, France.

For the purpose of checking the effects of new drugs on calcification induction, we developed a model of artificial bone tissue. This tissue was prepared from human osteoblast cultures. These cells were cultivated from human spongy bone after enzyme digestion. They were characterized as osteoblasts by different methods :

-the presence of alkaline phosphatase demonstrated by an histochemistry method,

-an amount of type III collagen divided by 3 in comparison with human fibroblasts,

-a large increase of the cAMP concentration after stimulation by PTH,

-the presence of osteocalcin in the culture medium.

The osteoid tissue was prepared by culture of these cells into a collagen gel. It can be carried out in presence or absence of calcium and ß glycerophosphate. It can be or not submitted to tensile forces which are well known to play an important role in bone formation. After 3 weeks of culture, it was demonstrated by electron microscopy that calcification occurred "in vitro" : hydroxyapatite deposits were present around the collagen fibers under three differents conditions :

-the tissue under free retraction after addition of calcium and ß glycerophosphate,

-the tissue submitted to tensile forces after addition of calcium only,

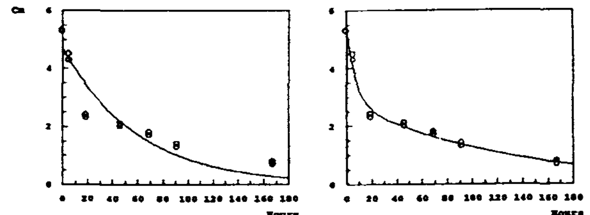
-the tissue submitted to tensile forces after addition of calcium and ß glycerophosphate.

This model can be used to study the regulation phenomena involved in calcification and bone formation.

CE 105 A MATHEMATICAL STUDY OF THE RETRACTION KINETIC IN COLLAGEN LATTICES POPULATED BY FIBROBLASTS, Jacques M. Frey, Aline Rattner, Annette M. Chamson, Department of Biochemistry, Medical School, 42023 Saint-Etienne, France.

The collagen lattices were made according to the E. Bell's method which consists of a fibroblast culture into a collagen gel. The fibroblasts organize and retract such a gel. The preparation was made in a 60 mm petri dish and the retraction kinetic was studied by measuring the diameter of the gel in fonction of time.

The kinetic of the retraction is an exponential decay. We looked at several kinds of exponential curves to know the function best correlated with the experimental points.



$y = A_0.e^{-kt}$ $\chi^2 = 0.0390$ $y = A_0(1).e^{-k(1)t} + A_0(2).e^{-k(2)t}$ $\chi^2 = 0.0059$

The χ^2 test showed that a double exponential decay with or without offset was the most probable function correlated with the gel retraction. We choiced a double exponential function without offset to anticipate the dimension of the lattice during the retraction.

This mathematical model allowed to design a device adapted to the preparation of a defined three dimensional tissue.

CE 106 A CULTURE SYSTEM FOR THE THREE-DIMENSIONAL GROWTH OF CARTILAGE, Daniel Grande*, Craig Halberstadt, James Linton, Gail Naughton, Marrow-Tech Incorporated, La Jolla, CA 92037, *Northshore University Hospital, Manhasset, NY 11030

A three-dimensional culture system is being utilized to support the growth of chondrocytes and the natural deposition of a cartilaginous matrix *in vitro*. Isolated chondrocytes are seeded onto various biocompatible matrices enclosed in Teflon bags. Pre-treatment of the support material with RGD peptides or incubation with serum proteins enhances the initial attachment of cells and their expansion across pore openings. After 24-48 hours, additional media is injected into each bag and individual units are attached to a peristaltic pump system for continuous flow feeding of the developing tissue. This proprietary culture system allows for the maintenance of sterility, optimum uniformity of growth from sample to sample, and takes advantage of growth factors secreted throughout the system. As the chondrocytes divide, grow and fill in the space of the support framework, they secrete a variety of matrix components and growth factors. Proteoglycan synthesis and matrix protein composition are being characterized by pulse labeling and specific immunohistochemical staining, respectively. The closed system allows for the regulation of oxygen tension, hydrostatic pressure and ionic concentrations, factors which have been shown to be integral to physiological cartilage formation. Tensile strength determination (trouser tear test) and creep testing are being utilized to assess the structural characteristics of the cultured cartilage. A physiological cartilage model has important applications in the study of the mechanism of various joint diseases, in addition to utility as a tissue for articular cartilage resurfacing.

CE 107 THE DEVELOPMENT, CHARACTERIZATION AND CLINICAL USE OF A THREE-DIMENSIONAL DERMAL EQUIVALENT, Craig Halberstadt, Sandra Slivka, Frank Zeigler, Michael P. Zimber, Ronnda Bartel, Marrow-Tech Incorporated, La Jolla, CA 92037

A cultured dermal equivalent has been developed in which human neonatal dermal fibroblasts secrete an entirely human dermal tissue matrix on a bioabsorbable mesh scaffolding. After seeding dermal fibroblasts on a polyglactic mesh, cells attach, extend their processes and establish a tissue matrix over a 19 day culture period. Fibroblasts remain metabolically and mitotically active. The dermal equivalent that is formed has been termed "Dermagraft™" and has been characterized for a variety of matrix proteins and growth factors. The Dermagraft tissue is cultured in a closed-system to optimize conditions of sterility, to help take advantage of recirculation of naturally-secreted growth factors, and to result in the manufacture of a highly reproducible living tissue. Immunohistochemical and immunofluorescence studies have indicated that this dermal equivalent contains human fibronectin, a variety of glycosaminoglycans, and collagen types I and III. PCR techniques have been utilized to identify a variety of growth factors secreted by the dermal equivalent. To date, a number of growth factors have been identified including PDGF and insulin-like growth factors I and II. Studies are continuing to further characterize factors secreted by this system. The dermal equivalent is being used in the treatment of a variety of wounds, including full-thickness burns and chronic skin ulcers. To date, over 50 patients with full-thickness burns have been grafted using Dermagraft and meshed split-thickness autografts. The dermal equivalent, which has been shown to act as a physiological tissue, rapidly vascularizes, takes in the wound bed and supports epithelial migration, attachment and differentiation. Immunofluorescence has shown the deposition of basement membrane constituents, which are a distinct advantage in wound healing. Clinical studies are continuing to assess the efficacy of Dermagraft in healing burn wounds and a variety of skin ulcers. Future applications of this cultured human dermal equivalent may include use in corrective and cosmetic surgery as well.

CE 108 OSTEOGENESIS WITH BONE MARROW CELLS AND GELFOAM, Paul A. Lucas, Steven Troum, James Shoptaw, Scott Bowerman, and Henry E. Young, Department of Surgery, Mercer University School of Medicine, Macon, GA 31207

Obtaining sufficient bone to heal orthotopic defects is a continuing problem in orthopedics. Bone marrow contains osteogenic cells that induce bone formation at ectopic sites in rats when incorporated into porous ceramics, which serve as a substrate for the osteogenic cells. However, porous ceramics are not biodegradable. Gelfoam, an insoluble type I collagen matrix, is biodegradable and we have used it as a substrate for marrow cells. Bone marrow obtained from the long bones of 6-week old rats was dissociated enzymatically and the cells reconstituted in rat serum. 30×10^6 cells in 0.1 ml were then adsorbed into 1 cm^3 pieces of Gelfoam and implanted into ectopic sites in syngeneic rats. The implants were retrieved and analyzed histologically for bone formation at 3, 4, 6, and 8 weeks post-implantation. Woven bone formation was seen as early as 3 weeks and persisted through 8 weeks. In some locations the bone seemed to be laid on the Gelfoam, but in most cases the bone apeared to have been formed by intramembranous ossification and was between the pieces of Gelfoam, with cells lying between the bone and the Gelfoam. No cartilage was observed. Osteoclasts first appeared at 3 weeks, peaked in numbers at 4 weeks, and seemed to degrade the Gelfoam as well as the woven bone. By weeks 6 and 8 only small isolated islets of Gelfoam remained, surrounded by dense woven bone. Gelfoam can serve as a substrate for osteogenic cells in bone marrow. While porous ceramics offer initial mechanical strength, Gelfoam has the advantage of being biodegradable and allows its replacement by new bone.

CE 109 REINDUCTION OF ENDOTHELIAL FENESTRAE IN COCULTURES OF ENDOTHELIAL CELLS AND MDCK CELLS IN THREEDIMENSIONAL COLLAGEN GELS. V. Mironov and W. Risau, Max-Planck-Institut für Psychiatrie, D-8033 Martinsried, Germany

Endothelial cells are functionally and morphologically heterogenous. It was proposed that differences in the local microenvironment are responsible for this heterogeneity. Fenestrations in endothelial cells represent a unique organotypic differentiation present in many, mainly endocrine organs. They are typically found in endothelial cells that are in close proximity to adjacant epithelium *in vivo*. This suggests that interaction with epithelial cells are involved in fenestra induction.

Adrenal capillary endothelial cells (ACE) are fenestrated *in vivo* but lose this property during *in vitro* culture. In a coculture system with epithelial cells (MDCK) in threedimensional collagen gels ACE changed their monolayer organisation and formed capillary-like tube structures with clusters of fenestrae. Capillary-like structures were often located in close proximity to organotypic cyst-like structures formed by MDCK cells in collagen gels. The analysis of serial ultrathin sections did not demonstrate tight or gap junctions in the region of close contact between MDCK cells and ACE. Epithelial cell conditioned medium or MDCK matrix could not reproduce these effects. In cocultures separated by porous filters no reinduction of fenestrae was observed.

We propose that reinduction of fenestrae is mediated by fenestra inducing factors produced by MDCK cells. These are likely to act over a short distance, and a well developed extracellular matrix may be needed for the maintenance of the biological activity of these putative factors. The *in vitro* model may be instrumental in the biochemically characterization of inducing factors.

CE 110 HEMATOPOIESIS ON SUSPENDED NYLON SCREEN STROMAL CELL MICROENVIRONMENTS, Brian A. Naughton*, Benson Sibanda, Julia San Roman, Craig Halberstadt, Marrow-Tech Incorporated, La Jolla, CA; *Medical Laboratory Sciences Dept., Hunter College School of Health Sciences, New York, NY 10010

A three-dimensional culture system for the growth of primate and rodent bone marrow was developed in our laboratory. This method involves the seeding of stromal cells onto a nylon screen. After stromal cell processes have extended across three to four out of every five mesh openings, the cultures are inoculated with fresh or cryopreserved bone marrow hematopoietic cells. Stromal cells attach, grow, and secrete matrix proteins. They then create a microenvironment which supports multilineage hematopoiesis. This process was observed for >270 days in the rat model and for >12 weeks in the human system, as evidenced by flow cytometry analysis and *in vitro* clonogenic assays. The mesh system has been adapted for growth in a closed perfusion system which allows for the regulation of oxygen tension, flow rate, and recirculation of naturally secreted growth factors.

These cultures can also be used as substrates for cytotoxicity measurements. For example, treatment of rat bone marrow cultures with cytosine ß-D arabinofuranoside, cyclophosphamide, 5-fluorouracil, or methotrexate resulted in a dose-dependent decrease in CFU-C numbers and altered phenotypic distribution of hematologic cells in the adherent zone. A modification of this method utilizing rat bone marrow-derived natural killer cells has been studied. These cultures produced large numbers of active cytolytic cells after >75 days culture. Preclinical studies with NK cells generated by this system are underway. Suspended nylon screen bone marrow cultures also have other potential applications, i.e. genetic insertion and blood component therapy, graft vs. host disease studies, evaluation of *ex vivo* purging programs and in marrow expansion for transplantation.

CE 111 A THREE-DIMENSIONAL SYSTEM FOR THE LONG-TERM CULTURE OF HEPATIC PARENCHYMAL CELLS, Benson Sibanda, Brian A. Naughton*, Julia San Roman, Craig Halberstadt, Marrow-Tech Incorporated, La Jolla, CA; *Medical Laboratory Sciences Dept., Hunter College School of Health Sciences, New York, NY 10010

The ability of adult Long-Evans rat hepatic cells to proliferate and to retain the capacity to synthesize albumin and metabolize drugs *in vitro* was studied. A three-dimensional culture method was employed, one similar in design to the stromal-based system employed to grow hematopoietic cells in our laboratory. Stromal support cells isolated from the tissue of choice were inoculated onto a nylon filtration screen template and suspended in liquid medium. Liver stroma consisting of fibroblast-like cells, vascular and bile duct endothelial cells, and Kupffer cells were isolated on discontinuous Percoll gradients and grown in monolayer cultures. Stroma were lifted from monolayer and transferred to nylon screens which were pre-treated to enhance cellular attachment. Stromal cells grew rapidly across the 210 µm mesh interstices to create a living stromal support tissue. When the majority of mesh openings were covered by the stromal cells, 10^7 freshly isolated parenchymal cells were inoculated onto the tissue. Included in the inoculum were 20 - 30 µm basophilic cells with high N:C ratios as well as larger acidophilic cells with lower N:C ratios and prominent multiple nucleoli. After inoculation, the mixed cultures were suspended in liquid medium. Rat albumin production in culture was assessed utilizing an ELISA assay and cytochrome P-450 enzyme activity was determined cytofluorographically, by the ability of discrete populations of cells to convert non-fluorescent monoethoxyfluorescein ethyl ester (EFEE) to fluorscein. Flow cytometry was utilized after propidium iodide labeling of RNase-treated isolated nuclei and ^{3}H-thymidine incorporation into adherent zone cells of the screen culture were determined. The results revealed that by 6 weeks of culture, native albumin synthesis was still evident and cells retained their ability to metabolize EFEE for this period. DNA studies also revealed a substantial number of S phase hepatocytes, a result that was consistent with the percent ^{3}H-thymidine incorporation. A high rate of proliferation was observed in cells produced in co-cultures inoculated with the large acidophilic cells; they also possessed the ability to convert EFEE and synthesize albumin. Cells derived from these cultures may potentially be useful for gene manipulation and transplantation. Preliminary studies also indicate that they can be employed for the determination of cytotoxicity.

CE 112 A NOVEL FORMATION METHOD OF ORGANOIDS COMPOSED OF MESENCHYMAL CELLS AND EPITHELIAL CELLS IN VITRO, Toshiaki Takezawa and Yuichi Mori, Japan Research Center, W. R. Grace & Co.-Conn., 100 Kaneda, Atsugi, Kanagawa 243, Japan

It has been known that epithelial cell specific functions might be expressed by interaction with mesenchymal cells. A novel method for the preparation of spheroids containing two types of cells (hetero-spheroid) has been successfully developed by utilizing a collagen-conjugated thermo-responsive polymer, poly-N-isopropyl acrylamide (PNIPAAm), as a cell substratum. PNIPAAm solidifies above its lower critical solution temperature (LCST, about 30°C), and instantly dissolves into the culture medium below its LCST[1]. We firstly seeded and cultured human dermal fibroblasts as mesenchymal cells on the substratum up to a confluent state and then seeded rat primary hepatocytes or human epiderml keratinocytes as epithelial cells onto the fibroblast monolayer. The hetero-spheroid was prepared by detaching the epithelial cell-attached mesenchymal cell monolayer at a temperature below the LCST and culturing it on the non-adhesive substratum. In the former case bile canaliculus-like structures and desmosomes were clearly observed between the hepatocytes, and Disse's space-like structures were seen between the hepatocytes and fibroblasts by TEM. Hepatocytes also expressed albumin for up to at least 3 weeks. In the latter case, keratinocytes segregated outward with differentiation and covered the spheroid composed of fibroblasts. These findings suggest that the above-described method is useful to make a hetero-spheroid, an organoid that morphologically and functionally resembles *in vivo* tissues or organs. 1) T. Takezawa *etal*. Bio/Technology 8, 854-856, 1990.

CE 113 MECHANICAL PROPERTIES OF DEVELOPING SKELETAL MYOTUBES GROWN *IN VITRO*

Miroslav Tolar, Charles P. Ordahl and Roger Cooke
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While there exist numerous studies on the contractile properties of postnatal and adult skeletal muscle fibers, there is little information regarding the development of contractile properties in embryonic muscle fibers. Embryonic myotubes undergo numerous transitions in contractile protein isoforms which may affect the development of their contractile properties. We have been developing techniques to analyze the mechanical properties (stiffness, force, contraction velocity, etc.) of individual embryonal myotubes cultured for different periods *in vitro*. Breast or limb muscle tissues from day 12 chick embryos were mechanically dissociated and added to medium containing collagen type I (0.3 mg/ml) and Matrigel (Collaborative Research; 2.7 mg/ml) at 4°C. When incubated at 37°C the medium forms a gel within which myotubes form after 3-4 days. Within 7-10 days spontaneous contractions occured and by 14-18 days strongly cross-striated, individual myotubes were visible by phase contrast microscopy. At different times in culture, individual myofibers were dissected away from matrix components and fibroblast-like cells, and then transferred to a tensiometer. The contractile properties of such fibers will be presented as well as the matrix, and other components determined to facilitate the isolation and analysis of individual, cultured myofibers.

Growth and Differentiation in Vitro

CE 200 FURTHER DIFFERENTIATION OF HYPERTROPHIC CHONDROCYTES IN CULTURE. Ranieri Cancedda, Fiorella Descalzi, Chiara Gentili, Paola Manduca. Istituto Nazionale Ricerca Cancro, Viale Benedetto XV, 10 - 16132 GENOVA - ITALY.

It has been postulated that also growth plate hypertrophic chondrocytes might contribute to the formation of a bone matrix. In this work we have defined conditions for promoting further differentiation of hypertrophic chondrocytes obtained in culture starting from chick embryo tibiae as previously described (Castagnola P., G. Moro, F. Descalzi, and R. Cancedda, 1986 J. Cell Biol. 102:2310-2317). When hypertrophic chondrocytes, isolated as single cells after 3 weeks in suspension culture, were transferred to substrate dependent culture in the presence of ascorbic acid, they underwent profound changes in their morphology and biosynthetic activity suggesting a further maturation to an osteoblast-like stage. Cells acquired an elongated or star shaped morphology expressed alkaline phosphatase and eventually mineralized. Type II and X collagen synthesis was halted and replaced by type I collagen synthesis. In addition the cells started producing and secreting a large amount of a protein with an apparent molecular weight of 82KDa in reducing conditions and 63 KDa in unreducing conditions. This protein is soluble in acidic solutions, does not contain collagenous domains and is glycosilated. The Ch21 protein, a protein belonging to the superfamily of proteins binding small hydrophobic molecules, was synthesized throughout the culture. Calcium deposition in the extracellular matrix occurred regardless of the addition of beta glycerophosphate to the culture medium. Comparable results were obtained when the cells were plated both at a low density and at confluence. When retinoic acid was added to the hypertrophic chondrocyte culture between day 1 and day 5, the conversion of chondrocytes from stage II to the osteoblast-like stage was highly accelerated.

CE 201 CELLS ON ROTATING FIBERS. Robert Clyde, Clyde Engineering, POB 740644, New Orleans, Louisiana, 70174

When cells are immobilized on high area fibers and rotated, fast reactions occur. Several scientists have grown plant cells on fibers and we are now designing a reactor for Taxol, designated by the National Cancer Institute as emergency priority. Mammalian cells also grow on fibers to produce valuable products. Skin for burn victims and internal organ tissue can be grown so there is no rejection. Cosmetics can be checked. Metals such as lead, Cr6 and uranium can be removed quickly from wastewater and chlorinated compounds degraded. When an RBC (rotary biological contactor) is run half full and a light shone in the top, the light hits a thin moving film. Sugar is fermented to ethanol in 10-15 minutes. All these concepts are covered by patents which are available for license.

CE 202 INHIBITION OF MYOBLAST ADHESION TO LAMININ BY AN ENDOGENOUS LECTIN, L-14.

Douglas N.W. Cooper, Steven M. Massa, and Samuel H. Barondes, Langley Porter Institute, University of California, San Francisco, CA 94143-0984

L-14 is a soluble lactose-binding lectin with dimeric subunits of 14 kD. It is expressed at high levels in extracellular matrix of many mesodermal tissues, where it is presumed to bind to complementary glycoconjugates and thus influence cell-cell or cell-matrix interactions. In skeletal muscle, expression of L-14 peaks at the time of myoblast fusion. It is then secreted in an unusual manner and accumulates in basement membrane formed around myotubes. L-14 shows particular affinity for polylactosaminoglycans, including laminin, the major glycoprotein of basement membranes. We present evidence for functional interaction of L-14 and laminin. L-14 secreted during differentiation of C2C12 muscle cells is deposited in nascent basement membrane by binding to complementary glycoconjugates, and laminin is the primary glycoprotein ligand for L-14 in these cells. Furthermore, myoblast adhesion to a laminin substrate is inhibited by L-14 either added exogenously or by cell transfection with a vector directing constitutive L-14 secretion. Such inhibition is due to lectin binding to laminin polylactosamine chains, because inhibition of adhesion is blocked by the competitive sugar, thiodigalactoside, or by cleaving laminin polylactosamines with endo-beta-galactosidase. We propose that developmentally regulated expression and secretion of L-14 participates in regulating myoblast detachment from laminin during formation of tubular myofibers. In other tissues, L-14 might similarly modulate adhesion or other cell interactions with laminin.

CE 203 SERUM-FREE PRIMARY CULTURE OF NORMAL FOETAL MAMMARY EPITHELIAL CELLS WITHIN BASEMENT MEMBRANE MATRIX.

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Data concerning the development and culture of the fetal mammary gland in small mammals can be only partly extrapolated to the human female. It therefore appeared to us necessary to study the conditions of development of the human fetal mammary gland (obtained in accordance with the laws in force).

A serum-free primary culture system has been developed which allows for three dimensional growth and differentiation of normal human fetal mammary epithelial cells (HFMEC) within an extracellular matrix preparation. HFMEC were isolated from mammary glands of human female fetus, 17 to 40 weeks-old and the organoids embedded within a reconstituted basement membrane matrix prepared from the ENGELBRETH-HOLM-SWARM sarcoma according to HAM and IP. Organoids were grown either in serum-free media or in media with fetal bovine serum (FBS). The organoids proliferated over a 2 to 3 weeks culture period and remained viable for one or two months within a basement membrane matrix. Several types of colonies were observed including the alveolar-like budding cluster obtained in cultures of fetal mammary gland of over 20 weeks age, unit with ductal-like projections and "stellate colonies". Cell proliferation was dependent on culture medium (with FBS no proliferation was obtained) and also on the substratum : without matrix, significantly less growth and development occurred. These types of colonies are obtained when a glandular differentiation from the budding of the malpighian epithelium is observed. A study on light microcopy and transmission electron microscopy was realised at the time of culture.

This unique system obtained with normal fetal mammary epithelial cells should thus serve as a model in which the regulation of human mammary development can be investigated.

CE 204 KIDNEY TUBULOGENESIS IN TISSUE CULTURE.

H.D. Humes and D.A. Cieslinski, VA Medical Center and University of Michigan, Ann Arbor, Michigan.

Since renal proximal tubule cells have the ability to regenerate after nephrotoxic or ischemic injury to form a fully functional differentiated epithelium, renal proximal tubule stem cells likely exist in the adult kidney. Since cells with stem cell-like characteristics in other epithelia have been shown to be resistant to maneuvers which induce premature terminal differentiation in tissue culture, experiments were undertaken utilizing primary cultures of adult rabbit renal proximal tubule cells in hormonally-defined, serum free media. Renal proximal tubule cells were grown on plastic to near confluency in primary culture, then treated with retinoic acid (RA, 0.1 uM) as a differentiation factor and epidermal growth factor (EGF, 0.01 uM) as a growth promoter. After treatment with RA and EGF for 24-96 hours, cells were passed onto plastic utilizing hormonally-defined, serum free media containing RA and EGF. Under these growth conditions, these cells are continuing to grow through the eighth passage. More importantly, by the third passage the cells developed the ability to form in collagen gels monolayers of cells with centralized lumens. These lumens are bordered by polarized epithelial cells with well defined microvilli and tight junctional complexes along the luminal border. Since a stem cell is defined as a cell with a high capacity for self renewal and the potential to produce differentiated progeny, these results suggest that these growth conditions are ablé to progressively enrich a population of renal proximal tubule cells with stem cell-like characteristics.

CE 205 A QUANTITATIVE ASSAY DEMONSTATES THE ROLE OF G-PROTEINS IN MORPHOGENESIS,

Robert J. Klebe, Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, TX 78284

Events involved in morphogenesis can be studied with a new quantitative assay. Normal human fibroblasts form swirling patterns that merge with one another to form structures that we term organizing centers. The assay presented here involves simply counting the number of organizing centers in a standard microscopic field.

We show that macroscopically visible organizing centers form by the progressive merger of smaller organizing centers. Formation of organizing centers involves the alignment of cells into parallel arrays. While parallel orientation of cells on plastic substrata requires cell-to-cell contact, parallel orientation on collagen gels takes place without cell-to-cell contact. On collagen gels, the spatial orientation of cells is determined by the orientation of collagen fibrils.

Treatment of transformed cells with agents that increase cyclic-AMP levels or modify guanine nucleotide binding proteins results in the morphogenesis of organizing centers. Via quantitative assays for morphogenesis, it was demonstrated that cholesterol precursors involved in isoprenylation of G-proteins are involved in morphogenesis.

The simple assay described here should permit the analysis of morphogenesis at the molecular and cellular levels.

CE 206 EVIDENCE OF TIGHTLY BOUND TGF-BETA IN RECONSTITUTED EHS TUMOR EXTRACELLULAR MATRIX, Frank J. Mannuzza, Tom L. Collins, Kathy Foster, Collaborative Biomedical Products, Inc., Becton Dickinson Labware, Two Oak Park, Bedford, MA 01730

Reconstituted basement membrane components from the EHS tumor including laminin, entactin, collagen IV and proteoglycans form a structured gel at elevated temperatures. Cells express differentiated phenotype on Matrigel and in some instances this response is known to be caused by growth factors entrapped in the gel. The concentration of TGF-Beta in Matrigel is approximately 4 ng/ml; this can be decreased by repeated precipitations using 20% $(NH_4)_2SO_4$. We have measured the partitioning of ^{125}I TGF-Beta after $(NH_4)_2SO_4$ precipitations and found an unexpected amount of label in the precipitated fractions suggesting that TGF-Beta is bound to one or more of the insoluble components. The apparent complex of TGF-Beta and its binding component(s) appears to be extremely stable as evidenced by the lack of dissociation in the pH range from 3 to 7. The failure of the complex to inhibit the incorporation of ^{3}H thymidine in CCL-64 cells further substantiates that the complex is highly stable. Acid/alcohol extraction of Matrigel resulted in the dissociation of the TGF-Beta from the complex. The data confirms that TGF-Beta is present in Matrigel at biologically significant levels but is tightly bound to one or more components of the matrix (S. Vukicevic, et.al. unpublished results).

CE 207 RETINOIC ACID AND TGF-BETA DECREASE THE YIELD OF CELLS EXPRESSING ALKALINE PHOSPHATASE. Muschler GF, Scerbin V, Miller RR, McDevitt C. The Section of Musculoskeletal Biology, Biomedical Engineering, The Cleveland Clinic Foundation. Cleveland, Ohio 44195.

Pluripotential bone marrow stromal cells will express a well characterized osteoblast-like phenotype *in vitro* including alkaline phosphatase expression. We report a clinically applicable method for isolating osteoblastic cells from bone marrow aspirates and the evaluation of agents which modify the harvest, proliferation, and differentiation of osteoblastic stem cells *in vitro*.

METHODS: Bone marrow (2ml) is aspirated from the human iliac crest. 2.5×10^6 nucleated cells are plated per 10 cm^2 dish and cultured in alpha-MEM, 10% FBS, dexamethasone (10^{-8}M), 50mg/ml ascorbate, 10 mM beta glycerol phosphate. 15-150 colonies are generated. By day 17, over 90% of colonies strongly express alkaline phosphatase. Osteocalcin is expressed and mineralization occurs between 24-45 days.

EXPERIMENTAL DESIGN: The effect of TGF-Beta (5 ng/ml) and retinoic acid (10^{-8}M) (given day 1-10, day 10-17, or day 1-17) on alkaline phosphatase yield was evaluated using six wells in each group.

RESULTS:

1. Transformation growth factor beta (5 ng/ml) added day 10-17 significantly decreased total alkaline phosphatase ($p<.02$). Earlier exposure (day 1-10) did not influence total alkaline phosphatase yield. This is consistent with inhibition of a differentiated osteoblastic phenotype.
2. Retinoic acid (10^{-8}M) added day 1-10 increased alkaline phosphase expression at day 10, but resulted in diminished yield of alkaline phosphatase activity on day 17 ($p<.01$). This is consistent with acceleration of osteoblastic differentiation and limitation of proliferation in harvested osteoprogenitor cells.

DISCUSSION: Simple bone marrow aspiration is a reproducible method for isolation of human osteoblastic cells and should be useful in defining clinically applicable means of selective harvest and proliferation of osteoblastic cells for tissue engineering applications.

CE 208 SCANNING TUNNELING MICROSCOPY OF MATRIX MOLECULES, Vincent B. Pizziconi, Darren L.Page and Pamela A. Diamond, Department of Chemical, Bio & Materials Engineering, Arizona State University, Tempe, Arizona 85287-6006. Scanning tunneling microscopy (STM) is a relatively new type of high resolution microscopy that is capable of imaging viable, biomolecular materials directly in aqueous solutions at nanometer and possibly atomic resolution at ambient pressure. Thus, STM may be a valuable tool in further characterizing the molecular structure of matrix molecules in their near native, fully hydrated state. STM was used to image and characterize laminin and fibronectin, two of the extracellular matrix (ECM) cellular adhesive proteins involved in cellular self-assembly. STM experiments were carried out at room temperature and under ambient pressure conditions using an Angstrom TAK 3.0 STM microscope. Approximately 100 µl of Tris buffer solution containing 15 µgm/ml of ECM protein was placed into a custom HOPG-ZYB graphite well assembly prior to tunneling. Fresh graphite surfaces were created prior to well assembly by carefully cleaving the exposed graphite surface layer with scotch tape. Apiezon-coated platinum-iridium and tungsten STM probes were manually advanced to within close proximity of the surface followed by automatic approach until a constant tunneling current of 1 nanoampere was achieved. Using a tip bias voltage of -0.1 volts, high resolution STM images of laminin and fibronectin were obtained which correlated well with the reported molecular structure and dimensions obtained from electron microscopy studies. A remarkably similar cruciform structure was obtained for laminin while fibronectin depicted its characteristic 'cotter pin' structure. Additionally, the molecular features of these matrix molecules are much more evident with STM particularly in the 3-dimensional image mode. For example, the contrast (height) data of the STM images of laminin appear to be coincident with the reported cell binding domains located at the intersection of the long and short arms and at the terminal end of the long arm (A chain). Thus STM is a potentially valuable tool to gain a deeper understanding of the structure -function role that ECM molecules play in molecular and cellular assembly. Studies are currently underway to image other matrix molecules important in tissue assembly.

CE 209 VASCULOTROPIN/VEGF DRIVEN HYPERSECRETION OF ENDOTHELIN : A PARACRINE LOOP INVOLVED IN HYPERTENSION? Jean Plouët, Centre de Recherche de Biochimie et Génétique Cellulaires du CNRS, 118 Route de Narbonne, 31062, Toulouse.

Vasculotropin/VEGF (VAS) is a new angiogenic cytokine related to the sis oncogene family. Since this cytokine seems to act through a paracrine pathway, we examined its putative role in the vessel wall.

Cultured bovine aortic smooth muscle cells secreted but did not respond to VAS. By contrast cultured aortic endothelial cells did not synthesize VAS but proliferated, migrated and differentiated in response to VAS.

Aortic smooth muscle cells cultured from the genetically spontaneously hypertensive -SHR- and their normotensive littermates Wistar Kyoto -WKY- rat strains. SHR cells secreted constitutively 12 folds more VAS than WKY cells (10^5 and 0.8×10^4 molecules per cell per day). Phorbol esters and basic Fibroblast Growth Factor increased this secretion. VAS increased the level (x2.5) of Endothelin 1, a potent growth factor for smooth muscle cells, secreted by the endothelial cells and therefore might contribute trough an indirect mechanism to the SHR smooth muscle cells hyperproliferation.

Taken together these results suggest that VAS overexpression by SHR cells might constitute an important step in the cascade of events leading to hypertension.

CE 210 FIBROBLAST CONTRACTION OCCURS ON RELEASE OF TENSION IN ATTACHED COLLAGEN LATTICES.

James J. Tomasek, Department of Anatomical Sciences, University of Oklahoma, Oklahoma City, OK 73190

The generation of tension in granulation tissue undergoing contraction is believed to be a cell-mediated event. In this study we used attached collagen lattices as a model system for studying the cellular mechanisms of tension generation by fibroblasts in an extracellular matrix. Attached collagen lattices are manufactured such that collagen fibrils are tethered to the underlying plastic substratum in contrast to previously described free-floating collagen lattices. Over time, fibroblasts reorganize the lattice and reduce its height but not its diameter. Collagen fibers and cells become oriented parallel to the substratum. In addition, fibroblasts in attached collagen lattices develop stress fibers, surface associated fibronectin fibrils and a fibronexus-like transmembrane association interconnecting the two structural components. Release of the attached collagen lattice from its points of attachment results in a rapid, symmetrical contraction of the collagen lattice. Rapid contraction occurs within the first 10 minutes after release of the lattice from the substratum, with greater than 70% of the contraction occurring within the first two minutes. Rapid contraction results in a shortening of the elongate fibroblasts and compaction of the stress fibers with their subsequent disappearance from the cell. Cytochalasin D treatment prior to release disrupts the actin cytoskeleton and completely inhibits rapid contraction. The removal of serum prior to release inhibits rapid contraction, while the re-addition of serum restores rapid contraction. These results demonstrate that fibroblasts can develop tension in an attached collagen lattice and that upon release of tension the fibroblasts undergo contraction resulting in a rapid contraction of the collagen lattice. Fibroblast contraction is dependent upon an organized actin cytoskeleton and is promoted by the presence of serum. Cell traction has been proposed to generate the force responsible for contraction of free-floating lattices. Fibroblast contraction is distinct from tractional force. These results suggest that the rapid contraction of a released collagen lattice is a different process than slow contraction of a free-floating collagen lattice.

CE 211 MAINTENANCE OF HAIR FUNCTION IN CULTURE.

Daniel J. Waldon and Allen E. Buhl. Dermatology, Upjohn Laboratories, Kalamazoo MI 49001

Our efforts at in vitro hair growth have demonstrated hair formation in follicle explants grown with simple culture methods. Extensive work with media and supplements to create conditions that maintained hair growth was unsuccessful despite the structural integrity of the follicles which contained both dermal and epidermal tissue, held in proper tertiary structure and surrounded by a protective connective tissue. Cultured follicles have been shown in previous work to respond to specific drugs, such as minoxidil, by increased expression of histone, c-fos, and c-myc, plus incorporation of ^{3}H-thymidine, ^{35}S-cysteine and ^{3}H-glycine. Cultured follicles have indicated hair growth by techniques including *in situ* hybridization, a gene marker in transgenic mice, gel electrophoresis of keratins and physical measurements of the hair shaft. Effects of culture conditions on hair growth can quickly and quantitatively be measured for a hair specific gene using our transgenic mice. Cultured follicles expressed the hair specific gene for several days and expression declined rapidly with in the first two hours. Minoxidil reduced the decline but could not prevent it. Explanted follicle cells expressed the hair gene but cell from successive passages showed reduced hair specific gene activity. Transformed cells from follicle explants also showed reduced hair gene activity with repeated passage. We conclude that follicles can not maintain their hair growth, with or with out minoxidil, and have a finite hair growth potential after removal from the animal. This termination of hair growth is not telogen but a failure of the follicle to continue the proliferation and specific differentiation in culture. These cultured follicles can support limited epithelial proliferation and hair keratin formation with drug regulated effects but fail to support normal levels of hair specific epithelial differentiation.

CE 212 HUMAN ORAL KERATINOCYTES DIFFERENTIATE IN VITRO TO RESEMBLE INTACT MUCOSA.

David M. Williams, Alan T. Cruchley, Lakshmi Balasubramaniam, Harshad A. Navsaria, Irene M. Leigh, and Christopher A. Squier*, Departments of Oral Pathology and Experimental Dermatology, London Hospital Medical College, Whitechapel, London E1 2AD, UK and *Dows Institute for Dental Research, University of Iowa, Iowa, IA 52242.

The mechanism by which the site specific structure of oral epithelium is established and maintained is poorly understood. Although epithelial - mesenchymal interactions may be important in embryogenesis, their influence on the local differentiation pattern in the adult is uncertain. To elucidate this issue the capacity of adult oral keratinocytes to stratify and differentiate in the absence of connective tissue has been investigated. Keratinocytes were obtained by trypsinisation of non-keratinised human buccal mucosa and primary cultures established using gamma irradiated 3T3 fibroblasts as a feeder layer. Pure keratinocytes at passage 2 or 3 were seeded, in the absence of fibroblasts, at a density of 2x105 cells / ml onto inert collagen membranes (Cellagen Discs, ICN Biomedicals), allowed to reach confluence and then grown as either submerged (n=5) or interface cultures (n=5) in a supplemented DMEM / F12 medium for 10 days. Epithelial sheets on their supporting Cellagen Disc were processed for a comparative ultrastructural study and permeability measurements. Submerged and interface cultures both showed evidence of orderly stratification forming 5 to 8 cell layers, compared with up to 40 in intact tissue. Morphological differentiation was evident: basal cells were rounded, an intermediate spinous cell layer was seen and the superficial layer was flattened. Desmosomes were present but hemidesmosome formation was absent in the basal cells. Tonofilament distribution was similar to that seen in intact mucosa with bundles of filaments in the basal cells and disaggregation in the superficial cells. Membrane coating granules (MCG) identical in morphology, density and distribution to those found in normal non-keratinised epithelium were present in the distal part of the superficial cells. These structural similarities were reflected in the permeability of the cultured epithelium to water - there was no significant difference in the permeability coefficients for $3H_2O$ between intact control buccal mucosa and either submerged or interface cultures. We conclude that normal adult human buccal mucosal keratinocytes have the capacity to express a site-associated phenotype in the absence of connective tissue.

Stem Cells, Cell-Matrix and Mechanical Stress and Properties

CE 300 TWO CELL LINES FROM BONE MARROW STROMA DIFFER IN COLLAGEN SYNTHESIS AND OSTEOGENIC CHARACTERISTICS, Gary Balian[1,2], David R. Diduch[1], Michael R. Coe[1], Clive Joyner[3], and Maureen E. Owen[3], Departments of Orthopaedics[1] and Biochemistry[2], Univ. of Virginia School of Medicine, Charlottesville, VA 22908, [3]MRC Bone Research Lab, Nuffield Orthopaedic Centre, Univ. of Oxford, Oxford, OX3 7LD U.K.
Two cloned cell lines were isolated from mouse bone marrow cell cultures. One of the lines, D1, exhibits osteogenic properties and synthesizes type I collagen, $(\alpha 1)_2\alpha 2$. The second cell line, D2, is not osteogenic and produces a collagen homotrimer, $(\alpha 1)_3$. The triple helical domain of the α1 chain of homotrimer has the same cyanogen bromide peptide map and the same hydroxyproline content as α1 type I. However, hydroxylysine content is considerably higher, at 36%, in homotrimer α1 and the melting temperature of the collagen homotrimer is 3-5° higher than that of type I. Whereas the extracellular matrix of D1 cell cultures contains striated collagen fibrils, presumably comprising type I collagen, the homotrimer producing D2 cells do not demonstrate striated collagen fibrils, but instead have thin filaments without detectable striations. The addition of sodium ascorbate stimulates collagen synthesis at the transcriptional level in both D1 and D2 cells. The bone producing characteristics of D1 in vitro include high levels of alkaline phosphatase activity, increased cyclic AMP upon treatment with parathyroid hormone, and expression of osteocalcin mRNA. D1 cells, unlike D2, produce a mineralized matrix in vitro as demonstrated by histological staining, dispersive x-ray analysis for calcium and phosphate, and x-ray diffraction crystallography. Mineralization in the D1 cultures occurs in nodules of increased cell density which also contain the cells with the highest concentrations of collagen mRNA as shown by in situ hybridization. When D1 cells are implanted in a diffusion chamber in vivo a mixture of both osteogenic and adipogenic tissues is formed. This indicates that the D1 cell line is derived from an early marrow stromal precursor which is multipotential.

CE 301 INDUCED MUSCULAR ATROPHY IN THE CHICK EMBRYO HINDLIMB ALTERS GENE EXPRESSION IN LONG BONE

J.A. Germiller, M. Wong, S.A. Goldstein, J. Bonadio. Orthopaedic Research Laboratories, Section of Orthopaedic Surgery, Department of Pathology, and the Howard Hughes Medical Institute, University of Michigan, Ann Arbor 48109.

Ablation of the lumbosacral spinal cord of the 3-day old chick embryo significantly alters the growth and morphogenesis of the hindlimb skeleton. In the absence of a normal functional environment, the femur and tibia of treated embryos at 18 days were shorter and straighter than control and possessed smaller chondroepiphyses. In addition, the microstructure of the trabeculated bone of the femur was found to be more porous (p=.003) and more isotropic (p=.076) than control. To investigate whether this altered skeletal phenotype was associated with a change in the pattern of expression of structural and regulatory genes, Northern blot hybridizations were performed on mRNA from 18-day control and denervated hindlimb whole bones, using probes for 3 isoforms of the transforming growth factor-ß (TGF-ß) and Type I collagen.

Interestingly, each TGF-ß isoform studied responded differently to the denervation, although changes were mild. TGF-ß1 mRNA remained constant, TGF-ß3 was moderately increased, and TGF-ß4 was moderately decreased in the long bone of the day-18 denervated hindlimb. The message level for Type I collagen was found to be increased in the denervated hindlimb bones, despite the smaller size of these bones. Similar Northern analyses are in progress on mRNAs from earlier stages of limb development, using probes for collagen types I and II, TGF-ß1-4, and aggrecan core protein. Our results support the hypothesis that epigenetic cues, such as mechanical stresses and strains due to muscle action, may have the ability to influence skeletal phenotype by altering the pattern of expression of regulatory and structural proteins.

CE 302 EXPANSION OF HUMAN HEMATOPOIETIC PROGENITORS WITH SYNERGISTIC CYTOKINE COMBINATIONS IN A PERFUSION BIOREACTOR, Manfred R. Koller,[1,2] James G. Bender,[2] William M. Miller,[1] and E. Terry Papoutsakis,[1] [1]Department of Chemical Engineering, Northwestern University, Evanston, IL 60208-3120 and [2]Applied Sciences, Baxter Healthcare Corporation, Round Lake, IL 60073.

Hematopoiesis is the tightly-regulated process by which mature cells in the circulating blood are generated from common pluripotent stem cells residing in the bone marrow. Although bone marrow transplantation can restore hematopoiesis in patients who have undergone irradiation or chemotherapy, in many cases the marrow has been damaged from prior anti-cancer treatment or may have malignant involvement. Therefore, attempts have been made to expand and/or purge bone marrow cells *in vitro* prior to transplantation. Present methods for hematopoietic cell growth on stromal layers in long-term hematopoietic culture (LTHC) do not result in the expansion of progenitor cell numbers, and this may be due in part to the poor growth environment which exists in these static cultures. It is therefore evident that improvements in LTHC techniques are required if the goal of *in vitro* stem and progenitor cell expansion is to be attained. In cord blood LTHC, we have demonstrated that total cell and progenitor cell numbers are increased as much as 5-fold and 12-fold, respectively, by culture under 5% O_2 as compared with culture under 20% O_2. The beneficial effects of 5% O_2 were then combined with the potent synergistic cytokine combinations of IL-3 and IL-6 (IL-3/IL-6) and IL-1 and IL-3 (IL-1/IL-3). IL-3/IL-6 provided a greater expansion of both total and progenitor cells than IL-1/IL-3 in these cultures. IL-3/IL-6 also maintained a higher level of progenitors throughout the 8 week culture period while progenitors disappeared earlier from cultures with IL-1/IL-3. This indicates that an earlier cell type was affected by IL-3/IL-6, and/or that IL-3/IL-6 favored self-renewal while IL-1/IL-3 induced differentiation. IL-3/IL-6 was therefore used in a perfusion bioreactor system for the culture of human hematopoietic cells. The bioreactor system expanded progenitor cell numbers to a higher level more rapidly than in the corresponding static cultures. The resulting hematopoietic culture system which utilizes low oxygen, synergistic growth factors, and continuous perfusion therefore provides a substantial improvement over conventional Dexter-type cultures for the expansion of human hematopoietic progenitors.

CE 303 HISTOLOGICAL STRUCTURE OF THE HYBRID ISLET ORGANOID, Chao Y. Kuo, Henry G. Herrod, George A. Burghen, and Royce Joyner, Department of Pediatrics, University of Tennessee, Memphis and LeBonheur Children's Medical Center, Memphis, TN 38163
The development of an organoid *in vivo* (Thompson et al. Proc Natl Acad Sci, USA 86:7928-32, 1989) suggests a new prospect for cells/tissue transplantation. We are evaluating the organoid as a potential model for pancreatic islet transplant. We have developed a rat organoid that is capable of supporting the growth of human pancreatic islet tissue transplants. The organoid was constructed of expanded polytetrafluoethylene fiber (the angel hair, W.L. Gore and Associates, Flagstaff, Arizona) coated with collagen and endothelial cell growth factor and became a neo-organ upon the ingrowth of host tissue. The organoid was well vascularized in 4-5 weeks. Islets were isolated from cadervaric human pancreas by collagenase digestion procedure. After transplantation of human islet into the vascularized rat organoid, the glandular and duct-like cells were grown/developed inside the organoid. Immunocytochemical staining showed that insulin positive cells were identified within the pancreatic-like glandular tissue. The cellular structure and organization were resembled to normal islet tissue. Although there were polymorphonuclear leukocytes and single cell necrosis within the islet-like structures. However, there was no evidence of lymphocytic infiltration into this area. The possibility of creating an immunologically privileged site inside organoid is suggested, yet it remains to be studied. The observation that the endothelial cell growth factor induced neovascularization inside the organoid was sufficient to support the growth of transplanted pancreatic islet tissue offers a new approach for future islet transplantation.

CE 304 CULTURE OF MONKEY BRAIN ENDOTHELIAL CELLS AND THE DEVELOPMENT OF AN *IN VITRO* BLOOD BRAIN BARRIER (BBB),

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The BBB is formed by specialized properties of brain endothelial cells, including complex tight junctions that restrict intercellular transport of solutes from the lumen of the blood vessels to the brain parenchyma. Cultures of endothelial cells were generated from both rhesus and cynomolgus monkey cerebral microvessels for the development of an *in vitro* BBB. The cells were characterized by endothelial morphology, uptake of acetylated LDL, the presence of non-thrombogenic surfaces and the expression of Factor VIII associated antigen. Cells were cultured on permeable membranes and the integrity of the barrier was assessed by the diffusion of two BBB-excluded molecules, sucrose and dextran (ave. M.W. 70KD), as previously described for a human model (1). In addition, the degree of resistance to hydrostatic pressure by the cellular monolayer was determined. The cultures were confluent within six days following an initial plating density of 20% but did not develop resistance to hydrostatic pressure until day 9. The slowest diffusion of both sucrose and dextran was demonstrated after 13 to 15 days in culture. When Endothelial Growth Mitogen (EGM) was omitted from the culture medium after the cells reached confluency, there was a significant reduction in the passive diffusion of both solutes tested. The permeability of the monolayers was also assessed on membranes that were precoated with basement membrane components. A significant increase in the barrier to passive diffusion was observed in the presence of both laminin and fibronectin, while collagen IV coating resulted in a leakier barrier.

1. McCarroll, L. A., *et al*, (1991) Journal of Cellular Biochemistry, Supplement 15F:241

CE 305 INDUCTION OF MORPHOGENETIC CHANGES IN THE EPIDERMIS OF SKIN EQUIVALENTS BY HAIR FOLLICLE DERMAL PAPILLA CELLS,

G. Philip M. Moore, Sally A.J. Watson, Pilas Pisansarakit, Mark Eisenberg[1] and Kathy Isaacs. CSIRO, Division of Animal Production, P.O. Box 239, Blacktown, N.S.W. 2148, Australia; [1] School of Biochemistry, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033, Australia.

Dermal equivalents composed of fibroblasts and collagen and covered with an epidermis have been developed as substitutes for skin in mammals. These constructs have been used successfully as grafts and appear to function as normal skin. However, they do not contain cutaneous appendages, such as hair follicles and skin glands. We have explored the possibility of using skin equivalents to study the processes of follicle morphogenesis. To accomplish this we have focused our attention on the part of the follicle called the dermal papilla. This small group of cells, derived from the mesenchyme, is generally believed to have a central function in follicle development and fibre specification. Certainly, transplantation of isolated papillae to mouse skin induces epidermal proliferation and results in the formation of new follicles (Pisansarakit *et al*., 1986, J. Emb. exp. Morph. 94: 113-9). We have implanted hair follicle papillae between an epidermis isolated from mouse skin and a dermal equivalent composed of fibroblasts and bovine collagen (Devro). Histological examination of implanted equivalents maintained *in vitro* for 5 days showed that the epidermis in the vicinity of the papilla had thickened and the basal cells were large and cuboidal in appearance. These features were not evident in equivalents which lacked papilla implants nor in those where the papillae were separated from the epidermis. Implanted equivalents which had been grafted to nude mice showed evidence of more extensive epidermal reorganisation adjacent to the papilla than those maintained *in vitro*. The basal cells had actively proliferated and epidermal downgrowths were evident around the papilla. These structures were similar to those stages of follicle development in which the papilla enters an invagination at the base of the follicle. In some sections, parts of the papilla had become completely surrounded by epidermal cells. Current work is centred on defining those features of the equivalents required to permit more advanced stages of follicle formation to be attained.

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CE 306 STUDIES ON ENZYMATIC GENE CO-POLYMERIZATION: THE PREPARATION OF GENETICALLY PROGRAMMED POLYMERS WITH POTENTIAL APPLICATION IN THE RECONSTRUCTIVE SURGERY

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With the aim of preparing new surgical implant materials with controlled resorption properties, we are studying routes to the preparation of genetically engineered fibrous protein materials The basis for these studies is the polymerization of DNA segments encoding structural motifs of known fibrous proteins. Following previous studies on the construction of multimeric bradykinin genes[1] we have prepared polymeric genes encoding motifs derived from collagen type I.

A simple approach consisted in polymerizing a DNA sequence for the core motif $(GXY)_{15}$, where G = glycine, X, Y = defined amino acids. Products containing 25 or more repetitions of this sequences were isolated.

Different strategies of gene polymerization and parameters governing this process will be discussed.

[1] Ramalho Ortigao, J.F. and Seliger, H (1986) Chemical synthesis and cloning of a fragment coding for bradykinin Proceedings of the GBF workshop "Gezielte Mutagenese and Protein-Design" (Blöcker, H., ed.) GBF, Braunschweig, 33.

CE 307 **MESENCHYMAL STEM CELLS, AN IN VITRO MODEL FOR CELLULAR DIFFERENTIATION,**

Henry E. Young, Jennifer C. Smith, Paul A. Lucas, and Donna C. Morrison, Division of Basic Medical Science, Department of Surgery, Mercer University School of Medicine, Macon, GA 31207

Populations of putative mesenchymal stem cells have been isolated from the connective tissue matrices associated with skeletal muscle and skin using either mechanical dissociation or enzymatic digestion. Culturing these putative stem cells using multiple parameters, including various initial plating densities, medium serum contents, and/or concentrations of dexamethasone, elicited morphologies expressing differentiation markers for five mesenchymal phenotypes, i.e., adult sarcomeric myosin (muscle), saturated neutral lipid (fat), chondroitin sulfate-containing fibrillar extracellular matrix (fibroblasts), keratan sulfate/chondroitin sulfate-containing pericellular matrix (cartilage), and a calcium phosphate-containing extracellular matrix (bone). Since these apparently quiescent stem cell populations might contribute cells to the wound healing process, the current project was undertaken to determine their potential cytodifferentiative responses to factors postulated to be involved during wound healing. Incubation with insulin at pharmacological doses elicited statistically significant adipogenic and myogenic responses, whereas incubation with insulin-like growth factors I (IGF-I) and II (IGF-II) at pharmacological doses elicited only a statistically significant myogenic response. No statistically significant responses were noted when physiological doses were used for insulin, IGF-I, or IGF-II. Studies are currently underway to assess what affects other growth factors, known to influence the wound healing process, have on the subsequent cytodifferentiation of these putative stem cells.

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Late Abstracts

RAPID TRANSFER OF SUBCONFLUENT KERATINOCYTES TO WOUNDS USING SYNTHETIC POLYMER FILMS. Y. Barlow, A. Ballam-Davies, D. Pearce, A. Burt*, N. Bontoft, M.D. Leek. Smith and Nephew Research Limited, Gilston Park, Harlow, Essex, U.K. * Rayne Institute, University College Hospital, London, U.K.

A synthetic polymer film/skin grafting system has been developed and used to transfer actively growing human keratinocytes to wounds on burned patients within 3 - 4 days of receiving the tissue specimen for culture [1,2] instead of the more usual 20 - 30 days [3]. This has allowed very rapid coverage of the burned patients with autologous cells on a bacteria impermeable, non adherent dressing, which permits moist wound healing and results in good graft take.

The optimum degree of cell confluency and time of transfer of cells to the patients, in order to maximise graft take, has not been established. Using a porcine keratinocyte culture system, cultured graft take onto full thickness, granulating wounds has been assessed in the pig.

Graft take onto pig wounds in vivo was variable and it was difficult to determine the effect of cell confluence on wound closure. However, using immunocytochemical markers, the apparent quality of epithelium on wounds grafted with keratinocytes grown on polymer films and the dermal repair beneath these films was considerably better than using traditional "Dispased" grafts.

1. Barlow Y, Burt A, Clarke J et al (1992) J.Tiss.Viab. 4, (in press).
2. Barlow Y, Lang S. International Patent Application GB91/00329.
3. Green H, Kehinde O, Thomas J (1979) Proc Natl Acad Sci 76, 5665-8.

EXPRESSION OF DESMOSOMAL CADHERINS DURING DIFFERENTIATION OF HUMAN EPIDERMIS, Anthony I. Magee[1], Kate Sullivan[1], Roger S. Buxton[1], Ian A. King[2] and Joachim Arnemann[1], [1]National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K; [2]CRC, Harrow, Middx., U.K.

Desmosomal junctions are abundant in epidermis and are composed of two classes of glycoprotein as well as several non-glycosylated proteins. The transmembrane glycoproteins belong to two subfamilies of the cadherin superfamily of Ca^{2+}-dependent cell adhesion molecules, namely the desmogleins and the desmocollins. The desmoglein subfamily includes HDGC, Pemphigus vulgaris antigen (PVA) and DGI while the desmocollin subfamily includes DGII/III and DGIV/V. We have used *in situ* hybridisation and immunofluorescence of sections of human skin to study the expression of these genes and their products in epidermis. We find the desmogleins to be spatially and temporally expressed in the order HDGC-PVA-DGI, and the desmocollins to be expressed in the order DGII/III - DGIV/V. The resultant changes in desmosomal composition and structure are presumably related to the need for changes in cell adhesion during stratification, terminal differentiation and desquamation.

PHORBOL ESTER-INDUCED PROMYELOCYTIC LEUKEMIA CELL ADHESION TO MARROW STROMAL CELLS INVOLVES FIBRONECTIN SPECIFIC α5β1 INTEGRIN RECEPTORS. M Lanotte[1], MC. Gendron[1], F. Hogervorst[2], C. Figdor[3] and V. Martin-Thouvenin[1]. (1) INSERM. U301. Institute of Hematology. Centre Hayem. Hopital Saint-Louis. 75010 PARIS. France. (2) Department of Immunohematology. Central Laboratory of the Netherlands Red Cross Blood Transfusion Service. and (3) Division of Immunology. The Netherlands Cancer Institute. 1066 CX Amsterdam. The Netherlands.

The human promyelocytic cell line NB4 exhibited a weak adhesion capacity for bone marrow-derived stromal cells and their extracellular matrices (5 to 15% of adherent cells). Adhesion was enhanced by pulse-treatment of cells with phorbol-ester (PMA 10^{-7} M). Adhesion was induced within minutes. was fibronectin-specific and affected up to 100% of the treated cells. This biological response to PMA resulted from the activation of protein kinase C (PKC). since PKC inhibitors (staurosporine. sphingosine. CGP 41251 and calphostin C) prevented the phenomenon. Phenotypical analysis of integrin receptor expression (particularly FN receptors VLA-4 and VLA-5) at the membrane of untreated or PMA-treated cells revealed that PMA induced no significant modification of the level of expression of these receptors. However. inhibition studies carried out with anti-VLA monoclonal antibodies demonstrated that the FN-specific adhesion triggered by PKC involved the α5β1 FN-specific receptors (VLA-5). We showed that the binding of NB4 cells to fibronectin was RGD-dependent. PMA-induced adhesion was not correlated to phosphorylation of the VLA-5 receptor. These findings may partially explain the malignant behaviour of these cells : the loss of their capacity to adhere to stromal cells may arrest differentiation and explain the large number of leukemic cells in the circulation.