MOLECULAR BIOLOGY OF THE ENDOTHELIAL CELL

Organizers: Thomas Edgington, David Loskutoff and Thomas Maciag

January 16 - 23, 1994; Keystone, Colorado

Sponsored by Genentech, Inc., and Zeneca Pharmaceuticals Group

Plenary Sessions *Page*

Poster Sessions

Growth Factors and Cytokines

EZ 001 EVALUATION OF THE BIOLOGICAL ROLES OF PDGF RECEPTORS USING CHIMERIC MICE, Daniel F. Bowen-Pope and Karen L. Deyerle, Department of Pathology, University of Washington, Seattle WA 98195.

Transgenic and mutant/knockout animals can provide a powerful method for studying gene function *in vivo*. However, they can have problems/limitations for the study of genes which play critical roles in development. For example, the *Patch* mutant mouse (deletion of the PDGF receptor α-subunit gene) cannot be used to investigate the role of the PDGF system in adult vascular pathology because the animals die before birth. One solution to this problem would be to study mutant and transgenic phenotypes as "chimeras" in which some of the cells in the chimeric individual were wild type and others were mutant. The rationale is that the wild type component is available to contribute to lineages that are developmentally dependent on PDGF receptor expression so that the general embryonic environment will remain relatively normal. Quantitative information about developmental roles of PDGF can then be derived by determining changes in the relative proportions of receptor-positive vs receptor-negative genotypes in different cell types and tissues. The roles of PDGF in adult pathologies can be evaluated by comparing the responses, within a tissue, of adjacent receptor-positive vs receptor-negative cells. Since the receptor-positive and receptor-negative cells are adjacent in the same animal, this eliminates animal-to-animal variation and indirect (systemic) effects of the gene deletion. We have constructed such chimeras and will describe our findings using them.

EZ 002 STRUCTURE-FUNCTION STUDIES OF FIBROBLAST GROWTH FACTOR-1, Wilson H. Burgess, Pauline Wong, Brian Hampton, Anne Shaheen, Ewa Szylobryt, Patrick J. Donohue, and Jeffrey A. Winkles, Department of Molecular Biology, Holland Laboratory, American Red Cross, Rockville, MD 20855.

We reported previously that the mitogenic activities of FGF-1 (acidic FGF) could be dissociated from its receptor-binding activities by site-directed mutagenesis of lysine 132 to a glutamic acid[1]. The mutant FGF-1 binds to the high-affinity tyrosine-kinase receptors, stimulates tyrosine-kinase activity and promotes transcription of immediate-early genes. The mutant is not equivalent to wild-type FGF-1 in its ability to promote delayed early gene expression. In addition, the protein is not mitogenic for a variety of tested cell lines. Interestingly, the mutant FGF-1 is capable of other functions associated with the wild-type protein such as promotion of mesoderm formation in *Xenopus* animal caps. The mutant exhibits a reduced apparent affinity for heparin-Sepharose compared to wild-type protein. The relationship between this reduced heparin affinity and lack of mitogenic activity of this mutant is not clear. Recent data indicates the relationship is not as simple as reduced stability of the protein. When NIH 3T3 cells are transfected with expression vectors encoding either wild-type or mutant FGF-1, a transformed phenotype can be seen in cells overexpressing the wild-type FGF-1 whereas cells overexpressing mutant FGF-1 appear normal. Analysis of lysates of these cells indicates that a tyrosine-kinase cascade, distinct from the high-affinity cell surface receptors, has been activated in the wild-type transfected cells but not in the mutant transfected cells. The wild-type transfectants are refractory to exogenous FGF-1 whereas the mutant transfectants respond normally. Both cell types contain normal cell surface receptors as judged by cross-linking studies. Together these results support an intracellular role of wild-type FGF-1 in mediating certain of its functions. In addition, they demonstrate that certain functions of the growth factor can be dissociated at the structural level.

The reduced heparin-affinity of the lysine 132 mutant described above indicated that this region of the protein may represent a heparin-binding domain. This has been confirmed through a series of studies using site-directed mutagenesis and synthetic peptide analogues. The additional mutagenesis studies have resulted in the identification of mutants with heparin-binding or mitogenic deficiencies that do not correlate as well as those of the 132 mutant. It appears that the inactivity of the lysine 132 mutant is related, in part, to cysteine 131.

1. Burgess, W.H., Shaheen, A.M., Ravera, M., Jaye, M., Donohue, P.J. and Winkles, J.A. "Possible Dissociation of the Heparin-binding and Mitogenic Activities of Heparin-binding (Acidic Fibroblast) Growth Factor-1 from Its Receptor-binding Activities by Site-directed Mutagenesis of a Single Lysine Residue." J. Cell Biol., 111:2129-2138 (1990).

EZ 003 TGFß REGULATION OF EPITHELIAL CELL PROLIFERATION *IN VITRO* AND *IN VIVO*, Harold L. Moses, Mark Alexandrow, Donald F. Pierce, Jr., and Rosa Serra, Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232

The transforming growth factor ßs (TGFßs), are potent inhibitors of cell proliferation and are usually secreted in a latent form. TGFß1, TGFß2, and TGFß3 are expressed in distinct but overlapping patterns in most tissues, including the developing mouse mammary gland. To study the role of transforming growth factor ß1 (TGFß1) in normal mammary development and in mammary neoplasia, we have constructed three transgenic mouse lines that express a simian TGFß1$^{S223/225}$ mutated to produce a constitutively active product under the control of the MMTV enhancer/promoter. Expression of the transgene, as confirmed by *in situ* hybridization, immunohistochemistry and Northern blot analysis, was associated with marked suppression of the normal pattern of mammary ductal tree development in female transgenics. Reduction in total ductal tree volume was observed at six weeks as estrous begins and was most apparent at 13 weeks, as ductal growth in the normal mammary gland declines. This effect was seen in all three lines. However, during pregnancy, alveolar outgrowths developed from the hypoplastic ductal tree, and lactation occurred so that transgenic females could feed full litters. Unlike many other transgenic mouse models in which expression of growth factors, including TGFα or oncogenes under control of the MMTV promoter leads to mammary epithelial hyperplasia and increased tumor formation, the MMTV-TGFß1$^{S223/225}$ transgene causes conditional hypoplasia of the mammary ductal tree and no spontaneous tumors have been detected in the MMTV-TGFß1$^{S223/225}$ transgenic animals. Preliminary studies involving crossbreeding of MMTV-TGFα and MMTV-TGFß1$^{S223/225}$ transgenic mice indicate that expression of the TGFß1 transgene markedly decreases the incidence of mammary carcinoma development relative to the MMTV-TGFα transgenic mice, indicating that TGFß agonists may be useful in breast cancer prevention. The mechanisms of TGFß1 growth inhibition have been investigated. In skin keratinocytes, TGFß1 rapidly suppresses c-*myc* expression at the level of transcriptional initiation and expression of c-*myc* was shown to be necessary for proliferation of these cells. Overexpression of c-*myc* using an inducible construct blocks growth inhibition by TGFß1. In 11.5 day *p.c.* lung bud organ cultures, TGFß1 inhibits tracheobronchial epithelial development including branching morphogenesis. The tracheobronchial epithelia express N-*myc* but not c-*myc* at this stage of development. TGFß1 was shown to markedly inhibit N-*myc* expression in epithelia of the lung bud organ cultures and N-*myc* gene knockout experiments by others have shown that N-*myc* is required for branching morphogenesis of the tracheobronchial tree as well as other epithelial structures. The data indicate that the TGFßs are important negative autocrine growth regulators *in vivo* and suggest that suppression of expression of either N-*myc* or c-*myc* may play a role in TGFß growth inhibition.

EZ 004 MECHANISMS AND CONSEQUENCE OF TGF-ß FORMATION BY VASCULAR CELLS.
Daniel B. Rifkin, Department of Cell Biology, New York University Medical Center, New York, NY 10016

Tranforming growth factor ß (TGF-ß) has been shown to have profound effects on vascular cells. TGF-ß is an inhibitor of migration and division of both endothelial cells (EC) and smooth muscle cells (SMC). It also induces dramatic changes in the proteolytic balance of these cells as well as increasing the expression of collagens and fibronectin. Although TGF-ß is produced by both EC and SMC, the molecule as normally released is inactive due to the non-covalent association of the TGF-ß propeptide with the mature growth factor. In this state the complex is latent and cannot interact with cellular high affinity receptors for TGF-ß. Latent TGF-ß can be converted to TGF-ß in co-cultures of EC and SMC. Activation appears to require cell-cell contact, surface proteases, interaction with the mannose-6-phosphate/insulin-like growth factor II cation-independent receptor, tissue transgultaminase, and the TGF-ß binding protein as a complex cell surface assemblage. The formation of TGF-ß is self-regulating as TGF-ß influences its own formation via the induction of plasminogen activator inhibitor-1 (PAI-1) which blocks the protease required for activation. Under certain conditions cultures of EC can be induced to activate latent TGF-ß. However, this requires the addition of exogenous factors such as bFGF, PMA, G-CSF, or retinoids. The properties of latent TGF-ß activation in homotypic cultures are similar to those seen in cocultures. Conversely, endotoxin blocks latent TGF-ß activation. Details of these results as well as aspects of the biochemical reactions will be discussed.

Receptors and Signal Transduction (Joint)

EZ 005 HETEROMERIC KINASE RECEPTORS FOR THE TGF-ß SUPERFAMILY
Jeffrey L. Wrana, Liliana Attisano, Juan Carcamo, Fernando Lopez-Casillas, Franscesc Ventura, Frances Weis and Joan Massague, Cell Biology and Genetics Program and Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York City, New York 10021

The TGF-ß superfamily of cytokines represents one of the largest groups of growth and differentiation factors. Studies based on affinity crosslinking of various superfamily members to the cell surface has demonstrated that these ligands generally interact with pairs of membrane receptors that have been designated as type I and type II receptors. In addition, TGF-ß interacts with a cell surface proteoglycan, betaglycan (type III receptor) that functions to control ligand access to the signalling receptors. Type II receptors for activin, TGF-ß and bone morphogenetic proteins (BMPs) have been identified in mammals, Drosophila and C. elegans. These receptors bind their respective ligands with high affinity and share certain structural features. They are composed of a short, cysteine-rich extracellular domain followed by a single transmembrane domain and an intracellular region containing a kinase domain with predicted ser/thr specificity. More recently type I receptors for TGF-ß, activin and BMPs have been identified in mammals and Drosophila. Interestingly, these receptors share common structural features with the type II receptors, including an intracellular kinase domain. Thus the receptors for TGF-ß-related factors are grouped into a superfamily of related transmembrane kinases representing a new class of signalling receptors.

To explore receptor requirements for signalling we have investigated both the activin and TGF-ß systems. Both activin and TGF-ß type II receptors bind their respective ligands with high affinity, while the type I receptors require the presence of their corresponding receptor II in order to interact with ligand. Receptors I and II form stable complexes and while neither type I nor type II receptors can signal when expressed alone, signalling can be restored when both receptor types are coexpressed. Several different type I receptors for TGF-ß and activin have been characterized. Two of these type I receptors, TßR-I and ActR-IB contain kinase domains that are over 90% identical. Interestingly, expression of these receptors in non-responsive cell lines that express activin and TGF-ß type II receptors restored biological responses that were similar for both ligands. In contrast, expression of a second set of type I receptors for activin and TGF-ß (ActR-I and TSR-I, respectively) was unable to restore any of the common responses. These data demonstrate that signalling receptors for the TGF-ß superfamily are composed of heteromeric complexes of related transmembrane receptor kinases. Moreover, the presence of multiple, distinct activin and TGF-ß receptor complexes may represent one way in which the common and distinct biological responses observed for these factors are generated.

Transcriptional Regulation in Vascular Cells (Joint)

EZ 006NF-κB AND IκB-α: AN INDUCIBLE REGULATORY SYSTEM IN ENDOTHELIAL ACTIVATION, Tucker Collins, Margaret Read, Maryann Whitley, and Amy Williams, Vascular Research Division, Department of Pathology, Brigham and Women's Hospital, 221 Longwood Avenue, Boston, Massachusetts.

Induction of new endothelial cell surface proteins is an important early step in the development of an inflammatory infiltrate. Cytokine-induced expression of endothelial-leukocyte adhesion molecule-1 (E-selectin or ELAM-1) may mediate neutrophil adhesion to endothelium, whereas induced expression of vascular cell adhesion molecule (VCAM-1) may be important in the adhesion of mononuclear cells. Both the corresponding genes are transcriptionally regulated by the inflammatory cytokines. Structural analysis of the promoters of these genes reveals binding sites for the transcription factor nuclear factor κB (NF-κB). These NF-κB sites are necessary but not sufficient for cytokine responsiveness. Endothelial cells express transcripts encoding the p50/p105 (NFKB1) and p65 (RelA) components of NF-κB and the rel related proto-oncogene c-Rel; steady state levels of these transcripts are transiently increased by tumor necrosis factor-α (TNFα). Western blotting revealed that stimulation of endothelial cells with TNFα resulted in nuclear accumulation of the p50 and p65 components of NF-κB. UV crosslinking and immunoprecipitation demonstrated binding of the p50 and p65 components of NF-κB to the E-selectin κB site. Endothelial cells express an inhibitor of NF-κB activation, IκB-α (MAD-3). Protein levels of this inhibitor fall rapidly after TNFα stimulation. In parallel, p50 and p65 accumulate in the nucleus and RNA transcript levels for IκB-α are dramatically upregulated. Recombinant p65 stimulates expression of E-selectin promoter-reporter constructs. IκB-α inhibits p65 or TNFα-stimulated E-selectin promoter-reporter gene expression in transfected endothelial cells. Since multiple genes relevant to the pathobiology of endothelial activation have functional NF-κB sites, activation of this pleiotropic mediator in endothelial cells could coordinate the expression of numerous endothelial products which are important in endothelial activation. Increased expression of IκB-α decreases NF-κB activation and diminishes expression of κB dependent genes. This regulatory mechanism insures that the induction of NF-κB is transient and that the activated endothelial cells returns to a quiescent state. This dynamic balance may be offset during the initial onset of vascular pathobiology.

EZ 007 TNF INDUCED GENES. Vishva M. Dixit, Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109.
Programmed cell death (PCD) is of fundamental importance in cancer, development of the immune system, and embryogenesis. In development, it is critical to the establishment of proper neuronal connections and the formation of the immune repertoire. Inhibition of PCD has been associated with the development of a number of malignancies including follicular lymphoma and breast cancer. We have utilized a subtractive hybridization strategy to clone genes capable of conferring resistance to PCD induced by the cytokine tumor necrosis factor(TNF). Significantly, one of the genes capable of inducing resistance to PCD, designated A20, represents a novel class of zinc finger proteins. Interestingly, A20 is induced by a number of viral gene products known to inhibit PCD including the LMP-1 protein of the Epstein-Barr virus and the *tax* gene product of the HTLV-I virus which causes adult T-cell leukemia. The initial characterization of the regulation of A20 and other TNF-induced genes will be discussed.

Membrane Regulation of Cell Function

EZ 008 MECHANISMS OF CHAPERONE-ASSISTED PROTEIN FOLDING; F.U. Hartl, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, N.Y. 10021.

Cellular protein folding is mediated by so-called molecular chaperones, mostly constitutively-expressed stress proteins which occur in the cytosol and within subcellular compartments. We are interested in understanding the function of members of the hsp70 and hsp60 families of heatshock proteins in de novo protein folding.

Recent evidence suggests that hsp70 and hsp60 components can act sequentially in protein folding determined by their differential specificity for structural elements exposed at different stages of the folding pathway. Hsp70 recognizes completely unfolded polypeptide chains as they emerge from ribosomes or at the trans-side of membranes following translocation into organelles such as mitochondria. The newly-made proteins, adopting the conformation of partially folded intermediates, are then transfered to hsp60 which is required for their folding and assembly to the native state. Proteins appear to acquire stable tertiary structure by an ATP-dependent folding process within the central cavity of the hsp60 double-ring. The eukaryotic cytosol contains the recently discovered TCP-1 ring complex (TRiC) which may have hsp60-like function in this compartment.

Using the isolated heatshock proteins of *E. coli*, we have reconstituted hsp70/hsp60 mediated protein folding reactions. These experiments show that hsp70 (the DnaK protein of *E. coli*) functionally depends on two additional heatshock proteins, DnaJ and GrpE. DnaJ and DnaK cooperate in stabilizing the polypeptide chain, presumably as a compact folding intermediate. GrpE, in the presence of DnaJ, stimulates the ATPase activity of DnaK which allows the transfer of the associated substrate to hsp60 (the groEL protein of *E. coli*). This hierarchical action of chaperone components may represent a major route for the folding of newly-synthesized proteins.

We are currently testing this model of chaperone action using cell-free translation systems. We find that the chaperone DnaJ is able to bind to short ribosome-associated polypeptide chains preventing their folding and aggregation during elongation. Upon completion of synthesis, folding to the native state can occur in the presence of DnaK, GrpE and ATP.

In contrast to the bacterial cytosol, relatively little is known about the chaperone components required for protein folding in the cytoplasm of eukaryotic cells. Based on measuring the refolding of denatured firefly luciferase, we have established that reticulocyte lysates contain a potent system for the ATP-dependent refolding of this protein. Several distinct chaperone activities participate in this reaction.

EZ 009 ROLE OF E-CADHERIN IN THE ESTABLISHMENT OF EPITHELIAL CELL POLARITY, W. James Nelson[1,2], Lindsay Hinck[1,2], Inke Näthke[1], Jackie Papkoff[2,3], and James A. Marrs[1], [1]Department of Molecular and Cellular Physiology, Beckman Center for Molecular and Genetic Medicine, Stanford University School of Medicine, Stanford, CA 94305-5426; [2]Cancer Biology Program, Stanford University, Stanford CA 94305; [3]Sugen Corp., Redwood City, CA 94063.

Cell-cell adhesion is at the top of a molecular cascade of protein interactions that leads to the remodelling of epithelial cell structure and function. Cadherins are a family of calcium-dependent cell adhesion proteins that regulate epithelial cell interactions. The function of cadherins is modulated by a group of cytoplasmic proteins, termed catenins (α-, β-, plakoglobin), that bind to the highly conserved cytoplasmic domain of cadherins. We are interested in the regulation of assembly and function of the catenin/cadherin complex, and the role of cell adhesion in the development of cell surface polarity of epithelial cells. Studies of the assembly of the catenin/cadherin complex show that assembly of α-catenin probably occurs at the cell surface after delivery of β-catenin/cadherin or plakoglobin/cadherin complexes. Assembly of β-catenin appears also to be modulated by expression of Wnt-1. In Drosophila, the homologs of Wnt-1 (*wingless*) and β-catenin (*armadillo*) are segment polarity genes that participate in a signal transduction pathway important for cellular boundary formation in embryonic development, but functional interactions between these proteins are unknown. We find that Wnt-1 expression post-transcriptionally regulates the accumulation of β-catenin and plakoglobin. In addition, binding of β-catenin to cadherin is stabilized, resulting in a concomitant increase in the strength of calcium-dependent cell-cell adhesion. To examine the consequences of cell adhesion on cell organization, we have analyzed the role of cadherins and membrane-cytoskeleton assembly in regulating Na/K-ATPase distribution in kidney and choroid plexus epithelial cells. In polarized epithelial cells the cell surface distribution of the same subunits of Na/K-ATPase may be restricted to either the apical (eg. choroid plexus and retinal pigmented epithelium) or basal-lateral (eg. kidney) membrane domain. Results show that in the kidney, E-cadherin mediated adhesion and induction of membrane-cytoskeleton assembly is sufficient to restrict Na/K-ATPase distribution to the lateral membrane domain. In choroid plexus and retinal pigmented epithelium, Na/K-ATPase and membrane-cytoskeleton proteins are also co-localized indicating that the membrane-cytoskeleton may restrict Na/K-ATPase. However, B-cadherin, the predominant cadherin expressed in the choroid plexus and retinal pigmented epithelium, appears not to have the potential to influence Na/K-ATPase distribution. Interestingly, transfection of E-cadherin, but not B-cadherin, into retinal pigmented epithelial cells and fibroblasts results in co-expression and co-localization of Na/K-ATPase, ankyrin and fodrin at sites of E-cadherin induced cell-cell contacts. Together, these results demonstrate that regulation of E-cadherin expression and function plays critical roles in determining the strength of adhesion between cells and the subsequent reorganization of proteins in the development of epithelial cell surface polarity.

Special Address (Joint)

EZ 010 MOLECULAR GENETIC ANALYSIS OF CELL ADHESION, Richard Hynes, Howard Hughes Medical Institute, Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139

A multiplicity of cell surface adhesion receptors and adhesive extracellular matrix proteins contributes to the adhesion of cells during normal development and in physiological and pathological processes in adults. Extensive molecular and *in vitro* cellular studies of these molecules have suggested roles for these adhesive proteins. However, since there are apparently overlapping specificities and functions of the different receptors, counterreceptors and ligands, it is necessary to determine which molecules contribute to which biological processes *in vivo*. Genetic analyses provide one good way to investigate these questions.

Integrin adhesion receptors and some matrix proteins are known to exist in invertebrate species which have been the traditional systems for genetic analyses. A certain amount of genetic dissection of the functions of these molecules during development of fruit flies and nematodes has been conducted and the roles of several adhesive proteins in specific developmental presses have been analyzed. However, in order to analyze the functions of these proteins in developmental processes and diseases characteristic of mammals, it would be desirable to conduct genetic analyses in a mammal; the obvious choice is the mouse.

In recent years techniques have been developed for introducing targeted mutations in genes of interest and establishing mutant strains of mice. In the past two to three years, mutations have been generated in several extracellular matrix proteins (tenascin, fibronectin, thrombospondin, collagens), several integrins, selectins, cadherins and Ig superfamily adhesion receptors. Some of these mutations produce embryonic lethality (eg. fibronectin, integrins, cadherins) and provide insights into the functions of these proteins in early mammalian development. Other mutations (eg. selectins, ICAM-1, β_2 integrin) are viable but have defects in inflammatory responses and yet others (eg. tenascin, thrombospondin) do not exhibit obvious phenotypic defects.

Examples of mutant phenotypes will be discussed to illustrate the types of information which can be gleaned by this approach, some of the complications of interpretation and potential avenues for future research to address these complications.

Regulation of Angiogenesis

EZ 011 MATRIX MODULATION OF SURFACE MOLECULES DURING ANGIOGENESIS by Joseph A. Madri, Sabita Sankar and Theresa Lu, Yale University School of Medicine, New Haven, CT, USA 06510.

During vasculogenesis and angiogenesis endothelial cells come into contact with a complex number of matrix components having dynamic organizational patterns. Dynamic transient interactions of local endothelial cells with matrices of differing compositions and organizations play important roles in determining the surface expression of a number of endothelial cell surface molecules including growth factor receptors and adhesion moleucles, which, in turn, have profound effects upon cell behavior.

Specifically, adherence and spreading to fibronectin, a matrix component present during the early stages of angiogenesis, elicits high phosphorylation of $pp125^{FAK}$, a transient tyrosine phosphorylation of PECAM-1 and low levels of PKC activity. Adherence and spreading on type I collagen, a matrix component present during the later stages of angiogenesis, elicits a lower phosphorylation of $pp125^{FAK}$, a lower transient tyrosine phosphorylation of PECAM-1 and high levels of PKC activity. These differences correlate with differences in proliferative and migratory rates of endothelial cells, suggesting a bi-directional matrix-mediated modulation of endothelial phenotype via several signalling pathways, involving matrix-specific responses, likely mediated by engagement of specific integrin heterodimeric pairs in "outside to inside" signalling and specific kinase and phosphatase stimulation in "inside to outside" signalling.

In addition to these composition-induced responses, the organization of the extracellular matrix also elicits dramatic changes in endothelial cell behavior. Upon culture in three-dimensional collagen gels endothelial cells lose surface PDGF receptors and their responsiveness to PDGF isoforms. In addition, their responsiveness to TGF-ß1 changes. Cells cultured **in** three-dimensional gels exhibit dramatic changes in fibronectin PAI, allpha 5 integrin and PECAM-1 expression compared to cells cultured **on** matrix components. These findings are consistent with interrelationships of complex matrix-related signalling pathways which are of importance in development, maintenance and repair of the vascular system.

Developmental Processes (Joint)

EZ 012 EMBRYONIC DEVELOPMENT OF THE ENDOTHELIAL NETWORK STUDIED IN THE AVIAN MODEL, Françoise Dieterlen-Lièvre, Dominique Luton and Luc Pardanaud, Institut d'Embryologie cellulaire et moléculaire du CNRS et du Collège de France, 49bis, av. de la Belle Gabrielle, 94736 Nogent s/Marne cedex, France.

The blood system is a derivative of the mesoderm. We have undertaken to define its early history, i.e. which of the mesoderm substructures are capable of giving rise to endothelial cells and whether a hemangioblastic anlage, endowed with both endothelial and hemopoietic capacities, can be identified at an early step of the segregation process. These questions are best approached in the avian model, because it is possible to perform orthotopic exchanges of selected rudiments between two species, quail and chick, and trace the progeny through a structural cell marker (quail nuclear marker) (1) or monoclonal antibodies (for instance the quail hemangioblastic marker, QH1) (2). We have previously determined that the two sheets of the lateral plate mesoderm have definitely different capacities, only the splanchnopleural or internal layer giving rise to endothelial and hemopoietic cells (3). We now show that the segmental plate, i.e. the paraxial mesoderm that divides into somites, has an important endothelial potential. Segmental plate and somites also appear capable of producing hemopoietic cells, although in our experimental conditions they are less productive than splanchnopleura (4). Whether there is a common progenitor to the two lineages is now studied through an expression analysis of two protooncogenes, c-ets1 and c-myb, respectively linked to the amplification process of endothelial versus hemopoietic progenitors (5, 6).

1 - Le Douarin, N.M. 1969. Particularités du noyau interphasique chez la Caille japonaise (Coturnix coturnix japonica). Utilisation de ces particularités comme 'marquage biologique' dans les recherches sur les interactions tissulaires et les migrations cellulaires au cours de l'ontogenèse. Bull. biol. Fr. Belg., 103: 435-452.
2 - Pardanaud, L., Altmann, C., Kitos, P. & Dieterlen-Lièvre F. 1987. Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. Development, 100, 339-349.
3 - Pardanaud, L., Yassine, F. & Dieterlen-Lièvre, F. 1989. Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. Development, 105: 473-85.
4 - Pardanaud, L. & Dieterlen-Lièvre, F. 1993. Emergence of endothelial and hemopoietic cells in the avian embryo. Anat. & Embryol., 187: 107-114.
5 - Vandenbunder, B., Pardanaud, L., Jaffredo, T., Mirabel, M.A. & Stéhelin, D. 1989. Complementary patterns of expression of c-ets1, c-myb and c-myc in the blood-forming system of the chick embryo. Development, 106: 265-274.
6 - Pardanaud, L. & Dieterlen-Lièvre, F. 1993. Expression of c-ets1 in early chick embryo mesoderm: relationship to the hemangioblastic lineage. Cell Adhesion & Communication (in press).

EZ 013 ABERRANT INFLAMMATORY RESPONSE IN TGF-BETA-1-DEFICIENT MICE, Tom Doetschman[1], Ron Diebold[1], Mike Eis[1], Ann Kier[2], Greg Boivin[2], Ingrid Grupp[3], Stacy Smith[4], Paul Allen[4], Marcia Shull[1], and Jeff Saffitz[4], [1]Department of Molecular Genetics, [2]Department of Pathology, [3]Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, [4]Department of Internal Medicine, Washington University School of Medicine, St. Louis.

TGF-ß1 knockout mice develop an acute, multifocal, mixed cell inflammatory disease followed by wasting and death usually within the week after weaning. Among the affected organs are stomach, liver, pancreas, heart, lung, diaphragm, salivary, and lacrimal glands. Other disorders such as cardiomyopathy, hyperplasia, and stomach ulcers often accompany the inflammation. To distinguish inflammatory from developmental effects of the TGFß1 deficiency, several approaches have been undertaken: 1) FACS and immunohistochemical techniques are being used to determine whether thymus development and thymocyte activity is abnormal. 2) The time course of inflammation is being monitored so that possible developmental abnormalities can be observed prior to the onset of inflammation. 3) Genetic and therapeutic approaches to inhibit the inflammatory response are being pursued to determine which disorders persist. Our initial results are as follows. Firstly, activated thymocytes appear in the thymus in pre-symptomatic animals and increase to high levels in symptomatic (wasting) animals. Peripheral thymocytes appear activated but exhausted in symptomatic animals. These results suggest abnormal thymic development, improper thymocyte activation, or peripheral activation followed by abnormal trafficking into the thymus. Secondly, inflammation first occurs in many of the affected tissues in general during the second week of life, with some tissues being affected earlier than others. Consequently, abnormalities detected in the first week of life should be independent of inflammation. Thirdly, our genetic approach is to combine the TGFß1 mutation with the *scid*, *lps*, and *beige* mutations to determine the role played by lymphocytes, macrophages, and natural killer cells, respectively, in the inflammatory process. TGFß1/SCID mutant mice have just been born and are being analyzed. Finally, anti-integrin (LFA-1) therapy inhibits inflammation in several tissues revealing disorders that appear to have other origins such as growth control deficiency, impaired ventricular relaxation, and reduced ß-adrenergic response and receptor density. Using these approaches we hope to separate the developmental from the inflammatory disorders and thereby shed light on the function of TGFß1 at the whole animal level.

EZ 014 REGULATION OF VASCULOGENESIS AND ANGIOGENESIS, Werner Risau, Max-Planck-Institute, D-61231 Bad Nauheim, Germany.

Angioblasts and hemopoietic precursor cells differentiate from the mesoderm and form the blood islands of the yolk sac. The development of blood vessels from these early in situ differentiating endothelial cells is called vasculogenesis which can be induced in avian embryonic stem cells by fibroblast growth factors in vitro. Tyrosine kinase receptors, such as the VEGFR-2 (flk-1) and tie-2, specific for the endothelial lineage, are concomitantly induced in angioblasts. Angiogenesis, the formation of blood vessels by sprouting from preexisting vessels, is observed during embryonic development, e.g. organogenesis of the brain. The expression of VEGF and its cognate receptors VEGFR-1 (flt-1) and VEGFR-2 (flk-1) as analyzed by northern blot and in situ hybridization suggests a paracrine control of vasculogenesis, angiogenesis and endothelial cell proliferation during mouse development. Furthermore, the VEGF/VEGFR signaling system is necessary for pathological angiogenesis and tumor growth of human and rat glioblastoma.

EZ 015 **SMOOTH MUSCLE DIVERSITY AS PART OF THE VASCULAR RESPONSE TO INJURY.** Stephen M. Schwartz, Volkhard Lindner, Edward O'Brien, Rick Johnson, Michael A. Reidy & Cecilia M. Giachelli. University of Washington, Seattle, WA 98195

We have hypothesized that the arterial wall may be comprised of at least two smooth muscle cell (SMC) types. When SMCs from the arteries of two week old pup rats are put into culture and compared with SMC grown from the arteries of adult rats, a number of pup-specific differences appear. Cells with similar properties have been isolated from the artery of the adult rat in 3 ways. First, if the vessel is subjected to balloon angioplasty, a neointima forms and cells from this structure put into culture display the full range of properties seen in pup cells. Cells with similar pup/intimal (π) properties can be derived from the adult vessel wall either by cloning or by selectively isolating SMC in the absence of PDGF.

We have used differential hybridization to identify genes whose expression stably marks the two types of SMC. The growing list of π genes include cytochrome P450 IA1, PDGF-B, type I (alpha 1) collagen, elastin, differential tenascin and osteopontin (OP). Genes which appear unique to the adult SMC phenotype in culture include versican and PDGF-alpha receptor.

The differential libraries made from π cells have proven to be useful in identifying genes showing patterns of overexpression in response to injury or in atherosclerosis Among genes first identified in cultured π cells in vitro, several have shown selective expression in the neointima in vivo as well. These include PDGF-B chain, elastin, cyp1a1, and OP. OP is also a prominent feature of atherosclerosis, especially in areas of calcification. OP, however, is not found in pre-atherosclerotic intima, implying that its expression is somehow chronically controlled within the plaque, either by changes in lineage or by chronic expression of factors that stimulate its expression. OP may play a critical role in formation of atherosclerotic lesions via functions in chemotaxis of leukocytes and smooth muscle cells. Moreover in examining the role of OP in microvascular injury we have found that OP protein is prominent in vessels undergoing malignant arteriosclerosis as well as being a common early component of tubulointerstitial injury in the kidney where it could serve as a chemotactic/adhesive factor for monocytes. In light of its close association with biomineralization in the bone and its striking co-localization with ossified areas of atherosclerotic plaque, it is interesting to speculate that osteopontin may be an important modulator of calcium deposition in a diverse panel of responses to injury.

In summary, constitutive expression of a set of genes by intimal or pup arterial smooth muscle cells in vitro appears to identify markers either of a subset of cells important in vascular response to injury or a common response of vascular smooth muscle cells at injury sites.

Adhesion Pathobiology: Molecular Genetic Approaches (Joint)

EZ 016 MOLECULAR GENETIC MANIPULATION OF MURINE E-SELECTIN EXPRESSION IN ADULTS AND DURING DEVELOPMENT, David S. Milstone, Peter O'Donnell, Vannessa Davis, and Michael A. Gimbrone, Jr. Vascular Research Division, Department of Pathology, Brigham and Women'sHospital, Boston, MA 02115. E-Selectin (endothelial-leukocyte adhesion molecule-1, ELAM-1) belongs to a family of cell surface glycoproteins the members of which share a unique polypeptide domain organization and play key roles in leukocyte adhesion to vascular endothelium. Expression of E-selectin in adult tissues is largely restricted to cytokine-activated postcapillary venular endothelial cells where it is thought to be involved in early leukocyte adhesive events. Although a large body of *in vitro* functional evidence and both *in vitro* and *in vivo* gene expression data support this proposed function, few stringent tests of this hypothesis have been reported. Murine embryonic stem (ES) cells differentiate *in vitro* to form embryoid bodies (EB) containing vascular channels which are lined by morphologically identifiable endothelial-like cells, contain nucleated blood cells and may be analogous to embryonic blood islands. To compare the properties of these endothelial-like cells with those of mature vascular endothelium, we are examining the expression pattern and function of E-selectin, and other adhesion molecules, in ES cells differentiated *in vitro*. E-Selectin mRNA is not induced by LPS in undifferentiated ES cells but is first induced, in a time and concentration-dependent manner, in stages of EB differentiation when vascular channels are not prominent. We are currently determining the relationship of E-Selectin induction to emergence of the endothelial lineage in EB, whether E-Selectin inducible cells are precursors of the endothelial-like cells formed in EB, and whether induction of E-selectin may thus provide a useful marker of the vascular endothelial lineage during development. We have cloned a portion of the murine E-Selectin gene and introduced into ES cells, *via* homologous recombination, a predicted null allele to test the hypothesis that E-Selectin expression is required for differentiation and function of the endothelial lineage in EB. We are also establishing, *via* blastocyst-mediated transgenesis, lines of mice bearing this mutation to test the hypothesis that E-Selectin expression is required for endothelial-neutrophil interactions at sites of acute inflammation and for evolution of a normal acute inflammatory response *in vivo*.

EZ 017 MOLECULAR BASIS FOR L-SELECTIN FUNCTION IN COMPARISON WITH P- AND E-SELECTIN, Thomas F. Tedder[1,2], Kim B. Saunders[2], Geoffrey S. Kansas[2], Klaus Ley[3], Andreas Zakrzewicz[3], Rosemary M. Gibson[4], Barbara C. Furie[4], and Bruce Furie[4],

[1]Duke University Medical Center, Durham, NC 27710, [2]Dana Farber Cancer Institute and Harvard Medical School, Boston, [3]Institute for Physiology, Free University Berlin, Berlin, Germany, [4]Tufts University School of Medicine, New England Medical Center, Boston.

The selectin family of adhesion molecules mediates the initial interactions of leukocytes with endothelium. L-selectin is expressed by most leukocytes, while P- and E-selectin are expressed by activated endothelial cells. The extracellular region of each selectin contains an amino-terminal lectin domain, followed by an epidermal growth factor (EGF)-like domain and multiple short consensus repeat units (SCR). L-selectin mediates lymphocyte attachment to high endothelial venules (HEV) in lymph nodes and also mediates leukocyte attachment to cytokine-activated endothelial cells through an induced endothelial ligand which is independent of both E- and P-selectin. Transfection of L-selectin cDNA into cells which do not normally bind to endothelium confers adhesion and the ability to roll in rat mesenteric venules *in vivo*, demonstrating that expression of L-selectin alone is sufficient to mediate leukocyte rolling. However, removal of the cytoplasmic domain of L-selectin completely abrogates attachment and rolling of transfected cells suggesting that cytoskeletal associations or signals generated through the cytoplasmic domain are involved in regulating L-selectin function. Since the affinity of L-selectin for its ligand is transiently increased following leukocyte activation, this event may also be regulated through the cytoplasmic tail. The increase in affinity can be recapitulated by the binding of a unique mAb to the EGF-like domain of L-selectin, suggesting the involvement of the EGF-like domain in this process. Therefore, the role of the selectin extracellular domains in cell adhesion was examined using a panel of chimeric selectins generated by exchange of domains between L- and P-selectin and between L- and E-selectin. The binding ability of chimeric selectin molecules expressed by cDNA-transfected cells was assessed in two ways. L-selectin function was assessed by the ability of transfected cells to bind HEV and to roll in mesenteric venules. P- and E-selectin-like activities were assessed by the ability of the chimeric receptor to bind the myeloid cell line HL60, that is not bound by L-selectin. Exchange of only the lectin domains between selectins successfully conferred the adhesive and ligand recognition functions of the lectin-derived molecule. However, chimeric selectins which contained both the lectin domain of L-selectin and the EGF-like domain of P-selectin exhibited dual ligand-binding specificity, supporting adhesion both to myeloid cells and to HEV and mesenteric venules *in vivo*. Comparable experiments with L- and E-selectin chimeric molecules provided similar results. In all cases, L-selectin-like function was always correlated with the presence of the L-selectin lectin domain, with no apparent functional contribution provided by the L-selectin EGF-like domain. Exchange of the SCR domains between selectins had no detectable effect on receptor function or specificity. These results suggest a unique role for the EGF-like domains of P- and E-selectin and indicate that the EGF-like domain is in part responsible for ligand recognition and leukocyte adhesion mediated by these selectins, either by binding ligand directly or by contributing to the binding specificity of the lectin domain.

EZ 018 P-SELECTIN-DEFICIENT MICE, Denisa D. Wagner, New England Medical Center, Division of Hematology-Oncology, and Tufts University School of Medicine, Boston, MA 02111.

Our laboratory studies the response of endothelial cells to vascular injury and inflammatory stimuli. One of our main interests is the expression and function of the adhesion receptors P- and E-selectin. P-selectin is stored in the endothelial storage granules called Weibel-Palade bodies from where it can be rapidly expressed on the cell surface. We have shown that after surface expression P-selectin is rapidly internalized and is directed into newly formed Weibel-Palade bodies. This means that when necessary this molecule can be reexpressed on the plasma membrane without requiring de novo synthesis. E-selectin in contrast is not stored and after expression on the plasma membrane by de novo synthesis is routed into lysosomes where it is degraded. P- and E-selectin mediate adhesion to monocytes and neutrophils and some cancer cells. To study the role of these molecules in normal and pathological processes, we have prepared mice lacking P-selectin by gene targeting in embryonic stem cells (Mayadas et al., 1993), and we are currently preparing animals lacking both P- and E-selectin by the same methodology. The P-selectin-deficient animals are fertile, appear grossly normal and under the conditions of routine mouse husbandry appear generally healthy at one year of age. We have found major changes in leukocyte behavior in the P-selectin-deficient animals. There was a complete absence of spontaneous leukocyte rolling in the mesenteric venules and a delayed recruitment of neutrophils to sites of inflammation. Peripheral neutrophil counts in blood were elevated in P-selectin-deficient animals compared to wild type, likely the result of defective interaction of the neutrophils with the vessel wall. We are now examining the role of P-selectin in later stages of inflammation, in neutrophil demargination, and in wound healing. Since P-selectin is also found in platelet α-granules, the P-selectin-deficient platelets that can be obtained in normal numbers from the mutant animals will be an excellent tool to study the role of this receptor in platelet functions.

T.N. Mayadas, R.C. Johnson, H. Rayburn, R.O. Hynes, and D.D. Wagner. Leukocyte Rolling and Extravasation are Severely Compromised in P Selectin-Deficient Mice. Cell 74:541-564, 1993.

Special Address (Joint)

EZ 019 AN *IN VIVO* LOOK AT MICROCIRCULATION IN HEALTH AND DISEASE, Rakesh K. Jain, Harvard Medical School and Massachusetts General Hospital, Department of Radiation Oncology, Boston, MA 02114.

The possibility to grow endothelial cells in culture from various organs has increased our understanding of the structure and function of these cells at the molecular level. It has also provided novel hypotheses about the molecular mechanisms of various diseases involving vascular pathophysiology, e.g., cancer, inflammation, and atherosclerosis. A prerequisite for testing these hypotheses *in vivo* is the availability of tissue models which permit non-invasive, continuous and long term monitoring of microvascular events. To this end, we have utilized two chronic preparations: modified Sandison rabbit ear chamber (1) and modified Algire mouse dorsal skin chamber (2) to study microcirculation in normal and neoplastic tissue. The former has the advantage of superior optical qualities and the latter of being able to work with immunodeficient and transgenic species. These preparations have allowed us to test hypotheses generated from *in vitro* studies and to formulate new ones in the following areas: angiogenesis and blood flow in tumors (2-4); metabolic microenvironment in tumors (5,6); transvascular and interstitial transport in normal and tumor tissues (7-11); and leukocyte-endothelial interactions in normal and tumor vasculature (12,13). In this presentation I will highlight our findings in these general areas and focus on parameters which can only be measured *in vivo*. I will also discuss the implications of our findings for cancer detection and treatment (14).

1. T.E. Dudar and R.K. Jain, *Microvascular Research* **25**, 1-21 (1983).
2. M. Leunig, F. Yuan, M. Menger, Y. Boucher, A. Goetz, K. Messmer and R.K. Jain, *Cancer Research*, **52**, 6553-6560 (1992).
3. T.E. Dudar and R.K. Jain, *Cancer Research* **44**, 605-612 (1984).
4. R.K. Jain, *Cancer Research* **48**, 2641-2658 (1988).
5. G.R. Martin and R.K. Jain, *Microvascular Research* **46**:216-230 (1993).
6. I.P. Torres-Filho, M. Leunig, F. Yuan, M. Intaglietta, and R.K. Jain, *Proceedings of the National Academy of Sciences*. (In Press).
7. L.E. Gerlowski and R.K. Jain, *Microvascular Research* **31**, 288-305 (1986).
8. F. Yuan, M. Leunig, D. Berk and R.K. Jain, *Microvascular Research* **45**:269-289 (1993).
9. L.J. Nugent and R.K. Jain, *Cancer Research* **44**, 238-244 (1984).
10. S.R. Chary and R.K. Jain, *Proceedings of the National Academy of Sciences* **86**, 5385-5389 (1989).
11. M.A. Clauss and R.K. Jain, *Cancer Research* **50**, 3487-3492 (1990).
12. A. Sasaki, R.J. Melder, T.L. Whiteside, R.B. Herberman, and R.K. Jain, *Journal of National Cancer Institute* **83**, 433-437 (1991).
13. C. Ohkubo, D. Bigos and R.K. Jain, *Cancer Research* **51**, 1561-1563 (1991).
14. R.K. Jain, *Cancer and Metastasis Reviews* **9**, 253-266 (1990).

Proteases and Their Receptors as Effectors of Cell Function

EZ 020 THROMBIN RECEPTOR STRUCTURE AND FUNCTION, Shaun R. Coughlin, Cardiovascular Research Institute, University of California, San Francisco, CA 94143-0524.

Thrombin, a multifunctional serine protease generated at sites of vascular injury, is a robust activator of cellular events important in cardiovascular function. It is the most potent agonist for platelet activation and has a variety of proinflammatory and proliferative effects on monocytes, neutrophils, endothelial, and vascular smooth muscle cells. A recently cloned thrombin receptor has provided a framework for understanding how thrombin, a protease rather than a classical ligand, talks to cells and has also generated new tools for defining thrombin's role in the cell biology. The thrombin receptor is a member of the seven transmembrane domain receptor family, but is activated by a novel mechanism. Thrombin binds to then cleaves its receptors' extracellular amino terminal extension to unmask a new amino terminal. This new amino terminal then functions as a tethered peptide ligand, binding to as yet unidentified sites within the body of the receptor to effect receptor activation. This formulation raises a number of important questions. How does thrombin, if it is acting enzymatically, elicit concentration-dependent responses via this receptor? If the receptor is liganded irreversibly, how is it shut off? Can manipulation of the shut off mechanisms modulate a cell's responsiveness to thrombin? Evolving answers to these and other questions will be presented.

EZ 021 **MOLECULAR BIOLOGY AND VASCULAR PATHOBIOLOGY OF TISSUE FACTOR,** Thomas S. Edgington, The Scripps Research Institute, La Jolla CA 92037.

Tissue Factor (TF) is the requisite cell surface cofactor and high affinity receptor for factor VIIa. The complex TF•VIIa initiates the coagulation serine protease cascades resulting in generation of thrombin and factor Xa and cellular activation via the thrombin receptor and Xa receptor as well as the formation of fibrin polymers. Based on sequence homology TF is considered a member of the Cytokine Receptor Superfamily. Coagulation is initiated by assembly of the catalytically and proteolytically active TF•VIIa complex. TF is a β protein likely organized as two seven strand domains that are critically interactive. From the Cα coordinates of the homologous Growth Hormone Receptor a three dimensional model of TF has been built. Using directed scanning alanine mutagenesis key residues and local structures are proposed to account for: i) high affinity binding of ligand, ii) induction of catalytic activity in the bound protease domain of VIIa, and iii) regulation of ternary complex assembly and overall proteolytic function. Inhibition of TF *in vivo* in primates abolishes activation of coagulation pathways in primates. These studies suggest the presence of a number of discrete substructures that regulate the function of this receptor and may be generalized to other members of the cytokine receptor family. TF is relatively silent in endothelial cells and monocytes, but transcription can be induced by certain cytokines and/or endotoxin. An enhancer has been identified 5' to the initiation of transcription, and serves to translate signals derived by engagement of the cognate agonist receptors on these cells. *In vivo* TF appears to account for the initiation of the coagulation pathways associated with infusion of recombinant VIIa or endotoxin challenge of chimpanzees analyzed by use of neutralizing monoclonal antibodies to the substrate coordination substructure of TF or by monoclonal antibodies to factor VIIa that neutralize the TF•VIIa functional complex. In baboons the mortality and morbidity of lethal *E. coli* sepsis is diminished by neutralizing TF with appropriate monoclonal antibodies. Interruption of the pathways distal to TF in the thrombogenic pathways fails to interrupt the lethality, whereas proximal interruption is effective, suggesting the duality of inflammatory vs. thrombogenic roles of TF in vascular pathobiology.

EZ 022 EVIDENCE FOR A SECOND RECEPTOR INVOLVED IN SIGNAL TRANSDUCTION. Salvatore V. Pizzo, Charleen T. Chu, Govind Gawdi, Gayle C. Howard, and Uma K. Misra, Duke University Medical Center, Durham, NC 27710.

α_2-Macroglobulin (α_2M)-methylamine binds to purified low density lipoprotein receptor-related protein (LRP), and it is assumed that this protein functions as the α_2M receptor *in vivo*. Previous studies from this laboratory demonstrated that binding of α_2M-methylamine to murine peritoneal macrophages elevates intracellular calcium ($[Ca^{2+}]_i$), inositol phosphates and cyclic AMP (Misra et al., Biochem J 290:885-891, 1993). In the present study, human α_2M-methylamine and the recently cloned and expressed carboxyl terminal receptor binding fragment (RBF) of rat α_1M were employed to further characterize signal transduction mechanisms in murine peritoneal macrophages. Either ligand (40 nM) was found to induce a rapid rise in $[Ca^{2+}]_i$. The application of either ligand immediately after application of the other ligand demonstrated little or no increase in $[Ca^{2+}]_i$. These results are consistent with previous studies which showed that RBF and α_2M-methylamine bind to the same site. We then studied the effect of the 39 kDa α_2M receptor associated protein (RAP), a protein which binds LRP and blocks ligand binding, on signalling. Exposure of the cells to RAP (200 nM) caused no change in $[Ca^{2+}]_i$. In addition, pretreatment of the cells with RAP (200 nM) failed to block the increase in $[Ca^{2+}]_i$ seen upon exposure to α_2M-methylamine or RBF. Since the signalling events observed are usually associated with G protein-coupled receptors, RBF was employed to probe for the possible role of G proteins in this signal transduction pathway. RBF binding to macrophage receptors resulted in a transient increase in inositol 1,4,5-triphosphate (IP_3) generation by 1.5-2.0 fold in 30 s. RBF was also found to induce a transient increase in cyclic AMP levels. These effects were comparable to the effects of α_2M-methylamine on a molar basis. In permeabilized cells, GTP-γ-S and GPP(NH)p were found to potentiate and sustain the increase in IP_3 resulting from binding of RBF to cells, and the effects of GTP-γ-S could be drastically reduced by preincubating the permeabilized cells with GDP-β-S. Exposure of RBF stimulated cells to pertussis toxin failed to result in any ADP-ribosylation of the 41 kDa G protein present in the plasma membranes of the cells; however, exposure to cholera toxin resulted in ADP-ribosylation of the 43 kDa G protein. These data suggest that the α_2M-receptor is coupled to one or more G proteins. Given that the sequence of LRP contains only one transmembrane domain, it is unexpected to find these G protein signalling characteristics. We conclude from our studies that murine peritoneal macrophages contain a second α_2M receptor which is G protein coupled.

EZ 023 ROLE OF THE α_2M RECEPTOR/LOW DENSITY LIPOPROTEIN RECEPTOR RELATED PROTEIN (LRP) IN REGULATION OF PROTEINASE ACTIVITY. Dudley K. Strickland, Maria Z. Kounnas, Suzanne E. Williams, and W. Scott Argraves. American Red Cross, Rockville MD 20855.

LRP, a member of the LDL receptor family, is a large cell surface receptor that consists of a 515 kDa heavy chain which contains the ligand binding domains, and an 85 kDa light chain which contains the transmembrane domain. These two subunits are noncovalently associated on the cell surface. LRP is widely expressed by many types of cells, including hepatocytes, fibroblasts, macrophages, and neurons. During the purification of this receptor, a 39 kDa receptor associated protein (RAP) was identified. RAP binds with high affinity to multiple sites on LRP. Once bound to LRP, RAP antagonizes ligand binding to the receptor, and thereby prevents cellular internalization and subsequent degradation of LRP ligands. The carboxyl-terminal portion of RAP contains the LRP binding domain. LRP binds and mediates the internalization of α_2-macroglobulin-proteinase complexes, pregnancy zone protein inhibitor complexes, complexes of tissue type (tPA) and urinary-type (uPA) plasminogen activator with plasminogen activator inhibitor type I (PAI-1). Interestingly, LRP also binds and internalizes certain proteinases, such as tPA and uPA. Our studies indicate that pro-urokinase (pro-uPA) and two chain urokinase (tc-uPA) bind directly to purified LRP, and that LRP mediates their internalization and degradation in Hep G2 cells. Both the internalization and degradation are completely blocked by RAP or affinity purified LRP antibodies, indicating that LRP is mediating this process. These processes are also blocked by an amino-terminal fragment of uPA which blocks binding of uPA to the urokinase receptor, but not to LRP. These results suggest that the favored pathway for u-PA metabolism is initial binding to the urokinase receptor, followed by ligand transfer to LRP, then internalization leading to degradation. Thus, it appears that LRP can function to reduce cell surface uPA activity by facilitating its internalization and subsequent degradation. The ability of LRP to bind and rapidly internalize both proteinases and proteinase-inhibitor complexes suggests a major role for this receptor in regulating proteinase activity.

Pathophysiologic Aspects of Proteases and Their Inhibitors (Joint)

EZ 024 THE LDL RECEPTOR GENE FAMILY - ROLES IN PROTEASE REGULATION, LIPID METABOLISM AND ATHEROSCLEROSIS, Shun Ishibashi[1], Dennis Burns[2], Thomas Willnow[1], Hideo Otani[1], Robert E. Hammer[3], Michael S. Brown[1], Joseph L. Goldstein[1], and Joachim Herz[1]; [1] Departments of Molecular Genetics and Internal Medicine, [2]Department of Pathology, [3] Howard Hughes Medical Institute and Department of Biochemistry, University of Texas, Southwestern Medical Center, Dallas TX 75235

Four structurally closely related proteins that belong to the low density lipoprotein (LDL) receptor gene family are currently known in mammals. They comprise the LDL receptor, the LDL receptor-related protein (LRP), the Heymann's nephritis antigen gp330 and the very low density lipoprotein (VLDL) receptor. All these constitutively recycling cell surface receptors are composed of the same four structural motifs: complement-type cysteine-rich repeats, epidermal growth factor (EGF) precursor-homologous domains, a single membrane-spanning segment and a short cytoplasmic tail that contains between one and three 'NPxY' endocytosis signals. LRP and gp330 are approximately five times larger than the LDL receptor and the VLDL receptor and a structurally perfectly conserved homologous protein has been found in the evolutionarily distant organism *Caenorhabditis elegans*. LDL receptor and LDL receptor-related proteins function in biologically diverse processes which include the metabolism of lipoproteins, lipoprotein lipase, plasminogen and plasminogen activators and plasminogen activator inhibitor, α_2-macroglobulin, Pseudomonas exotoxin A, lactoferrin and vitellogenin. The activity of LRP and of gp330 can be modulated in vitro by a small vesicular protein, called receptor-associated protein (RAP). A physiological role of RAP as a fast-acting modulator of LRP activity has been proposed. To study the physiological functions of the different members of this gene family we have created strains of mice in which individual genes have been disrupted. Observed phenotypes vary depending on the gene defect and include increased plasma cholesterol levels and increased susceptibility for the formation of atherosclerotic lesions in LDL receptor deficient mice and early embryonic lethality in LRP-deficient mice. Furthermore, we have used somatic cell gene transfer to study the effect of overexpression of the transferred gene in normal and genetically altered mice.

EZ 025 PAI-1 GENE EXPRESSION IN HEALTH AND DISEASE by David J. Loskutoff, Ph.D., Department of Vascular Biology, The Scripps Research Institute, La Jolla, CA 92037.

Plasminogen activator inhibitor 1 (PAI-1) is a rapid and specific inhibitor of both t-PA and urokinase and may be the primary regulator of plasminogen activation in vivo. A primary hypothesis of our work is that the expression of PAI-1 in vivo is under strict regulation, and that the inappropriate expression of this inhibitor may suppress the normal fibrinolytic system of the tissues and create a local prothrombotic state. This condition may result in pathological fibrin deposition following injury and/or tissue factor expression. In order to test this hypothesis, we have begun to study PAI-1 gene expression in mouse and human tissues, using Northern blotting, in situ hybridization, and immunohistochemistry. PAI-1 mRNA and antigen were detected in most murine tissues from normal animals suggesting a vascular origin. Although no PAI-1 mRNA was detected in endothelial cells (ECs), it was detected in smooth muscle cells (SMCs) throughout the vasculature. Endotoxin (LPS) induced PAI-1 mRNA in most tissues and this increase resulted from specific induction in vascular ECs. The level of PAI-1 mRNA in SMCs was rapidly and dramatically reduced by LPS. PAI-1 was elevated locally in a variety of disorders. For example, it was dramatically increased in the kidneys of mice with glomerulonephritis, in atherosclerotic human vascular tissue, and in the pulmonary arteries of individuals with non-resolving pulmonary emboli. In most instances, the pattern of PAI-1 induction resembled the pattern of fibrin deposition. Thus, the abnormal expression of PAI-1 in tissues may inhibit the clearance of fibrin and contribute to the ongoing pathologies of these disorders.

Strategies to Modify Gene Expression (Joint)

EZ 026 ADENOVIRUS-MEDIATED GENE TRANSFER INTO VASCULAR CELLS, Robert D. Gerard and Robert S. Meidell, Departments of Biochemistry and Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75235-8573

In an effort to develop gene-based strategies to intervene in the pathogenesis and progression of vascular disease, we have employed recombinant, replication-defective adenoviruses to introduce foreign genes into mammalian cells *in vitro* and *in vivo*. Viruses have been generated which carry genes encoding either reporter enzymes (luciferase, β-galactosidase) or physiologically important proteins (tissue plasminogen activator, plasminogen activator inhibitors, uPA receptor, LDL receptor, apolipoprotein A1, bFGF) expressed from a variety of promoters of viral or cellular origin. Efficient genetic modification has been observed following infection of a wide range of mammalian cell types in culture and several different tissues in living animals, including liver parenchyma, pancreatic islet cells, renal tubular epithelium, skeletal myocytes, and vascular endothelial and smooth muscle cells. Following intravenous administration of recombinant adenovirus to mice or rabbits, foreign genes are preferentially (>99%) expressed in the liver. Histochemical staining demonstrates expression in both hepatocytes (predominantly) and endothelial cells. Mice infected with a virus encoding the human LDL receptor express the protein at a 10-fold higher level than uninfected mice, and show significantly accelerated clearance and reduced circulating levels of cholesterol. Similarly, animals infected with a virus encoding human apolipoprotein A-I accumulate high (>200 mg/dl) circulating levels of ApoA-I and show increased circulating HDLc levels, suggesting the potential for gene-based therapeutic strategies to reduce cardiovascular risk. Delivery of foreign genetic material to vascular endothelial and smooth muscle cells has been examined using several catheter-based strategies. Intraluminal dwell of high-titer purified stocks of recombinant adenovirus, either by direct instillation into a surgically isolated vessel segment or infusion through a double-balloon catheter, results in efficient and preferential genetic modification of vascular endothelium. In contrast, infusion of recombinant virus stock into the vessel wall under pressure using perforated or Hydrogel-coated balloon catheters targets foreign genes to medial smooth muscle cells, although the efficiency of genetic modification is relatively low. Vascular endothelial cells infected with recombinant adenoviruses encoding human tPA and PAI-1 demonstrate marked overexpression of these proteins. Efficient, directed genetic modification of cells resident in the vessel wall offers the potential to intervene in important pathophysiologic processes, including angiogenesis, vascular thrombosis and restenosis following angioplasty. While adenovirus mediated gene transfer offers the potential of therapeutic application, several important limitations, including cell-type specific targeting, stability of foreign gene expression and the effects of host immune response, remain to be addressed.

Signal Transduction

EZ 100 OVEREXPRESSION OF THE SMALL GTP-BINDING PROTEIN $p21^{Ha\text{-}ras}$ INHIBITS IL-1 OR TNFα INDUCED EXPRESSION OF E-SELECTIN IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS, P. Adamson, *H.F. Paterson and J.D. Pearson, Vascular Biology Research Centre, King's College London, Campden Hill Road, London W8 7AH and *Chester Beatty Laboratories, Institute of Cancer Research, Fulham Road, London, SW3 6JB, UK.

Human umbilical vein endothelial cells (HUVEC) were isolated and seeded onto gelatin coated 13mm glass coverslips. Expression of E-selectin was monitored using immunohistochemical staining with an anti-human E-selectin monoclonal antibody (MAb). HUVEC showed no basal expression of E-selectin but treatment with IL-1α, Il-1ß or TNFα (100U/ml) for 6h resulted in marked expression of the adhesion molecule. Cytosolic microinjection of functional $p21^{Ha\text{-}ras}$ recombinant protein (1mg/ml) 1h before cytokine treatment completely inhibited E-selectin expression. Microinjection of inactive mutant (Cys →Ser186) $p21^{Ha\text{-}ras}$ protein did not inhibit cytokine mediated induction of E-selectin. When mammalian expression vectors containing $p21^{Ha\text{-}ras}$ cDNAs under the control of an SV40 promotor were injected (50µg/ml plasmid DNA) into the nuclei of HUVEC 16h prior to treatment with cytokines, induction of E-selectin was inhibited following injection of cDNA coding for functional $p21^{Ha\text{-}ras}$ but cDNA for $p21^{Ha\text{-}ras}$ (Cys→Ser186) had no effect on inducible E-selectin expression. Injection of $p21^{Ha\text{-}ras}$ cDNA coding for a Ser→ Asn17 mutated protein (which is a dominant negative inhibitor of endogenous ras) or injection of the ras-neutralising MAb Y13-259 (10mg/ml) did not inhibit cytokine-mediated induction of E-selectin. We have also demonstrated by ELISA that growth factors which are capable of activating $p21^{ras}$ can reduce the extent of cytokine-induced increases in E-selectin expression. These results support the hypothesis that $p21^{Ha\text{-}ras}$ is not essential for cytokine induction of E-selectin expression but activation or overexpression of functional $p21^{Ha\text{-}ras}$ is capable of blocking the cytokine mediated induction of this adhesion molecule.

EZ 101 **ENDOTHELIAL AND MEGAKARYOBLAST GROWTH FACTOR RECEPTORS IN ANGIOGENESIS**

Kari Alitalo, Juha Partanen, Jaana Korhonen, Katri Pajusola, Luca Tamagnone, Satu Vainikka, Arja Kaipainen, Tuija Mustonen and Olga Aprelikova Molecular/Cancer Biology Laboratory, University of Helsinki, POB21, 00014 Helsinki, FINLAND

In order to get insight into the growth regulation of hematopoietic and leukemia cells we have cloned novel tyrosine kinase cDNAs from human leukemia cells with a bipotential erythroid/megakaryoblastoid differentiation potential. This resulted in the identification of several novel receptor tyrosine kinases including a potential anti-oncogene and members of the fibroblast growth factor (FGF) and FLT1/KDR/FLK-1 receptor families. One of the novel receptor tyrosine kinases (Tie) is expressed in fetal endothelial cells. In 8.5 day embryos, *tie* expression was observed in differentiating angioblasts of the head mesenchyme, in splanchnopleure, in dorsal aorta as well as in migrating endothelial cells of the developing heart. A *tie* signal was also obtained from hemangioblasts in the blood islands of the yolk sac. In adults, Tie mRNA expression is enhanced during neovascularization associated with the ovulatory cycle and wound healing. - Ligands binding to these receptors are being characterized. These receptors may be involved in tumor angiogenesis and hematopoiesis. They may provide a potential to prevent the growth of several types of solid tumors by strategies that will be discussed.

Proc. Natl. Acad. Sci. 87: 8913, 1990; ***EMBO J.*** 10: 1347, 1991; 11: 2919, 1992; ***Oncogene*** 6: 2013, 1991;8: 2009, 1993; in press; ***Blood*** 80: 2548, 1992; ***Nucl. Acids Res.*** 19: 5096,1991; ***Mol. Cell Biol.*** 12: 1698, 1992; ***Cancer Res.*** 52: 746, 5738; 1992; ***Progr. Growth Factor Res.*** 4: 69, 1992.

EZ 102 ANATOMICAL AND FUNCTIONAL ASSOCIATIONS BETWEEN ENDOTHELIAL CELLS AND OSTEOBLASTS.

Claude D. Arnaud, Emilio L. Khoury, Gus del Puerto, John McAdams, Katalin Nyiredy, Caterina Mathews and Kin-Kee Pun. Program in Osteoporosis and Bone Biology, University of California, San Francisco, CA 94143-1610

The endothelial cell's regulatory repertoire includes stretch induced autocrine and paracrine interactions with vascular smooth muscle cells at its antiluminal surface and the ability to mount cell-cell interactions with the circulating immune system at its luminal surface. Endothelial cells populate bone but little attention has been paid to their possible role in its metabolism. We studied the anatomical and functional relationships among endothelial cells, osteoblasts and the bone marrow immune system. Using monoclonal antibodies (MAb), we immunostained unfixed cryostat sections of undecalcified bone from patients (n=12, age 23-73 years) undergoing bone surgery. Osteoblasts were identified with alkaline phosphatase (AP) MAb, endothelial cells with Factor VIII MAb, and MHC Class II molecule expression with HLA DR, DQ and DP MAbs. Fusiform shaped cells that double stained for Factor VIII and MHC Class II were found to overlie linear arrays of osteoblasts that lined trabeculae and to occupy Haversian canals. AP positive osteoblasts did not double stain with MHC Class II MAbs. Receptors for endothelin, the potent endothelial cell-derived vasoconstrictor, were identified on osteoblast-like (OB) cells (ROS 17/2.8 and chick primary OB cultures) using classical ligand binding techniques and photoaffinity labeling. Endothelin bound specifically to high affinity sites on both cell types, and photoaffinity labeling showed a binding component of 50,000 kDa, the same as found in other endothelin receptor bearing cells. Northern analysis showed that endothelin (5 ng/ml) stimulated collagen type I α1 mRNA expression 2 fold in chicken OB and 3 fold in ROS 17/ 2.8 cells. We conclude that endothelial cells are strategically positioned in bone, as in blood vessel walls, to carry out cell-cell interactions antiluminally with osteoblasts and luminally with marrow immune cellular elements. Our results suggest that those interactions could involve the transduction of stretch forces into paracrine signals (i.e., endothelin) that regulate the bone forming activities of osteoblasts and the homing to and activation of T lymphocytes at sites destined for bone remodeling.

EZ 103 **PHEOCHROMOCYTOMA (PC12) CELL-DERIVED VASCULAR GROWTH FACTOR(S).**

D.H. Damon and H. Tang. Department of Pharmacology, The University of Texas Health Science Center, San Antonio, Texas 78284-7764.

The sympathetic nervous system affects the growth of blood vessels. The mechanism(s) underlying this effect are not well understood. We used PC12 cells, a model for sympathetic neurons, to study sympathetic regulation of rat aortic endothelial (EC) and vascular smooth muscle (VSM) growth. Media conditioned (3 days) by undifferentiated (PC12) or differentiated (PC12N - exposed to nerve growth factor for 7 days) cells stimulated vascular cell growth. Maximal increases in EC number were 482±85% (mean±s.e.) and 484±185% for PC12 and PC12N medias, respectively. Corresponding increases for VSM were 422±109% and 446±47%. PC12N cells produced more EC growth stimulatory activity (143±70 units/10^6 cells) than PC12 cells (40±8 units/10^6 cells) ($p<0.05$). PC12N also produced more VSM growth stimulatory activity (65±10 units/10^6 cells versus 27±8 units/10^6 cells; $p<0.05$). The growth stimulatory activity derived from PC12 cells was partially but not totally heat sensitive; incubating the conditioned media at 65°C for 10 minutes reduced VSM growth stimulatory activity 57%. Adrenergic antagonists (both α and β) did not affect the ability of PC12-conditioned media to stimulate VSM growth. These data indicate that 1) sympathetic-like cells produce mitogen(s) for vascular cells; 2) the expression of this mitogen(s) is regulated by neuronal differentiation and/or nerve growth factor; 3) the mitogen is at least partially heat-sensitive and is not a catecholamine.

EZ 104 ISOLATION AND CHARACTERIZATION OF AN FGF-1-INDUCIBLE GENE ENCODING A NOVEL PUTATIVE SERINE/THREONINE PROTEIN KINASE, Patrick J. Donohue, Gregory F. Alberts, Brian Hampton and Jeffrey A. Winkles, Department of Molecular Biology, Holland Laboratory, American Red Cross, Rockville, MD 20855

Fibroblast growth factor-1 (FGF-1), also known as acidic FGF, is one member of a family of nine structurally-related polypeptides. It is a potent mitogen for endothelial cells and smooth muscle cells *in vitro* as well as *in vivo*. One approach for therapeutic intervention of FGF-mediated vascular cell proliferative diseases is to interrupt the FGF signalling pathway. Genes that are activated when FGF is added to quiescent cells may encode proteins that participate in this pathway. Therefore, we have used a RT-PCR differential display technique to isolate cDNA clones representing FGF-inducible mRNAs. Briefly, serum-starved NIH 3T3 cells were either left untreated or treated for various times with FGF-1 and heparin. RNA was isolated and random-primed cDNA prepared. Degenerate oligonucleotides based on conserved structural domains found in various classes of proteins were used as PCR primers. PCR amplification products were resolved by agarose gel electrophoresis and those representing FGF-1-inducible mRNAs were cloned. Nucleotide sequence analysis of one of the cDNA clones, named FR-2, indicates that the FR-2 gene encodes a novel member of the serine/threonine protein kinase family. FR-2 mRNA is rapidly and transiently induced in NIH 3T3 cells by FGF-1, FGF-2, serum, PDGF and PMA. FGF-1 regulation of FR-2 mRNA expression is due to transcriptional activation and can occur without *de novo* protein synthesis. Preliminary studies indicate that FGF-1 can also induce FR-2 expression in vascular cell cultures.

EZ 105 THE TEK ENDOTHELIAL RECEPTOR TYROSINE KINASE IS REQUIRED FOR MOUSE, DEVELOPMENT, D.J. Dumont[1], G.J. Gradwohl[2], M. Puri[1,3], G.H. Fong[1], K. Alitalo[4], A. Joyner[1,3] and M.L. Breitman[1-3]. [1]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada; [2]Institut de Chimie Biologique, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg, France; [3]Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario, Canada;[4] Molecular/Cancer Biology Lab., Dept. of Pathology, Unv. of Helsinki, P.O.B. 21 (Haartmaninkatu 3).

Recent studies have identified several receptor tyrosine kinases (RTKs) whose expression is restricted to cells of the endothelial cell lineage. These endothelial-specific RTKs belong to two structurally distinct classes, one of which includes the vascular endothelial growth factor (VEGF) receptors, flt-1 and flk-1, and another which includes tie and tek, the latter which we identified using a PCR-based screen for RTKs expressed in murine embryonic heart.

Primary structural analysis has indicated that tek is similar to tie, containing a split catalytic domain and an exceptional multidomain extracellular region comprised of three fibronectin type III-like repeats linked to two immunoglobulin loops that are in turn separated by three epidermal growth factor-like repeats. Despite the extensive structural homology between these two receptors, tek and tie show only modest sequence similarity in their carboxy-terminal tails, suggesting that they probably utilize non-identical signalling pathways. In addition examination of tek expression by in situ hybridization revealed that tek transcription begins between E7.5 and E8.0, approximately 0.5 to one day before detectable expression of tie, and that, in addition to the mesoderm cell layer of the amnion, tek marks vascular endothelial cells as well as their von Willebrand factor-negative progenitors, the angioblasts.

To investigate the biological function of tek, we disrupted the gene by homologous recombination in ES cells using a strategy that deletes the coding sequences of the first exon. Mice heterozygous for this mutation appear phenotypically normal; however, to date, no homozygous offspring have been detected among the progeny of heterozygous matings, suggesting that disruption of the tek locus results in embryo lethality and that tek probably plays a critical role in development of embryonic vasculature.

EZ 106 CHARACTERIZATION OF THE Tek SIGNALLING PATHWAY. Guo-Hua Fong[1], Daniel J. Dumont[1], Gérard Gradwohl[2] and Martin L. Breitman[1,3]. [1]Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave., Toronto, Ontario, Canada M5G 1X5; [2]Institut de Chemie Biologique, Faculté de Médicine, 11 rue Humann, 67085 Strasbourg, France; [3]Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario, Canada

Recent evidence suggests that the receptor tyrosine kinase Tek, along with four others including Tie, Flk-1, Flt-1 and Flt-4, may play pivotal roles in the development of the cardiovascular system. These and other receptor tyrosine kinases are thought to regulate cellular growth and differentiation by signal transduction processes in which ligand binding to the extracellular domain results in the autophosphorylation of the cytoplasmic domain, thereby promoting interaction of the receptor with SH-2 domain containing proteins. Different RTKs interact with non-identical, though often overlapping, sets of SH-2 proteins. Such specific interactions provide a molecular basis for the unique roles played by different members of the large RTK family.

In order to understand the functional mechanism of the Tek signalling pathway in endothelial cells, we have investigated the interactions between Tek and intracellular signalling molecules. To that end, we established conditions where Tek can be specifically stimulated to undergo autophosphorylation. We have then investigated the role of the tyrosine residue 1101, which is located in the carboxyl tail. The sequence context of Y1101, YLNT, suggests that Y1101 may mediate interaction of Tek with Grb2, an SH2 domain containing protein, since similar sequence motifs have been shown to be involved in mediating interaction of other RTK's with Grb2. Mutation of Y1101 to F1101 dramatically reduced autophosphorylation of Tek without diminishing its catalytic activity, suggesting that Y1101 may indeed be an autophosphorylation site. Studies of Tek-Grb2 interaction, and the possible role of this interaction in neovascularization and angiogenesis is currently in progress.

EZ 107 ANGIOTENSIN IV AND BASIC FGF EXHIBIT A SYNERGISTIC EFFECT ON THE ENHANCEMENT OF DNA SYNTHESIS IN CULTURED CORONARY MICROVASCULAR ENDOTHELIAL CELLS, Keith L. Hall, Subramaniam Venkateswaran, Jodie M. Hanesworth, M.E.Schelling and Joseph W. Harding, Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, Washington State University, Pullman, WA 99164-6520

A new class of angiotensin binding sites has recently been identified in many mammalian tissues that exhibits high specificity and affinity for the hexapeptide fragment of angiotensin II, angiotensin IV (AngIV). The distribution of this binding site (now termed the AT4 receptor) has also been shown to include both cultured vascular endothelial and vascular smooth muscle cells. Using coronary microvascular endothelial cells (CVEC) as an in vitro model for angiogenesis, we report that when added simultaneously, AngIV and bFGF induce a mitogenic response that is significantly greater than that produced by bFGF alone. In addition, we find that AngIV does not bind to bFGF, heparin or the FGF receptor and conversely, bFGF is unable to compete with AngIV at the AT4 receptor. These data suggest that AngIV and bFGF bind to distinct receptors on CVEC and that their synergistic effect on mitogenesis is mediated through events subsequent to receptor binding. DNA synthesis was determined by the incorporation of [^{3}H]thymidine into quiescent (2% FBS) CVEC monolayers following exposure to 10nM AngIV and/or 10ng/ml bFGF for 1,3,5,7,9, or 11 days. A significant enhancement of DNA synthesis (AngIV with bFGF vrs bFGF alone, $p < .01$) was seen following 3,5,7,9 and 11 days exposure. Binding of [^{125}I]AngIV to S2 fractions of CVEC exhibited high affinity (KD=1.2nM, single site model, no cooperativity) and specificity (AngIV > AngIII > AngII > AngII(4-8), DUP 753 and CGP 42112A were noncompetitive) for the AT4 receptor.

EZ 108 CALRETICULIN MODULATES ENDOTHELIAL CELL PROLIFERATION AND NUCLEAR TRANSPORT,
Victor B. Hatcher, Barry M. Kaplan, Christina A. Samathanam, Marek Michalak and Peter M. Buttrick, Departments of Biochemistry and Medicine, Albert Einstein College of Medicine, Bronx, New York 10461.
Calreticulin (CRT) functions as a major Ca^{2+} binding protein in the lumen of the endoplasmic reticulum. The potential role of CRT in cell proliferation and nuclear transport was evaluated. Human umbilical vein endothelial cells (HUVEC) were grown in complete medium (C) containing acidic fibroblast growth factor (FGF-1) and heparin for 24 hours and then the media was changed to either C, C without FGF-1, C with antisense (AS) DNA oligomers, or C with sense DNA oligomers. AS was a phosphorothiolated 18 base oligomer complementary to -2 to +16 relative to the transcription start site of CRT while sense was identical to bases +60 to +78. Cell counts after 4 days indicated that the response to AS was dose dependent with mean ($\pm$ SE) growth inhibition of 25 $\pm$ 2% with 1 μm, 51 $\pm$ 4% with 5 μM and 87 $\pm$ 5% with 10 μM. Sense oligomers caused a 6 $\pm$ 3% growth inhibition. Northern blot analysis using a cDNA probe for CRT revealed significant reduction in CRT mRNA levels in cells grown with AS. Western blots utilizing goat anti-calreticulin revealed significant reduction in CRT expression. A nuclear import model in digitonin permeabilized NRK cells and HUVEC was set up using the fluorescent allophycocyanin (APC) conjugated to a synthetic peptide containing the SV40 large T antigen and rhodamine labelled FGF-1. Calreticulin promoted the import of APC-CGGGPKKKRKVED and rhodamine labelled FGF-1 in NRK cells and HUVEC. These observations suggest that CRT is more than a Ca^{2+} binding storage protein and may well be involved in cell proliferation and nuclear transport.

EZ 109 THE SYNTHESIS OF FIBROBLAST GROWTH FACTOR (FGF)-2: FGF-2 DOMAIN CHIMERIC PROTEIN DEFINE THE REGION RESPONSIBLE FOR HEPARIN-DEPENDENT MITOGENIC ACTIVITY,
*1Toru Imamura, *1Yoshihito Tokita, *1Shuichi Oka, *2Stan Friedman, *2Xi Zhan and *2Thomas Maciag, *1National Institute of Bioscience and Human-Technology, Tsukuba, Ibaraki 305, Japan and *2Jerome H. Holland Laboratory, American Red Cross, Rockville, MD 20855, USA
We have constructed heparin-binding fibroblast growth factor (FGF)-1 and -2 genes in an array of four-cassettes format so that the cassettes can be interchanged each other. These gene constructs were subjected to a variety of chimeric arrangements. The chimeric genes were translated into proteins using pET T7 RNA polymerase system. The chimeric proteins were systematically named where FGF-C[1111] translates to FGF-1 and FGF-C[2222] translates to FGF-2. We previously reported that the deletion of N-terminal cassette from FGF-1 reduced its activity and heparin-binding, and that FGF-1 with the N-terminal domain interchanged with FGF-2 (FGF-C[2111]) had full mitogenic activity. We presently report the results of additional FGF chimeras. Among them are the two with the heparin-binding domain interchanged with each other, FGF-C[1211] and FGF-C[2122]. Interestingly, FGF-C[1211] has full mitogenic activity comparable to the FGF-1 construct in the presence of heparin. Furthermore, in the absence of heparin, FGF-C[1211] is found to be as potent as in the presence of heparin, a characteristic distinct from FGF-1 but similar to FGF-2. Thus, the heparin-binding domain of FGF-2 confers FGF-1 molecule with a heparin-related characteristic of FGF-2. In the case of FGF-C[2122], however, although it demonstrated an affinity for heparin, the protein showed little activity as a growth factor. Thus, the sequence defined by the second FGF-2 cassette defines the region for heparin-dependent mitogenesis in FGF-1.

EZ 110 ***IN VIVO*** **AND** ***IN VITRO*** **REGULATION OF ICAM-1 AND MCP-1 EXPRESSION BY INTERLEUKIN-10**
Michael L. Jones, Ara A. Vaporciyan, and Peter A. Ward, Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109-0602
The emigration and subsequent sequestration of leukocytes into an inflammatory lesion play an integral role in the development of injury. Attachment of leukocytes to endothelium via adhesion molecule interactions, such as Intercellular Adhesion Molecule-1 (ICAM-1) and it's ligand LFA-1, are considered the first stage in the response of leukocytes to an inflammatory stimulus. During stimulation, chemoattractants such as the Monocyte Chemoattractant Protein-1 (MCP-1) are expressed, which play a significant role in the directed migration of leukocytes across the endothelial junctions into the site of inflammation. In this study we investigated the ability of recombinant IL-10 (kindly supplied by Dr. Maureen Howard, DNAX, San Jose, CA) to regulate the *in vivo* and *in vitro* expression of ICAM-1 (message and protein) and *in vitro* expression of MCP-1 message. Human Umbilical Vein Endothelial cells (HUVEC) cultured in the presence of 20ng/ml Tumor Necrosis Factor-α (TNF-α) and IL-10 revealed a time (1, 4, 8, 12, 18 hour) and dose (.01, 0.1, 1, 10ng/ml) dependent, relative decrease in ICAM-1 and MCP-1 message. The maximum inhibition of message for ICAM-1 (30%, $p<0.05$) and MCP-1 (43%, $p<0.01$) occurred at 4-8 hours with 10ng/ml of IL-10. IL-10 alone had no effect on ICAM-1 or MCP-1 message in concentrations up to 50ng/ml at any time point. Utilizing a cell ELISA assay with a specific anti-ICAM-1 monoclonal antibody, TNF-α-stimulated HUVEC cultured for six hours in the presence of 10ng/ml IL-10 expressed 43% ($p<0.05$) less ICAM-1 than TNF-a-stimulated alone. Furthermore, *in vivo* rat studies using IgG-immune complex-induced alveolitis ICAM-1 upregulation showed a 109% ($p<0.01$) reduction in ICAM-1 expression, in comparison to the negative control, by using an ^{125}I-labeled anti-rat ICAM-1 monoclonal antibody. These combined studies suggest that IL-10 can play an important regulatory role in the expression of ICAM-1 and MCP-1 message and protein.

EZ 111 MODULATION OF THE PRODUCTION AND THE ACTIVATION OF LATENT TGF-ß BY RETINOID AND LIPOPOLYSACCHARIDE IN BOVINE ENDOTHELIAL CELLS,
Soichi Kojima* and Daniel B. Rifkin§ *Laboratory of Gene Technology and Safety, Tsukuba Life Science Center, RIKEN, Tsukuba Ibaraki 305, Japan and §Department of Cell Biology and Kaplan Cancer Center, New York University Medical School and Raymond and Beverly Sackler Foundation Laboratory, New York, NY 10016.
The conversion of high molecular weight latent transforming growth factor-ß (LTGF-ß) to 25-kD active TGF-ß, namely, the activation of LTGF-ß, is dependent upon both cellular plasminogen activator (PA)/plasmin and transglutaminase (TGase) levels. Therefore, retinoids, which enhance EC production of PA , TGase, and LTGF-ß, induce activation of LTGF-ß in ECs and mature TGF-ß formed acts on ECs in autocrine manner, that we have previously reported. This fact suggests that formation of TGF-ß is regulated by PA/plasmin, TGase, and LTGF-ß levels in ECs. Since lipopolysaccharide (LPS) has been reported to reduce EC surface plasmin activity by increasing the production of PA inhibitor-1 and its deposit on the cell surface, we have tested the possibility that LPS might suppress retinoid-induced activation of LTGF-ß in ECs. ECs were treated with 2 µM retinol and/or 10 ng/ml LPS and the expression and/or the amount of PA, surface plasmin, TGase as well as active and latent TGF-ß were measured. Interestingly, the down-regulation of surface PA/plasmin levels with LPS was accompanied by a profound decline of both TGase and LTGF-ß expressions, thus, resulted in the suppression of surface activation of LTGF-ß. The effect was dependent on the concentration of LPS and on treatment time. These data suggest that formation of TGF-ß in ECs might be modulated, in general, by factors which change cellular PA and TGase levels.

EZ 112 DIFFERENTIAL EXPRESSION OF GROWTH FACTORS BY ENDOTHELIUM AND SMOOTH MUSCLE CELLS IN INJURED ARTERIES, Volkhard Lindner, Cecilia Giachelli and Michael A. Reidy, Department of Pathology, University of Washington, Seattle, WA 98195

Endothelial cells (EC) and smooth muscle cells (SMC) in normal rat arteries are quiescent with replication rates of less than 0.05%/day. After injury, however, EC at the wound edge and up to 20% of the medial SMCs undergo rapid replication and migration. SMCs migrating from the media into the intima show high replication rates which return to quiescence within 6 weeks after injury. We used an arterial balloon injury model to study differences in growth factor expression in replicating versus nonreplicating EC and SMCs using en face techniques. In situ hybridization and immunocytochemistry were carried out on en face preparations with rat specific probes. mRNA for basic fibroblast growth factor (bFGF) were found in replicating but not in quiescent EC and SMC although bFGF protein was present in both growth states. FGF receptor-1 mRNA was expressed by SMCs at all times but was seen in EC only when they were replicating. PDGF-B mRNA was expressed by endothelial cells at the leading edge within 8 hours after injury and expression was still seen after cells were no longer replicating but no expression was detectable in endothelium of normal arteries. PDGF-B mRNA was also expressed by a subpopulation of SMCs (7-11% of the luminal SMCs) that had migrated from the media onto the luminal surface within 2 weeks after balloon injury. At 6 weeks after denudation, when the intimal lesion was no longer growing only 0.4% of the luminal SMCs were found to express PDGF-B. SMCs expressed the PDGF receptor ß-subunit all times but no expression was detectable in EC. Our data suggest that bFGF/FGFR-1 may be involved in growth of both EC and SMC while expression of PDGF-B by EC may stimulate underlying SMCs in a paracrine way. Our findings also demonstrate the heterogeneity of SMCs in the vessel wall with regard to PDGF-B expression.

EZ 113 EXPRESSION OF RECOMBINANT HUMAN $VEGF_{189}$ IN BACULOVIRUS INFECTED INSECT CELLS. Georg Martiny-Baron, Karin Weindel and Dieter Marmé. Institute of Molecular Cell Biology, University of Freiburg, D-79108 Freiburg, FRG.

Vascular endothelial growth factor (VEGF) has been described as a specific growth factor for endothelial cells which is released from pituitary folliculostellate cells as well as from a variety of tumor cell lines. The fact that VEGF is secreted and is specific with respect to the target cells had led to the idea that VEGF is an important regulator of angiogenesis under physiological and pathophysiological circumstances. Up to now four different VEGF isoforms have been discovered which probably arise from alternative RNA splicing. They are named by their number of amino acids after cleavage of the signal peptide $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$. While the effects of $VEGF_{165}$ have been documented in various biological systems and VEGF receptors have been identified with purified $VEGF_{165}$, only little information about the other isoforms is available.

For this reason we decided to produce different recombinant VEGF isoforms as tools for further characterization. The cDNA of human $VEGF_{189}$ was obtained from astrocytoma cells SNB-19 by PCR cloning and cloned into the baculovirus transfer vector pVL1392. Recombinant baculovirus was used to infect SF158 insect cells. Production of recombinant $VEGF_{189}$ was confirmed by Western blot analysis. Experiments are on the way to purify the recombinant protein.

The use of purified $VEGF_{189}$ will hopefully help us to get new insights into the role of the different VEGF isoforms.

EZ 114 **THE ROLE OF PROTEIN KINASE C (PKC) IN E-SELECTIN EXPRESSION BY HUVEC,** Michael J. May and Jeremy D. Pearson, Vascular Biology Research Centre, King's College London, Campden Hill Road, London W8 7AH.

The adhesion molecule E-selectin, expressed by endothelial cells at sites of inflammation, is involved in the initial rolling and tethering of leukocytes to the vascular endothelium. The regulation of E-selectin expression by HUVEC was studied using an enzyme-linked immunosorbant assay (ELISA) employing the anti-E-selectin antibody 1D2. Unstimulated HUVEC did not express E-Selectin, however, pretreatment with either IL-1α, IL-1ß or TNFα induced expression in a dose and time dependent manner. Pretreatment with 100 units/ml of each cytokine induced maximal expression after 4-8 hours and after a further 24-48 hours expression returned to basal levels. Pretreatment with the PKC-activating phorbol ester phorbol 12-myristate, 13-acetate (PMA; 100nM) also induced E-selectin expression. Maximal PMA-induced expression occurred after 4-8 hours exposure and was 60% of maximal expression observed with cytokines. The inactive PMA analogue 4α-phorbol 12,13-didecanoate had no effect. Combinations of maximal doses of cytokines did not increase E-selectin expression over that observed with individual cytokines, whereas combinations of cytokines and PMA increased expression to 115-157% of the levels observed with cytokines plus αPDD. The role of PKC was examined by pre-incubating HUVEC with one of five separate PKC inhibitors prior to activation with IL-1α or PMA. The inhibitor H7 had no effect on E-selectin expression induced by IL-1α or PMA at any concentration tested (10^{-9}-10^{-4}M). However, PMA induced expression was dose dependently inhibited by staurosporine (STS), bisindolylmaleimide (BIM) and the BIM derivatives Ro31-7549 and Ro31-8220. The effects ranked according to half maximal inhibitory concentrations (IC_{50}; in brackets) were: STS (15-50nM) $\geq$ Ro31-8220 (20-500nM) > BIM (80-350nM) > Ro31-7549 (2.5-6.0μM). Only Ro31-8220 inhibited IL-1α induced E-selectin expression, however, the IC_{50} of 2-3μM was approximately 100 fold higher than that for inhibition of PMA induced expression. These results suggest that E-selectin expression by HUVEC induced by PMA involves activation of PKC whereas IL-1α induced expression involves a PKC-independent signalling pathway.

EZ 115 FACTORS RELEASED BY SPINDLE CELLS FROM AIDS ASSOCIATED KAPOSI'S SARCOMA CELLS (AIDS-KS CELLS) INDUCE ANGIOGENESIS AND EDEMA. Shuji Nakamura[1], Kaoru Murakami-Mori[2,1], Shinsaku Sakurada[3], Buddi Rajeeve[4], Narsing Rao[4], Herbert Weich[5] and Syed Zaki Salahuddin[2,1], [1]Institute of Molecular Medicine & Technology, Huntington Memorial Hospital, Pasadena, CA., 91105, [2]University of Southern California, Los Angeles, CA., University of Tokyo, Japan, [4]Doheny Eye Institute, University of Southern California, Los Angeles, CA., [5]GBS-Gasellschaft fur, Biotechnologische Forscunh mbH, Germany.

Angiogenesis and in many cases edema are two major components of AIDS associated Kaposi's sarcoma (AIDS-KS). Spindle cells isolated from KS-lesions of patients with AIDS have been cultured in vitro initially by us and now by several other laboratories. We culture these cells in the presence of factors in the conditioned medium (CM) of a CD4+ T cell line. The critical factor in this CM is Oncostatin M (OM). The AIDS-KS cells transplanted in nude mouse induce KS-like lesion of murine origin. We report here that AIDS-KS cells produce and secrete two different mediators with important consequences for vascular cells. One of the factors is related to vascular endothelial growth factor (VEGF) and the other to basic fibroblast growth factor (bFGF). AIDS-KS cell CM induces vascular hyperpermeability (VHP) in vivo and also proliferation of endothelial cells in vitro. These activities secreted by the AIDS-KS cells totally bind to heparin sephrose. The bound material can be eluted in stepwise manner with 0.5M NaCl (VEGF) and 2.0M NaCl (bFGF). Neutralizing antibody to BFGF completely eliminates the activity in 2.0M NaCl fraction. Similarly, polyclonal antibody to VEGF only neutralizes the activity in 0.5M NaCl fraction. In immunoprecipitation assay, anti-bFGF gave a 18 kd band on SDS gel. We estimate that approximately 10% of total bFGF is secreted from intact AIDS-KS cells. Although the activities in these fractions are distinguishable, both induce angiogenesis in rabbit cornea. In vivo the 0.5M NaCl fraction induced VHP. This was tested in our nude mouse and hairless guinea pig models. Significant level of VEGF specific mRNA is present in AIDS-KS cells (3.9 kd band). VHP factor appears to be related to VEGF. In summary, it appears that AIDS-KS cells produce factors VEGF and bFGF, that mediate two important features of AIDS-KS, edema and angiogenesis.

EZ 116 IDENTIFICATION OF THE TYPE III TGF-β RECEPTOR ON MICROVASCULAR ENDOTHELIAL CELLS, Maureen D. O'Connor-McCourt*, Jean Pierre Morello*, Barbara Meyrick#, *Receptor Group, Biotechnology Research Institute, Montreal, Quebec, Canada H4P 2R2, #Dept. of Pathology, Centre for Lung Research, Vanderbilt University Medical Centre, Nashville, TN 37232

We have previously demonstrated, using quantitative radioautography, that microvasccular endothelial cells are the major site of binding for intravenously administered ^{125}TGF-β1 in vivo. This result was unexpected since it had been reported that endothelial cells in culture express low to moderate numbers of only the Type I and II TGF-β receptors. However, in these studies only macrovascular endothelial cells had been examined. In order to determine which TGF-β receptor type may be responsible for the in vivo endothelial binding, we have characterized the TGF-β receptor types which are expressed on both macro- and microvascular endothelial cells in culture. As expected, we find that macrovascular endothelial cells express predominately the Type I and II receptors. However, on both primary bovine lung microvascular endothelial cells and EGJ cells (a microvascular endothelial cell line) we find, in addition to low levels of the Type I and II receptors, high levels of the Type III TGF-β receptor. This indicates that not all endothelial cells lack the Type III TGF-β receptor, and that there appears to be a distinction between the TGF-β receptor profile of macro- and microendothelial cells in culture.

EZ 117 INDUCTION BY LPS AND CYTOKINES OF ENDOTHELIAL CELL GLYCOSPHINGOLIPIDS AND THEIR RELATIONSHIP TO HUMAN DISEASE, Tom G. Obrig, Chandra B. Louise, Susan A. Kaye and Clifford A. Lingwood, Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY, 14642 and Hospital; for Sick Children, Toronto M5G 1X8, Canada.

Endothelial cells are capable of synthesizing neutral glycosphingolipids. However, the functional role of these compounds in endothelial cell physiology remains to be determined. In one case, globotriaosylceramide (Gb_3) has been identified as the high-affinity receptor for the bacterial Shiga toxins. These toxins have been implicated as causal agents in *E. coli* O157:H7-related hemolytic uremic syndrome, with their putative target being renal microvascular endothelial cells. Recently, we demonstrated that bacterial lipopolysaccharide or the cytokines TNF-alpha or IL-1 beta sensitized human umbilical vein endothelial cells (HUVEC) to Shiga toxin. The present study was conducted to determine if the sensitization of HUVEC to the toxin could be due to induction of Gb_3 and other glycosphingolipids by LPS, TNF, or IL-1.

The results indicate that LPS, TNF and IL-1 individually induced HUVEC glycosphingolipids of the Gb_3 pathway. This induction was both dose- and time-dependent. During the 24 h induction period, their was a 2 to 10-fold increase in glucosylceramide, lactosylceramide, Gb_3, and globotetraosylceramide content of HUVEC as determined by HPLC analysis. All three inducing agents appeared to favor the accumulation of Gb_3 vs. the other glycosphingolipids. Removal of LPS, TNF or IL-1 from the induced HUVEC cultures resulted in a partial decay of the glycolipids within the following 48 h period. Signal transduction mechanisms involved in the induction process and the role of these glycosphingolipids in endothelial cell physiology are presently under investigation.

EZ 118 ENERGETICS OF LIGAND-RECEPTOR BINDING INTERACTIONS IN THE FGF SYSTEM: THE BINARY BINDING REACTIONS OF FGFR1 WITH BFGF, FGFR1 WITH HEPARIN, AND BFGF WITH HEPARIN. M.W. Pantoliano, R.A. Horlick, B. Springer, L.D. Thompson, D.E. Van Dyk, W.F. Herblin, & W.P. Sisk. Crystallography & Biophysical Chemistry, The Du Pont Merck Pharmaceutical Co., Wilmington, DE 19880.

The extracellular binding component, D(II)-D(III), of the *flg* fibroblast growth factor receptor (FGFR1) was cloned from cultured human endometrial carcinoma cell mRNA and expressed using baculovirus infected Sf9 insect cells. Recovery of this soluble truncated receptor through an affinity chromatographic strategy yielded ~15 mg of purified receptor/L of culture media. A biophysical examination of the binding interactions of the three primary reactants, FGFR1, bFGF, and heparin/heparan sulfate (HS) of the FGF system was explored through the use of isothermal titrating calorimetry. The binding reactions were dissected into three fundamental binary reactions: (1) FGFR1 + bFGF <-> FGFR1/bFGF; (2) FGFR1 + HS <-> FGFR1/HS; and (3) bFGF + HS <-> bFGF/HS; and characterized by dissociation constants, K_1 = 41 (±12) nM, K_2 = 104 (±17) μM (when HS = low MW heparin, 3 kDa; or 11 (±3) μM for HS = pentosan polysulfate), and K_3 = 480 (±20) nM (low MW heparin), respectively. The binding of bFGF to FGFR1 was found to be a simple binary binding reaction characterized by a single equilibrium constant, K_1, and does *not* require the presence of HS. Furthermore, the large free energy of binding for this reaction, ΔG° = -10.2 (±0.2) kcal/mol, identified reaction (1) as the central binding reaction for the FGF system. The three binary binding reactions revealed, however, that each one of the three reactants is multivalent: FGFR1 binds growth factor *and* HS, bFGF binds receptor *and* HS, and HS binds FGFR1 *and* bFGF. The multivalent nature of each primary reactant suggests that many ambiguous facets of this growth factor system that relate to the role of HS in "high affinity" binding and the mechanism of the biological activities of bFGF may be understood in terms of well studied classical multiple ligand binding mechanisms that allow for the cooperative energetic coupling of the secondary HS binding reactions, reactions (2) and (3) with the central bFGF/FGFR1 binding event, reaction (1).

EZ 119 TRANSFORMING GROWTH FACTOR BETA (TGF-ß) AUTO-REGULATES THE PROLIFERATION OF VASCULAR SMOOTH MUSCLE CELLS DERIVED FROM SPONTANEOUSLY HYPERTENSIVE RATS, Andreina Poggi, Anna Di Sciullo, Giuseppe Bonapace, Mimmo Nasuti, Andria D'Orazio, Department of Vascular and Tumor Cell Biology, Consorzio Mario Negri Sud, 66030 Santa Maria Imbaro, Italy.

TGF-ß is a multifunctional cytokine that regulates cell proliferation and differentiation. We evaluated the possible role of TGF-ß on the hyperproliferative state of vascular smooth muscle cells (SMC) obtained by explants of the thoracic aortas from 6 month old spontaneously hypertensive rats (SHR) in comparison with SMC from normotensive rats (WKY) of the same age. Cells were grown to confluency and incubated with DME without serum for 48 h. The presence of TGF-ß in the cell-conditioned medium was evaluated either as biological activity, measuring the ability to inhibit ^{3}H-thymidine incorporation by MV1-Lu cells, or with immunological techniques, using a specific antibody to human TGF-ß (R&D, Minneapolis, Mo.). Conditioned medium from both cell types contained an inhibitory factor, identified as TGF-ß by western blot. The biological activity of this material was about 1-2ng TGF-ß equivalents per μg of protein. Slot-blot analysis showed that the immunoreactive material was 50% greater in SHR than in WKY. Treatment with TGF-ß (1-10 ng/ml) inhibited in vitro proliferation of WKY but not of SHR cells. Moreover, in experiments of wound repair after in vitro injury, we observed that SHR SMC grew faster than WKY SMC to cover the injured area. The regrowth rate, measured 48 hours after the injury, was of 1200 μm/h for SHR SMC and of 750μm/h for WKY.In conclusion, SMC from hypertensive rats showed a reduced response to the inhibitory effect of exogenous TGF-ß and an increased production of immunoreactive TGF-ß in the conditioned medium, that seems to allow an autocrine regulation of their growth.

This work was supported by Agenzia per la Promozione dello Sviluppo del Mezzogiorno (6168-PR3).

EZ 120 EXPRESSION OF VASCULAR PERMEABILITY FACTOR (VPF) mRNA BY HUMAN MELANOMA CELLS AND BY MELANOMA XENOGRAFTS IN NUDE MICE, Andy J.G. Pötgens, Nicolette H. Lubsen, Greet van Altena, John G.G. Schoenmakers, Dirk J. Ruiter, Robert M.W. de Waal, Institute of Pathology, University Hospital Nijmegen, and Department of Molecular Biology, University of Nijmegen, The Netherlands.

Vascular permeability factor (VPF), also known as vascular endothelial growth factor (VEGF), is a potent regulator of vascular function. It promotes angiogenesis, augments vascular permeability, and induces endothelial proliferation *in vitro*. We investigated the expression of VPF in a panel of human melanoma cell lines which are all tumorigenic upon intradermal injection into nude mice. The tumors developing from these cell lines differ however, with respect to vascularization grade and metastatic frequency. *In vitro*, the highly metastatic lines had much higher levels of the VPF messenger than the low-metastatic lines. Surprisingly, the clear differences in expression found *in vitro*, were not present *in vivo*. All melanoma xenografts synthesized similar high levels of human VPF mRNA, suggesting that the *in vivo* environment induces high level VPF expression in the low-metastatic lines. Our data suggest that if VPF contributes to the different angiogenic and metastatic properties of the melanoma xenografts, it does so only in the early stages of development, when the melanoma lines still differ clearly in VPF expression. Melanoma cell lines with a low metastatic capacity showed a dramatic increase in the VPF mRNA levels when cultured under a low oxygen tension. The upregulation of the VPF gene expression found *in vivo* may therefore be caused by hypoxia in the tumor tissue.

EZ 121 MODULATION OF SMOOTH MUSCLE CELL PHENOTYPE BY ENDOTHELIAL CELLS AND TGF-β_1, Powell RJ, Fillinger MF, Cronenwett JL, Wagner RJ, Section of Vascular Surgery, Dartnouth-Hitchcock Medical Center, Lebanon, NH 03756

Mechanisms controlling SMC phenotype and growth pattern are not well defined. SMCs in culture assume a synthetic phenotype and exhibit a "hill and valley" growth pattern. This study examined the effect of endothelial cells (ECs) and transforming growth factor-β_1 (TGF-β_1) on SMC morphology and growth pattern *in-vitro*. Bovine ECs and SMCs grown to 3-5 passages were plated on opposite sides of a 13 μm thick permeable membrane (0.45 μm pores) to allow potential EC-SMC cross-membrane communication. ECs and SMCs were identified by immunohistochemical staining with anti-Factor VIII and α-actin respectively. SMCs were studied in co-culture (5 wells/ group) opposite confluent ECs (EC/SMC) and alone (SMC ONLY; controls). After four days of culture in DMEM/2.5% calf serum, SMCs were harvested for protein and DNA measurements. The ratio of SMC protein/DNA (an index of SMC synthetic phenotype) decreased from 175 ± 9 (SMC alone) to 115 ± 7 μg protein/μg DNA in EC/SMC co-culture ($p < 0.001$), indicating less synthetic phenotype in the EC/SMC group. Cell surface area, measured by planimetry, was reduced from 5.9 ± 0.3 x 10^3 μm^2 (SMC alone) to 0.9 ± 0.1 x 10^3 μm^2 by EC/SMC co-culture ($p < 0.001$), also indicating less synthetic phenotype. In the SMC ONLY cultures, SMC hypertrophy and hill and valley growth formation was confirmed by light and electron microscopy. In contrast, SMCs from the EC/SMC group exhibited a spindle-shaped, contractile phenotype and evenly distributed macroscopic growth features without hill and valley formation. Growth features of SMCs were further defined by measuring the surface area of the membrane that became devoid of SMCs as hill and valley growth occurred [bare surface area (BSA)]. Hill and valley growth as indexed by BSA was greatest in SMCs cultured alone (BSA 2.6 ± 0.5 mm^2) and absent in SMCs co-cultured with ECs (BSA 0.0 ± 0.0 mm^2, $p < 0.001$). Hill and valley growth in SMCs cultured alone was reduced by addition of either a neutralizing antibody to TGF-β_1 (BSA 0.9 ± 0.3 mm^2, $p < 0.001$) or the protease inhibitor aprotonin (BSA 0.0 ± 0.0 mm^2, $p < 0.001$) which prevents plasmin mediated activation of TGF-β_1. Addition of TGF-β_1 (5 ng/ml) to SMCs cultured with ECs resulted in hill and valley growth. Thus, EC presence maintained SMCs in a contractile phenotype and regulated SMC macroscopic growth characteristics. ECs may regulate SMC growth features via release of mediators which inhibit activation of latent TGF-β_1.

EZ 122 IDENTIFICATION OF THE PROMOTER OF THE ENDOTHELIAL CELL SPECIFIC RTK, tek, M. Puri[1,2], D.J. Dumont[1], G. J. Gradwohl[3], and M.L. Breitman[1,2]. [1]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada; [2]Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario, Canada; [3]Institut de Chimie Biologique, Faculté de Médicine, 11 rue Humann, 67085 Strasbourg, France.

Recent studies have identified several receptor tyrosine kinases (RTKs) expressed in endothelial cells, including flt-1, flt 4, flk-1, tie and tek. We have demonstrated that the tek receptor, which we identified using a PCR based screen for RTKs expressed in murine embryonic heart, is restricted in its expression to the endothelial lineage, including the endocardium, the leptomeninges, and the endothelial lining of the extra- and intra-embryonic vasculature at the earliest stages of their differentiation. This result suggests that Tek may play a critical role in the development and/or maintenance of the embryonic vasculature.

We have identified the tek promoter and have shown that this DNA fragment can direct the expression of a lacZ reporter gene specifically to endothelial cells in transgenic embryos and are currently testing shorter transgene constructs in founder embryos. Moreover, primer extension analysis of total RNA obtained from the tek expressing endothelial cell line, Py4-1, has identified the start site of transcription as position -157 or -156 relative to the first ATG codon.

We are also currently testing the possibility of utilizing tek/lacZ transgenic mouse line as an indicator line for the processes of angiogenesis and vasculogenesis in vivo. To this end, we have staged tek/lacZ expression during development relative to the expression of the endogenous tek gene and the other endothelial RTKs. In addition, in an attempt to visualize the process of tumor mediated angiogenesis, tek/lacZ mice have been crossed to other transgenic lines which develop a variety of vascularized tumors.

EZ 123 SIGNAL TRANSDUCTION BY THE VEGF RECEPTOR Flk1

Timothy P. Quinn and Lewis T. Williams, Cardiovascular Research Institute, UCSF, San Francisco, CA 94143

Vascular endothelial growth factor (VEGF) is a 46kD secreted polypeptide that induces endothelial proliferation in vitro and angiogenesis in vivo, and may increase vascular permeability. Recent reports suggest that VEGF may play a role in embryonic vascular development, tumor angiogenesis, and uterine angiogenesis. We are investigating the receptors and intracellular signalling pathways that mediate the actions of VEGF. The tyrosine kinase receptor Flt1 was previously shown to be a high affinity receptor for VEGF, and VEGF stimulation induces calcium efflux from Xenopus oocytes injected with Flt1 mRNA. However, when stably expressed in 3T3 or CHO cells Flt1 binds VEGF but does not undergo autophosphorylation or stimulate mitogenesis. Therefore the function of Flt1 remains unclear. Recently we showed that Flk1, a receptor tyrosine kinase structurally related to Flt1, is an functional VEGF receptor whose expression is restricted to vascular endothelium and endothelial cell precursors. In situ hybridization of mouse tissues showed Flk1 expression in yolk sac and intraembryonic mesoderm at E7.5; in endocardium, large and small vessel endothelium, and presumptive angioblasts at E12.5; and in the renal glomerulus of adult mice. VEGF bound to Flk1 with subnanomolar affinity, and induced tyrosine autophosphorylation of the receptor in Flk1-transfected 3T3 cells and calcium efflux from Xenopus oocytes expressing Flk1. These results suggest that VEGF may act through Flk1 to regulate endothelial differentiation, proliferation, migration or permeability. In order to study Flk1 signalling in endothelial cells possessing native VEGF receptors, we constructed a retroviral vector expressing a chimeric receptor fusing the Fms extracellular and transmembrane domains to the cytoplasmic domain of Flk1. HUVEC, bovine adrenal and brain endothelial cells, and 3T3 cells have been stably transfected with the FmsFlk chimera. Stimulation of the FmsFlk chimera with the Fms ligand M-CSF, or stimulation of wild type Flk with VEGF induced rapid tyrosine phosphorylation of the receptor. Activated Flk1 associated with and phosphorylated PLC-gamma, which stimulated phosphoinositidol turnover. In addition, Flk1 phosphorylated several other unknown cellular substrates. However, activation of Flk1 did not induce mitogenesis in endothelial or 3T3 cells, and did not activate Raf-1 kinase or MAP kinase, which are activated by other growth factor receptors such as the FGF and PDGF receptors. These results suggest that the signalling pathways activated by the Flt1 and Flk1 receptors may differ from other tyrosine kinase receptors.

EZ 124 NEOINTIMAL FORMATION FOLLOWING BALLOON CATHETER INJURY: THE ROLE OF HYALURONAN (HA) AND THE HA RECEPTOR RHAMM Rashmin C. Savani, Chao Wang, Yufang Shi, Chris Kaplan, Rob Panek, Robert Stern and Eva A. Turley. Dept. of Pediatrics and Manitoba Institute of Cell Biology, Winnipeg, Canada, Parke-Davis Research Development, Ann Arbor, MI and Dept. of Pathology, UCSF, San Francisco, CA.

Adherence of inflammatory cells, smooth muscle cell proliferation-migration into the intimal space, and excess production of extracellular matrix contribute to the stenosis seen in injured arteries after balloon angioplasty. We have previously shown that the interaction of hyaluronan (HA) with the HA receptor RHAMM (Receptor for HA-Mediated Motility) is necessary for the migration of smooth muscle cells following single-scratch wounding in vitro. Membrane-association and expression of a novel 70 kDa isoform of RHAMM coincided with increased migration. We therefore investigated RHAMM and HA expression in balloon-catheterized rat carotid arteries, an injury that affects smooth muscle cells. In control vessels, constitutive expression of RHAMM was noted in the endothelium and in the medial smooth muscle cells. Two hours after injury, neutrophils and macrophages adhered to the site of injury and strongly expressed RHAMM. By six hours, a subpopulation of medial smooth muscle cells that demonstrated increased expression of RHAMM could be identified next to the internal elastic lamina. Forty eight hours after de-endothelialization, a layer of smooth muscle cells, staining for both RHAMM and HA, could be seen adjacent to the lumen. From 7 to 14 days after injury, cells within this neointima continued to express high levels of RHAMM, but expression of HA was restricted to cells at the internal elastic lamina. Two isoforms of RHAMM (65 and 84 kDa) were noted in control arteries. Coincident with the increased staining for RHAMM and HA observed in injured arteries, a 70 kDa isoform, that may represent a membrane form of RHAMM, appeared. Sham operated controls showed no neointimal formation or changes in the expression of HA or RHAMM. In vitro, macrophage and neutrophil chemotaxis was inhibited by both an anti-RHAMM antiserum and a peptide encoding a RHAMM HA-binding domain. Smooth muscle cell migration following single-scratch wounding in vitro was also inhibited by these reagents. These results implicate HA and RHAMM in the migratory responses to vascular injury.

EZ 125 DELETION DOMAIN ANALYSIS REVEALS A CYTOSOLIC RETENTION SIGNAL NEAR THE CARBOXYL TERMINUS OF FIBROBLAST GROWTH FACTOR-1, Jianping Shi, Stanley Friedman, Xi Zhan, and Thomas Maciag, Department of Molecular Biology, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855

Fibroblast growth factors (FGFs) are potent mitogens for a wide variety of cells derived from the mesoderm and neuroectoderm. FGF-1 (acidic FGF) is a prototype member of the FGF family and lacks a classical signal sequence for secretion. Previous studies have shown that a nuclear translocation sequence resides near the N-terminus of FGF-1 (residues 21-26 NYKKPK) and this sequence can direct ß-galactosidase (gal) to the nucleus. However, FGF-1 itself or a FGF-1:gal fusion protein remains localized within the cytosol. Interestingly, in contrast to the failure of endogenous FGF-1 to localize in the nucleus, extracellular FGF-1 undergoes nuclei localization in a receptor dependent manner. Thus it is possible that endogenous FGF-1 contains a regulatory sequence for cytosolic retention. In order to identify this putative cytosolic retention signal within FGF-1, a variety of FGF-1:gal fusion constructs were made containing various deletions in the FGF-1 sequence. All deletion mutants lacking the C-terminal domain of FGF-1 (residues 112-154) were able to direct gal to the nucleus as determined by ß-gal staining and immunofluorescence. In addition, the deletion mutant which contains the nuclear translocation sequence (NYKKPK) and the C-terminal domain can also localize ß-gal to the nucleus. However, only the mutant which contains NYKKPK fused to the C-terminal half of FGF-1 (residues 77-154) remained cytosolic and these results were confirmed by confocal fluorescence microscopy and subcellular fractionation. These data suggest that there is a regulatory sequence within the C-terminal-half of FGF-1 (residue 77-154) which may enable intracellular FGF-1 to remain cytosolic.

EZ 126 TGF-β REDUCES ENDOTHELIAL IL-8 PRODUCTION AND NEUTROPHIL TRANSENDOTHELIAL MIGRATION.

William B. Smith, Leanne M. Noack, Mathew A. Vadas, Jennifer R. Gamble, The Hanson Centre for Cancer Research, I.M.V.S., Frome Road, Adelaide, South Australia.

We have previously shown that TGF-β exerts anti-inflammatory effects on endothelial cells by antagonising the TNF-α induced adhesiveness of EC for neutrophils and lymphocytes. Inhibition of leukocyte adhesion is mediated by a selective inhibition of induction of E-selectin with little or no effect on VCAM or ICAM-1 expression.

We and others have previously shown that neutrophil transmigration through TNF activated endothelium is mediated at least in part by interleukin-8 (IL-8). We now show that TGF-β reduces the production of interleukin-8 (IL-8) by endothelial cells stimulated with TNF-α or interleukin-1. Treatment of EC with 0.2ng/ml of TGF-β for 24 hours reduced TNF-α induced IL-8 production, measured by ELISA in HUVEC supernatants, by up to 90%. A decrease was seen at all doses of TNF-α, with the maximum percentage reduction seen at 1U/ml. Reduction in TNF-α induced mRNA for IL-8 was also seen in TGF-β pretreated EC.

TGF-β inhibited transmigration of neutrophils through EC monolayers. Adhesion of neutrophils to EC was increased by treatment of the EC with TNF for 4 hours, as was migration of neutrophils beneath the EC monolayer. After pretreatment of the EC with TGF, adhesion in response to TNF was reduced by 22%, whilst transmigration was reduced by 79%. These findings further extend the mechanisms of the anti-inflammatory effects of the cytokine TGF-β, and are consistent with the observation that mice lacking TGF-β die of widespread inflammatory disease.

EZ 127 POST-TRANSLATIONAL REGULATION OF PDGF RELEASE BY THROMBIN IN BOVINE AORTIC ENDOTHELIAL CELLS (EC) OVEREXPRESSING HUMAN PDGF B CHAIN (c-*sis*) cDNA, Abigail A. Soyombo and Paul E. DiCorleto, Department of Cell Biology, The Cleveland Clinic Research Institute, Cleveland, OH 44195

The study of post-transcriptional events in the regulation of PDGF production by EC has been hampered by the low level of expression of this protein making methods such as metabolic labeling and pulse-chase studies technically difficult. We have therefore transfected bovine aortic EC with a full length c-*sis* cDNA driven by a viral promoter. Control cells were transfected with the parental vector lacking c-*sis*. Cells were transfected using $CaPO_4$/DNA precipitate and individual colonies resistant to geneticin were selected and propagated. Northern analysis revealed a several fold increase in B chain mRNA in cells transfected with the c-*sis* plasmid over control cells and PDGF released into the media of c-*sis* transfected EC was 5 to 10 fold greater. Metabolic labeling of c-*sis* transfected EC revealed a very inefficient rate of basal PDGF secretion into the conditioned media, with the majority of newly synthesized PDGF remaining cell- or matrix-associated. Incubation of cells with thrombin, a key protease of the coagulation cascade, led to a dramatic increase in the amount of PDGF released into the media. This release was demonstrated to be due to the selective cleavage of cell-associated PDGF precursors by thrombin. In the absence of any stimulation, cell-associated PDGF B was internalized and degraded. We have demonstrated by pulse-chase studies using c-*sis* overexpressing EC that newly synthesized PDGF B chain is stably expressed as a cell-associated precursor protein, from where it is either inefficiently released into the medium, cleaved by proteases such as thrombin or it may be internalized for degradation. Modulation of PDGF secretion by selective cleavage of preformed precursors may represent a significant mechanism for acute regulation of PDGF activity independent of changes in the rate of synthesis.

(Supported by HL-29582 & AHA-NEO).

EZ 128 THE HEPARIN BINDING SITE OF BASIC FIBROBLAST GROWTH FACTOR Barry A. Springer, Leo D. Thompson, Michael W. Pantoliano, Drew Van Dyk. Department of Chemical and Physical Sciences, Crystallography and Biophysical Chemistry, DuPont Merck Pharmaceutical Company, Wlmington, DE 19880-0228.

Fibroblast growth factors (FGF's) interact on cell surfaces with "low affinity" heparan sulfate proteoglycans (HSPG) and "high affinity" FGF receptors (FGFR) in a ternary complex to initiate cell proliferation. Previous reports have implicated the binding of heparin, or heparan sulfate, to FGF as essential for FGF mediated signal transduction and mitogenicity. However, the molecular recognition events which dictate the specificity of this interaction have remained elusive. Amino acids on the surface of bFGF were chosen as potential heparin contacts based on the position of sulfate anions in the X-ray crystal structure of bFGF and on a modeled heparin-bFGF complex. Identified amino acids were replaced with alanine by site-directed mutagenesis, and mutant proteins were characterized for differences in binding to a low molecular weight heparin (≈ 3,000 Da) by isothermal titrating calorimetry, and for differences in [NaCl] dependent elution from a heparin sepharose affinity column. The combination of site-directed mutagenesis and titrating calorimetry permitted determination of the energetic contributions of individual basic FGF (bFGF) residues in the binding of heparin to bFGF. The key amino acids which comprise the heparin binding domain on bFGF constitute a discontinuous binding epitope. The interaction of low molecular weight heparin with wild-type and mutant bFGF's is enthalpically dominated. Addition of the observed $\Delta\Delta G$ of binding for each single site mutant accounts for >95% of the free energy of binding, of which four amino acids comprise ≈ 56 % of the total binding free energy. Although the heparin binding domain consists mostly of positively charged residues, a 7-fold increase in [NaCl] only decreases the affinity of wild-type bFGF binding to heparin 40-fold (K_d at 0.1 M NaCl = 465nM vs. K_d at 0.7 M NaCl = 17.2 μM) suggesting that nonionic interactions dominate as analyzed by polyelectrolyte theory.

EZ 129 FUNCTIONAL COMPARISON OF KDR AND FLT1: RECEPTORS FOR THE VASCULAR ENDOTHELIAL GROWTH FACTOR **Johannes Waltenberger[1,3], Lena Claesson-Welsh[1], Agneta Siegbahn[2] and Carl-Henrik Heldin[1], [1]Ludwig Institute for Cancer Research, Uppsala Branch, [2]Department of Clinical Chemistry, Uppsala University, Uppsala, Sweden, and [3]Department of Medicine II, Ulm University, Ulm, Germany**

Vascular endothelial growth factor (VEGF) is a homodimeric peptide growth factor, that has been shown to bind to two different receptor tyrosine kinases denoted Flt1 and KDR . In order to study the function and signal transduction of VEGF, the human VEGF-receptors Flt1 as well as KDR were stably expressed in porcine aortic endothelial cells. Binding analysis using ^{125}I-VEGF revealed different affinities for the two receptors matching the affinities of VEGF binding sites on cultured human umbilical vein endothelial cells (HUVEC). The KDR expressing cells showed striking changes in cell morphology as well as actin reorganization and membrane ruffling upon VEGF stimulation. Furthermore KDR mediated ligand-induced chemotaxis and mitogenicity, whereas Flt1 expressing cells were lacking such a response.

KDR was found to undergo ligand-induced autophosphorylation *in vivo*, and both Flt1 and KDR were autophosphorylated using an *in vitro* kinase assay. The coprecipitated and phosphorylated molecules were considerably different for both receptors and different to those coprecipitating with the PDGF-β-receptor suggesting distinct sets of signaling molecules. Phosphatidylinositol 3' kinase did not associate with the two receptors after ligand stimulation, whereas protooncogene products of the Src family changed their level of phosphorylation upon VEGF stimulation.

The ligand-induced responses in KDR expressing cells were seen at much higher concentrations of VEGF compared to HUVEC, i.e. in the presence of both Flt1 and KDR, suggesting heterodimerization of KDR and Flt1 *in vivo*.

EZ 130 **PLACENTA GROWTH FACTOR: ISOLATION AND CHARACTERIZATION OF A HEPARIN-BINDING FORM AND REGULATION OF THE GENE IN HUMAN VASCULAR ENDOTHELIAL CELLS BY CYTOKINES,** **Herbert A. Weich, Stefanie Hauser*, Dieter Marmé* and Bernhard Barleon*, Dept. Gene Expression, Gesellschaft für Biotechnologische Forschung, D-38124 Braunschweig and *Inst. of Molec. Cell Biol., University of Freiburg, D-79108 Freiburg, Germany.**

Placenta Growth Factor (PlGF) is a recently discovered mitogen for vascular endothelial cells and based on its high homology to vascular endothelial growth factor (VEGF) it can be classified as a new member of this growth factor family. Using the polymerase chain reaction technique we have examined human umbilical vein endothelial cells and placenta tissue and discovered a second species of PlGF, named PlGF-2. This new molecular form contains a 21-amino acid insertion relative to the described form PlGF-1 including a highly basic insertion very similar to that described for $VEGF_{189}$. Infection of insect cells with recombinant baculovirues specific for the two forms showed that PlGF-1 and PlGF-2 are efficiently secreted but that only PlGF-2 binds to heparin with high affinity. Northern blot studies demonstrated, that the PlGF gene is only expressed in a very limited number of cell types and tissues, but in all kinds of human vascular endothelial cells, suggesting an autocrine loop for growth stimulation in these cells. Moreover, we have investigated the transcriptional regulation of the PlGF gene in human umbilical vein endothelial (HUVE) cells. Stimulation with 15 different cytokines and growth factors indicated a time-dependent and differential regulation of this gene. Similar to VEGF, PlGF can occur as a free secretable and a heparin-binding form and HUVE cells show regulation of the PlGF gene this gene when stimulated with different cytokines.

EZ 131 MITOGENIC AND CHEMOTACTIC RESPONSE OF ENDOTHELIAL CELLS TO HUMAN RECOMBINANT $VEGF_{121}$, $VEGF_{165}$, PLGF-1 AND PLGF-2, Karin Weindel*, Georg Martiny-Baron*, Herbert A. Weich# and Dieter Marmé*. *Institute of Molecular Cell Biology, University of Freiburg, D-79108 Freiburg and #Dept. of Gene Expression, Gesellschaft für Biotechnologische Forschung, D-38124 Braunschweig

Both, VEGF and PlGF, belong to a still growing family of PDGF-related growth factors. Until today, four different VEGF ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$) and two PlGF (PlGF-1 and PlGF-2) isoforms have been discovered, which probably arise from alternative RNA splicing. All VEGFs as well as PlGFs described so far exist as homodimeric, glycosylated proteins of about 40 to 56 kD and share the ability of being a specific mitogen for cultured endothelial cells. Additionally, $VEGF_{165}$ has proofed to be an agiogenic factor *in vivo*.

We cloned the human cDNAs of $VEGF_{121}$, $VEGF_{165}$, PlGF-1 and PlGF-2 and expressed the recombinant proteins with the baculovirus expression system. To investigate their mitogenic and chemotactic activity, all the different growth factors were purified to near homogeneity. All four growth factors were able to stimulate proliferation and migration of various endothelial cells . $VEGF_{121}$ and $VEGF_{165}$ behaved similar in the proliferation as well as in the migration assay. PlGF-1 and PlGF-2 had less mitogenic activity but they were indistinguishable from $VEGF_{121}$ and $VEGF_{165}$ with respect to stimulation of cell migration.

Our data reveal that the recombinant $VEGF_{121}$ is at least as active as $VEGF_{165}$ *in vitro* and therefore might be another important angiogenic factor *in vivo* as well. The role of the two PlGF isoforms with respect to angiogenesis remains speculative, but the characterization of the recombinant proteins is another step forward in our understanding of the mode of action of all growth factors belonging to this new PDGF-related family.

Transcription

EZ 200 DIFFERENTIAL REGULATION OF THE TWO VEGF RECEPTOR GENES FLT-1 AND KDR BY CYTOKINES AND GROWTH FACTORS, Bernhard Barleon, Herbert A. Weich and Dieter Marmé, Inst. of Molecular Cell Biology, University of Freiburg, D-79108 Freiburg; Dept. Gene Expression, GBF, D-38124 Braunschweig, Germany

Vascular endothelial growth factor (VEGF) is a newly identified growth and permeability factor with a unique specificity for endothelial cells. The biological activities of VEGF are mediated by at least two high affinity receptor tyrosine kinases located on vascular endothelial cells and can be found in various fetal and adult tissues. One of them, flt-1 is expressed in a variety of normal tissues of human and rat with a high expression level in human placenta. The mRNA of the second receptor tyrosine kinase KDR is expressed in bovine and human vascular endothelial cells but not in smooth muscle cells and fibroblasts. KDR is also expressed in placental tissues of different species but to a significant lower level.

We have investigated the transcriptional regulation of the flt-1 and KDR genes in human umbilical vein endothelial cells stimulated with 15 different cytokines and growth factors under low-serum conditions by Northern blot analysis and binding studies. We found a time-dependent and differential regulation of both receptor types. For example, both receptor genes are up-regulated by bFGF and TPA. On the other hand flt-1 and KDR are down-regulated by TNF-α1 after a 24 hour treatment period. A differential effect can be seen after treatment with Il-4: the flt-1 gene is slightly up-regulated whereas the KDR gene is significantly down-regulated.

In conclusion our data indicate that the expression of both receptors in vascular endothelial cells is differentially regulated and that cytokines can up- and down-regulate both VEGF receptor genes.

EZ 201 REGULATION OF THE IkBa PROMOTER BY NFκB.

R. de Martin, Q. Cheng, C. A. Cant, B. Vanhove, V. Csizmadia*, H. Winkler*, E. Hofer and F. H. Bach*. Vienna International Research Cooperation Center, A-1235 Vienna, Austria, and Sandoz Center for Immunobiology, Harvard Medical School, Boston, MA 02215.

Activation of endothelial cells (EC) by inflammatory cytokines leads to up-regulation of several genes encoding cell adhesion molecules, interleukins and components of the coagulation cascade. In many cases, the pathways leading to their expression involve the transcription factor NFκB. We have found, using a differential screening approach, that as part of the activation process there is up-regulation of a gene encoding an IκBα-like protein, an inhibitor of NFκB (1). Similar observations have been made in other cell types such as T cells and keratinocytes (2, 3), suggesting common regulatory pathways. It is important in this context that an inhibitor of NFκB, the antioxidant PDTC, prevents up-regulation of IκBα (1).

We demonstrate here, by electrophoretic mobility shift analysis (EMSA), that the promoter of the IκBα gene contains several *in vitro* functional NFκB binding sites within the first 250 bp upstream of the transcription start site. In EMSA, these sites differentially bind individual *in-vitro* translated members of the NFκB family as well as NFκB from stimulated EC. In transient transfection experiments, the p65 subunit of NFκB is sufficient to drive the expression of IκBα promoter-luciferase reporter fusion genes. These results demonstrate that NFκB can directly interact with the IκBα promoter to induce the expression of its inhibitor, and suggest a novel regulatory feedback mechanism to shut down NFκB activity.

(1) de Martin, R., Vanhove, B., Cheng, Q., Hofer, E., Csizmadia, V., Winkler, H. and Bach, F. H. (1993). EMBO J. 12 (7), 2773-2779.
(2) Sun, S.-C., Ganchi, P. A., Ballard, D. W. and Greene, W. C. (1993). Science 259, 1912-1915.
(3) Brown, K., Park, S., Kanno, T., Franzoso, G. and Siebenlist, U. (1993). Proc. Natl. Acad. Sci. USA 90, 2532-2536.

EZ 202 THE 36KD ENDOTHELIAL CELL HYPOXIA ASSOCIATED PROTEIN IS GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE,

Krista K. Graven, Hardy Kornfeld and Harrison W. Farber, Pulmonary Center, Boston University School of Medicine, Boston, MA 02118.

Endothelial cells (EC) exposed to acute and chronic hypoxia upregulate a unique set of stress proteins of MWs 34, 36, 39, 47 and 56kD, termed hypoxia associated proteins (HAPs). These cell-associated proteins are distinct from heat shock and glucose-regulated proteins when assessed by MW and pI, and appear to be unique to EC and the stress of decreased O_2 tension. They are constitutively expressed, and are upregulated to the greatest extent in bovine EC cultured in 3%O_2 and then exposed to acute hypoxia (0%O_2). We report here the identification of the 36kD protein as the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by limited N-terminal sequence analysis. Using subcellular fractionation and SDS-PAGE, the protein was purified, and subjected to Edman degradation. The resultant 21 amino acid sequence was 95% homologous to human and 90.5% homologous to rat GAPDH. Northern blot analysis of bovine aortic and pulmonary artery EC RNA using a human GAPDH cDNA probe showed upregulation of GAPDH mRNA 4-4 1/2 fold following exposure of both types of EC to acute hypoxia. EC grown chronically in 3%O_2 and then exposed to acute hypoxia upregulated GAPDH mRNA 2-2 1/2 fold. This data together with previous in vitro translation data suggested that GAPDH expression was, at least in part, transcriptionally regulated. This was explored further using nuclear run-off experiments. EC exposed acutely 0% O_2 upregulated GAPDH transcription rates 2 to 3 fold above rates in normoxic cells. The potential role of GAPDH upregulation in hypoxic EC is unclear. A number of studies have linked GAPDH to nuclear structures. Interestingly, we find that the level of this protein increases in both the nuclear and cytoplasmic fractions of chronically hypoxic EC while its enzymatic activity increases only in the cytoplasmic fraction of these cells. Thus, GAPDH may mediate events other than glycolysis, particularly in hypoxic EC. The upregulation of GAPDH during hypoxia and its potential non-glycolytic functions may contribute to the relative hypoxia tolerance of ECs.

EZ 203 INDUCTION OF THE TISSUE FACTOR GENE IN PORCINE AORTIC ENDOTHELIAL CELLS,

E. Hofer, T. Moll, M. Czyz, H. Holzmüller and F.H. Bach, Department of Transplantation Immunology, VIRCC, A-1235 Vienna, Austria

We have been interested in the regulation of the tissue factor (TF) gene in endothelial cells from the perspective of the observed expression of TF during inflammatory processes and within organs transplanted between discordant species as porcine to primate transplants. It has been anticipated that the procoagulant properties mediated through the expression of tissue factor contribute significantly to the consequences of endothelial cell and organ damage in both cases. To test this hypothesis we have treated primary porcine aortic endothelial cells (PAEC) with lipopolysaccharide (LPS) or human serum (HS) and tested functional activity of tissue factor. Both, LPS and HS induced a strong increase in the competence to trigger clot formation in a clotting assay performed with the PAEC and human plasma. We have used factor VII deficient plasma and neutralizing anti-factor VII antibodies to demonstrate that the increased competence to trigger clotting is primarily mediated via the factor VII/TF dependent extrinsic pathway of coagulation, indicating increased expression of TF on the surface of the treated PAEC. To be able to study transcriptional regulation we have isolated the porcine TF cDNA and gene. Following stimulation of the cells with either LPS or HS a strong increase from nearly undetectable levels has been directly demonstrated for TF message and NFκB binding activity in nuclear extracts using the cDNA and promoter probes in Northern blots and electrophoretic mobility shift assays, respectively. In contrast, a strong constitutive binding to the dimeric form of the TF AP1 site was observed already in untreated cells, which was not or only slightly increased during the treatment of the cells with LPS or HS. This appears to differ from the described inducibility of binding of nuclear proteins from monocytic cells to the corresponding site of the human gene (1) and might indicate a partly different role of this site in monocytic and endothelial cells. The data indicate that LPS and human serum are both strong inducers of TF gene transcription in PAEC, mediated at least in part through the transcription factor NFκB and resulting in the expression of relatively high levels of TF on PAEC. This suggests the TF gene as a possible target for interference with problems of inflammatory disorders and xenotranplantation.

(1) N. Mackman, K. Brand and T.S. Edgington (1991). J. Exp. Med. 174, 1517-1526.

EZ 204 POSITIVE AND NEGATIVE CONTROL OF THE E-SELECTIN PROMOTER BY DIFFERENT SPLICE FORMS OF TRANSCRIPTION FACTOR ATF-a, Rob Hooft van Huijsduijnen, James Whelan, Wiweka Kaszubska, Rosanna Pescini, and John F. DeLamarter, GLAXO Institute for Molecular Biology, Geneva, Switzerland.

We have been studying the regulation of the E-selectin promoter by specific transcription factors. By screening a HeLa cDNA expression library with a regulatory element of the E-selectin promoter, NF-ELAM1/δA, we isolated the transcription factor ATF-a and a cDNA encoding a variant called ATF-a0. Relative to full-length ATF-a, the ATF-a0 cDNA contains a large in-frame deletion of 525 bp that removes the P/S/T-rich putative transactivation domain. ATF-a0 appears to be a splice variant similar to the one found for ATF-2, its closest homologue in structure and function. Using RT-PCR and Northern blot hybridization to characterize ATF-a0 expression, we found that putative mRNAs for ATF-a0 and ATF-a are present at varying ratios in different tissues. Full-length ATF-a is a transcriptional activator for the NF-ELAM1/δA site of the E-selectin promoter. In contrast, we show ATF-a0 has no measurable transactivating function on this element. Moreover we demonstrate that co-expressed ATF-a0 and ATF-a preferentially heterodimerize. In the heterodimer ATF-a0 is a dominant inhibitor that completely blocks the transactivating activity of ATF-a on the E-selectin promoter. Taken together, our results suggest that ATF-a0 is an important ATF family member with a negative regulatory role which may be operating in the transactivation of the E-selectin promoter.

EZ 205 DIFFERENTIAL ROLE OF THE ENDOGENOUS NF-κB p65 SUBUNIT IN REGULATING CYTOKINE ACTIVATION OF CELL ADHESION MOLECULE GENE EXPRESSION IN HUMAN VASCULAR ENDOTHELIAL CELLS. Bobby V. Khan, Nobuyuki Marui, Craig Rosen*, Charles Kunsch*, and Russell M. Medford, Division of Cardiology, Emory University School of Medicine, Atlanta, GA 30322 and *Human Genome Sciences, Rockville, MD 20850

Through DNA transfection analysis of chimeric promoter-reporter genes, NF-κB like transcriptional factors have been implicated as important mediators in the activation of vascular endothelial cell adhesion molecule (CAM) gene expression by diverse inflammatory signals. The precise identity of these transcriptional factors and to what degree they mediate endogenous CAM expression are unknown. An antisense oligonucleotide approach was used to determine the role of the NF-κB subunit p65 expressed in human umbilical vein endothelial (HUVE) cells in activating endogenous ICAM-1, VCAM-1 and E-selectin gene expression in response to the inflammatory cytokine tumor necrosis factor-α (TNFα). HUVE cells were transfected for five hours using lipofectin with either p65 sense (Sp65, 10-400nM) or antisense (Ap65, 10-400nM) phosphorothioate oligonucleotide. After 48 hours in fresh media, cells were either exposed or not with TNFα (100U/ml) and six hours later measured for cell surface CAM expression by ELISA assay. The ability of TNFα to induce ICAM-1 and VCAM-1 gene expression was unaffected by either Sp65 or Ap65 at all concentrations. In contrast, Ap65 significantly suppressed TNFα induced E-selectin expression in a dose-dependent manner with a maximal suppression of 65% at 400nM Ap65 ($p<0.05$). At all concentrations, Sp65 had no effect on E-selectin expression. These data suggest that cytokine activation of endogenous E-selectin in HUVE cells, unlike that of VCAM-1 or ICAM-1, may be at least partially dependent on endogenous expression of the p65 subunit of the NF-κB transcription factor.

EZ 206 TRANSCRIPTIONAL REGULATION OF HUMAN FGF-1: POTENTIAL INTERACTION BETWEEN POSITIVE AND NEGATIVE REGULATORY PROTEINS. René L. Myers, Maqsood A. Chotani and Ing-Ming Chiu, Department of Internal Medicine and Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210

We have shown that the expression of alternatively spliced human acidic fibroblast growth factor (FGF-1) transcripts is regulated in a tissue/cell line specific manner via multiple promoters (Oncogene 8: 341-349, 1993). The brain specific promoter (aFGF 1.B) is a non-TATA promoter and contains multiple transcription start sites. In order to identify the cis-regulatory elements in the aFGF 1.B proximal promoter, we constructed a series of sequential 5' deletions fused to the luciferase reporter gene. The transcriptional activity of these recombinants was determined following transfection into an aFGF 1.B positive cell line, U1240MG and a 1.B negative cell line, U1242MG. Results of transient transfections indicate several elements which are involved in the positive and negative regulation of aFGF 1.B expression. The core promoter is located in a 40 bp region, showing transcriptional activity in U1240MG but not in U1242MG cells. Two positive and two negative regulatory elements are located in the 831 bp region 5' to the start site. Electrophoretic mobility shift assays (EMSA) using radiolabeled probes from the 1.B promoter region have identified 2 sequence-specific binding sites for nuclear proteins. These proteins most probably function to enhance aFGF 1.B transcription. These proteins are present in all cell lines tested, despite these regions showing different functional activities in transient transection experiments. It is possible that additional regulatory elements, functioning in concert with these regions, are necessary for the tissue specific activation of the 1.B promoter. The transcription may be regulated, in part, by protein/protein interactions. Additional EMSA and footprinting analyses are ongoing to further characterize the sequence specific binding sites important in the transcriptional regulation of this gene. Mutational analyses of these elements are in progress to test for abrogation of function to provide further, more direct evidence for the functional role of these regions in the transcriptional regulation of aFGF 1.B.

EZ 207 REGULATION OF THE HUMAN TISSUE FACTOR GENE IN ENDOTHELIAL CELLS EXPOSED TO CYTOKINES AND LPS. Graham C. Parry, Thomas S. Edgington and Nigel Mackman. Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037.

Tissue factor (TF), initiates the coagulation protease cascade by binding plasma Factor VII/VIIa. Endothelial cells *in vivo* are normally antithrombotic but can be induced to express TF in animal models of septic shock. In this study, we have investigated the transcriptional regulation of the TF gene in cultured human endothelial cells exposed to either LPS or the cytokines TNF and Il-1β. Stimulation of HUVEC with these agonists induced a rapid, dose-dependent increase in procoagulant activity, TF mRNA and rate of transcription of the TF gene. Transfection of plasmids containing a 5' deletion series of the TF promoter, cloned upstream of a luciferase reporter gene, revealed that LPS and cytokine induction was mediated by a region, -278 to -111, which includes an LPS response element that contains two AP-1 sites and a κB-like site. Gel-shift assays using nuclear extracts from stimulated and unstimulated HUVEC demonstrated that AP-1 binding activity was constitutively expressed whereas NF-κB/Rel proteins were activated these agonists. The κB-like site, 5'-CGGAGTTTCC-3', was able to bind *in vitro* expressed p65 and c-Rel, but not p50 or NF-κB (p50/p65). Co-transfection of pTF(-278)LUC with plasmids expressing p50 or p65 resulted in *trans*-activation of the TF promoter. Currently, we are characterizing the nuclear complex from stimulated endothelial cells that binds to the TF κB-like site. Elucidation of mechanisms by which the prothrombotic activity of endothelial cells is increased by the expression of TF may establish novel therapies for the treatment of thrombotic diseases.

EZ 208 THE TRANSENDOTHELIAL TRANSPORT OF SMALL PORE PROBES IS MODULATED BY NEM.
Dan Predescu, Sanda Predescu, Reinhard Horvat and George E. Palade, Division of Cellular and Molecular Pathology, School of Medicine, University of California, San Diego, CA 92093-0651.
Work done in our laboratory, shows that molecules ranging in size from albumin (Mr 66 000 D) to myoglobin fragments (Mr 2 000 D) leave the lumina of perfused murine heart capillaries via plasmalemmal vesicles (P.V). For detection the following molecules: albumin (A), orosomucoid (Om), myoglobin (My), myoglobin fragments (MyF), and lactalbumin (L) were dinitrophenylated and their location in transit across the endothelium was investigated with anti-dinitrophenol (anti-DNP) antibodies followed by a gold conjugated reporter antibody. This approach provides satisfactory morphological preservation and allows the collection of large tissue samples. With the exception of A, all of these molecules qualify as small pore probes, according to their molecular weight and three-dimensional conformation. The evidence obtained indicates that PVs are involved in the transcytosis of small as well as large pore probes. So far there is no substantial and consistent evidence of exit through intercellular junctions of the small pore probes used by us..On account of these findings, current ideas about the nature and location of small pores in the continuous type of microvascular endothelium should be reassessed. Transcytosis by PVs implies frequent and repetitive membrane fusion-fission. In other cell types membrane fusion of vesicular carriers has been shown to depend on a NEM sensitive factor (NSF, Mr of 70 000 D) which operates along the exocytotic and endocytotic pathways. To find whether NEM affects PVs transcytosis across the continuous endothelium, we have perfused mouse hearts with the DNP tagged tracers. In the case of DNP-A , the *"in situ"* perfusion of the tracer, in the presence of 1mM NEM followed by quantitative assay (ELISA, dot blot) revealed an inhibition > 80% of DNP-A transport to the tissue. Immunocytochemical detection of the DNP-A at the electron microscopy level confirmed the extensive inhibition of transcytosis. Work in progress is designated to asses NEM effect on small pore probes transport. Future work will aim at the identification and localization of NSF and/or related molecules in the continuous vascular endothelium. Supported by NIH Grant HL17080 to G.E.P.

EZ 209 A *SCHIZOSACCHAROMYCES POMBE* EXPRESSION CLONING STRATEGY FOR THE ISOLATION OF TRANSCRIPTION FACTORS INVOLVED IN ENDOTHELIAL CELL-SPECIFIC EXPRESSION.
Jacques Remacle and Danny Huylebroeck, Laboratory of Molecular Biology (CELGEN), University of Leuven, Belgium.

The mechanism of transcriptional activation is well conserved between yeast and mammals, suggesting that yeast is a suitable organism for cloning mammalian transcription factors. We have chosen *Schizosaccharomyces pombe* as a suitable organism for developing an expression cloning strategy that allows us to isolate new transcription factors.
We fused several endothelial cell-specific promoters (Endothelin 1, Vcam1, Elam1, Thrombomodulin, P-selectin, von Willebrand factor, angiotensin converting enzyme, VEGF flk receptor, TIE2) which we isolated ourselves and/or were kindly provided by collaborating groups, to the neomycin resistance gene. These constructs were stably integrated in the *S.pombe* genome by homologous recombination and the transformants were checked for their resistance on G418-containing medium. The yeast containing a silent reporter construct is transformed with an episomal plasmid expressing an endothelial cDNA library under control of the *S.pombe* PHO4 regulatable promoter. After induction of the PHO4 promoter, we select the positives clones on a medium containing G418. The clones that are able to grow under antibiotic selection originates from a yeast cell wherein the endothelial cell-specific promoter has been turned-on by a transcription factor expressed from the cDNA library. In order to make the cloning of repressors possible, we constructed a cDNA library whereby the cDNA is fused to the powerful transcription activation domain of the Herpes Simplex Virus VP16 protein. It is one of the strongest activators known. The addition of such strong activation domain was shown to change a transcription repressor into an activator. Several promoter elements (like GATA-binding sites) are involved in the tissue specific expression of the Endothelin, P-selectin, VCAM1 and von Willebrand genes. However, the protein that binds to this site (GATA2) is produced by a variety of cells and therefore does not explain the primordial expression of these target genes in endothelial cells. We also developed a cloning strategy that allows us to search for transcription factors that cooperate with GATA2 to activate the target promoter in *S.pombe*. In this case, we stably intregrated in the *S.pombe* genome, a GATA2 expression cassette.

EZ 210 RECONSTITUTION OF REGULATED EXOCYTOSIS OF VON WILLEBRAND FACTOR IN PERMEABILIZED HUMAN ENDOTHELIAL CELLS,
Binks W. Wattenberg. Cell Biology Unit. The Upjohn Company. Kalamazoo, MI 49001.
Endothelial cells store extremely high concentrations of von Willebrand Factor (vWF) in large secretory organelles known as Weibel-Palade bodies. The Weibel-Palade body is also the intracellular storage site for the adhesion molecule P-selectin. Upon stimulation with physiological agonists such as histamine or thrombin, or experimental secretogogues such as phorbol ester or calcium ionophore, the Weibel-Palade bodies fuse with the plasma membrane, releasing vWF into the serum and exposing P-selectin on the endothelial cell surface. To begin a molecular description of this exocytic event we have reconstituted regulated secretion in human umbilical vein endothelial cells that have been permeabilized with digitonin. This secretion is calcium dependent and proceeds with a time course that is virtually identical to that of intact cells treated with secretogogue. Exocytosis can proceed normally when permeabilized cells have been depleted of greater than 80% of their normal content of cytosol. We have been unable to detect any components of the secretory apparatus that are sensitive to the thiol reagent N-ethylmaleimide. The ability to introduce both high and low molecular weight reagents into the cytosol of permeabilized endothelial cells with a functionally intact secretory apparatus will allow us to probe for components the molecular machinery regulating the exocytic process.

EZ 211 INHIBITION OF E-SELECTIN TRANSCRIPTION BY AN α-GLOBULIN,
H. Winkler, K. Stuhlmeier, Q. Cheng, V. Csizmadia and F.H. Bach, Sandoz Center for Immunobiology, Harvard Medical School, Boston, MA 02215
During the analysis of interactions of human serum with porcine aortic endothelial cells (PAECs), as part of a study of xenotransplantation, Stuhlmeier et al. discovered that human serum inhibits the induction of E-selectin expression by lipopolysaccharide (LPS), tumor necrosis factor a (TNFα) and other stimuli. The inhibitory factor is a heat-stable protein in the α-globulin fraction of human serum. This abstract deals with molecular aspects of this finding. The α-globulin inhibits mRNA accumulation not only for E-selectin but also for VCAM-1 and ICAM-1. Inhibition is selective in that IL-1, IL-8 and PAI-I mRNA accumulation is not inhibited under the same conditions. Nuclear run-off analysis reveals that the α-globulin blocks expression of E-selectin by inhibiting transcription. To test whether NFκB, a transcription factor essential for E-selectin expression, is inhibited by α-globulins, we analyzed nuclear extracts isolated from PAEC treated with LPS in the presence or absence of α-globulins. The results of electrophoretic mobility shift assays (EMSAs) using the porcine E-selectin NFκB site as a probe, show that a-globulins do not suppress induction of NFκB activity in the first 4 hours following stimulation with LPS, at which time E-selectin expression is inhibited. Thus, since in the presence of α-globulins there is no evidence that NFκB activity is inhibited concomitant with E-selectin inhibition, we conclude that inhibition of NFκB does not account for the suppression of E-selectin transcription by α-globulins. Further, given the finding that the α-globulins inhibit at the level of transcription, we suggest a different transcription factor must be involved.

Gene Expression I

EZ 300 EXPRESSION OF SPARC IN THE CHORIOALLANTOIC MEMBRANE: ROLE OF PROTEIN DEGRADATION IN THE PRODUCTION OF ANGIOGENIC PEPTIDES. Luisa Iruela-Arispe, Timothy F. Lane, and E. Helene Sage; Department of Biological Structure, University of Washington, Seattle, WA, 98195.

SPARC is a copper-binding protein previously shown to modulate cell shape and to regulate endothelial cell proliferation. SPARC protein is expressed at high levels by cultures of endothelial cells and has been shown to increase during the organization of capillary networks *in vitro*. Expression of SPARC by endothelial cells *in vivo* is restricted; it is not transcribed by mature vessels, whereas new vessels that arise during wound healing or developmental angiogenesis transcribe SPARC mRNA and secrete the protein. To gain a better understanding of the role of SPARC during angiogenesis and vessel remodeling, we have characterized its expression during the development of capillaries in the chicken chorioallantoic membrane (CAM). High levels of SPARC protein and mRNA were detected in the CAM from day 2 throughout development. Maximal expression was correlated with the time of exponential growth. Immunohistochemical localization in conjunction with confocal microscopy revealed preferential accumulation of SPARC in small capillaries and in areas of vessel branching. After day 11, breakdown products of SPARC indicated extracellular processing. SPARC is in fact cleaved by several extracellular proteases, such as plasmin. Zymographic analysis to detect areas of plasmin activity showed correlation with areas of SPARC expression. We also tested the ability of SPARC and peptide fragments of SPARC to facilitate the vertical invasion and proliferation of capillaries into meshes of type I collagen. Although SPARC did not increase the total number of capillaries, certain peptide fragments of SPARC potentiated neovascularization in a dose-dependent manner. This activity was restricted to domain II, a cationic region of SPARC that binds Cu^{+2}, peptides from other regions did not promote similar effects. Specifically, angiogenic activity was confined to the tetrapeptide KGHK, which can be released by proteolytic clevage of SPARC with trypsin or plasmin. We propose that at sites of neovascularization, specific degradation of SPARC releases bioactive peptides that regulate Cu^{+2} availability and facilitate endothelial cell remodeling into capillary networks.

EZ 301 INHIBITION OF ANGIOGENESIS IN VIVO BY ANTI IL-1 ANTIBODIES AND LACK OF INHIBITION BY ANTI bFGF ANTIBODIES, David BenEzra*, Brenda Griffin+, Genia Maftzir* and Olga Aharonov*, Ocular Angiogenesis Laboratory, Hadassah University Hospital, Jerusalem, Israel* and Alcon Laboratories, Fort Worth, Texas, U.S.A.+

Purpose: To study the involvement of basic fibroblast growth tumor (bFGF) and interleukin-1 (IL-1) in the in vivo angiogenic processes observed in the rabbit cornea.
Method: Angiogenesis was induced by insertion of Elvax-40 implants sequestering 500 ng bFGF or bacterial lipopolysaccharide (LPS) in the midstroma of the rabbit cornea at a distance of 2.5 mm from the limbus. The influence of IgG polyclonal antibodies against bFGF or IL-1 was tested by different protocols, as follows: 1) mixing the respective antibodies with each stimulant (bFGF or LPS) prior to preparing implants of the antigen-antibody complex; or 2) placing the stimulants and the antibodies in separate implants. The two implants were implanted in the same cornea either at the initiation of the experiment or, the implants sequestering the antibodies were inserted within the cornea 48 hours before insertion of the stimulating implants. The extent of corneal angiogenesis was recorded daily as the surface of the growing neovascular bed in a double masked manner (1).
Results: In all experiments, antibodies against IL-1 inhibited the extent of angiogenesis induced by either bFGF or LPS. Antibodies against bFGF had no influence on the extent of neovascularization induced by either of the stimulants.
Conclusions: These results are interpreted to indicate the possible direct involvement of interleukin-1 and the apparent lack of involvement of bFGF in the cascade of events leading to angiogenesis in vivo.

Reference:
1. D BenEzra: Am. J. Ophthalmol. 86:455-461, 1978

EZ 302 CD31: HOMOTYPIC ADHESION STUDIES AND BIOLOGICAL FUNCTION ON ENDOTHELIAL CELLS.

I. N. Bird, J. Fawcett*, J. Saunders, C. Holness*, J. H. Spragg and D. L. Simmons*

Yamanouchi Research Institute, Littlemore Hospital, Oxford OX4 4XN and *Imperial Cancer Research Fund, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom.

CD31 (PECAM-1) is a member of the immunoglobulin superfamily of adhesion molecules with an interesting cellular distribution, being found on all endothelial cells, platelets, PMNLs, monocytes and some lymphocytes. Adhesion experiments using a series of soluble recombinant CD31-Fc constructs immobilised on plastic and COS-7 cells expressing full length CD31 demonstrated that CD31 was capable of homotypic binding. However, only the full length construct significantly bound CD31-transfected COS-7 cells. This suggests that CD31 homotypic adhesion occurs by anti-parallel alignment of molecules.

Rabbit polyclonal antisera were raised against the extracellular domain of CD31. Whole antibody or Fab fragments inhibited the CD31:CD31 interaction in the COS-7 binding assays. The permeability of HUVEC monolayers formed in the presence of anti-CD31 Fab fragments on Costar Transwell filters was increased and EM studies showed that a disorganised monolayer was formed in the presence of Fab fragments. These results show that CD31 is capable of homotypic binding and plays a role in the formation/organisation of endothelial monolayers.

EZ 303 CLONING AND CHARACTERISATION OF GENES WHICH ARE MODULATED DURING ANGIOGENESIS

Gillian W Cockerill, Jennifer R Gamble and Mathew A Vadas, Hanson Centre for Cancer Research, Institute of Medical and Veterinary Science, PO Box 14 Rundle Mall, Adelaide, 5000, Australia.

The ability to retard the process of angiogenesis in developing tumours, and to ameliorate this process during wound healing, would be a significant clinical advantage. We have used an *in vitro* model of angiogenesis to investigate genes which are modulated during differentiation of the endothelial cell.
Endothelial cells, when seeded onto a basement membrane gel, form capillary-like tubes. This process requires transcriptional activation within the first few hours of contact of the cells with the gel. We have generated a subtracted cDNA library of messages which are upregulated during this time.
Analysis of this library has identifies a gene (PHL) similar to the alpha subunit of prolyl hydroxylase (4-PH), a tetrameric protein which catalyses the formation of 4-hydroxyproline in collagens. The subtracted clone shows 55% overall homology to the reported sequence and has the conserved histidine residues (His I, and His II) required for iron binding:

His I	PHFDHATYQ	PHL
	PHFDFARKD	4-PH
His II	HAGCPVLVGDKWVANKWIHE	PHL
	HAGCPVLVGNKWVSNKWLHE	4-PH

The expression of this gene will be discussed.

EZ 304 INDUCTION OF α-SMOOTH MUSCLE ACTIN EXPRESSION IN BRAIN PERICYTES BY TGFβ1, Robert M.W. de Waal, Marcel M. Verbeek, Irene Otte-Höller, Pieter Wesseling and Dirk J. Ruiter, Department of Pathology, University Hospital Nijmegen, The Netherlands.

Pericytes are cells localized at the abluminal side of the microvascular endothelium. Although separated by a basement membrane, pericytes are in close contact with endothelial cells and are probably involved in regulation of endothelial cell functions. Previous studies suggested a role for pericytes in microvascular proliferation in tumors. To study this cell type, we used human brain pericytes (HBP) isolated from microvessel segments derived from autopsy brain tissue. The cells were characterized *in vitro* using a panel of monoclonal antibodies (mAb). HBP were reactive with mAb directed against the high molecular weight-melanoma associated antigen (HMW-MAA) and ICAM-1, but only a minority of the cells expressed α-smooth muscle actin (α-SMA, 0-10%) or VCAM-1 (10-50%). In histologically normal human brain microvessels *in situ*, pericytes consistently failed to stain with these four markers. Tissue with microvascular proliferation, however, showed a marked pericyte staining for both α-SMA and HMW-MAA, indicating that the expression of these molecules is regulated by external stimuli. The expression of α-SMA *in vitro* could be slightly upregulated by incubation with serum-containing medium. An increase in α-SMA expression up to 40% of the total cell population was seen when pericytes were treated with TGFβ1, whereas bFGF slightly inhibited α-SMA expression. Incubation with other factors (PDGF-AA, heparin, IFNγ, TNFα) had no effect on the α-SMA expression. TGFβ1 thus induces smooth muscle-like differentiation in pericytes *in vitro*, and might play a role in the activation of pericytes during angiogenesis *in vivo*. This research was financed by a grant from the Janivo Foundation, The Netherlands.

EZ 305 BASIC FIBROBLAST GROWTH FACTOR (bFGF) INDUCES LESIONS IN MICE RESEMBLING KAPOSI'S SARCOMA (KS) AND ANTISENSE OLIGONUCLEOTIDES AGAINST THIS CYTOKINE INHIBIT THE GROWTH AND ANGIOGENIC ACTIVITY OF KS SPINDLE CELLS B. Ensoli[1], P. Markham[2], V. Kao[1], M. Raffeld[3], R. Gendelman[1], G. Barillari[1], G. Zon[4] and R. C. Gallo[1]. [1]Laboratory of Tumor Cell Biology and [3]Laboratory of Pathology, National Cancer Institute, NIH, Bethesda, MD 20892; [2]Advanced BioScience Labs., Inc., Kensington, MD 20895; [4]Lynx Therapeutics Inc., Foster City, CA 94404

KS is a proliferative disease of vascular origin and the most common malignancy of HIV-1-infected individuals (AIDS-KS). Histologically KS is characterized by the proliferation of spindle-shaped cells considered the "tumor" element of KS, and of normal vascular cells forming new blood vessels (angiogenesis). Previous studies have suggested that bFGF, a potent angiogenic factor, plays a key role in the histogenesis of AIDS-KS, including results indicating that this cytokine is overexpressed in spindle cells of KS lesions *in vivo*. Here we show that injection of bFGF into nude mice induces angiogenic lesions closely resembling human KS as well as the lesions induced in mice by the inoculation of AIDS-KS-derived spindle cells (AIDS-KS cells). In view of these results, we investigated the effects of phosphorothioate oligonucleotides directed against bFGF RNA (AS bFGF) on the growth of AIDS-KS cells and on the angiogenic-related activities produced by the cells. Antisense oligomers targeting the first splice donor-acceptor site of bFGF RNA blocked AIDS-KS cell growth and inhibited the angiogenic activity of these cells such as the stimulation of normal endothelial cell growth, migration and invasion. These effects were specific, dose-dependent and achieved at low (0.5-1 μM) oligomer concentration and were associated with a reduction of the number of bFGF expressing cells and with a lower cytoplasmic and nuclear content of the cytokine. These data indicate that the antisense directed against bFGF may represent a prime candidate in the therapy of this disease.

EZ 306 REGULATION OF ANGIOGENESIS BY ANTI-INTEGRIN ANTIBODIES, J Gamble, L Matthias, G Meyer*, L Noack, G Cockerill, P Kaur and M Vadas, Hanson Centre for Cancer Research, Institute of Medical & Veterinary Science, Adelaide, South Australia, 5000; *Department of Anatomy and Human Biology, University of Western Australia, Perth, Western Australia, 6000.

The formation of new blood vessels is important during embryogenesis, wound healing, menses, and in the formation of solid tumours. It is dependent on soluble factors (termed angiogenic factors) and also on the adhesivity of the extracellular matrix. We have extended these findings to show that the adhesive nature of endothelial cells for their extracellular matrix can profoundly effect the extent of capillary tube formation. Endothelial cells plated onto a collagen gel in the presence of phorbol myristate acetate (PMA) are induced to invade into the gel and form an anastomosing network of capillary tubes. In the presence of antibodies directed to the major collagen receptor on EC (the integrin, $\alpha_2\beta_1$) the number, length and width of capillary tubes is enhanced. The lumen structure and size are also changed by such antibodies converting the lumina from predominantly intracellular to lumina formed from multiple cells. The antibodies maintained the EC in a rounded morphology and inhibited subsequent EC proliferation on collagen but not on fibronectin laminin or gelatin matrices. Similar enhancement of capillary tube formation was seen using an antibody to the fibrinogen receptor ($\alpha_v\beta_3$) with EC added to fibrin gels. In contrast an antibody directed to the β_1 integrin which results in integrin activation and enhancement of EC adhesion to the ECM. results in inhibition of capillary tube formation. Under such conditions EC proliferation is enhanced. Inhibition of the contact between cells and their matrix converts the EC from a proliferative phenotype towards differentiation resulting in enhanced capillary tube formation. Increasing EC-matrix interactions leads to a proliferative rather than a differentiated phenotype. The signalling events which accompany such regulation of integrin activity will be discussed. Our results suggest therefore that angiogenesis may be regulated by the adhesivity of EC for their extracellular matrix.

EZ 307 EXPRESSION OF THE *flt* TYROSINE KINASE RECEPTOR IN MICROVESSEL ENDOTHELIAL CELLS ISOLATED FROM HUMAN LUNG AND ADIPOSE TISSUE

Peter W Hewett and J Clifford Murray, Endothelial Biology Group, CRC Gray Laboratory, Mt Vernon Hospital, Northwood, Middlesex, UK

The growth of solid tumours is dependent on their ability to evoke a blood supply. Vascular endothelial growth factor (VEGF) has recently been identified as a key factor in tumour neovascularisation, exerting its effects *via* several tyrosine kinase receptors. We have studied the expression of one of these receptors, *flt*, on microvessel endothelial cells isolated and cultured from human lung and adipose tissue, and large vessel endothelial cells from umbilical vein. mRNA for *flt* was detected by the polymerase chain reaction, using specific primers. There was no observable difference in the level of receptor expression between microvessel endothelial cells from different capillary beds, and cells from the umbilical vein. In addition, all three cell types were found to proliferate in response to rhu-$VEGF_{165}$. Long-term co-culture of endothelial cells with a range of cell lines derived from human breast adenocarcinomas and small cell lung cancers did not alter levels of *flt* mRNA. We are currently examining the effects of low oxygen tensions on *flt* expression.

Supported by the Cancer Research Campaign of the UK

EZ 308 Extracellular FGF-1 Decreases Expression of Decay Accelerating Factor on the Surface of Primary Human Fibroblasts: *J.D. Kerby, S.R. Opalenik, J.T. Shin, J.A. Thompson, Department of Surgery, The University of Alabama at Birmingham, Birmingham, Alabama 35294*

Decay Accelerating Factor (DAF) is a phosphatidylinositol anchored cell surface protein belonging to a family of complement regulators which serve to protect host tissues from complement-mediated damage. DAF both prevents the formation and increases the breakdown of C3 and C5 convertases, thereby preventing deposition of terminal complement components on host cell surfaces. Recent studies have shown that several transformed cell lines show decreased levels of DAF and are subsequently more susceptible to complement-mediated damage. Acidic fibroblast growth factor (FGF-1) is a potent angiogenic polypeptide that requires an extracellular autocrine/paracrine loop to induce its full transforming potential. This study was designed to investigate the effects of FGF-1 on the cellular expression of DAF. Primary human foreskin fibroblasts stably transduced with retroviral vectors conferring expression of either intracellular (FGF-1) or extracellular (hst/FGF-1) growth factor were analyzed. Compared to controls, cells expressing intracellular FGF-1 demonstrated similar levels of DAF mRNA (RT-PCR) and cell surface associated protein (FACS). FACS analysis of hst/FGF-1 cells demonstrated that the level of DAF was reduced to less than 6% of that found in controls. RT-PCR analysis of total mRNA extracted from hst/FGF-1 cells exhibited a four-fold decrease in the level of DAF mRNA transcripts. Biological stress, which is known to induce secretion of FGF-1, resulted in significantly decreased levels of both cell surface DAF and DAF mRNA. The absence of DAF on the surface of cells transformed by FGF-1 suggests that these cells would be more susceptible to complement-mediated damage and may represent a mechanism whereby transformed cells are more easily destroyed by the host's natural defenses. Even though transcriptional regulation of DAF is modulated by extracellular FGF-1, post-translational modification may also be involved in this response.

EZ 309 ISOLATION OF NORMAL- AND TUMOR-ASSOCIATED ENDOTHELIUM FROM NORMAL AND CANCEROUS PROSTATE TISSUE, Kenneth J. Pienta and Jeffrey E. Lehr, Wayne State University and The Michigan Cancer Foundation, Detroit, MI 48201

Angiogenesis, or the process of the formation of new blood vessels, is an essential event for the successful growth of tumors *in vivo*. Inhibition of angiogenesis, therefore, would be a logical step in prevention of primary cancers as well as metastatic disease. Currently utilized angiogenesis assays such as the colloidal gold motility assay and chicken chorioallantoic membrane (CAM) assay monitor the actions of endothelium as it reacts to experimental treatments, however, these systems do not mimic tumor associated angiogenesis *in vivo*. Consequently, we have developed an assay to isolate tissue specific endothelium from the tissues where cancers arise utilizing immuno-magnetic and Percoll gradient techniques. Endothelial cells from normal rat prostates and induced prostate tumors were effectively isolated and grown in culture. These cells formed monolayers with a cobblestone-like appearance and stained positive for factor VIII antigen. Normal endothelial cells and tumor-associated endothelial cells appear to react differently to different cytokines such as bFGF, TGFbeta, and IL-6. Further studies to characterize the differences between normal- and tumor-specific endothelium and their response to growth and inhibitory factors may prove to be an exciting tool to explore the process of angiogenesis and how interactions between tumor cells and endothelium can be utilized in cancer prevention and treatment.

EZ 310 ADENOVIRUS-MEDIATED GENE TRANSFER INTO VASCULAR ENDOTHELIAL CELLS OF RECOMBINANT SECRETED AND NON-SECRETED FORMS OF ACIDIC FIBROBLAST GROWTH FACTOR, Roberto Pili, Judith Mühlhauser, Marsha J. Merrill, Hiroyuki Maeda, Joan Chang, Tony Passaniti, Ronald G. Crystal and Maurizio C. Capogrossi, Pulmonary Branch, NHLBI; Laboratory of Biological Chemistry, NIA and Surgical Neurology Branch, NINDS, NIH, Bethesda, MD 20892

Angiogenic factors hold promise as potential therapeutic agents in the treatment of ischemic disorders. However, efficient and localized delivery of such factors in vivo is technically difficult. This problem may be overcome by gene transfer with recombinant adenovirus vectors. We have constructed two replication-deficient recombinant adenovirus vectors coding respectively for the human form of acidic FGF_{1-154} which is non-secreted (AdCMV.aFGF) and for a recombinant form of $aFGF_{1-154}$ which encodes the signal sequence of FGF-4 and is secreted (AdCMV.sp+aFGF). The adenovirus vectors were engineered by homologous recombination of the plasmid pJM17, containing the Ad5 genome, and of the plasmids pS5.aFGF and pS5.sp+aFGF, which contain the CMV promoter and the cDNA for either growth factor. Human umbelical vein endothelial cells (HUVEC) were infected either with AdCMV.aFGF, AdCMV.sp+aFGF or with the control vector Ad.RSVβgal (20 pfu). HUVEC were grown under low-serum conditions and cell number was assessed at 4 days after infection. Cultures exposed to either AdCMV.aFGF or AdCMV.sp+aFGF contained 3-4 fold more cells than those exposed to Ad.RSVβgal or uninfected controls. Cell differentiation was assessed with HUVEC infected with the adenovirus vectors 3 days prior to plating on Matrigel in 1% serum. Under these conditions only HUVEC infected with AdCMV.sp+aFGF formed capillary-like structures; neither uninfected cells, nor HUVEC infected either with Ad.RSVβgal or with AdCMV-aFGF formed capillary structures. Thus, under our experimental conditions, both AdCMV-aFGF and AdCMV-sp+aFGF enhance endothelial cell survival and growth in low-serum conditions while only the adenovirus vector coding for the secreted form of $aFGF_{1-154}$ causes HUVEC differentiation. Both adenovirus vectors may provide a novel approach to induce angiogenesis in vivo.

EZ 311 VEGF DEPENDENT TUMORAL PROGRESSION: STIMULATION BY ANTI VEGF IDIOTYPIC ANTIBODIES.

Plouët J., Jonca F., Ortega N., Say J., Laboratoire de Biologie Moléculaire Eucaryote, 118 Route de Narbonne, 31062, Toulouse, (Fr).

VEGF is an angiogenic lymphokine secreted by many tumoral cell lines and might therefore promote the tumoral angiogenesis. We tested its participation to the progression of a prostatic tumor that we propagated in nude mice by the use of antibodies.

A neutralizing antibody directed against VEGF was produced in rabbits. It was used to elicit anti idiotypic antibodies in rabbits. In vitro, the purified anti idiotypic immunoglobulins acted as agonists of the VEGF receptors. Despite the lack of internalization of the receptors, they induced the proliferation and the migration of aortic endothelial cells.

In vivo the prostatic tumor had a doubling time of 15 days in the presence of preimmune Ig, 35 days in the presence of anti VEGF 165 Ig and 8 days in the presence of anti idiotypic Ig.

These results demonstrate that the immunoneutralization of VEGF reduces the growth rate of the prostatic tumor whereas the overstimulation of VEGF receptors enhances the tumor progression.

EZ 312 VASCULAR ENDOTHELIAL GROWTH FACTOR INHIBITS E-SELECTIN AND VCAM-1 EXPRESSION *IN VITRO* AND *IN VIVO*, W. Gregory Roberts and Michael P. Bevilacqua, Howard Hughes Medical Institute, Cellular and Molecular Medicine, University of California San Diego, La Jolla, CA 92093

Most solid tumor therapies in use today are designed to exploit distinctive characteristics of tumor cells, such as rapid proliferation. Despite substantial successes, many tumors ultimately escape therapy. It is now widely accepted that the enlargement of solid tumors beyond 2-3 millimeters in diameter depends upon the ingrowth of host blood vessels (angiogenesis). Adhesion molecules expressed by tumor vessel endothelium are expected to play critical roles in the diverse cell-cell interactions involved in angiogenesis, permeability, and immunological responses to tumors. In this study we assessed the effects of a tumor-secreted angiogenic factor, vascular endothelial growth factor (VEGF) on the expression of two endothelial cell surface adhesion molecules: E-selectin and VCAM-1. Our data demonstrate that murine tumor vessels express little or no VCAM-1 after intravenous injection of either IL-1 or lipopolysaccharide (LPS), while normal vessels in the periphery are positive for VCAM-1. In addition, VEGF-transfected CHO cells generate highly vascularized, progressive "tumors" in nude mice which also are unable to express VCAM-1 after i.v. injection of either IL-1 or LPS. Moreover, VEGF inhibits the induction of E-selectin and VCAM-1 on endothelium in response to LPS *in vitro*. With as little as 1 hour of VEGF incubation, endothelial cell surface protein expression and mRNA expression are reduced. Similar effects were observed with endothelial cell growth factor (ECGF) suggesting that a general stimulus for growth may inhibit expression. These results may explain the observation that tumors often escape destruction from the host immune system and contribute to our understanding of the dynamics involved in developing and maintaining tumor vessels.

EZ 313 ANTI-FGF RECEPTOR MONOCLONAL ANTIBODY MODULATION OF ANGIOGENESIS, Margaret E. Schelling, S. Venkateswaran, Vincent Blanckaert, and Andries Zijlstra, Department of Genetics & Cell Biology, Washington State University, Pullman, WA 99164-4234.

Anti-Fibroblast Growth Factor (FGF) receptor monoclonal antibodies (Mabs) were produced against the native *flg* FGF receptor. Mab treatment of endothelial cells (ECs) resulted in blocking of ^{125}I-FGF binding, modulation of the proliferation of ECs and their differentiation into tubules ("in vitro angiogenesis"), and phosphorylation of the FGF receptor at 120 kDa. Both the bivalent F(ab')$_2$ and monovalent Fab' Mab fragments inhibit ^{125}I-FGF binding. The monovalent Fab', however, does not result in receptor autophosphorylation. Addition of anti-F(ab')$_2$ antisera, which crosslink the Fab'-receptor complexes, restores receptor autophosphoryl-ation. These data suggest that receptor occupancy by the ligand is not sufficient for signal transduction, and that FGF receptor dimerization or clustering is required.

EZ 314 REGRESSION OF EXPERIMENTAL BURKITT'S LYMPHOMA INDUCED BY EBV IMMORTALIZED B CELLS, Cecilia Sgadari, Anne Angiolillo, Kazuyuki Taga, Lori A. Love, Kishur Bathia and Giovanna Tosato, Laboratory of Immunology, CBER, FDA, Bethesda, MD 20892

When injected subcutaneously into young irradiated athymic mice, most Burkitt's lymphoma cell lines give rise to progressively growing subcutaneous tumors. In the same experimental system, EBV-immortalized cell lines survive only transiently, presumably due to host immunity. When EBV-immortalized and Burkitt's lymphoma cells were simultaneously injected in the same subcutaneous site of irradiated young athymic mice, the resulting tumors regularly underwent regression. In addition, treatment of established subcutaneous Burkitt's tumors with EBV-immortalized cells, almost always resulted in tumor regression. Furthermore, intratumor injections with serum-free supernatants of EBV-immortalized cell lines resulted in the regression of Burkitt's tumors.

Tumor regression in this system followed characteristic patterns, with initial formation of a central scar that progressively enlarged to cover the entire tumor mass followed by loss of scar tissue and new skin formation. Histologically, regressing tumors displayed central necrosis, often surrounding a blood vessel, extending to the epidermis. Variable numbers of lymphoblasts with prominent nucleoli, presumably the Burkitt's cells, surrounded the necrotic tissue, but tumor infiltration with inflammatory cells was not prominent. Early during tumor regression, there was evidence of vascular endothelium damage and necrosis surrounded by viable-looking tumor tissue. Preliminary in vitro experiments have shown that supernatants of EBV-immortalized cells but not supernatants of Burkitt's lymphoma cell lines contain an inhibitor of endothelial cell growth. These results suggest the possibility that tumor regression in this system is due to the secretion of an angiogenesis inhibitor by EBV-immortalized cells.

EZ 315 PLATELET-DERIVED GROWTH FACTOR (PDGF) AND FIBROBLAST GROWTH FACTOR (FGF) ARE IMPLICATED IN THE PATHOGENESIS OF PROLIFERATIVE DIABETIC RETINOPATHY AND MAY ACT SYNERGISTICALLY, Afshin Shafiee, Lesley C. McIntosh and John V. Forrester, Department of Ophthalmology Aberdeen University Medical School, Aberdeen AB9 2ZD, Scotland

Proliferative diabetic retinopathy (PDR) is thought to occur from local release of angiogenic factor (FGF) from ischaemic retina as a result of diabetic microvascular endothelial cell (EC) damage. We have previously shown that patients with PDR contain in their serum an activity which induces migration of retinal microvascular endothelial cells *in vitro*. In the present study, we have shown that the migration stimulatory activity correlates with serum levels of (PDGF) and antibodies to PDGF significantly reduce the stimulatory effect of serum from patients with PDR. Purified PDGF however, does not stimulate endothelial cell migration in our assay system while FGF is effective. In contrast PDGF and FGF act synergistically to stimulate EC migration to a level commensurate with stimulatory activity in diabetic serum. We suggest that systemically released PDGF and locally released FGF act together to initiate retinal angiogenesis by stimulating EC migration in the early phase after EC damage during ischaemia.

EZ 316 SERUM STARVATION INDUCES SECRETION OF FGF-1 FROM PRIMARY FIBROBLASTS, *J. T. Shin, S. R. Opalenik, V. K. Mahesh, S. Nan, and J. A. Thompson, Department of Surgery, University of Alabama School of Medicine, Birmingham, AL 35294.*

The full mitogenic potential of acidic fibroblast growth factor (FGF-1) is generally believed to involve an extracellular autocrine/paracrine loop involving: (i) interaction with cell surface receptors, (ii) hyper-tyrosine phosphorylation of specific polypeptides, (iii) and nuclear localization of both ligand and receptor. FGF-1 lacks a classical signal peptide (SP) sequence which has made it difficult to study the extrinsic pathways regulated by this mitogen. Alternative mechanisms for the extracellular presentation of FGF, including cellular death/damage, extraction from the extracellular matrix, and biological stress (UV, heat shock), have been described *in vitro*. To further define alternative mechanisms whereby this SP-less growth factor is released from cells, construction of retroviral vectors containing either a wild-type reporter gene alone or in combination with wild-type human FGF-1 (F) permitted gene transfer into primary murine embryonic fibroblasts (EFs). FACS selected (ß-gal) F-transduced EFs (F-EFs) produced increased levels of biologically active intracellular FGF-1 (Western analysis). When maintained in media containing reduced serum (0.5% FCS), F-EFs exhibited both an increased proliferative index (BrdU) and a growth advantage with delayed kinetics. *In situ* analysis of serum starved F-EFs demonstrated intense nuclear staining for FGF-1. Both the increased labeling index and nuclear localization of FGF-1 could be reversed by addition of neutralizing antibody specific to the growth factor. Compared to controls, serum starved F-EFs demonstrated hyper-tyrosine phosphorylation of FGF-specific polypeptides. Western analysis of conditioned medium from serum starved F-EFs required ammonium sulfate precipitation to detect the appearance of immunoreactive FGF-1. Trypan blue exclusion and measurement of LDH levels suggest that the extracellular appearance of FGF-1 is not related to cell death. Collectively, these results support the premise that, in response to biological stress, FGF is secreted as a complex with an as yet unidentified molecule. Additional characterization of this pathway may identify molecular mechanisms involved in the autocrine/paracrine stimulation of angiopathology and define strategies to mitigate the inappropriate mitogenic effects of FGF in disease.

EZ 317 VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) INDUCTION IN PRIMARY PULMONARY HYPERTENSION, R. Tuder[1] and N. Voelkel[2], Departments of Pathology[1] and Medicine[2], University of CO Health Sciences Center, Denver, CO 80262-0216

The pulmonary arteries in severe pulmonary hypertension (PPH) demonstrate a peculiar proliferation of vascular channels known as plexiform lesions. In an index case of PPH, we determined that the plexiform lesions are composed of proliferated, Factor VIII positive, endothelial cells, with no expression of muscle specific actin and desmin. Moreover, the EC had strong expression of the mesenchymal intermediate filament vimentin, whose expression is increased during cell proliferation. **We propose that VEGF may play a role in the vessel remodeling in PPH by means of activating EC growth and permeability.** We detected increased VEGF mRNA levels in the case of PPH with plexiform lesions when compared with normal lung. By *in situ* hybridization VEGF mRNA localized to the bronchiolar lining cells and alveolar macrophages. The intensity of the VEGF signal in PPH was higher than that in the normal lung. On the other hand, there was a decrease in the levels of the VEGF-receptors kdr and flt in the PPH lung over that present in the normal lung by Northern blot analysis. Because hypoxia might have stimulated VEGF synthesis in the PPH lung, we examined the effect of hypoxia on the expression of VEGF and VEGF-receptors mRNA in the lungs of rats exposed to chronic hypoxia. Chronic hypoxia caused an initial drop (24 hr) followed by a progressive increase of VEGF mRNA expression which paralleled the progressive pulmonary vascular remodeling. In conclusion, the increased expression of VEGF in PPH and in hypoxia-induced PH, suggests that this factor has a role in the growth of EC and in pulmonary vascular remodeling. (Source of support: Pulmonary Hypertension Center Grant from the UCHSC.)

EZ 318 CELL CYCLE KINETICS OF CULTURED ENDOTHELIAL CELLS EXPOSED TO ETHANOL, D.Way[1], G. Ramirez[1], P. Borgs[1], M. Witte[1], M. Fiala[2], C. Witte[1]. [1]Dept. of Surgery, The University of Arizona, Tucson, AZ, and [2]Dept. of Neurology, University of California Los Angeles, CA.

EtOH is a potent teratogen (fetal alcohol syndrome) and possible carcinogen for the liver and digestive tract. Because endothelial cells (EC) have been implicated as a target of EtOH in these syndromes, we examined the effects of binge-like blood levels of EtOH on cell cycle kinetics in a variety of EC populations. Tissue culture EC isolates (marker positive for Factor VIII-related antigen and Ulex europaeus agglutinin) of rat liver sinusoids, both primary (RSE-P) and a tumor cell line (RSE-1), and human AIDS-Kaposi sarcoma (AIDS-KS) as well as umbilical vein (HUVEC) were grown to confluence *in vitro* and exposed to EtOH in sealed wells. The cell cycle compartments $G_{1/0}$, G_2M and S percentages were determined by flow cytometry and proliferative potential by serial cell counts. EtOH increased $G_{1/0}$ while reducing G_2M (*$p<0.002$ c.f. control) in RSE-P, RSE-1 and HUVEC but not AIDS-KS. EtOH also markedly suppressed proliferation of RSE-P, RSE-1 and HUVEC with a near cessation of cell division during exposure. In RSE-1 but not HUVEC, the effect was rapidly reversed by withdrawal of EtOH with maximal cell density restored by 4 days (control EC 10-12 days). Thus, EtOH directly affects EC cell cycle progression by acting as a specific $G_{1/0}$ blocking agent in EC types other than AIDS-KS where resistance may represent transformation or transdifferentiation. Thus, cycles of EtOH consumption producing *in vivo* cycles of EC proliferation arrest and alternating proliferative bursts in combination with cellular resistance to EtOH's suppressive effect, generates selection for the "fittest" cell populations. This cell selection may be key to exuberant angiogenesis, teratogenesis, neoplasia and carcinogenesis associated with chronic alcohol abuse.

EZ 319 *IN VITRO* MODELS OF ANGIOTUMORIGENESIS, M. Witte[1], D. Way[1], M. Bernas[1], M. Weinand[1], C. Witte[1], and M. Fiala[2], [1]Dept. of Surgery, The University of Arizona, Tucson, AZ and [2]Dept. of Neurology, University of California, Los Angeles, CA.

Angiotumorigenesis - the growth of benign and malignant tumors of lymphatic and blood vessel origin - has received scant attention. Propagation of endothelial cell (EC) cultures from common benign (angiomas) and rarer malignant vascular tumors (angiosarcomas) has heretofore been unavailable and the cell of origin and nature of the process remain unresolved. To explore these issues, we isolated EC marker positive cells from various dysplastic or neoplastic vascular lesions (human lymphangioma, hemangioma and AIDS-Kaposi sarcoma) and an immortal rat liver sinusoidal EC line comparing the findings with "normal" endothelium derived from human umbilical vein, neural, omental, and dermal microvasculature and bovine mesenteric artery, vein and lymphatic. Neoplastic endothelium characteristically exhibited a more heterogeneous cell population of multiple and at times focal phenotypes occasionally with areas of "piling up", likely denoting a transdifferentiated phenotype. Lymphangioma and cystic hygroma cells formed EC lined cyst-like spaces surrounded by dense, thickened almost tubular aggregates of bipolar elongate, spindle-like cells. F8RAg was occasionally seen deposited extracellularly. Cell cycle abnormalities (a shift to higher G_2M and S phase percentages) occurred in several of the tumor isolates. These findings support that EC populations can be isolated from vascular tumors and propagated *in vitro* while retaining some morphologic characteristics of the original lymphatic or blood vascular endothelium. These *in vitro* systems allow not only better delineation of EC structure and function but also help define the biologic behavior of transdifferentiated or transformed endothelium and also provide an *in vitro* test system to examine the influence of putative angiostatic and angioinhibitory agents on lymphatic and blood vascular EC proliferation and morphogenesis.

Gene Expression II

EZ 400 DOWNREGULATION OF LYMPHOCYTE L-SELECTIN ON BINDING TO HIGH ENDOTHELIUM; A PREREQUISITE FOR TRANSMIGRATION? Ann Ager[1], Graham Preece[1] & Alan C. Wood[2] [1]National Institute for Medical Research, London and [2]University of Manchester , U.K.

L-selectin plays a crucial role in the homing of lymphocytes to peripheral lymph nodes, however, its precise role in the binding and subsequent migration of lymphocytes across high endothelium is undetermined. We have developed an *in vitro* model of lymphocyte homing which uses primary cultures of high endothelial cells (HEC) derived from rat lymph nodes. Lymphocytes were incubated with HEC layers for 60 min, adherent lymphocytes were detached using PBS/EDTA and analysed for l-selectin expression. The number of positive cells was reduced from 77% in the plated population to 23% in the adherent population. The mean fluorescence intensity (MFI) of l-selectin$^+$ cells was also reduced from 118 to 14. Non-bound lymphocytes expressed slightly lower levels of l-selectin than the plated population (60% positive; MFI 70). L-selectin expression returned to control levels within 60 minutes after detachment from HEC. The loss of l-selectin may be crucial for transendothelial migration of lymphocytes and was therefore investigated further. Down-regulation induced by binding to HEC was not inhibited by inclusion of up to 10μg/ml phosphomannan sugar PPME, which binds to l-selectin, in the adhesion assay. L-selectin expression was not altered following adhesion of lymphocytes to either immobilised VCAM-1 or CS1 peptide. Together these results suggest that surface l-selectin is down-regulated on lymphocytes which bind to HEC and that the mechanism is independent of both l-selectin and VLA-4 engagement on the lymphocyte surface by ligands presented in the endothelial layer.

EZ 401 AQUAPORIN CHIP, THE MOLECULAR WATER CHANNEL OF CAPILLARY ENDOTHELIA, Peter Agre, Barbara L. Smith, Gregory M. Preston, and Søren Nielsen, Departments of Biological Chemistry and Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205; Department of Cell Biology, University of Aarhus, Denmark, DK-8000

Although fenestrated capillaries are thought to contain structural pores, the molecular basis of water permeability by nonfenestrated capillaries was previously unknown. CHIP is a 28 kDa integral membrane protein purified from red cells and renal tubules. Expression of CHIP cRNA in *Xenopus* oocytes and reconstitution of pure CHIP in proteoliposomes conferred membranes with water channel activity. Immunohistochemistry of rat tissues demonstrated CHIP in nonfenestrated capillaries and postcapillary venules throughout the body. Expression was found to reflect different developmental patterns. CHIP mRNA was weak in fetal lung until birth. Expression in cardiac endothelium was strong during fetal development but subsided after birth. The abundance of CHIP in alveolar capillaries suggests a role in maintenance of reduced interstitial space necessary for optimal gas exchange. The presence of CHIP in mucosal lacteals and submucosal capillaries implies a role in the transport of water absorbed by the gut. The distribution of CHIP within interstitial capillaries indicates that it permits water secretion by salivary, lacrimal, and sweat glands; this also suggests that CHIP may be involved in the maintenance of interstitial fluid by facilitating water reentry into capillary lumens driven by the higher oncotic pressure within. It is also hypothesized that CHIP-mediated water movements may be found in several pathological states such as inflammation and fresh water drownings. Together these studies underscore the hypothesis that CHIP is likely to play a major role in endothelial fluid balance throughout the body.

EZ 402 VASCULAR AND HEMATOPOIETIC DEVELOPMENT IN ATTACHED CULTURES OF DIFFERENTIATING ES CELLS, Victoria L. Bautch, Scott Russell, Rob Byrum, and Tracy Futch, Dept. of Biology, University of North Carolina, Chapel Hill, NC 27599

Murine embryonic stem (ES) cells undergo a programmed differentiation in suspension culture to form cystic embryoid bodies that support vascular and hematopoietic development. To further study these processes, embryoid bodies were allowed to attach to the substratum and monitored for differentiation. ES cells that remained attached and were allowed to differentiate did not show vascular or hematopoietic development. In contrast, the embryoid bodies after 2-4 days in suspension were able to support limited development of both systems upon attachment. This was shown morphologically by the presence of structures resembling blood islands that had round refractile non-adherent cells surrounded by elongated and interconnected cells. The refractile cells were identified as hematopoietic because they stained for hemoglobin with benzidine. Likewise, the elongated cells were identified as endothelial by uptake of DiI-Ac-LDL. The presence of RNAs for the erythroid markers fetal and adult β globin and the endothelial markers ACE, ICAM-1, and ICAM-2 is consistent with the model that primitive blood islands develop in the attached cultures. Electron microscopy showed that the blood islands formed between two other cell layers that migrated from the center of undifferentiated ES cells. The identity of these cells and the molecular requirements of this system are under current investigation.

EZ 403 THE EXPRESSION OF THE ADHESION MOLECULES PECAM, ICAM-1, ICAM-2, ICAM-3, E AND P SELECTIN IN BREAST CANCER ENDOTHELIUM. SB Fox, G. Turner, KC Gatter, AL Harris. Nuffield Dept. Pathology & ICRF Molecular Oncology, John Radcliffe Hospital, University of Oxford, UK OX3 9DU.

Angiogenesis is essential for tumor growth. Sequential interaction by tumor cells with the endothelium of this neovasculature is now believed to be one of several significant steps in tumor metastasis. Increasing evidence suggests that many inducible endothelial surface molecules of the immunoglobulin and selectin families are intimately involved in this process. Therefore, using an immunohistochemical approach we examined the expression of the adhesion molecules, PECAM (CD31), ICAM-1 (CD54), ICAM-2, ICAM-3, VCAM, E and P (CD62) selectins in a series of invasive breast carcinomas using the antibodies JC70, 65B-5, CBRIC2/2, KS128, 4B2, 12B-6 and 11.8 respectively. Expression of PECAM, ICAM-1, ICAM-2, ICAM-3 and VCAM was present in 64/64 (100%), 44/64 (69%), 57/60 (95%), 9/62 (15%) and 3/31 (10%) respectively of endothelium. E and P selectins were also expressed in 32/61 (52%) and 32/54 (59%) respectively in the endothelium of tumor associated vessels. Expression of these adhesion molecules was not restricted to a particular vessel type but was observed in the endothelium of arterioles, venules and small capillaries. ICAM-3 and VCAM expression was usually focal and weak whilst PECAM, ICAM 1, ICAM-2, E and P selectin demonstrated more widespread and intense immunoreactivity. Furthermore, whereas PECAM immunoreactivity was consistently observed in endothelium throughout the tumor, expression of the other adhesion molecules, especially the selectins, was often more prominant at the tumor periphery. Some immunoreactivity of ICAM-1 and both selectins was also observed in 22/64 (34%), 4/61 (7%) and 20/54 (37%) respectively of the neoplastic element of the tumors. The differential spacial expression of these adhesion molecules within the various tumor elements demonstrated in this study suggests that tumors might be using the properties of these molecules to promote tumor dissemination and metastasis.

EZ 404 EXPRESSION AND INDUCIBILITY OF ADHESION RECEPTORS UPON IN VITRO DIFFERENTIATION OF MURINE ES CELLS, Scott A. Heyward and Victoria L. Bautch, Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

One role of adhesion molecules in the vasculature is to bind circulating lymphocytes and facilitate their passage across the vessel so as to access an area of injury or infection. Expression of these molecules has been shown to be inducible by various cytokines in cultured cells derived from adult organisms.

The expression and inducibility of adhesion molecules was studied in a developing model. Cultured embryonic stem cells go through a developmental program of growth, under proper culture conditions, to form cystic embryoid bodies (CEB's). These bodies form primitive vascular structures, blood islands, and beating structures. An expression analysis was performed on CEB's at various stages of development, and upon stimulation with lipopolysaccharide (LPS). RNA was prepared from CEB's and used in reverse transcriptase polymerase chain reactions (RT-PCR) and in RNase protection assays. RNA for intercellular adhesion molecule (ICAM-1) was present from day 12 onward. Moreover, ICAM-1 expression upon exposure to LPS was elevated as early as day 6. These results show that this adhesion receptor is expressed and cytokine regulated developmentally. Current studies involve localization of ICAM-1 expression as well as studies with other adhesion molecules.

EZ 405 REGULATED SECRETION IN HUVEC PROCEDES VIA A RAB3 INDEPENDENT PATHWAY.
Ronald R. Hiebsch,* Jerry L. Slightom,& Earl G. Adams,& and Binks W. Wattenberg,* Cell Biology,* Molecular Biology,& The Upjohn Company, Kalamazoo MI 49001.

The Rab family of proteins represents a large group of ras-like GTP binding proteins that have been demonstrated to regulate specific vesicular transport processes in eucaryotic cells. More specifically, members of the Rab3 subfamily of proteins have been implicated in the regulated secretion from neuronal cells and adipocytes. Human umbilical endothelial cells (HUVEC) synthesize and store Von Willebrand factor and P-selectin in dense secretory bodies known as Weibel-Palade bodies (WP bodies). Upon stimulation by a thrombin, histamine, cytokines or a number of other physiological factors WP bodies fuse with the cell surface resulting in release of VWF and the surface expression of P-selectin. We have initiated this study to determine if a member of the Rab3 family is involved in this regulated secretory event. Antibodies specific for Rab3 proteins failed to recognize any rab3 from HUVEC. PCR experiments failed to amplify any rab3 related sequences from HUVEC cDNA. No GTP binding proteins co-fractionate with WP bodies when isolated on percol gradients. A low stringency screen of a HUVEC cDNA library with a rab3 probe resulted in the isolation of known human rab1 and rab10 sequences as well as a previously unreported rab1 related gene, but no rab3. We therefore must tentatively conclude that a rab3 like protein is not involved in the regulated exocytosis of WP bodies thus distinguishing the regulated secretory pathway in HUVEC from the mechanisms described in other cell types.

EZ 406 *SCHISTOSOMA MANSONI* EGG ATTACHMENT TO CULTURED HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS: AN IN VITRO MODEL OF AN EARLY STEP OF PARASITE EGG EXCRETION. Eric A. Jaffe, Justinian R. Ngaiza, and Michael J. Doenhoff, Division of Hematology-Oncology, Department of Medicine, and Specialized Center for Research in Thrombosis, Cornell University Medical College, New York, NY 10021 and School of Biological Sciences, University of Wales, Bangor, Gwynedd LL57 2UW, Wales, UK

Adult schistosomes normally reside in the mesenteric vasculature where they attach to endothelial cells and lay eggs. These eggs are responsible for continuing the parasite's life cycle and inducing pathology in the host. A large percentage of the eggs pass through the blood vessel wall and into the gut lumen. To model the attachment of *Schistosoma mansoni* eggs to the endothelium of the mesenteric vasculature, the interaction between *S. mansoni* eggs and cultured human umbilical vein endothelial cells (HUVEC) in vitro was investigated. *S. mansoni* eggs rapidly attached to monolayers of both HUVEC and bovine aortic endothelial cells but more slowly to monolayers of cultured fibroblasts and smooth muscle cells. While both native and glutaraldehyde-fixed eggs attached equally well to HUVEC, eggs attached only to live, metabolically active HUVEC and attachment was inhibited by cytochalasin B and colchicine. Attachment was enhanced by both serum and plasma factors. In addition, platelet release products increased egg attachment by 75%. Preincubation of *S. mansoni* eggs with soluble egg antigens (SEA) promoted attachment (to HUVEC. In contrast, preincubation of HUVEC with SEA inhibited egg attachment. These results suggest that the interaction of *S. mansoni* eggs with HUVEC is an active process which can be modulated by molecules secreted by the egg (SEA) and by platelets during the process of egg extravasation.

EZ 407 **NITRIC OXIDE SYNTHASE (NOS) IN THE ENDOTHELIUM OF PULMONARY ARTERY TRUNKS OF LAMBS WITH CONGENITAL DIAPHRAGMATIC HERNIA (CDH).** HL Karamanoukian, PL Glick, DT Wilcox, J Rossman, RG Azizkhan. Buffalo Institute of Fetal Therapy (BIFT), The Children's Hospital of Buffalo, Department of Surgery and Pediatrics, SUNY at Buffalo, School of Medicine and Biomedical Sciences.

INTRODUCTION: The pathophysiology of CDH results from a combination of pulmonary hypoplasia, pulmonary hypertension and surfactant deficiency. We have previously shown that inhaled nitric oxide (NO), a known vasodilator, only improves oxygenation and decreases pulmonary artery pressures when the lamb model of CDH is pretreated with exogenous surfactant. NOS in endothelial cells is responsible for the production of NO, which is a mediator of smooth muscle cell relaxation. Pulmonary hypertension in CDH may result from a defect in the production of NO. Our aim was to determine if main pulmonary artery trunks in CDH lambs have NADPH-Diaphorase activity, and if it colocalizes with NOS immunoreactivity.

METHODS: Cryostat sections of both glutaraldehyde-fixed specimens of pulmonary artery and aortic rings from 10 CDH lambs and 5 control lambs were processed for NADPH-Diaphorase activity. Immunolocalization of NOS was studied in paraformaldehyde-fixed sections alone and compared to serially cut specimens from identical rings that were tested for NADPH-Diaphorase.

RESULTS: Intense NADPH-Diaphorase activity was present in the intimal layer of the pulmonary artery and aortic rings of both CDH and control lambs. This activity colocalized with NOS immunoreactivity in all specimens.

CONCLUSION: NOS is present in the main pulmonary arterial trunks of CDH lambs. We can only speculate whether this activity is preserved in the microvascular bed of the abnormally developed lung in CDH. To our knowledge, this is the first report of NOS immunoreactivity in CDH.

EZ 408 EFFECTS OF ACETYLSALICYLIC OR EICOSAPENTANOIC ACID ON PROSTANOID SECCRETION IN CULTURED ENDOTHELIAL CELLS, Charles D.Lox, Dept. Ob/Gyn., Texas Tech Univ.HSC. Lubbock, Tx.79430

It has recently been shown that sera from women with severe hypertension of pregnancy (pre-eclampsia, P-E), contains a cytotoxic factor that alters prostanoids in cultured human umbilical vein endothelial cells (HUVEC). Recently utalized treatment modalities includes a daily cyclooxygenase inhibitor, baby aspirin (acetylsalicylic acid, ASA) thought to suppress production of the potent vasoconstrictor thromboxane (TX); which is known to be elevated in P-E. In this experiment, we decided to evaluate a specific thromboxane synthetase inhibitor, eicosapentanoic acid (EPA) influence on secretion of TX and prostacyclin (PC) in HUVEC from normal and P-E gestations. Cultured cells were incubated with media containing 10% sera from normal or P-E women in addition to no, 10, 100, or 1000 pgs/ml of ASA or EPA. Control cell lines had significant increases in radioimmunoassayble PC and TX when exposed to 10 pg/ml EPA. HUVEC from P-E women exposed to media with normal sera were not significantly altered. However, P-E HUVEC incubated with P-E sera had a significant decrease in TX and an increase in PC when exposed to EPA but not ASA, which clinically is a favorable response. The data suggest that not only is P-E sera cytotoxic, but interacts with HUVEC in a differential manner dependent upon the pathophysiologic status of the maternal cell line.

EZ 409 INDUCTION OF VCAM-1 GENE EXPRESSION BY DOUBLE STRANDED RNA IN HUVE CELLS IS ASSOCIATED WITH A PARTIAL TRANSLATIONAL BLOCK. Margaret K. Offermann[1], Reneé Shaw[1] and Rosemary Jagus[2], [1]Emory University, Atlanta Ga 30322 and [2]Center of Marine Biotechnology, Baltimore, Md. 21202

Viruses that contain double stranded RNA or that replicate through double stranded RNA intermediates are among the pathogenic agents encountered by endothelial cells. VCAM-1 expression is a major determinant of binding of leukocytes that express the VCAM-1 counterreceptor, VLA-4, and these include cells such as lymphocytes that are important in the antiviral response. We have previously reported that both viral and synthetic double stranded RNA (poly (I:C)) directly induce VCAM-1 mRNA expression in human umbilical vein endothelial cells (HUVE), and this is likely to be an important component of the host response to viral infection. This induction of VCAM-1 by poly (I:C) is not a consequence of expression of class I IFNs in response to poly (I:C). The VCAM-1 mRNA levels that are induced by poly (I:C) are much higher and more sustained than mRNA levels in response to IL-1β or TNFα in HUVE cells. However, the VCAM-1 protein levels that are induced by double stranded RNA are dramatically less than would be predicted based on the mRNA levels. This suggests that there may be a partial translational block of VCAM-1 mRNA induced by poly (I:C). We demonstrate that HUVE cells constituitively express the double stranded RNA-activated protein kinase, PKR, and this kinase is activated by poly (I:C) in HUVE cells. The eukaryotic initiation factor for translation, eIF-2α, is a substrate for this enzyme, and phosphorylation of eIF-2α by PKR inhibits translation by disrupting the GTP/GDP exchange cycle required for eIF-2 function. Thus, translation of VCAM-1 mRNA is reduced by the poly (I:C)-mediated activation of PKR. Removal of the poly (I:C) after high levels of VCAM-1 mRNA are induced leads to higher levels of VCAM-1 protein, probably because the inhibition of translation is removed. Thus, the kinetics of induction of VCAM-1 by double stranded RNA differ dramatically at both the mRNA and protein levels from induction by agents such as IL-1β and TNFα. This suggests that in vivo, direct induction of VCAM-1 by viruses would lead to more sustained VCAM-1 protein expression than induction by cytokines, with a late peak of VCAM-1 protein expression occurring after clearance of the virus.

EZ 410 IL2R AS A NOVEL ENDOTHELIAL COSTIMULATOR MOLECULE FOR T CELLS, Charles G. Orosz, Jacqueline Ward, Patrick W. Adams and Claudia J. Morgan, Dept. of Surgery, The Ohio State University College of Medicine, Columbus, OH 43210

We have used cultured human umbilical vein endothelial cells (HUVEC) and gonadal vein endothelial cells (GVEC) to investigate T lymphocyte-endothelial interactions. Initial studies using RT-PCR analyses, nucleotide sequencing and flow cytometric analyses revealed that all tested GVEC and HUVEC lines expressed mRNAs and surface proteins for IL2Rβ, but not for IL2Rα or IL2Rγ. Treatment of GVEC with IL2 did not alter their surface expression of MHC class I, MHC class II, ICAM-1, VCAM-1 or IL2R molecules, but did cause the GVEC to become strongly mitogenic for CTLL-20 cells, a murine IL2-dependent cell line. This suggests that endothelial cells may use IL2Rβ to tether IL2 for use during leukocyte-endothelial interactions. Additional studies revealed that allogeneic HUVEC and GVEC could stimulate the proliferation of relatively large numbers (frequency: approx. 1/500) of CD4+ T cells, provided that a submitogenic concentration (2.5 U/ml) of human recombinant IL2 was present. HUVEC and GVEC do not express MHC class II molecules, and do not normally stimulate CD4+ T cells. The CD4+ T cell proliferation was not observed if recombinant IFNγ was used in place of IL2, or if allogeneic fibroblasts were used in place of allogeneic VEC. An advantage of GVEC is the availability of autologous leukocytes, and we further observed that autologous GVEC could readily replace allogeneic GVEC for this response. Hence, GVEC can provide signals to T cells that synergize with IL2 to promote T cell proliferation. Further, allogeneic MHC-TcR interactions are neither sufficient nor necessary for delivery of these signals. Perhaps adhesion molecules (CD2/LFA3?) mediate this effect. Nevertheless, endothelial cells may facilitate such responses by tethering locally produced IL2 via IL2Rβ.

EZ 411 FUNCTIONAL CHARACTERIZATION OF SALAMANDER FGFR2 ISOFORMS. Kevin M. Patrie, Matthew L. Poulin and Ing-Ming Chiu. MCDB Program and Department of Internal Medicine, The Ohio State University, Columbus OH, 43210.

The receptors for the fibroblast growth factor (FGF) family of polypeptide mitogens are encoded on four distinct genes within the human genome. At least for two of these receptors, FGFR1 and FGFR2, alternative splicing in the second half of the carboxy terminal Ig-like domain leads to receptor isoforms that differ in their ligand binding specificities. The isolation of two isoforms of the amphibian newt (*Notophthalamus viridescens*) FGFR2, *bek* and KGFR, has enabled us to study the function of these receptors. Utilizing mammalian FGFs as ligand, saturation binding kinetics and Scatchard analysis reveal that the two Ig-like domain variant of amphibian KGFR (stably maintained in Chinese hamster ovary cells) binds to aFGF and KGF with a K_d of 1-5 nM. Crosslinking studies reveal two bands migrating on SDS-PAGE at 145 and 125 kD. The faster migrating crosslinked product is the size expected of the receptor construct whereas the identity of the slower migrating band has not been determined. The ability of the human and bovine FGF ligands to bind to the amphibian receptor with a relatively high affinity underscores the high degree of evolutionary conservation within this growth factor/receptor system.

EZ 412 EMBRYONIC ENDOTHELIAL CELLS ARISE FROM SOMITES ONLY WHEN INDUCED BY FIBROBLAST GROWTH FACTOR (FGF), Thomas J. Poole, Department of Anatomy and Cell Biology, SUNY Health Science Center at Syracuse, 766 Irving Avenue, Syracuse, NY 13210

We have been working on the origin of the endothelial precursor cells, angioblasts, and their assembly into the earliest blood vessels in avian embryos. We have found good evidence for the induction of angioblast differentiation from mesoderm by growth factors. The basic facts are these:

1. No angioblasts differentiate from somites during normal development.
2. A quail somite transplanted into the head of a chick embryo and cultured overnight differentiates into many endothelial cells; whereas, a quail somite transplanted in place of a chick somite forms only a few.
3. Quail somite mesodermal cells, dissociated with trypsin, cultured for 20 hours with basic fibroblast growth factor (bFGF) show a ten-fold increase in the number of cells labelled with a monoclonal antibody which identifies angioblasts. During this time period there is less then a single doubling of cell number.
4. After grafting a quail somite into the head of a chick embryo, the injection of bFGF increases the number of labelled cells and the injection of neutralizing antibodies specific to bFGF decreases the number of labelled cells.
5. Injection of bFGF increases the number of labelled cells arising from a quail somite transplanted in place of a chick somite and appears to stimulate their migration.

This assay of single quail somite transplantation into either the head or trunk of chick embryos can be used to study angioblast and endothelial cell induction and differentiation by using molecular markers of the somite and endothelial cell lineages. In addition, we are currently using a PCR-based subtrative hybridization protocol to isolate genes that are up or down regulated in somite mesoderm in response to bFGF.

EZ 413 DIFFERENTIAL EXPRESSION OF RECEPTOR TYROSINE KINASES, tie-1, tie-2/tek, flk-1, IN THE EARLY EMBRYONIC VASCULAR SYSTEM. Ying Qin and Thomas N. Sato, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110

Molecular mechanisms underlying development and differentiation of early embryonic vascular endothelial cells have not been clearly defined. Recently, the vascular endothelial growth factor receptor, *flk-1*, was identified and its expression in developing vascular system seems to correlate with vasculogenesis and angiogeneisis (Millauer B. et al, Cell 72, 835-846, 1993). We and others have recently isolated two novel structurally related tyrosine kinases, *tie-1 and tie-2/tek* and found that both genes are specifically expressed in vascular endothelial cells during early embryonic stages (Sato, T.N. et al, PNAS in press). *Flk-1, tie-1 and tie-2/tek* are the first set of genes specifically expressed in developing vascular endothelial cells. Since all of these genes encode receptor tyrosine kinases, their ligand-receptor interactions and subsequent signalling cascades may govern key steps for the initial commitment to an endothelial lineage from progenitor cell populations and/or development of endothelial cells. In order to document the detailed temporal and spatial expression patterns of these three genes in developing vascular endothelial cells, their expression in the early embryonic vascular system was followed by in situ hybridization. In conclusion, *flk-1* is expressed in cells of earlier stages of endothelial cell lineage in cephalic mesenchyme and endocardium. The expression pattern of the three genes during later embryonic stages (E12.5 and later) is overlapping except that *tie-2/tek* expression in vascular endothelial cells of hyaloid artery around the developing lens is down regulated.

EZ 414 IDENTIFICATION OF THE VASCULAR ENDOTHELIAL CELL SPECIFIC PROMOTER OF *tie-2/tek* IN TRANSGENIC MOUSE. Thomas N. Sato, Ying Qin and Jeanne Magram*, Roche Institute of Molecular Biology, *Molecular Sciences, Roche Research Center, Nutley, NJ 07110

We and others have recently isolated two novel tyrosine kinases, *tie-1 and tie-2/tek* and found that both genes are specifically expressed in vascular endothelial cells during early embryonic stages (Sato, T.N. et al, PNAS in press). These genes define the set of early markers for endothelial cell lineage. Studying the mechanisms underlying the transcriptional regulation of the *tie* genes should help to define the key steps involved in differentiation of the vascular endothelial cell lineage. Transgenic mouse lines carrying a transgene construct consisting of the upstream 1.2kb fragment of the *tie-2/tek* gene and a β-galactosidase reporter gene were established. This upstream element was able to target the reporter gene expression specifically in vascular endothelial cells of yolk sac blood islands, endocardium, dorsal aortae in E8.5 embryos. This pattern of expression recapitulates the onset and the cell type specific expression of the *tie-2/tek* gene. In order to define precise upstream elements which confer this reporter gene expression, systematically deleted *tie-2/tek* promoter constructs are currently being tested in transgenic mice as well as in a hemangioma cell line known to express high level of *tie-2/tek*.

EZ 415 ISOLATION AND IMMUNOPHENOTYPING OF HUMAN BONE MARROW ENDOTHELIAL CELLS C.M. Schweitzer[1], C.E. van der Schoot[3], A.M. Dräger[1], P. van der Valk[2], A. Zevenbergen[1], and M.M.A.C.Langenhuijsen[1]. Departments of Hematology[1] and Pathology[2], Free University Hospital, Amsterdam. Department of Immunohematology[3], Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

In view of studying the adhesive interactions between endothelial cells and hematopoietic progenitor cells we have developed a method to isolate endothelial cells from human bone marrow. After density-gradient centrifugation of bone marrow aspirates the mononuclear cell fraction was depleted for T-cells, B-cells and myeloid cells by immunomagnetic separation. Further enrichment of endothelial cells was achieved by fluorescence activated cell sorting (FACS), using BNH9 or S-Endo1 monoclonal antibodies. These antibodies were found to react highly specifically with sinus endothelial cells as tested on bone marrow tissue sections by immunohistochemistry. After cell sorting, 98% of the cells showed strong von Willebrand factor (vWf) positivity. After one week of culture a monolayer of spindle shaped cells developed. Cells remained vWf positive after several passages. To determine the antigenic characteristics of the isolated endothelial cells an extensive panel of monoclonal antibodies including anti-VCAM and anti-ELAM1 was tested by means of an indirect immunoperoxidase technique. A comparison was made with endothelium of other origin. Specimens of tonsil, lymph node, colon, stomach and also of bone marrow aspirates were snap frozen and endothelial cells were recognized as sinus lining cells. Interestingly, anti-ELAM1 and anti-VCAM showed a positive reaction with bone marrow endothelium whereas anti-ELAM1 did not show a positive reaction and anti-VCAM showed only a weakly positive reaction with endothelium of other origin. In conclusion, the method described provides highly purified preparations of human bone marrow endothelium. Preliminary results implicate a constitutive expression of VCAM and ELAM1 on bone marrow endothelial cells in contrast to endothelial cells of other origin, possibly due to the high concentration of cytokines in the bone marrow.

EZ 416 HUMAN PLASMA IS NECESSARY FOR INDUCTION OF PERSISTENT E-SELECTIN EXPRESSION, Robert A. Swerlick, Norbert T. Sepp, Jens Giles, S. Wright Caughman, Lian-Jie Lie, Thomas J. Lawley, Department of Derm, Emory University, Atlanta, GA. 30322

E-selectin is an inducible adhesion protein which is expressed on the surface of endothelial cells (EC) after stimulation with proinflammatory cytokines. Initial *in vitro* observations suggested that E-selectin was only transiently expressed on EC after cytokine stimulation and stimulation was followed by a period of refractoriness to E-selectin induction. This transient expression suggested that E-selectin played an important role only in the early phases of inflammation *in vivo*. However, *in vivo* studies of chronic cutaneous inflammatory disorders have demonstrated that E-selectin is persistently expressed and these findings were difficult to reconcile with the transient expression observed *in vitro*. In order to examine whether E-selectin expression can be persistently inducted *in vitro*, we stimulated human dermal microvascular EC (HDMEC) and human umbilical vein EC (HUVEC)) with single or repetitive doses of IL-1, TNF, or LPS and examined for E-selectin expression. Initial studies demonstrated that HDMEC, but not HUVEC, stimulated with repetitive doses of IL-1, TNF, or LPS maintained maximal cell surface E-selectin expression for up to 72 hours. This expression was associated with persistent E-selectin mRNA expression and E-selectin mediated HL-60 binding. Further examination of E-selectin expression on HUVEC demonstrated that persistent E-selectin expression could be induced if cells were grown in media containing human serum or plasma, which is a normal constituent of media used to grow HDMEC. Repetitive stimulation in the absence of human serum or plasma, in serum free media, or in the presence of 20% calf serum did not result in persistent E-selectin expression. Maximal persistence of E-selectin required repetitive stimulation with cytokine in the presence of 30% human serum or plasma while lower concentrations resulted in intermediate levels of expression. Fractionation of human plasma by gel filtration on sephacryl S-200 demonstrated two peaks of activity. These studies suggest that a constituent of human plasma is critical to chronic E-selectin expression on endothelial cells.

EZ 417 MURINE VASCULAR CELL ADHESION MOLECULE PROTEINS ARE DIFFERENTIALLY TARGETED IN POLARIZED EPITHELIAL CELLS , Robert W. Terry, Gregorio Pirozzi and Mark A. Labow. Roche Research Center, Hoffmann-La Roche Inc. Nutley NJ 07110-1199.

VCAM-1 was originally identified as a cytokine inducible adhesion molecule expressed on endothelial cells which mediated binding to a variety of leukocytes in a VLA-4 dependent manner. We have recently characterized the murine VCAM-1 gene and shown that it is highly homologous to the human VCAM-1 gene. In the mouse, however, two forms of VCAM-1 protein are produced by alternatively spliced mRNAs. The largest mRNA encodes a transmembrane protein ($VCAM^{TM}$) containing seven immunogobulin domains. A small 1.6 kb mRNA encodes a GPI-anchored protein ($VCAM^{GPI}$) containing only the first three Ig-domains. The 1.6 kb mRNA is prefereintially induced by cytokines and LPS. One possible role for the different VCAM-1 proteins may be to allow for differential localization on the surface of polarized cells. This may be particularly relevant since since VCAM-1 is known to be expressed by two different polarized cell types, endothelial cells and kidney epithelial cells. In this study the localization of the different murine VCAM-1 isoforms has been examined in MDCK cells in culture. MDCK cells permanently expressing both VCAM isoforms were established. Indirect immunoflourescence was used to demonstrate that the two forms of VCAM were targeted to different surfaces of polarized epithelial cells. Specifically, $VCAM^{TM}$ was expressed on the basolateral surface while $VCAM^{GPI}$ was expressed on the apical surface. Quantitative domain selective botinylation was used to demonstrate that >95% of each isoform was present on their respective surface domains. The differential localization of VCAM-1 isoforms on the cell surface may contribute to the ability of VCAM-1 to mediate cell adhesion and transmigration.

EZ 418 TSP-1 AND ITS PEPTIDES BLOCK ANGIOGENESIS BY MAKING ENDOTHELIAL CELLS REFRACTORY TO STIMULI AND BY ENHANCING DIFFERENTIATION. Sara S. Tolsma, Olga Volpert, and Noel Bouck, Department of Microbiology-Immunology and Lurie Cancer Center, Northwestern University, Chicago IL 60611.

The matrix protein thrombospondin-1 (TSP-1) is a potent inhibitor of angiogenesis. TSP-1 is a large multidomain, multifunctional, protein found in platelet a-granules and in the matrix of many cell types including endothelial cells. In response to an angiogenic stimulus the endothelial cells in a capillary must migrate out of that structure toward the stimulus, a process inhibited by TSP-1 *in vivo* and *in vitro*. The inhibitory activity of TSP-1 was localized to a 50/70 kDa chymotryptic fragment from the stalk portion of TSP-1 which inhibited endothelial cell migration by 50% at a concentration (0.9 nM) that was not significantly different from that of the whole molecule (0.8 nM). Peptides from two of the three domains in the stalk region contained anti-angiogenic activity. A 7 amino acid peptide from the procollagen homology region and two 19 amino acid peptides from the malarial or type I repeats were able to block angiogenesis and endothelial cell migration at micromolar concentrations.

Endothelial cells grown *in vitro* are not associated with a capillary structure. However, if TSP was removed from endothelial cells by direct addition of neutralizing antibodies against TSP, >80% of the cells formed ring-like structures which stained positive for the lumenal marker angiotensin converting enzyme. This data suggests that the absence of TSP causes these cells to progress along a path which leads to new vessel formation. Using time lapse photography it was determined that these structures were formed by the fusion of vacuoles present in the cells. Similar vacuoles have been shown to contribute to vessel lumens *in vivo*.(Konerding, et al, 1992, Angiogenesis: Key Principles, p.40-58).

Thus TSP-1 contributes to vessel stability both by blocking migration and new vessel formation by endothelial cells in response to angiogenic factors and by enhancing their differentiation into lumen-containing cells.

EZ 419 AGE-RELATED INJURY TO HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS, James Varani, Carla G. Taylor, Michael K. Dame and Hedwig Murphy, Department of Pathology, University of Michigan, Ann Arbor, MI 48109

Human umbilical vein endothelial cells (HUVEC) in first and fourth passage were labeled with 51Cr and incubated for 6 hours in serum-free, growth factor-free basal medium. At the end of the incubation period, the percentage of released 51Cr was assessed and used as an indicator of cell death. First-passage cells survived incubation while a high percentage (routinely 50-60%) of the fourth passage cells died. Trypan blue exclusion and failure to replate confirmed lethal cell injury in the passage-4 cells. Protein kinase C activity was assessed in passage-1 and passage-4 HUVEC and found to be significantly higher in fourth passage cells. Interestingly, however, treatment of passage-4 cells with PMA completely prevented cell death and the protective effects of PMA were blocked by prior exposure of the cells to staurosporine. As expected, staurosporine inhibited PMA-induced protein kinase C activation. Efforts to understand the mechanism through which the late-passage cells were killed suggested that intracellular-derived oxidants were not primarily responsible for the cytotoxic effect. This was based on 1) failure to detect differences in intracellular DMPO-adduct formation in electron spin trapping experiments between first- and fourth-passage cells and 2) failure to inhibit cytotoxicity with of oxygen radical scavengers and iron-chelators. Conversely, inhibitors of endonucleases (zinc, pyrithione and aurintricarboxylic acid) as well as actinomycin D and cyclohexamide provided partial protection. Additionally, DNA fragmentation and margination was observed in fourth-passage HUVEC after incubation in basal medium for 1-2 hours but this was not observed when PMA was present. In these regards, age-related HUVEC injury is distinguishable from injury induced in HUVEC by inflammatory cells (Lab. Invest. 66:708) and by direct exposure to cytotoxic cytokines such as TNFα (Lab. Invest. 61:62). Rather, these findings are consistent with the suggestion that apoptosis may play an important role in the injury process.

EZ 420 IDENTIFICATION OF INDUCIBLE INFLAMMATORY LIGANDS FOR L-SELECTIN

Susan A. Veals, Philippe Hartl, Doug Hanahan, Jeffrey J. Bowden, Donald M. McDonald and Steven D. Rosen. Depts. of Anatomy and Biochemistry, Program in Immunology, HRI and CVRI, U.C. San Francisco, San Francisco CA 94143-0452.

Lymphocyte emigration from blood to peripheral lymph nodes (PLN) depends on the initial adhesive interaction between L-selectin on the lymphocyte and its cognate ligands, expressed on high endothelial post-capillary venules (HEV). Soluble factors are thought to regulate expression of these HEV ligands, recently identified as the mucin GlyCAM-1 and a glycoform of the mucin CD34 (Baumheuter *et al.* Science, *in press*). Consistent with the binding requirements for L-selectin, both these PLN HEV ligands are sulfated and sialylated.

L-Selectin is also involved in leukocyte migration to extralymphoid sites of inflammation, but ligands at these sites have not been defined. Using a soluble recombinant form of L-selectin as a probe, we found sulfated ligands on HEV-like vessels induced in infiltrated hyperplastic islets from pancreas of transgenic mice. With specific antisera, we identified these ligands as GlyCAM-1 and CD34. GlyCAM-1 expression was also induced on HEV-like vessels which appear in rat trachea during chronic *Mycoplasma pulmonis* infection. Thus, the L-selectin ligands which direct lymphocyte homing to PLN may also serve as inducible, inflammatory ligands. We are now comparing the ligands' synthesis in PLN with that at extralymphoid sites to identify any similarities or differences.

EZ 421 ROLE OF CADHERINS IN MAINTAINING THE INTEGRITY OF PY-4-1 ENDOTHELIAL CELLS,

Rong Wang and Victoria L. Bautch, Dept. of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3280.

Cell-cell adhesion plays a fundamental role in endothelial cell functions. Cadherins are a family of transmembrane molecules that mediate Ca^{++}-dependent cell-cell adhesion by homophilic interactions. E (epithelial)-cadherin, P (placental)-cadherin, and N (neural)-cadherin represent the classic cadherins. Although endothelial cells have cadherin-like activity, the type of cadherins and their function in endothelial cells are largely unclear. Recently cadherin-5, which is more restrictively expressed in endothelial cells, was cloned. Py-4-1, a murine endothelial cell line isolated from hemangiomas of Polyoma transgenic mice, was used as a model to investigate endothelial cadherins. When Py-4-1 cells were trypsinized under conditions that protect Ca^{++}-dependent cell adhesion (TC-treatment), the cells maintained a monolayer. When Py-4-1 cells were trypsinized under conditions that abolish Ca^{++}-dependent cell adhesions (TE-treatment), the monolayer was dissociated. This result suggests that Ca^{++}-dependent cell adhesion is important in Py-4-1 cell interactions. RT-PCR expression analysis showed that E, P and N cadherins were not expressed at detectable levels in Py-4-1 cells. Western blot analysis with two pan-cadherin antibodies identified a 120 kd band in Py-4-1 cell lysates and membrane fractions. This 120 kd band also reacted with a cadherin-5 antibody, suggesting that this protein is cadherin-5. The 120 kd band was sensitive to trypsinization when the cells were subjected to TE-treatment, and it was protected from trypsinization when the cells were subjected to TC-treatment. The function of the 120 kd murine cadherin is currently under investigation.

EZ 500 FUNCTIONAL EXPRESSION AND CHARACTERIZATION OF HUMAN THROMBIN RECEPTOR IN INSECT CELLS USING BACULOVIRUS EXPRESSION VECTOR, X. Chen, K. Earley, W. Luo, T.-C. Liang, S.-H. Lin and W. P. Schilling. Dept. of Molecular Physiology and Biophysics, Baylor College of Medicine, and Dept. of Molecular Pathology, M.D. Anderson Cancer Institute, Houston, TX 77030.

The functional expression of the human thrombin receptor (TR) was studied in *Spodoptera frugiperda* (Sf9) insect cells following infection with recombinant baculovirus (BV) containing the TR-cDNA. Thrombin or thrombin receptor peptide, SFLLRN ($TRP^{42/47}$), produced a dose-dependent and sustained increase in free cytosolic calcium concentration ($[Ca^{2+}]_i$), measured using fura-2, in Sf9 cells 40 hrs after infection with TR-cDNA recombinant BV. The EC_{50} values for thrombin and $TRP^{42/47}$ were 0.5 U/ml and 1 μM, respectively, which are similar to reported values in mammalian cells. No detectable change in $[Ca^{2+}]_i$ was observed in Sf9 cells infected with antisense TR-cDNA recombinant BV. Pertussis toxin pre-treatment (1 μg/ml for 30 hours) had no effect on the increase in $[Ca^{2+}]_i$ produced by thrombin or $TRP^{42/47}$, suggesting that the TR in Sf9 cells is coupled to a pertussis toxin-insensitive G-protein of the Gq type. In the presence of the Ca^{2+} influx blocker, La^{3+}, the response to thrombin or $TRP^{42/47}$ was transient suggesting that thrombin causes both the release of Ca^{2+} from intracellular stores and the influx of Ca^{2+} from extracellular space. Similar to mammalian cells, the TR expressed in Sf9 cells exhibits desensitization. Pre-exposure to $TRP^{42/47}$ (10 μM for 15 min) shifted subsequent dose-response curve for $TRP^{42/47}$ to the right, increasing EC_{50} without changing the maximum response to $TRP^{42/47}$. These results suggest that the human TR, functionally expressed in Sf9 cells, has characteristics similar to those seen in mammalian cells. The baculovirus-Sf9 cell expression system may be useful for evaluation of those structural features of the TR associated with ligand recognition, G-protein interaction and receptor desensitization.

EZ 501 ANALYSIS AND THERAPY OF VASCULAR TUMORS INDUCED BY TRANSGENIC ENDOTHELIAL CELLS,

Nathalie Dubois-Stringfellow and Victoria L. Bautch, Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

Py-4-1 cells have been isolated from hemangiomas induced by expression of the polyoma virus early region gene in transgenic mice. Although Py-4-1 cells have retained numerous vascular endothelial cell properties, they induced fatal hemangiomas when introduced into histocompatible mice.

Hematological and pathological analysis of mice at different time points after Py-4-1 cell inoculation showed that they developed many of the features of the Kasabach-Merritt syndrome. These include thrombocytopenia, anemia and splenomegaly. These mice may serve as an animal model to perform therapeutic studies. Because in vitro studies revealed that addition of plasminogen at 0.1 U/ml killed selectively Py-4-1 cells whereas control endothelial cells were not affected, we attempted hemangioma therapy. Intratumoral injection of plasminogen was performed on mice inoculated with Py-4-1 cells. Tumor treatment with plasminogen delayed the death of the animal and, in some cases, reduced significantly the size of the tumor. The mechanism of action of plasminogen toxicity in endothelial cells is currently under investigation.

EZ 502 FUNCTIONAL STUDIES OF AN INS/DEL POLYMORPHISM IN THE PAI-1 PROMOTER REGION WHICH IS ASSOCIATED WITH MYOCARDIAL INFARCTION AT A YOUNG AGE, Per Eriksson, Bengt Kallin, Ferdinand van 't Hooft and Anders Hamsten, Atherosclerosis Research Unit, King Gustaf V Research Institute, Department of Medicine, Karolinska Institute, Karolinska Hospital, Stockholm, Sweden.

A common polymorphism in the human plasminogen activator inhibitor - 1 (PAI-1) promoter has recently been described (Dawson, et al. J Biol Chem 1993; 268:10739-10745) which correlates according to genotype with the levels of plasma PAI-1 activity. The single base pair insertion/deletion polymorphism ($GACACGTG_{4 \text{ or } 5}$ AGT) is located 675 base pairs upstream from the start of transcription of the PAI-1 gene.

The prevalence of the *del* allele is significantly higher in 93 patients with myocardial infarction before the age of 45 than in 100 population-based controls (allele frequencies of 0.63 vs 0.53). Individuals in the control group who are homozygous for the *del* allele show two times higher plasma PAI-1 activity compared to individuals homozygous for the *ins* allele. In the patient group, PAI-1 activity is not significantly associated with genotype, probably because of a confounding effect of higher very low density lipoprotein (VLDL) levels in the *ins/ins* group.

Gelshift assay shows that both alleles bind a common factor present in nuclear extracts derived from either liver (HepG2) or endothelial (HUVEC) cells. DNA constituting the *ins* allele binds an additional protein. Footprinting studies using DNase I and methylation interference assays show that the common factor binds adjacent to the polymorphic site. The *ins* allele-specific factor binds to a DNA segment overlapping the binding site of the common factor.

Results from transfection assay using the *ins/del* polymorphic site cloned upstream of a minimal and heterologous promoter show that the DNA segment containing the *del* polymorphic site mediates a strong transcriptional activation. The *ins* sequence also mediates an activator activity, although to a lower extent. This suggests that the common factor is a transcriptional activator, while this activity is restricted by the *ins* allele-specific factor which thus acts as a transcriptional repressor. The absence of repressor activity could significantly increase the amount of plasma PAI-1 activity in subjects homozygous for the *del* allele and influences their risk of myocardial infarction. The identity of the two proteins is currently under investigation.

EZ 503 SPONTANEOUSLY HYPERTENSIVE RATS DISPLAY THROMBOMODULIN DIFFERENTLY THAN NORMAL RATS, Naomi L. Esmon[ab] Donald Houston[b], Toya Botchlet[a] and Paula Grammas[a], [a]Department of Pathology, University of Oklahoma Health Sciences Center and [b]Oklahoma Medical Research Foundation, Oklahoma City, OK 73104

In hypertension, there is an altered response of the vessel wall to vasoactive and growth promoting substances such as thrombin. Thrombomodulin (TM) is a high affinity endothelial receptor for thrombin and serves an important thrombin regulatory role. It catalyzes protein C (PC) activation with thrombin, directly inhibits thrombin fibrinogen clotting and enhances ATIII inhibition. We therefore investigated whether spontaneously hypertensive rats (SHR) have different TM properties than the normotensive (WKY) strain. Endothelial cells from these strains were tested in 3 systems for their ability to activate PC in the presence of saturating concentrations of thrombin: (1) the microvasculature exposed in the Langendorff heart perfusion, (2) isolated aortic rings and (3) cloned cells in monolayer culture. In the first two models systems, fresh tissue derived from SHR animals possessed 30-40% less PC activation potential than the normal WKY controls. Preliminary experiments with monolayer cultures indicate the SHR cells do not reduce TM expression in response to IL-1β as the WKY cells do (40% reduction after 5 hr incubation with IL-1β). Moreover, SHR cells may have structurally different TM. Although the SHR and WKY derived cells appeared to have similar PC activation activity, cells derived from SHR animals did not distinguish between native PC and PC lacking the gla-domain. WKY cells activated PC >6 fold faster than gla-domainless PC, a previously observed property of endothelial cells from several species. These data suggest that biochemical modification of TM or its control could underlie abnormal vessel responses to thrombin in hypertension.

EZ 504 LPS-STIMULATED, CRYOPRESERVED HUMAN MONOCYTES (MO) CONVERT PLASMA FIBRINOGEN TO FIBRIN AND SUBSEQUENTLY LYSE THE FIBRIN FORMED BY CONVERTING PLASMA PLASMINOGEN (Pg) TO PLASMIN (Pl) BY MO-UROKINASE RECEPTOR (uPAr) BOUND SINGLE CHAIN UROKINASE PLASMINOGEN ACTIVATOR (scuPA)
Peter Kierulf, Hilde Horge, Reidun Øvstebø, Liv Osnes and Åse-Brit Westvik. The Research and Development Group, Clinical Chemistry Department, Ullevål University Hospital, Oslo, Norway.

BACKGROUND: Human Mo harbours the capacity to initiate coagulation through its Tissue Factor (TF) synthezising capacity, and to express plasminogen activator (PA) activity (scuPA) upon maturation into macrophages. LPS stimulation upregulates Mo-TF, and leads to decreased Mo-PA activity. Upon culturing LPS-stim. Mo in heparinized overlay plasma, initial fibrin formation (day 0-1) occurs, followed by fibrin resolution (day 1-7).
AIM: To examine to what extent plasma (Pg) and Mo-scuPA and Mo-uPAr are involved in fibrin resolution.
MATERIALS & METHODS: Cryopreserved PBM were thawed, seeded (2.4×10^5 Mo/well, RPMI/5% AT-FCS), LPS-stim. (4 h, 2μg/well), medium removed, diluted overlay plasma (15 U Hep/ml) added, fibrin formation [fibrinopeptid A-(FPA) (RIA)] and resolution (Fibrinostika FbDP, microleliza system, specific for Factor XIII cross-linked fibrin, Organon Teknika) and plasma scuPA (Tint Eliza, Biopool) monitored (days 0, 1, 4, 7) ± Pg (Mab-solid phase removal) and ± Pg-inhibitor (EACA). Mo-scuPA and Mo-uPAr appearance was monitored by specific-Mab - FITC-Mab - Flow cytometry. Mo-scuPA mRNA (oligos to Exon V, VII) was semiquantified by RT-PCR. - agarose gel electrophoresis.
RESULTS & DISCUSSION: Fibrin formation (FPA) peaked on day 1, and fibrin resolution (FbDP) steadily increased from day 1-7, and was more than 90% inhibited by EACA or Pg-depletion. scuPA-antigen gradually disappeared from plasma, Mo-scuPA and Mo-uPAr flow cytometry intensity increased, and scuPA-mRNA increased with time. LPS stim. PBM, when operating in plasma and initiating fibrin formation, may resolve this fibrin by Mo-uPAr bound scuPA, which cooperates with (Mo-bound?) plasma plasminogen.

EZ 505 EFFECTS OF HIGH AFFINITY uPA RECEPTOR ANTAGONISTS ON ANGIOGENESIS IN AN IN VIVO RABBIT MODEL
Hye Yeong Min, Jennifer Stratton-Thomas, David Hwang*, Marc Shuman#, and Steven Rosenberg, Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608 and UCSF, Departments of Ophthalmology* and Hematology/Oncology#, San Francisco, CA 94143

Urokinase (uPA) is a serine protease which consists of three domains, an EGF-like domain (residues 1-50) comprising the receptor binding determinants, a Kringle domain (residues 51-140) and a serine protease domain (residues 180-411). uPA binds to the cell surface via a specific receptor (uPAR) and converts plasminogen to plasmin more efficiently on the surface of cells than in solution, suggesting that cell surface -bound uPA is of major importance in in vivo plasminogen activation. Recently, uPA and uPAR have been implicated as components of the angiogenic response because angiogenic agents up-regulate the expression of both uPA and uPAR in endothelial cells. We have expressed high affinity murine and human uPA receptor antagonists, consisting of the uPA EGF-like domains (residues 1-48), in yeast. These proteins compete for binding to the murine and human urokinase receptors with dissociation constants in the low nanomolar range. We have used these molecules to investigate the role of the uPA receptor in the rabbit cornea in vivo angiogenesis model.

EZ 506 DELAYED AND PERSISTENT LOSS OF ENDOTHELIAL ALKALINE PHOSPHATASE (AP) ACTIVITY INDUCED BY IONIZING RADIATION IN VIVO, Schultz-Hector S. , Balz K., Behrends U., Graf M., *Ikehara Y., *Misumi Y., GSF-Institut f. Strahlenbiologie, 85758 Neuherberg, FRG, *Department of Biochemistry, Fukuoka University, 814-01 Fukuoka Japan

Chronic radiation injury in normal tissues is dose limiting in tumor therapy. In tissues with postmitotically fixed parenchymal cells, the microvasculature is the likely target of radiation injury. We chose the rat myocardium as an in vivo model to study longterm radiation effects in capillary endothelial cells. Heart irradiation with a single dose of 20 Gy causes a focal loss of endothelial AP (EC 3.1.3.1). In untreated rat or human myocardium the AP reaction product and protein can be detected along the luminal and abluminal cell membrane as well as in cytotic vesicles of all capillary endothelial cells. Following heart irradiation clusters of completely enzyme negative capillaries develop. ^{3}H-thymidine labelling studies show that enzyme loss is associated with a regional threefold increase in endothelial cell proliferation in these areas. Enzyme negative areas rapidly increase in size and number, covering 79±10% of the myocardium by 120 days postirradiation. At 70 days foci of myocardial degeneration and necrosis start to form within enzyme negative areas. These also increase in size and number to a maximum of 23± 4% of the myocardium and cause congestive heart failure at 249±21 days. After a lower, sublethal radiation dose to the heart of 15 Gy, AP negative areas cover 20±8% of the myocardium and enzyme loss persists at this level throughout an observation time of 660 days. Alkaline phosphatase negative endothelial cells are ultrastructurally intact and the number of cytotic vesicles is unchanged. Northern blot hybridization reveals no change in AP specific RNA during development of alkaline phosphatase loss. Regional distribution of ALP mRNA will be studied by in situ hybridization. In summary, we report a radiation induced alteration of endothelial cell function, which is unrelated to cell death. It occurs after a delay of 25 days, persists throughout the remaining life-span of the animal and plays a key role in the development of radiation induced cardiomyopathy. Enzyme loss is associated with endothelial cell proliferation.

Supported by DFG Grant La 576/4-1

EZ 507 TARGETING OF SAPORIN TO UROKINASE RECEPTORS, Marco R. Soria[1], Ugo Cavallaro[1], Antonella del Vecchio[1], and Douglas A. Lappi[2], [1]Department of Biological and Technological Research, Scientific Institute H San Raffaele, Milano, Italy; [2]Department of Molecular and Cellular Growth Biology, The Witthier Institute for Diabetes and Endocrinology, La Jolla, CA.

We have synthesized a conjugate between human uPA and saporin (SAP), a ribosome-inactivating protein produced by *Saponaria officinalis*. Results of our cell killing assays show that uPA is very effective at targeting saporin specifically to uPAR-expressing cells. Mouse LB6 cells transfected with the cDNA for human uPAR and the human monocytoid cell line U937 were greatly sensitive to the conjugate, whereas non-transfected LB6 cells and the human T lymphocyte cell line Jurkat, expressing no uPARs, were not inhibited. Receptor-bound uPA is internalized only upon formation of a complex with one of its inhibitors (PAIs). However, our conjugate was highly cytotoxic even when the interaction between uPA and PAIs was prevented. The α_2-macroglobulin receptor (α_2-MR), that has been reported to mediate the internalization of uPA-PAI complexes, could be involved in cell killing caused by the conjugate. The mechanism of internalization might be different from that of unconjugated uPA complexed to PAIs, although still uPAR-mediated. Our results also suggest that α_2-macroglobulin and/or its receptor could mediate the internalization and cytotoxicity of unconjugated saporin, as it has been shown for other toxins. The cytotoxicity of the conjugate was also tested on the IST-KS2 cell line from a sporadic case of Kaposi's sarcoma and compared with that on EA hy 926, a human endothelial stabilized cell line. The conjugate's toxicity towards IST-KS2 cells was rather high, having an ID_{50} of 2×10^{-10}M. Similar cell killing was shown on EA hy 926 cells ($ID_{50} = 3 \times 10^{-10}$M). The results of these cell killing assays were quite similar to those obtained with a conjugate between SAP and basic Fibroblast Growth Factor, selectively toxic towards various cell types expressing the FGF family of receptors. *E. coli* expression of recombinant saporin has been achieved using inducible vectors carrying a strong phage promoter, and fusion proteins with cell-binding ligands have been obtained for clinical and experimental use.

References: Albini et al. (1992) *Int. J. Oncol.* 1, 723-730; Barthelemy et al (1993) *J. Biol. Chem.* 268, 6541-6548; Cavallaro et al. (1993) *J. Biol. Chem.*, in press.

EZ 508 TSG-6, AN ARTHRITIS-ASSOCIATED HYALURONAN BINDING PROTEIN, FORMS A STABLE COMPLEX WITH INTER-α-INHIBITOR VIA A GLYCOSAMINOGLYCAN CROSS-LINK, Hans-Georg Wisniewski, Wilson H. Burgess[1], Joel D. Oppenheim and Jan Vilček, NYU Med. Center, New York, NY 10016; [1]American Red Cross - Holland Lab., Rockville, MD 20855

TSG-6 is a secreted 35 kDa glycoprotein, inducible by TNF and IL-1, with 37-40% amino acid sequence homology to the lymphocyte homing/hyaluronan receptor CD44, cartilage link protein and the proteoglycan core proteins aggrecan and versican. High levels of TSG-6 are found in the synovial fluid of patients with rheumatoid arthritis and, to a lesser degree, other arthritic diseases. TSG-6 protein readily forms a 130 kDa complex with a protein present in human, bovine, rabbit and mouse serum. The complex is stable during SDS-PAGE under reducing conditions, and in the presence of 8M urea or 8M guanidinium HCl. The protein that binds TSG-6 was purified from human serum and identified as inter-α-inhibitor (Iαl) by N-terminal microsequencing. Antibodies directed against either TSG-6 or Iαl recognized the 130 kDa complex in Western blots. Experiments with pure recombinant TSG-6 and with Iαl purified from human serum show that the 130 kDa complex is rapidly formed in the absence of other proteins. The 130 kDa complex is cleaved by chondroitin sulfate ABC lyase from *Proteus vulgaris* but stable against treatment with hyaluronidase from *Streptomyces hyalurolyticus,* indicating that TSG-6 and the Iαl-derived polypeptide(s) present in the 130 kDa complex are linked by a chondroitin sulfate chain. Treatment of Iαl with chondroitin sulfate ABC lyase abolishes its ability to bind TSG-6 whereas TSG-6 is not sensitive to chondroitin sulfate ABC lyase treatment. In addition, no chondroitin sulfate could be detected in association with the 35 kDa TSG-6 protein, suggesting that the chondroitin-4-sulfate chain coupling the chains of Iαl also forms the link to TSG-6 protein. Complex formation with TSG-6, which is induced during inflammation, may explain fluctuations in the levels of Iαl or its components during various diseases.

Late Abstracts

KNOCK-OUT OF THE PDGF GENES IN MICE
Christer Betsholtz, Per Leveen and Milos Pekny, Department of Pathology, University of Uppsala, Sweden

Platelet-derived growth factor is a family of homo- and heterodimeric ligands built up by two polypeptide chains, A and B. The PDGFs interact with two different receptor molecules, *pdgfra* and *pdgfrb*. The A-chain appears to have affinity only for *pdgfra* whereas the B-chain can bind to both receptors. The current model of receptor activation involves receptor dimerization followed by autophosphorylation in *trans*, which permits subsequent interaction with exogenous substrates for the receptor protein tyrosine kinase. The PDGF chains as well as the PDGF receptors are expressed during embryonic development. For example, the appositional expression of PDGFA and *pdgfra* suggests a role for this ligand-receptor pair in the formation of mesenchymal structures. In support of this, the mouse *patch* mutation, a deletion which encompasses the *pdgfra*-gene (and possibly additional genes), leads in its homozygous state to severe connective tissue defects and to death before birth.

In order to increase our understanding of the *in vivo* roles of the PDGFs, we have knocked out the PDGF genes in embryonic stem cells with the aim of generating specific PDGF-isoform deficiencies in mice. Aspects of the PDGF B-chain-negative phenotype will be presented at the meeting.

GENOMIC STRUCTURE OF A CYCLIC NUCLEOTIDE-GATED K^+ CHANNEL.
Gary V. Desir, Paul Welling, David Engel, Xiaqiang Yao. Department of Medicine, Yale University and West Haven Veterans Administration Medical Center, New Haven CT.

We recently identified a novel gene that encodes a 562 a.a. protein (Kcn). Kcn RNA injected in *Xenopus oocytes* results in the expression of a cGMP-activated K channel. *Northern* analysis suggests that Kcn is differentially expressed since the message is detectable only in kidney, heart and thoracic aorta.

Examination of a genomic clone for Kcn indicate that the coding region (longest open reading frame) is intronless and resides on a single exon. The transcription initiation site (TIS) for Kcn was identified by primer extension assay using a 30 nucleotide-long primer . It is located approximately 470 nucleotides upstream to the first codon . The same TIS is used in both heart and kidney. We then examined a 1 kb region immediately upstream to the TIS for potential genomic regulatory elements. Sequence analysis of that region revealed the presence of consensus sequences for 3 glucocorticoid, 1 cAMP and 2 thyroxine responsive elements.

We conclude that the Kcn gene is intronless and that gene transcription may be regulated in a complex manner. Transfection studies will be needed to determine if all the consensus sequences identified are functionally active.

TYROSINE PHOSPHORYLATION OF ENDOTHELIAL PECAM-1 IS A EXTRACELLULAR MATRIX MODULATED-C-SRC MEDIATED EVENT, Theresa Lu, Linda Yan and Joseph A. Madri, Department of Pathology, Yale University School of Medicine, New Haven, CT 06510

Platelet-endothelial cell adhesion molecule-1 (PECAM-1), found at the cell-cell borders of endothelial cell monolayers, has been shown to be involved in cell migration. In migrating endothelial cells, PECAM localization is altered, becoming more diffusely distributed at the migrating front, thus raising the possibility of the coordination of PECAM activity with that of cell-substratum interactions. However, the mechanism by which PECAM function is regulated has not been well understood. We have found that PECAM-1 is rapidly and transiently tyrosine phosphorylated upon human umbilical vein endothelial cell attachment to fibronectin. This increase in tyrosine phosphorylation is inhibited by the kinase inhibitor herbimycin A, indicating that the increase in phosphorylation is a kinase mediated event. Kinase assays show that PECAM-1 is an in-vitro substrate for c-src. Furthermore, PECAM-1 from bovine aortic endothelial cells overexpressing c-src but not the kinase deficient mutant is more highly phosphorylated. These c-src transfected cells have been shown in the past to migrate more quickly than vector or mutant transfected controls. These results indicate that tyrosine phosphorylation on PECAM-1 may be mediated by c-src or a kinase activated by c-src in endothelial cells, and also raise the possibility that tyrosine phosphorylation is a means of regulating PECAM-1 function.

CULTURED BOVINE LYMPHATIC AND VASCULAR ENDOTHELIAL CELLS: COMPARATIVE IMMUNOLOCALIZATION OF FIBRONECTIN AND VON WILLEBRAND FACTOR, Franco Piovella, Carla Marchetti, Amalia Di Nucci, Silvia Serafini, and Maria Reguzzoni, Clinica Medica II, IRCCS Policlinico S. Matteo, Istituto di Istologia ed Embriologia Generale and Istituto di Farmacologia II, University of Pavia, 27100 Pavia, Italy.

Little is known about the structure and function of lymphatic endothelium. Few studies are available on the properties of cultured lymphatic endothelial cells (LEC), and data concerning the similarities and differences between these cells and vascular endothelial cells (VEC) are rare. In the present study we isolated and cultured both VEC and LEC from bovine mesentery, evaluated their morphological features, and compared their ability to express adhesive proteins, namely fibronectin (Fn) and von Willebrand factor (vWf). Endothelial cells were obtained by cannulating bovine mesenteric lymphatic and blood vessels (veins), and perfusing it with 0.1% collagenase solution. Cells were resuspended and cultured onto glass coverslips in multiwell plates, and incubated in 95% air, 5% CO_2 humid athmosphere, at 37°C. For immunofluorescent studies, cells were air dried and fixed with 95% ethanol for 10 min. After washings, slides were incubated with rabbit anti Fn or vWf polyclonal antisera for 30 min. After further washings, cells were incubated with TRITC-conjugated swine anti rabbit immunoglobulins. For Trasmission Electron Microscopic (TEM) studies, glass coverslips were omitted, cells were fixed in situ with 2.5% glutaraldehyde in 0.1 cacodylate buffer and processed by standard techniques.

LEC displayed the characteristic culture pattern already described for VEC, the only difference being the presence of elongated or "star shaped" cells, at subconfluence. In both cell types immunofluorescence detected Fn as an extracellular meshwork of fibers which progressively disappears to form the subendothelial matrix. vWf was detected in VEC as fine perinuclear particles and large cytoplasmic elongated granules. In LEC, vWf was inconstantly present, and showed a different pattern, being mainly localized in small, round cytoplasmic granules. TEM showed the same pattern in LEC and VEC, with the major difference in the shape of Weibel Palade bodies, which appeared round and less numerous in LEC in comparison to VEC.

DIFFERENTIAL REGULATION OF GENES INVOLVED IN MATRIX PRODUCTION AND DEGRADATION BY TGFβ1 IN MICROVESSEL ENDOTHELIAL CELLS, Sabita Sankar, Negar M. Brooks and Joseph A. Madri, Department of Pathology, Yale University School of Medicine, New Haven, CT 06510

Previous data has shown that TGFβ1 stimulates the reorganization of microvessel endothelial cells into capillary tubes when they are dispersed in a three-dimensional collagen gel. Our aim was to determine the role TGFβ1 plays in promoting this process of in vitro angiogenesis. Total mRNA were isolated from untreated and TGFβ1-treated rat epididymal fat pad microvessel endothelial cells (RFCs) grown in collagen gels and probed for genes involved in matrix production and degradation. Our results indicate that TGFβ1 dramatically increased fibronectin and alpha 5 integrin message levels whereas collagen I and plasminogen activator inhibitor 1 (PAI-1) message levels were reduced compared to the untreated controls. In addition, there was a concomittant increase in fibronectin protein production and decrease in PAI-1 protein levels in TGFβ1-treated cells. The plasminogen activator (PA) activity of TGFβ1-treated cells exhibited a decrease compared to untreated cells. TGFβ1 treatment of RFCs grown on collagen I matrix in a two-dimensional culture system has been shown to increase collagen I, fibronectin, alpha 5 integrin and PAI-1 message levels. Hence the selective upregulation of fibronectin and alpha5 integrin and downregulation of PAI-1 genes may be important in stabilizing capillary tube formation during in vitro angiogenesis.