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Symposium on Diabetic Retinopathy: Pathogenesis and Novel Therapies

S1

THE HISTOPATHOLOGY OF DIABETIC RETINOPATHY

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The retina is one of those tissues that appear selectively vulnerable to the metabolic milieu induced by diabetes mellitus with evidence of neural and vascular dysfunction manifest within days of the onset of hyperglycemia. Studies of early diabetic retinopathy (DR) in animal models have provided evidence of premature death in all the cellular components of the neurovascular complex. Indeed, since the neuroglial elements represent the functional parenchyma of the tissue, determining both the phenotype of the vasculature and its long-term survival, it is arguable that neural dysfunction represents the primary target in DR. Therefore, it is upon such a backcloth of neurovascular dysfunction that the downstream vasodegenerative and associated blood–retinal barrier changes that characterizes DR are superimposed. Nevertheless, it is these later vascular alterations that are responsible for the gross pathological features of hard exudates, cotton wool spots, and intraretinal hemorrhages that characterize the fundus picture of DR so familiar to clinical ophthalmologists. This review examines the classical features of DR as revealed by vascular digests and electron microscopy and draws attention to some hitherto poorly appreciated yet functionally crucial evidence for early vascular smooth muscle cell loss and the role of microaneurysms in major vasoocclusive events.

S2

RETINAL BLOOD FLOW REGULATION IN THE NORMAL AND DIABETIC EYE

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The human eye shows a complex vascular supply. The inner retina is nourished by the retinal blood vessels and the outer retina is nourished by the choroidal blood vessels. Regulation of retinal blood flow shows many similarities with cerebral blood flow. Most importantly, retinal blood flow is autoregulated in response to changes in perfusion pressure and is

metabolically regulated during changes in blood oxygen and carbon dioxide tension. In addition, neurovascular coupling is present in the retina, meaning that retinal blood flow is increased during neural stimulation as evidenced by experiments using flicker light. The present talk summarizes our understanding of the physiology of retinal blood flow regulation. Abnormalities of retinal blood flow regulation in diabetes are reviewed and the significance of these abnormalities in relation to the pathogenesis of diabetic retinopathy is discussed.

S3

INFLAMMATION AND VASCULAR REPAIR IN DIABETIC RETINOPATHY

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Diabetic maculopathy is the leading cause of visual loss in diabetic patients. The pathogenesis is not fully understood and a satisfactory therapy is currently not available. Malfunction of the blood–retinal barrier plays a central role in the disease and leads to retinal edema and secondary photoreceptor dysfunction. Diabetic vascular leakage and this diabetic macular edema are regulated by a distinct combination of direct paracellular transport, alterations in the cell–cell junctions of endothelial cells, and cell death of these endothelial cells. With increasing duration of diabetes, the relative relevance of these three components will vary, and with increasing duration of diabetes, the cumulative endothelial cell death will become more relevant. Leukocyte adhesion in the retinal vasculature is one of the earliest manifestations of diabetic retinopathy. We have previously shown that leukocytes are causally related to capillary nonperfusion, endothelial cell death, and vascular permeability in diabetes. Also, differentiated cells precursors could play a role in vessel repair and alteration in diabetes. We discuss the role of the recruitment of endothelial precursors and macrophages to sites of retinal neovascularization on the background of an altered basal membrane behavior.

S4

EXPERIMENTAL DIABETIC RETINOPATHY— NOVEL APPROACHES TO PREVENT VASOREGRESSION

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Vascular complications due to chronic hyperglycemia, including diabetic retinopathy, are an increasing therapeutic

challenge. The epidemiology of diabetic eye disease has been well described. However, due to the complex, multifactorial nature of the vascular damage of diabetes on vessels, it had been difficult to identify key targets for treatment or prevention. Novel techniques for studying molecules and mechanisms involved in retinal vessel development and vascular cell interactions improved the understanding of retinal cell biology and pathobiology. A unifying concept has been proposed that links hyperglycemia-induced mitochondrial overproduction of reactive oxygen species with long-known biochemical alterations, such as the formation of advanced glycation end products (AGE), or the activation of the protein kinase C pathway. Potential mechanism-based therapeutic agents were proposed, and classified into two classes of agents: metabolic signal blockers and catalytic antioxidants. Thiamine derivatives limit vascular damage to the diabetic retinopathy, both in a primary prevention, and in a secondary intervention approach by limiting the accumulation of toxic triose phosphates through activation of the enzyme transketolase, diverting intermediates into the nonoxidative branch of the pentose-phosphate pathway. Similar effects in experimental diabetic retinopathy were identified using inhibitors of the enzyme poly-ADP-ribose polymerase. R-alpha-lipoic acid belongs to the class of catalytic reactive oxygen scavengers and has several advantages over classic antioxidants. Its use in the diabetic rat model resulted in the prevention of acellular capillaries as the hallmark of experimental diabetic retinopathy, and in the prevention of pericyte loss. These benefits were associated with a reduction in retinal oxidative stress and NFkB, as well as a reduction in the expression of vascular endothelial growth factor (VEGF) and angiopoietin-2. Genetic deletion of the signal pathway initiated by the receptor for AGEs, RAGE, confers protection against hyperglycemia-induced endothelial cell damage. Together, available *in vitro* data and results from animal experiments suggest that novel mechanism-based pharmacological strategies have the potential for prevention of diabetic microvascular damage.

JDRF Sponsored Symposium:

S5

THE PATHOGENESIS OF DIABETIC RETINOPATHY: IS ANGIOGENESIS ALWAYS A BAD THING?

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Diabetic retinopathy is traditionally classified clinically into two forms; nonproliferative and proliferative. The nonproliferative form of the disease is by far the most common and, in a significant number of cases, it progresses to sight-threatening proliferative diabetic retinopathy (PDR) and macular edema, both of which constitute the sight-threatening stages of the condition. Ophthalmologists are currently seeking to limit neovascularization and reverse breakdown of the blood-retinal barrier by introducing agents that inhibit the bioactivity of VEGF and other angiogenic factors. While this

approach has shown promise and could provide immediate benefits for patients, it fails to address the progressive vasodegeneration and retinal ischemia that lie at the heart of PDR. Moreover, inhibiting VEGF in a moribund microvasculature could accelerate capillary closure in the resident vessels by depriving the endothelial cells of their main survival factor. This review presentation will cover the nature of retinal ischemia in diabetic retinopathy and highlight some key factors that may alter the way we view this complication and, perhaps, influence future directions of therapeutic intervention. Using evidence gleaned from clinical cases and experimental studies, questions are raised about the nature of vascular repair in the diabetic retina. The presentation also explores ways that intraretinal angiogenesis could be enhanced to reverse retinal ischemia, prevent hypoxia-induced elaboration of angiogenic/vasopermeabilizing growth factors, and thereby prevent the sight-threatening stages of diabetic retinopathy.

S6

VEGFR-1 AND γ -SECRETASE: UNIQUE REGULATORS OF ANGIOGENESIS

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The vascular endothelial growth factor (VEGF) family of growth factors plays a pivotal role in pathological angiogenesis and increased vascular permeability associated with diabetic retinopathy. VEGF exerts its functions on endothelial cells via interaction with the cellular receptors VEGFR-1 and VEGFR-2. While VEGFR-2 plays a pivotal role in angiogenesis, the role of VEGFR-1 is not clear. However, there is increasing evidence that VEGFR-1 is a potent negative regulator of VEGFR-2. Studies have shown that there is considerable intracellular "cross-talk" between VEGFR-1 and VEGFR-2 and that this plays a critical role in regulating VEGFR-2-mediated signaling. We have recently identified a novel regulatory pathway for VEGFR-1 by which VEGFR-1 can undergo regulated intramembrane proteolysis via γ -secretase and that the translocation of both cleaved and full-length VEGFR-1 acts as a potent negative regulator of VEGFR-2 signaling in retinal microvascular endothelial cells. Interestingly, PEDF, a potent endogenous antiangiogenic factor, appears to exert its effect by promoting γ -secretase-dependent translocation of VEGFR-1 that, in turn, inhibits VEGFR-2-induced angiogenesis and decreases vascular permeability. Both VEGFR-1 and γ -secretase are potent negative regulators of angiogenesis that may offer potential targets for pharmacological intervention in proliferative diabetic retinopathy.

S7

GENE THERAPY FOR DIABETIC RETINOPATHY: HYPE OR HOPE?

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Gene therapy offers the means to control the expression of pathogenic or therapeutic proteins in the retina in a way that is targeted, sustained, and potentially regulatable. The eye has a combination of properties that make it an ideal target organ for this approach. Potential targets for gene therapy of diabetic retinopathy include genes and proteins involved in several pathways, including oxidative stress, polyol accumulation, nonenzymatic glycation, ischemic neuronal degeneration, vascular hyperpermeability, and pathological angiogenesis. Retinal cell types can be targeted with some specificity by vector design and surgical delivery. Vector-mediated transfer of genes encoding angiostatic proteins can suppress the extent of neovascularization in experimental models. Challenges for the further development gene therapy for diabetic retinopathy include achieving efficient transduction of relevant cell types and appropriate control of transgene expression.

S8

ISCHEMIC VASCULAR DAMAGE CAN BE REPAIRED BY HEALTHY, BUT NOT DIABETIC, ENDOTHELIAL PROGENITOR CELLS

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Endothelial precursor cells (EPC) play a key role in vascular repair and maintenance, and their function is impeded in diabetes. We previously demonstrated that EPC isolated from diabetic patients have a profound inability to migrate in vitro. We asked whether EPC from normal individuals are better able to repopulate degenerate (acellular) retinal capillaries in chronic (diabetes) and acute (ischemia–reperfusion [I/R] injury and neonatal oxygen-induced retinopathy [OIR]) animal models of ocular vascular damage. Streptozotocin diabetic mice, spontaneously diabetic BBZDR/Wor rats, adult mice with I/R injury, or neonatal mice with OIR were injected within the vitreous or the systemic circulation with fluorescently labeled CD34⁺ cells from either diabetic patients or age- and sex-matched healthy controls. At specific times after administering the cells, the degree of vascular repair of acellular capillaries was evaluated immunohistologically and quantitated. In all four models examined, healthy human (hu) CD34⁺ cells attached and assimilated into vasculature in these areas, whereas cells from diabetic donors were uniformly unable to integrate into damaged vasculature. These studies demonstrate that healthy huCD34⁺ cells can effectively repair injured retina, and that there is defective repair of the vasculature in patients with diabetes. Defective EPCs may be amenable to pharmacological manipulation and restoration of the cells' natural robust reparative function.

Oral Communications:

OC1

BEVACIZUMAB DOES NOT INHIBIT GROWTH OF LS174t TUMORS OVEREXPRESSING VEGF_{165b}

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Vascular endothelial growth factor (VEGF-A) is a key contributor to colorectal carcinoma (CRC) growth. The anti-VEGF antibody, bevacizumab (Bz), increases median CRC survival by 30%, but no predictive markers of response (~20%) have been identified. Bz has a similar affinity for VEGF₁₆₅ and VEGF_{165b} (Varey, A.H.R., et al. (2006). *Microcirculation* 13:511–534), yet CRC tumors produce variable ratios of pro-angiogenic VEGF₁₆₅ to anti-angiogenic VEGF_{165b} (Varey, A.H.R., et al. (2006). *Colorectal Dis* 8:99–102), suggesting that the ratio may predict the response to Bz. To test this, LS174t human colon carcinoma cells (normally $5.7 \pm 0.3\%$ of their VEGF is VEGF_{165b}) were transfected to overexpress VEGF_{165b} ($109 \pm 16\%$ VEGF_{165b}), then 2×10^6 cells of either LS174t ($n = 12$) or the VEGF_{165b} derivative ($n = 14$) subcutaneously injected into nude mice. Then 24 h later, twice weekly intraperitoneal injections of either 50 μ g Bz or saline were commenced in each group and tumor growth was monitored. The control cells ($5.7 \pm 0.3\%$ VEGF_{165b}) formed smaller tumors when treated with Bz (357 ± 150 mm³ on day 22) compared with saline (853 ± 360 mm³, $p < .01$, ANOVA), yet Bz did not affect the growth of the VEGF_{165b} tumors (410 ± 239 mm³ compared with 506 ± 448 mm³ at day 34). Similar results were seen when established tumors were treated. Bz effectively inhibited growth of tumors predominantly expressing VEGF₁₆₅, but not VEGF_{165b}. Measuring the VEGF_{165b}:VEGF₁₆₅ ratio in CRC tumors may enable more accurate selection of patients most likely to benefit from Bz treatment.

OC2

STIMULATION OF TUMOR GROWTH AND VEGF-MEDIATED ANGIOGENESIS BY LOW CONCENTRATIONS OF ANTI-ANGIOGENIC $\alpha v \beta 3 / \alpha v \beta 5$ -INTEGRIN INHIBITORS

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Anti-angiogenic inhibitors of integrins $\alpha v \beta 3$ and $\alpha v \beta 5$ are in clinical trials for the treatment of cancer. However, recent studies show $\alpha v \beta 3 / \alpha v \beta 5$ -integrin inhibitors are less effective at repressing tumor growth and angiogenesis than originally predicted (Tucker (2003). *Curr Opin Investig Drugs* 4:722–731) and at low concentrations these inhibitors may even act as agonists of integrin function in vitro (Legler

(2001). *J Cell Sci* 114:1545–1553). We found that the growth of transplanted tumors in mice was enhanced significantly when plasma concentrations of $\alpha v\beta 3/\alpha v\beta 5$ integrin inhibitors were maintained at nanomolar levels (2 nM) using osmotic minipumps ($p < .05$ and $p < .01$ for B16F0 melanoma and Lewis lung carcinoma, respectively, Student's *t* test). Tumor angiogenesis in these mice was also enhanced ($p < .05$, Student's *t* test). Nanomolar (0.2–20 nM) concentrations of $\alpha v\beta 3/\alpha v\beta 5$ inhibitors significantly enhanced angiogenesis in the *ex vivo* aortic ring assay ($p < .01$, Student's *t* test) and promoted the expression of VEGF receptor 2 (VEGFR2) in primary endothelial cells ($p < .05$, Student's *t* test). Enhanced tumor growth and angiogenesis induced by nanomolar doses of $\alpha v\beta 3/\alpha v\beta 5$ inhibitors *in vivo* was abrogated by an antibody that blocks VEGFR2 function, DC101 ($p < .01$, Student's *t* test). These data show that nanomolar concentrations of $\alpha v\beta 3/\alpha v\beta 5$ -inhibitors can actually stimulate tumor growth and angiogenesis by promoting the expression of VEGFR2 in endothelial cells. This effect could compromise the overall anti-angiogenic activity of these drugs.

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OC3

ASSESSMENT OF ANGIOGENESIS, VASCULAR ENDOTHELIAL GROWTH FACTOR, AND TISSUE FACTOR IN HUMAN BREAST CANCER

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Tissue factor (TF) has been shown to stimulate angiogenesis, in part by upregulating vascular endothelial growth factor-A (VEGF). This study is the first to quantify angiogenesis, VEGF, and TF in the hyperplasia, preinvasive, invasive breast carcinoma sequence. A total of 133 serial sections of normal human breast, benign and premalignant hyperplastic tissue, and preinvasive and invasive breast cancer specimens were immunohistochemically stained for CD31, VEGF, and TF. Angiogenesis was quantified using Chalkley grid analysis (MVD), VEGF expression was assessed semi-quantitatively, and TF expression was graded as present or absent. A significant increase in MVD was seen between normal and hyperplastic/preinvasive breast cancer tissue ($p < .005$) and between preinvasive and invasive carcinomas ($p < .0005$). Both increases in MVD were associated with a significant increase in VEGF expression in breast epithelial and tumor cells, respectively ($p < .0005$). In contrast, TF was not expressed in normal or hyperplastic breast epithelial cells, or in preinvasive cancer cells, but was expressed in approximately 45% of invasive cancer specimens. VEGF was expressed in the endothelium of normal breast, while TF was not. However, VEGF and TF were both expressed in ECs in hyperplastic breast and in preinvasive and invasive cancer samples. These data indicate that angiogenesis is initiated at the earliest signs of dyspla-

sia and increases rapidly between preinvasive and invasive cancer. VEGF and TF expression patterns suggest that these factors play a role in this process.

OC4

CCR7 EXPRESSION RESCUES METASTATIC POTENTIAL OF NONMETASTATIC MELANOMA

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In malignant melanoma death results from metastatic disease, initially spreading through the lymphatic system. Metastatic A375 melanoma actively migrates toward lymphatic endothelial cell (LEC) conditioned media (CM) and not blood endothelial cell (BEC) CM, a response inhibited by a lymphatic secreted chemokine, CCL21, neutralizing antibody (Emmett et al. (2005). *Microcirculation* 12:677). To determine if expression of the receptor for CCL21, CCR7, could mediate metastatic potential we transfected nonmetastatic (NM) A375P cells with CCR7. Using a modified Boyden chamber, A375P-CCR7 cells showed a 14.7 ± 0.9 -fold increase in migration toward LEC CM, whereas A375P cells did not ($p < .05$, Bonferroni, $n = 3$). This response was specific to LEC not BEC CM. *In vivo*, 1×10^5 LECs or BECs in 100 μ L PBS were injected subcutaneously into 15 nude mice. Then 1×10^6 A375P-CCR7 or A375P (NM) cells were injected approximately 10 mm rostral to the EC injection site (marked with Monastral blue). Tumors were allowed to grow up to 12 mm in diameter before sacrifice. Macroscopic measurements were made from prosections of the tumor area relative to the endothelial cell injection site. A375P-CCR7 tumors migrate toward LECs ($57.9 \pm 20\%$ of tumor on EC side of injection) but not BECs ($29 \pm 8\%$). A375P (NM) cells did not migrate toward either LECs ($16 \pm 15\%$) or BECs ($43 \pm 4\%$). Although these populations are not significantly different, this may be due to loss of homogeneity in the transfected cells *in vivo*. CCR7 expression increases migratory/metastatic potential *in vitro* and possibly *in vivo*.

Supported by ScARF

OC5

FUNCTIONAL LYMPHATICS DRIVE TUMOR CELL HOMING TO LYMPH NODES VIA AUTOLOGOUS CHEMOTAXIS

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Lymphatics maintain tissue homeostasis by regulating interstitial fluid balance. Breast cancer spreads via the lymphatics but the mechanisms used by tumor cells to access lymphatics remain unclear. Furthermore, CCR7 expression has been linked with lymphatic metastasis but the significance of this is not yet known. Using a novel co-culture model that includes biophysical factors encountered by tumor cells, i.e., 3D matrix, interstitial flow (IF), and lymphatic endothelial cells (LECs), we report how, in the presence of IF, autocrine CCR7 signals direct tumor cell migration toward draining lymphatics.

Co-culture of tumor cells with LECs enhanced tumor cell migration in a CCR7-dependent manner (no LEC vs. LEC vs. LEC block; $0.3 \pm 0.1\%$, $1.09 \pm 0.03\%$, $0.5 \pm 0.08\%$ migration for metastatic cells, $p < .05$ ANOVA). A similar CCR7-dependent, but LEC-independent increase in tumor cell migration occurred in the presence of slow IF alone (static vs. IF vs. IF block; $0.11 \pm 0.03\%$, $0.22 \pm 0.05\%$, $0.28 \pm 0.08\%$, respectively, for nonmetastatic cells and $0.3 \pm 0.1\%$, $1.4 \pm 0.2\%$, $0.4 \pm 0.1\%$ for metastatic cells, $p < .001$ ANOVA). We observed that tumor cell-secreted chemokine alone (0.1 vs. 0.4 pg/1000 cells/day, non-met vs. met) could promote autocrine chemotaxis with IF. Furthermore, in response to IF or CCL21 cues, tumor cell cytoskeleton machinery directionally polarized. In summary, we show how tumor cells use IF to create and amplify autologously generated CCL21 gradients, and thus chemotact toward local lymphatics by virtue of their draining function. This work reveals the first evidence of autologous chemotaxis and illustrates an elegant mechanism explaining how tumor cells may be "guided" toward lymphatics early in metastasis.

OC6

PREVENTION OF DIRECTED METASTATIC MELANOMA CHEMOTAXIS TOWARD LYMPHATIC ENDOTHELIAL CELLS

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We previously showed that the chemokine ligand CCL21 mediated active migration and growth of melanomas to lymphatic endothelial cells (LEC) (Shields et al., *Oncogene*, in press). Chemotraps (Endocube, France) are the chemokine-binding domains of THAP proteins fused to IgG Fc antibody regions. They bind free chemokines and prevent chemokine receptor interactions. Metastatic melanoma cells (A375) expressing the CCL21 receptor CCR7 were transfected with expression vector (control) or containing Chemotrap 1 (active Chemotrap), or Chemotrap 189 (no chemokine binding activity). Expression was confirmed by Western blot. In *in vitro* migration assays A375 (8.1 ± 1.1 -fold), and Chemotrap 189 cells (8.8 ± 1.5 -fold) migrated toward LEC conditioned media (CM). Chemotrap 1 secretion inhibited this migration (1.1 ± 0.2 -fold, SNK post hoc $p < .01$). To demonstrate efficacy *in vivo*, nude mice were injected with 10^6 melanoma cells 1 cm rostrally to 10^5 LEC and injection sites tattooed. Growth direction was measured macroscopically, mapping tumor area growing toward the LEC injections. In the Chemotrap 1 group only $32.5 \pm 11.1\%$ of the tumor grew on the LEC side, compared with $83.5 \pm 3.1\%$ in the empty vector group, and $85.4 \pm 5.1\%$ in the Chemotrap 189 group (Bonferroni $p < .05$). Here we show *in vitro* and *in vivo* that metastatic melanoma cell migration can be prevented by Chemotraps. This identifies a potential therapeutic prevention of lymphatic melanoma metastasis.

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OC7

THE EFFECTS OF COMBRETASTATIN, A TUBULIN BINDING AGENT, IN PHYSIOLOGICAL ANGIOGENESIS

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Two forms of physiological angiogenesis, abluminal sprouting and capillary splitting, occur through an increase of shear stress in the microvasculature and via an as yet unknown abluminal signal, respectively. We investigated the role and importance of the microtubule and microfilament systems in these two forms of angiogenesis *in vivo*. Disodium combretastatin A-4 3-O phosphate (CA-4-P), a tubulin binding and vascular disrupting agent (VDA), was injected IP on alternate days into C57 BL10 male mice at a concentration of 30 mg/kg. These mice were split into three groups ($n = 6$ in each): a control group, a group undergoing prazosin (50 mg/L drinking water) treatment (capillary splitting model), and a third group that underwent unilateral extirpation of the tibialis anterior muscle (abluminal sprouting model). At day 14 femoral blood flow, arterial blood pressure, and heart rate were monitored. Capillary to fiber ratio was determined for the extensor digitorum longus (EDL) muscle, and used as an indicator of angiogenesis. A complete angiogenic blockade was observed in the muscle undergoing abluminal sprouting, with no effect on flow with respect to control mice. A partial, though significant, reduction in angiogenesis was observed in muscle undergoing capillary splitting, with a similar proportional decrease in flow when compared to mice undergoing prazosin treatment alone. These results suggest an anti-angiogenic effect of CA-4-P in abluminal sprouting, and an antivascular effect in capillary splitting.

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OC9

THERAPEUTIC ARTERIOGENESIS FOLLOWING HUMAN TISSUE KALLIKRIEN OVEREXPRESSION

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Therapeutic angiogenesis via administration of single recombinant growth factors or vector-mediated overexpression has been largely unsuccessful in part due to inadequacy of a single growth factor to induce all aspects of vascular growth. Modern therapy aims to address this by using combinations of growth factors or single pleiotropic factors such as tissue kallikrien (hTK). hTK was overexpressed in the adult rat mesentery, and intravital and confocal imaging was used to investigate the vessel function and vessel morphology, as previously described (Benest et al. (2006). *Microcirculation* 14:423-437). hTK overexpression increased the functional vessel area compared with control treated eGFP vessels (32 ± 4 vs. $457 \pm$

90%, $p = .02$), increased the number of proliferating vessels (33 ± 10 vs. $123 \pm 11 \text{ mm}^{-2}$, $p = .0017$) and also the microvessel density (321 ± 18 vs. 628 ± 45 , $p = .0003$). The induced angiogenesis was not associated with an increased sprout point formation (11 ± 3 vs. $7 \pm 2 \text{ mm}^{-2}$, $p = .34$), but hTK overexpression did increase branch point formation (134 ± 7 vs. $254 \pm 22 \text{ mm}^{-2}$, $p = .001$), suggestive of a nonsprouting form of angiogenesis. In addition, the fractional pericyte coverage was doubled (32 ± 4 vs. $64 \pm 6\%$, $p = .025$) and smooth muscle cell recruitment was observed (0 vs. $7.2 \pm 1.2\%$) in the periendothelium. These data provide an ultrastructural analysis of hTK-induced neovessel formation, and provide evidence for altered periendothelial composition, a reflection of the potential therapeutic arteriogenic effect of hTK. All data are presented as means \pm SEM, eGFP $n = 5$, hTK $n = 4$.

OC10

ANTINEUTROPHIL CYTOPLASMIC ANTIBODIES ENHANCE LEUKOCYTE-ENDOTHELIAL CELL INTERACTIONS IN VIVO WITH A CRITICAL ROLE FOR Fc γ RECEPTORS

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Antibodies against neutrophil cytoplasmic myeloperoxidase (MPO-ANCA) are implicated in small vessel vasculitis. We used a novel murine vasculitis model and intravital microscopy (IVM) to determine the effects of MPO-ANCA on leukocyte activation and define signaling mechanisms underlying pathology. Anesthetized (ketamine/xylazine) wild-type (WT) or FcR γ -chain^{-/-} mice were pretreated with intrascrotal TNF- α (500 ng) and the cremaster was prepared for IVM. MPO-ANCA (18 $\mu\text{g/g}$ bwt), generated by immunizing MPO-deficient mice (^{-/-}) with purified murine MPO, or control IgG was injected systemically 2 h post-TNF- α . Leukocyte-endothelial (L-E) cell interactions were observed in postcapillary venules. Circulating leukocyte numbers were also determined. Renal and pulmonary histology sections were assessed for injury. MPO-ANCA alone had no effect on L-E interactions in WT mice. However, when administered to TNF- α prestimulated WT mice, significantly reduced leukocyte rolling (41.6 ± 6 vs. 10.5 ± 4.2 ; $p < .05$) and increased adhesion and migration were observed. Systemic leukocyte numbers were also significantly reduced (3405 ± 400 pre-MPO-ANCA vs. 2063 ± 267 post-MPO-ANCA). Increased neutrophilic infiltrates, hemorrhage, fibrin deposition, and edema were observed in pulmonary tissue. These IVM and histological events were not observed in FcR γ -chain^{-/-} mice. We provide the first in vivo evidence that MPO-ANCA acts synergistically with inflammatory stimuli to potentiate L-E cell interactions in a murine model of vasculitis. A mechanistic role for FcR γ -chain was identified, pointing to new strategies to target vasculitis.

Supported by the BHF

OC11

INTERFERON BETA INCREASES BARRIER PROPERTIES OF HUMAN GLOMERULAR ENDOTHELIAL CELLS AND PODOCYTES IN CULTURE

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Treatment with IFN β has been shown to reduce proteinuria in three distinct animal models of nephritis (JASN 2005;16:29A). We hypothesized that the reduction in proteinuria was due to effects on cells of the glomerular filtration barrier. We used tissue culture inserts and an Electric Cell-Substrate Impedance Sensor (ECIS) to test barrier properties of glomerular endothelial cells (GEnC) and podocytes by transendothelial electrical resistance (TEER) and passage of FITC-labeled albumin in response to IFN β . We further defined effects of IFN β on GEnC and podocytes by Western blotting, immunofluorescence, and focused gene arrays. The actions of IFN-induced protein IP10 on cell monolayers were also studied in the insert system. IFN β increased TEER of GEnC and podocyte monolayers by 9.7 Ω (74%) and 9.0 Ω (30%), respectively. Resistance of GEnC by ECIS was increased by 38.4%. Passage of FITC-labeled albumin decreased by 55 and 39%. There was no effect on expression of adhesion molecules but VCAM was upregulated by IFN β treatment. Gene arrays confirmed upregulation of elements of the IFN β pathway. IP10 was strongly upregulated following treatment at 4 and 24 h. This finding was validated by Western blotting. IP10 increased TEER of GEnC and podocyte monolayers by 6.7 Ω and 2.2 Ω , respectively. These studies suggest that the direct cellular effects of IFN β may be responsible for reducing proteinuria in animal models, and preliminary studies indicate that IP10 contributes to this result. These are the first data dissecting the mechanism of this powerful anti-proteinuric pathway.

OC12

THE ROLE OF HISTAMINE IN THE RESPONSE OF THE RAT MESENTERIC MICROCIRCULATION TO NOCICEPTIN (N/OFQ)

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N/OFQ is an opioid like peptide that has endogenous ligand activity at the OP4 (NOP) receptor, causing hypotension and vasodilation. Previous studies using isolated vessels eliminated nitric oxide, muscarinic receptors, CGRP and α -adrenergic receptors as potential mechanisms for N/OFQ induced dilation. This study aimed to determine the effects of N/OFQ in the rat mesenteric microcirculation in vivo and the role of histamine in these responses. In vivo fluorescent intravital microscopy ($n = 24$) was used to study mesenteric arterioles and venules (20–50 μm) and determine the effects of N/OFQ on diameter, macromolecular leak and leukocyte-endothelial interactions following administration of FITC-BSA (0.25 mL/100 g).

N/O/FQ (0.6–60 nmol/kg IV) elicited significant ($p < .05$) hypotension (MAP, baseline: 117.78 ± 8.42 mmHg, 60 nmol/kg N/O/FQ: 79.99 ± 5.11 mmHg), vessel dilation (arterioles; baseline: 21.95 ± 1.01 μ m, 60 nmol/kg N/O/FQ: 24.62 ± 0.89 μ m, venules; baseline: 34.75 ± 1.16 μ m, 60 nmol/kg N/O/FQ: 40.13 ± 1.04 μ m), macromolecular leak (baseline: 48.5 ± 3.39 , 60 nmol/kg N/O/FQ: 172.92 ± 10.01) and leukocyte rolling (baseline: 7.33 ± 1.43 , 60 nmol/kg N/O/FQ: 39 ± 11.5) and adhesion (baseline: 4.83 ± 0.96 , 60 nmol/kg N/O/FQ: 12.83 ± 2.14). Pretreatment with the H₁ antagonist pyrilamine (1 mg/kg IV) completely inhibited dilation and leak ($p < .05$), as did pretreatment with the H₂ antagonist ranitidine (1 mg/kg, IV). In summary, N/O/FQ elicits vasodilation and inflammation via a histamine-mediated mechanism.

OC13

APOPTOTIC CELLS ENHANCE ENDOTHELIAL PROGENITOR CELL RECRUITMENT TO DAMAGED ENDOTHELIUM

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In the present study we have explored whether apoptosis in retinal microvascular endothelium can induce cytokine release and adhesion molecule expression and enhance recruitment and reparative function of endothelial progenitor cells (EPCs). Bovine retinal microvascular endothelial cells (RMEC) were treated with a photoreactive agent (verteporfin 0.5–1 μ g/mL; VPF), which upon light activation, induced synchronous apoptosis and denudation of the monolayer. The resultant apoptotic bodies were added to healthy RMECs to study gene expression of adhesion molecules. Conditioned media collected from apoptotic RMECs was assayed for chemotactic activity in EPCs. DiI-labeled EPCs were also added to circumscribed denuded regions of RMEC monolayers in which apoptotic cells had either been removed or left in situ. Treatment of healthy RMECs with apoptotic bodies upregulated expression of ICAM and E-selectin by 10- and 23-fold ($p < .001$), respectively, while VCAM was upregulated by 5-fold ($p < .05$). EPCs showed significant chemotactic response to apoptotic body-conditioned media. ELISA of the conditioned medium showed increased levels of following cytokines (pg/mL) over plain medium; VEGF (83.7 vs. 49.2), IL-8 (7.2 vs. 2.9), IL-6 (79.5 vs. 68.5) and TNF- α (50.5 vs. 38.5); SDF-1 (682.7) remained unchanged. The presence of apoptotic bodies enhanced EPC recruitment to areas of RMEC denudation up to 5-fold ($p < .05$), compared to washed lesions. The data indicate that ingestion of apoptotic endothelial cells by healthy neighbors induces cytokine, chemokine, and adhesion molecule expression that facilitates EPC recruitment to sites of endothelial injury.

OC14

HEPATIC ISCHEMIA REPERFUSION INJURY PROMOTES THE RECRUITMENT OF HEMATOPOIETIC STEM CELLS TO MURINE SINUSOIDAL MICROCIRCULATION IN VIVO

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Numerous studies suggest that hematopoietic stem cells (HSCs) can migrate to injured liver and aid tissue repair. This study aimed to determine whether hepatic ischemia-reperfusion (I/R) injury could promote HSC recruitment. Recruitment of a murine HSC line, HPC-7, was investigated in vivo using fluorescent intravital microscopy (IVM) and in vitro using frozen tissue section adhesion assays. Hepatic I/R injury was induced for 90 min in anesthetized (ketamine/xylazine) C57BL/6 mice. CFSE-labeled HPC-7s (1×10^6 cells; ia) were administered after 5 or 30 min postreperfusion. Intravital observations were made every 5 min for 60 min. Parallel in vitro studies were conducted on frozen tissue sections to quantify HPC-7 adhesion. HPC-7 cell adhesion was significantly raised in vivo at 30 min postreperfusion (13.27 ± 1.66 in I/R animals vs. 3 ± 0.94 in sham controls at 60 min; $p < .005$). Similar results were observed regardless of whether cells were introduced at 5 or 30 min postreperfusion. Adhesion was predominantly in sinusoidal capillaries rather than postcapillary venules. Significant in vitro HPC-7 adhesion to frozen tissue sections isolated from I/R animals was also observed compared to sham controls (9.4 ± 2.5 vs. 4.5 ± 0.5 , respectively; $p < .05$). These novel results illustrate that hepatic I/R injury can indeed act as a stimulus for HSC recruitment to murine sinusoidal microcirculation. Having established this model, future work will aim to identify the molecular mechanisms that govern HSC recruitment.

Supported by the MRC

OC15

INVESTIGATION OF ENDOTHELIAL PROGENITOR CELL INTERACTIONS WITH RETINAL ENDOTHELIAL CELLS DURING ANGIOGENESIS

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Marrow-derived endothelial progenitor cells (EPC) participate in angiogenesis in various tissues. However, little is known of the intimate associations of EPCs with vascular endothelial cells, especially in the retina. This study was undertaken to examine the interactions of EPCs of distinct lineage with retinal endothelial cells during active angiogenesis in vitro. Human EPCs from peripheral blood were cultured for 1 week and after DiI-labeling were added to confluent monolayers of bovine retinal vascular endothelial cells (RVEC). In separate experiments, EPCs from 1- and 3-week cultures were added to the secondary layer of a duplex Matrigel model of sprouting angiogenesis. After 48 h the cultures were stained with Alexa-488-B4 isolectin. Some monolayers were stained with anti-ZO1 to outline the tight junctions. Confocal microscopy revealed that DiI-labeled EPCs crossed the monolayers but most showed

little physical contact with the overlying endothelium. EPCs only occasionally incorporated into the monolayer. One-week EPCs stimulated profuse angiogenic sprouting from RVEC networks yet despite close association with the sprouts, no incorporation was observed. In contrast, EPC from 3-week cultures stimulated angiogenesis and incorporated within the tip cells. EPCs could be seen to communicate directly with RVEC via nanotubes over 30 μm long. This study has revealed that EPCs from both short- and long-term cultures are able to stimulate retinal angiogenesis *in vitro* but only those from long-term cultures show significant incorporation in vascular structures.

OC16

VEGF_{165b} INHIBITS DEVELOPMENT OF MAMMARY TISSUE IN VEGF_{165b} OVEREXPRESSION MICE DUE TO INHIBITION OF PHYSIOLOGICAL ANGIOGENESIS

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The postnatal life cycle of the mammary gland involves dramatic morphological and functional changes reflecting its role in providing nourishment for the young. These changes are angiogenesis dependent. VEGF plays a crucial role in physiological angiogenesis. Alternative exon splicing of the VEGF pre-mRNA results in different isoforms. Of these, VEGF_{165b} inhibits the angiogenic properties of VEGF₁₆₅. The roles of VEGF_{165b} on physiological angiogenesis and the development of mammary tissue in transgenic mice specifically overexpressing VEGF_{165b} in mammary epithelial cells were investigated. Both transgenic females (MVTg) and wild-type (WT) littermate controls were allowed to mate. Fate and size of litters and mammary tissue from both groups were compared. Two lines of mice were generated that contain the VEGF_{165b} transgene. Mice were viable and fertile. Most of the litters from female MVTg mating with male WT mice died within 1–2 days after birth due to lack of maternal milk (70% in MVTg vs. 18% in WT, $p < .05$). Pups from MVTg father and WT mother were phenotypically normal. In lactating female transgenic mice, the mammary tissue was smaller, and less erythematous than that in the WT. Also the litter size was smaller from MVTg mothers (3.33 ± 0.44 from MVTg vs. 5.78 ± 0.70 from WT, $p < .01$).

In summary, VEGF_{165b} overexpression inhibits mammary tissue development in MVTg possibly due to the inhibition of angiogenesis.

OC17

LONGITUDINAL ASSESSMENT OF MATERNAL ENDOTHELIAL FUNCTION AND MARKERS OF INFLAMMATION AND PLACENTAL FUNCTION THROUGHOUT PREGNANCY IN LEAN AND OBESE MOTHERS

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Our aim was to measure microvascular endothelial function in lean and obese pregnant women at intervals throughout their pregnancy and at 4 months after delivery. Plasma markers of endothelial function, inflammation, and placental function and their association with microvascular function were also assessed. Women in the first trimester of pregnancy were recruited, 30 with a body mass index (BMI) $< 30 \text{ kg/m}^2$ and 30 with a BMI $\geq 30 \text{ kg/m}^2$. *In vivo* endothelial-dependent and independent microvascular function was measured using laser Doppler at each timepoint. Plasma markers of endothelial activation (sICAM-1, sVCAM-1, vWF, and PAI-1), inflammation (IL-6, TNF α , C-reactive protein, and IL-10), and placental function (PAI-1/PAI-2 ratio) were also assessed. Endothelial-dependent vasodilation was significantly lower ($p < .05$) in the obese women at each trimester. At post partum the improvement in endothelial-dependent vasodilation persisted in the lean women but declined to near first-trimester levels in the obese (lean/obese difference 115%; $p < .01$). There was a small but significant difference in endothelial-independent vasodilation between the two groups, lean response being greater than obese ($p = 0.021$). In the first-trimester obese women had a significantly higher PAI-1/PAI-2 ratio (obese median [IQ range] 0.87 (0.54–1.21) vs. lean 0.30 [0.21–0.47], $p < .001$) reflecting the lower PAI-2 levels in obese pregnant women. Obese mothers have a lower endothelium-dependent and -independent vasodilation when compared to lean counterparts. There was a higher PAI-1/PAI-2 ratio in the first trimester in obese women, which improved later in pregnancy. Obese pregnancy is associated with chronic preexisting endothelial activation and impairment of endothelial function secondary to increased production of inflammatory Th2 cytokines.

OC18

ANGIOSTATIN (AS) IS ASSOCIATED WITH PERTURBED PLACENTAL VASCULAR GROWTH AND FETAL GROWTH RESTRICTION (FGR)

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Angiostatin_{4,5} (AS_{4,5}) is a 52-kDa proteolytic cleavage product of plasminogen that is liberated at the cell surface and is potently anti-angiogenic. We hypothesized that AS is capable of inducing FGR. Stereological analysis of placenta from *murine*

dams treated with AS_{4,5}, showed clear dose-dependent effects; at 20 mg/kg there was a marked reduction in fetal vascular volumes (38.4 m at 0 mg vs. 11.2 m at 20 mg) and the proportion of maternal blood vessels in the decidual layer, whereas the maternal blood vessels within the labyrinthine layer increased in length (30.5 m at 0 mg vs. 57.9 m at 20 mg) but showed a significant decrease in vessel diameter (20.9 μ m at 0 mg vs. 13.1 μ m at 20 mg). Fetuses showed clear evidence of FGR, including both retarded skeletal development and edema. In vitro studies also showed a dose-dependent effect of AS_{4,5} on apoptosis in two (1st and 3rd trimester) human trophoblast cell lines. Finally, dual immunostaining with β -actin, plasminogen or uPAR revealed an FGR-specific pattern of expression in human placenta of β -actin/uPAR. Taken together, these results provide strong evidence that AS causes a reproducible model of FGR and is involved in the pathogenesis of the human condition.

OC19

SUBPODOCYTE SPACES RESTRICT FLUID AND SOLUTE MOVEMENT ACROSS THE GLOMERULAR CAPILLARY WALL

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Subpodocyte spaces (SPS), labyrinthine regions between podocyte cell bodies/processes and the traditional glomerular filtration barrier (GFB), cover 50–65% of the outer GFB aspect (JASN 2005; 16: 1223–1235). To examine the role of SPS in glomerular permeability, single glomeruli ($n = 3$) were dissected from rabbit renal cortex, and the afferent arteriole was perfused with cell dyes (Hoechst 33342 & TMA-DPH), and solutes rhodamine-conjugated 10-kDa dextran and lucifer yellow (≈ 450 Da). The rate of change of solute fluorescence intensity [dI_f/dt] was examined in capillary lumina {[dI_f/dt]^{lumen}} and in regions of the capillary wall with {[dI_f/dt]^{covered}} and without {[dI_f/dt]^{naked}} abluminal cell cover (representing SPS-covered and naked GFB regions, respectively). The 10-kDa dextran-rhodamine flux, but not that of lucifer yellow, was significantly hindered in SPS-covered GFB regions, as compared with naked GFB regions [$d(\text{dextran } I_f)/dt$]^{covered} - [$d(\text{dextran } I_f)/dt$]^{lumen} = 0.74 ± 0.12 units s^{-1} , $p < .0001$, one-sample t test vs zero; all other indices $p > .05$ for one-sample t tests vs. zero]. Lucifer yellow flux (\sim fluid flux) through SPS was calculated to be 54–75% of that through naked GFB regions. These results indicate that SPS, downstream of the traditional GFB, are a novel GFB layer that restrict both solute and solvent flux, necessitating re-evaluation of previous GFB permeability characteristic estimates.

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OC20

HUMAN GLOMERULAR ENDOTHELIAL GLYCOCALYX CONTRIBUTES TO THE PERMEABILITY BARRIER TO PROTEIN IN VITRO AND IS DISRUPTED BY HUMAN HEPARANASE

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Glycocalyx coats the luminal surface of the glomerular capillaries and is composed of proteoglycans (PG). PG consist of a core protein and glycosaminoglycan chains. Human heparanase (HPSE-1) degrades heparan sulfate (HS) glycosaminoglycans and is upregulated in proteinuric states. In this study, we define the structure of the human glomerular endothelial (GEnC) glycocalyx, and examine its functional relevance. We used conditionally immortalized GEnC developed in our laboratory. The ultrastructure of glycocalyx was analyzed by electron microscopy (EM) and confocal microscopy using fluorescein-wheat germ agglutinin (WGA) lectin. Removal of glycocalyx by neuraminidase, heparinase III, and HPSE-1 was assessed using WGA and an anti-HS antibody. Barrier properties of GEnC monolayers were studied by trans-endothelial electrical resistance (TEER) and passage of labeled albumin. EM revealed a 200-nm-thick glycocalyx covering the GEnC, which was further demonstrated by WGA. Neuraminidase removed the majority of glycocalyx, which functionally corresponded to reduced TEER by 59% and increased albumin flux by 207%. Heparinase III and HPSE-1 cleaved HS: this caused no change in TEER, but increased the albumin passage across the monolayers by 40 and 39%, respectively. We have characterized the GEnC glycocalyx and shown that it contributes to the barrier to passage of albumin in vitro. These results signify the importance of GEnC glycocalyx in restriction of glomerular protein passage in vivo and suggest ways that HPSE-1 levels may be linked to proteinuria in clinical disease.

OC21

ULTRAFILTRATION COEFFICIENT IN ISOLATED INTACT GLOMERULI FROM PODOCYTE-SPECIFIC VEGF_{165b} OVEREXPRESSING TRANSGENIC MICE

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Vascular endothelial growth factor (VEGF) is expressed by podocytes within the glomerular filtration barrier and has been suggested to contribute to the high permeability of glomeruli to water. (Salmon J. (2006). *Physiology* 570:141–156). Differential splicing of the VEGF gene forms two families of isoforms: the pro-angiogenic family (VEGF_{xxx}), and the anti-angiogenic families (VEGF_{xxx}b). VEGF_{165b} has been shown to transiently (but not chronically) increase hydraulic permeability in individually perfused microvessels in the mesentery of frogs and rats (Glass J. (2006). *Physiology*

572:243–257). To determine the effects of VEGF_{165b} on glomerular permeability to water, transgenic mice overexpressing VEGF_{165b} under nephrin promoter (confined to podocytes) were established and the normalized ultrafiltration coefficient (L_pA/V_i) was measured in individual glomeruli. Three heterozygote nephrin-VEGF_{165b} C57/BL6 mice lines were produced. Glomerular L_pA/V_i ($\text{min}^{-1} \text{mmHg}^{-1}$) was not significantly different between lines (1.44 ± 0.20 , $n = 4$; 1.43 ± 0.19 , $n = 8$; 1.45 ± 0.18 , $n = 6 \text{ min}^{-1} \text{mmHg}^{-1}$; ANOVA, $p > .05$) allowing incorporation into one group. VEGF_{165b} overexpression reduced glomerular L_pA/V_i compared to littermate controls (heterozygous 1.44 ± 0.11 , $n = 18$; controls 1.93 ± 0.16 , $n = 8$; t test, $p = .0169$). These results suggest that altering the balance of VEGF_{xxx}/VEGF_{xxx}b in podocytes may alter glomerular permeability and have therapeutic potential.

Supported by the BHF

OC22

Ca²⁺-ACTIVATED Cl⁻ CURRENT IN RETINAL ARTERIOLAR SMOOTH MUSCLE

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The goal of the present study was to characterize the properties of the Ca²⁺-activated Cl⁻ current ($I_{Cl,Ca}$) in rat retinal vascular smooth muscle cells (VSMCs) using the amphotericin B perforated patch clamp technique. K⁺ currents were blocked using Cs-based patch solutions. Depolarizing voltage steps to -40 mV and greater activated a slowly developing current, and on stepping back to -80 mV large tail currents were observed. The slowly developing current and tail currents were reduced by $\sim 75\%$ following removal of extracellular Ca²⁺. The tail currents reversed close to 0 mV in symmetrical Cl⁻ concentrations. Substitution of 86 mM Cl⁻ with equimolar I⁻ or gluconate resulted in reversal potentials shifting by -23 ± 2.2 mV and $+16.2 \pm 1.3$ mV, respectively. Outward tail currents were sensitive to the Cl⁻ channel inhibitors 9-AC, DIDS and SITS (1 mM), but only DIDS produced a substantial ($>80\%$) block of inward tail currents. Addition of 10 mM caffeine produced large transient currents that reversed close to E_{Cl} and were blocked by 1 mM DIDS or 100 μM tetracaine. In current-clamp mode, 1 mM DIDS hyperpolarized the cell membrane potential from -41.3 ± 0.7 mV to -49 ± 1.3 mV. Application of 10 nM endothelin-1 in current-clamp mode induced a slow depolarization upon which DIDS-sensitive transient depolarizations were superimposed. We conclude that rat retinal VSMCs express Ca²⁺-activated Cl⁻ channels, which appear to be involved in setting the resting membrane potential. These channels may play an important role in mediating microvascular responses to endothelial-derived agents.

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OC23

ULTRASOUND IMAGING OF THE POPLITEAL VEIN DURING SYMPATHETIC ACTIVATION IN MEN

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Venous tone is modulated by sympathetic activity, but in humans active venoconstriction is usually observed in hand veins or inferred from whole limb volume changes. We compared calf volume measurements (strain gauge plethysmography) with direct measures of popliteal (deep) vein diameter (Doppler ultrasound), during sympathoexcitation in 8 male subjects (19 ± 1 yrs, BMI 24 ± 3 , mean \pm SD). Venous distension was induced 3 times by cuff inflation (50 mmHg, 6 min), one control (CON) and two with either isometric leg exercise (ILE) or mental stress test (MST) intervention between minutes 3 and 5. Calf volumes after 3 min distension were 4.5 ± 0.6 , 4.6 ± 0.6 , and $4.4 \pm 0.6 \text{ mL } 100 \text{ mL}^{-1}$ (mean \pm SEM) for CON, ILE, and MST. ILE and MST increased mean arterial pressure ($+16 \pm 6$, $+11 \pm 3 \text{ mmHg}$) and heart rate ($+20 \pm 4$, $+12 \pm 3 \text{ bpm}$) vs. CON ($+1 \pm 1 \text{ mmHg}$, $+1 \pm 1 \text{ bpm}$). During minutes 3–5, calf volume increased by $2.0 \pm 0.6\%$ in CON and $2.6 \pm 1.3\%$ with MST, due to fluid filtration. With ILE, calf volume decreased by $9.5 \pm 6.3\%$ ($p < .05$ vs. CON, MST), indicating active venoconstriction. Popliteal vein diameters after 3 min distension were 10.1 ± 1.1 , 9.6 ± 0.7 , and $10.0 \pm 1.0 \text{ mm}$ for CON, ILE, and MST. Vein diameter changes during ILE and MST ($+2.1 \pm 2.2$, $+3.0 \pm 1.6\%$) or CON ($-0.4 \pm 3.0\%$) were not significant. We conclude that the popliteal vein does not venoconstrict to sympathetic activation by exercise or mental stress. The reduced calf venous volume during exercise could represent superficial venous constriction or a contribution from smaller venous vessels included within the calf volume measurement.

OC24

FLOW MEDIATED DILATION IN SLE: ALTERED MICROVASCULAR HEMODYNAMICS OR LOCAL ENDOTHELIAL DYSFUNCTION?

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Impaired flow mediated dilation (FMD), a marker of endothelial dysfunction, occurs in disease states associated with atherosclerosis, including SLE. The primary hemodynamic determinant of FMD is wall shear stress, which is critically dependent on the arterial mechanics of the forearm microcirculation. We measured FMD and diastolic shear stress (DSS), and interrogated changes in the Doppler velocity waveform in 60 SLE patients and 32 age-matched controls. FMD performed as per guidelines (Corretti, M.C., et al. (2002). JACC

39:257–265). DSS measured as described (Mitchell, G.F., et al. (2004). *Hypertension* 44:134–139). Pulsed Doppler velocity waveforms during reactive hyperemia were analyzed for resistive index (RI), and interrogated using eigenvector decomposition. No significant difference in age, BMI, BP, fasting lipids, or glucose was observed between groups. FMD was reduced in SLE patients (median (range) 2.6% (-2.1–8.8%) vs. 7.3% (1.9–14.3%); $p < .001$). DSS (dyne/cm²) was reduced in SLE patients (median (range) 18.3 (3.9–39.1) vs. 23.0 (12.5–43.2); $p = .001$). Strong correlation between FMD and DSS, $r_s = 0.72$, $p < .001$. Significant differences in the lower-frequency sinusoidal components of the Doppler velocity waveforms ($p < .05$) during reactive hyperemia between groups but no difference in RI. In SLE patients, the impairment in FMD may be due to altered microvascular hemodynamics in the forearm and hence a reduction in stimulus for dilation rather than solely a local abnormality in brachial artery endothelial function.

OC25

DIABETES ALTERS THE MOLECULAR COMPOSITION OF BK CHANNELS IN RETINAL ARTERIOLAR SMOOTH MUSCLE

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Retinal vasoconstriction and reduced retinal blood flow signify the onset of diabetic retinopathy. The pathophysiological mechanisms that underlie increased retinal arteriolar tone during diabetes remain unclear. We have previously reported that diabetes reduces the coupling strength between Ca²⁺ release from the endoplasmic reticulum and activation of large conductance Ca²⁺-activated K⁺ (BK) channels in retinal vascular smooth muscle cells (VSMCs). Here we have investigated this pathology further. Spontaneous transient BK currents were evident at membrane potentials positive to -40 mV, but integrated current densities were considerably smaller in VSMCs from streptozotocin-induced diabetic rats (3-month disease duration; 0.55 ± 0.14 pC/pF/s in nondiabetics vs. 0.05 ± 0.02 pC/pF/s in diabetics at 0 mV). The peak amplitude of Ca²⁺ sparks was substantially larger in diabetic than in nondiabetic VSMCs (F/F₀: 0.92 ± 0.06 and 0.42 ± 0.03 , respectively), but the frequency and duration remained unchanged. The sensitivity of single BK channels to Ca²⁺ was markedly reduced in diabetic myocytes. The expression of the BK β 1, but not the BK α -subunit, was decreased in diabetic retinal arterioles. Consistent with this, the mean open times and the sensitivity of BK channels to 1 μ M tamoxifen were decreased in diabetic cells. We conclude that diabetes downregulates the expression of the BK β 1 subunit and consequently decreases Ca²⁺-dependent activity of BK channels in retinal VSMCs. Changes in the molecular composition of BK channels may contribute to retinal hypoperfusion in early diabetes.

Supported by the JDRF, The Wellcome Trust, and Fight for Sight

OC26

ADVANCED GLYCATION END PRODUCTS ACTIVATE THE TRANSCRIPTION FACTOR NRF2 AND INDUCE HEME OXYGENASE-1 EXPRESSION IN BOVINE AORTIC ENDOTHELIAL CELLS

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Hyperglycemia and diabetes are associated with increased formation of advanced glycation end products (AGE) and cell dysfunction arising from enhanced oxidative injury leading to the progression of diabetic vascular pathologies (Goldin et al. (2006). *Circulation* 114:597–605). The transcription factor Nrf2 mediates the induction of cytoprotective genes such as heme oxygenase-1 (HO-1) via activation of antioxidant response elements (ARE). HO-1 catabolizes the pro-oxidant heme to generate the vasodilator carbon monoxide and antioxidants biliverdin and bilirubin (Siow et al. (2007). *Redox Rep* 12:11–15). The present study has investigated whether AGE enhances superoxide generation measured by chemiluminescence, Nrf2 activation and induction of HO-1 protein expression assessed by immunofluorescence and western blot analyses in bovine aortic endothelial cells (BAEC), cultured in medium containing 5 mM glucose. Treatment of BAEC with AGE (0–100 μ g/mL, 0–24 h), but not unmodified bovine serum albumin, in medium containing 1% fetal calf serum, elicited a significantly enhanced generation of superoxide, nuclear translocation of Nrf2, and induction of HO-1 expression in a dose- and time-dependent manner. Our findings suggest that AGE may facilitate induction of adaptive antioxidant genes via the Nrf2/ARE pathway in endothelial cells and that enhanced generation of superoxide may contribute to altered cellular signaling and vascular dysfunction in diabetes.

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OC27

MUSCLE MICROVASCULAR FILTRATION CAPACITY AND BLOOD FLOW IN METABOLIC SYNDROME

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Metabolic syndrome is a combination of risk factors that increase risk of cardiovascular disease and diabetes. This study explores the relation between muscle microvascular filtration capacity (Kf), changes in resting blood flow (Qa) and metabolic syndrome-associated risk factors. Thirty volunteers (age 37–67 y; 11 male) with two or more features of the metabolic syndrome (central obesity, dyslipidemia, high blood pressure, and dysglycemia) were studied. All participants gave written informed consent. Kf and Qa were measured in the calf using noninvasive strain gauge plethysmography (Filtrass Angio, Compumedics. dwl, Germany) using a small

cumulative pressure step protocol. Kf was derived from the slope of calf volume change (Jv) vs. cuff pressure (Pcuff) and isovolumetric venous pressure (Pvi) from its intercept (Gamble et al. (1993). *J Physiol* 464:407–442). Insulin sensitivity (measured during insulin clamp) was positively associated with Kf ($3.79 \pm 0.26 \times 10^{-3}$ mL/100 mL/min/mmHg; mean \pm SD) and inversely with Pvi (19.6 ± 8.7 mmHg). Resting Qa (3.02 ± 1.7 mL/100 mL/min) fell as Pcuff was raised. The slope of the relationship (summary measure, SM -1.45 ± 0.35) was significantly greater than that that predicted using Darcy's equation (-1.07 ± 0.11 ; $p < .001$) and qualitatively similar to that found in patients with preeclampsia (Anim et al. (2001). *Cardiovasc Res* 50:603–609) and endothelial cell dysfunction. These preliminary results suggest an attenuation in the capacity for blood/tissue exchange and vasomotor control in these individuals. They further confirm that venous occlusion plethysmography is a sensitive tool with which to investigate endothelial dysfunction in this patient group.

Poster Communications:

PC1

CELLULAR AND MOLECULAR EFFECTS OF COMBRETASTATINS A1/A4 IN HYPOXIA AND NORMOXIA

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The combretastatin family are a group of vascular disrupting agents that target endothelial microtubules. Preclinically combretastatin A1 phosphate (CA-1-P) has shown greater efficacy than combretastatin A4 phosphate (CA-4-P) in vivo. We previously demonstrated the involvement of the stress activated protein kinase P38 pathway in the remodeling of the cytoskeleton and CA-4-P mechanism of action. Our studies aim to identify and compare the cellular and molecular effects of CA-4-P and CA-1-P (supplied by G.R. Pettit, Arizona State University). Due to heterogeneity of tumor oxygenation, studies were carried out in normoxia and hypoxia (0.1% oxygen). Hypoxia was confirmed by increased HIF-1 α expression in human umbilical vein endothelial cells (HUVECs). An increase in phosphorylation of heat shock protein-27 (pHSP27) and P38 (pP38) was identified following exposure to CA-1-P and CA-4-P in normoxia. CA-4-P induced greater pP38 and pHSP27 than CA-1-P. Reactive oxygen species (ROS) scavengers inhibited pHSP27 but not pP38. In hypoxia, a pP38-dependent increase in pHSP27 by CA-4-P was observed. Using carboxy-H₂DCFDA, we showed significant ROS production by CA-4-P/CA-1-P in HUVECs in normoxia. CA-4-P induced more extensive microtubule disruption and actin remodeling (membrane blebbing/stress fibres) than CA-1-P in both normoxia and hypoxia. In conclusion, we show CA-4-P to be more active than CA-1-P in vitro, and demonstrate the involvement of pHSP27 and ROS in mediating in vitro cytoskeletal remodeling.

Supported by Cancer Research UK

PC2

THE EFFECT OF ANGIOPOIETINS ON VASCULAR MATURATION IN TUMORS

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Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are important growth factors involved in vascular maturation, acting via the endothelial cell-specific receptor Tie-2. The immature phenotype of the tumor vasculature relates to susceptibility to vascular targeting drugs such as combretastatin (CA-4-P), a tubulin-binding microtubule depolymerizing agent. We investigated the role of angiopoietins in tumor response to CA-4-P in vivo. SW1222 human colorectal carcinoma cells were transfected with Ang-1 or Ang-2 cDNA, or with empty vector. Cell aggregates of stable transfectants were transplanted into window chamber-bearing mice, under Hypnorm and midazolam anesthesia, for intravital microscopy. Ang-1 tumors grew more slowly than Ang-2 or wild-type (wt) tumors. All tumors initially caused hemorrhage. However, with time, Ang-1 and wt tumors formed more stable and functional vessels than Ang-2 tumors, which contained extremely dense networks of leaky, poorly organized, and fragile vessels. Established Ang-2 tumors frequently lacked blood flow in central areas. All tumors were relatively insensitive to CA-4-P but Ang1 tumor vessels constricted the most, which may relate to presence of pericytes (studies in progress). There was significant vascular remodeling within 24 h of a single 30-mg/kg dose in Ang-2 and wt tumors but not Ang1 tumors. In conclusion, Ang1 stabilized tumor blood vessels and modified their response to CA-4-P.

Supported by Cancer Research UK

PC3

TIE-2 RECEPTOR EXPRESSION IN NORMAL, PREMALIGNANT, AND MALIGNANT BREAST CANCER IN VITRO

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Tie receptors elicit responses involved in cell survival, proliferation, and differentiation. Tie-2 acts as an endothelial cell surface receptor for the angiopoietins, and is overexpressed on invasive breast cancer endothelium. The aim of this study is to characterize Tie-2 expression in human normal, premalignant, and malignant breast cells and tissue in vitro. Western blot and real-time PCR analysis were performed on breast cell lines representing normal breast (MCF10A), atypical ductal hyperplasia (ADH; MCF10AT), ductal carcinoma in situ (DCIS; MCF10DCIS.com), breast cancer (MCF7, T47D, MDAMB436), and human breast microvascular endothelial cells (HuDMEC). Similarly, 114 surgical specimens consisting of normal breast tissue ($n = 6$), usual hyperplasia ($n = 25$), ADH ($n = 19$), and DCIS ($n = 64$)

were immunohistochemically stained and semi-quantitatively graded for Tie-2 expression. Significantly higher levels of Tie-2 mRNA and protein were observed in HuDMECs compared to preinvasive and cancer cell lines ($p < .001$). In tissue there was no significant increase of Tie-2 endothelial expression in breast lesions compared to normal breast tissue endothelium. Epithelial derived Tie-2 was significantly increased in all stages of preinvasive breast lesions compared to normal breast tissue epithelial cells ($p = .046$). These data confirm previous studies that Tie-2 receptors are present on endothelial cells, maintaining vessel quiescence in normal tissue. Upregulation of Tie-2 on premalignant breast epithelium may reflect a role for Tie-2 in carcinogenesis as well as angiogenesis.

PC4

ANGIOGENESIS DURING PREMALIGNANT STAGES OF BREAST CANCER IS DRIVEN BY HYPOXIA INDUCIBLE FACTOR 1 ALPHA

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Angiogenesis is essential for the growth and progression of tumors. Studies have also shown that angiogenesis is increased before the onset of cancer. Breast cancer is preceded by hyperplastic and in situ disease states. This study investigated the angiogenic activity and the molecular factors (hypoxia inducible factor 1 alpha [HIF-1 α] and vascular endothelial growth factor [VEGF]) potentially involved during the premalignant stages of breast cancer. Immunohistochemical staining was performed on sections of 6 normal breast specimens and 97 premalignant breast lesions (33 benign proliferative lesions, 54 non-invasive malignancies [ductal carcinoma in situ]) for the presence of HIF-1 α and VEGF in the tumor cells. Angiogenic activity was quantified using microvessel density (MVD) based on CD31 staining. Twenty-one of the 43 (48%) benign lesions and 38 of the 54 (70%) noninvasive cancer lesions were positive for HIF-1 α with a statistically significant difference ($p < .05$, χ^2 test) between the groups while no normal specimen was positive. All sections stained positive for VEGF. A significant correlation was found between the expression of HIF-1 α and MVD ($\rho = .664$, $p < .05$), but no correlation was noted between HIF-1 α and VEGF or VEGF and MVD. Increased angiogenesis in the premalignant stages of breast disease may be associated with increased expression of HIF-1 α ; however, factors other than VEGF may be responsible for this.

PC5

ANGIOGENESIS AND LYMPHANGIOGENESIS IN UROLOGICAL CANCERS

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This study was undertaken to evaluate the clinical usefulness of quantifying angiogenesis-associated markers CD105, TGF β -1, and TGF β -3 and the ligand receptor complexes in the plasma of patients with different types of urological cancer. ELISA procedures were developed to quantify the above markers in the plasma of patients with cancers of prostate ($n = 88$), kidney ($n = 29$), and bladder ($n = 55$). The data were analyzed to determine their prognostic value; none correlated with prognosis. Since carcinomas mainly metastasize through lymphatic vessels, an ELISA procedure was developed and utilized to quantify the levels of the lymphangiogenic factor vascular endothelial growth factor-C (VEGF-C) in the plasma of patients with urological cancers. The results showed that pretreatment levels of VEGF-C are correlated with shorter survival rates in patients with prostate, but not in those with renal or bladder cancers ($p < .001$; Spearman's rank).

PC6

ANGIOGENESIS AND LYMPHANGIOGENESIS IN HUMAN HEMATOLOGICAL MALIGNANCIES

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Angiogenesis is an important prognostic marker in solid human tumors, but little is known about its relevance in hematological malignancies. For this study, plasma samples from 176 children with newly diagnosed leukemia comprising common acute lymphoblastic leukemia (cALL; $n = 73$), acute myeloid leukemia (AML; $n = 24$), T-cell acute lymphoblastic leukemia (T-cell ALL; $n = 13$) and with various other types of leukemia and lymphoma ($n = 66$) were examined to quantify a panel of angiogenesis and lymphangiogenesis markers. These were CD105, its two ligands (TGF- β 1, TGF- β 3), receptor-ligand complexes (CD105/TGF- β 1, CD105/TGF- β 3) and vascular endothelial growth factor-C (VEGF-C), a marker of lymphangiogenesis. Plasma samples from normal children ($n = 79$) were used as controls. Analysis of data using ELISAs showed that median levels of CD105 (315 ng/mL, range 0–630), TGF- β 3 (127 pg/mL, range 0–1900), CD105/TGF- β 1 (31.7 units/mL, range 0–300), CD105/TGF- β 3 (52.5 units/mL, range 0–1395) complexes, and VEGF-C (16.4 ng/mL, range 0–102.5) were statistically significantly higher in patients with cALL compared to controls (1.83 ng/mL, range 0–82.4; 5.2 pg/mL, range 0–234.6; 0.33 units/mL, range 0–25.9; 0.0 units/mL and 2.1 ng/mL, range 0–25), respectively ($p < .0001$ in all cases, Mann-Whitney U test). Similarly, median values for TGF- β 1 were higher in same patients (362.1 pg/mL, range 0–2301) compared to controls (136.4 ng/mL, range 0–843.4) ($p < .03$). There were no significant correlations between the total white blood cell counts and levels of CD105, TGF- β 1, TGF- β 3, CD105/TGF- β 1, CD105/TGF- β 3, or VEGF-C.

PC7**IN VITRO EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND ITS RECEPTORS IN PREMALIGNANT AND MALIGNANT BREAST CELLS**

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Vascular endothelial growth factor (VEGF) is the most potent primary stimulator of angiogenesis identified to date and correlates with angiogenesis in breast cancer. VEGF mediates its effects by binding to receptor kinases, including VEGF-R1 and R2, and the recently identified neuropilins NRP-1 and NRP-2. As angiogenesis is initiated in premalignant lesions of the breast this study aims to evaluate the expression of VEGF, VEGF-R1, VEGF-R2, NRP-1, and NRP-2 in premalignant and malignant breast cells as well as in endothelial cells in vitro. Immunocytochemistry and Western blot analysis were performed on breast cell lines representing normal breast (MCF-10A), atypical ductal hyperplasia (MCF-10AT), DCIS (MCF-10DICS), low metastatic breast cancer (MCF-7 and T47D), high metastatic breast cancer (MDA-MB-436) and dermal microvascular endothelial cells extracted from human breast skin (HuDMEC). VEGF expression was detected in all cells, with an increase in expression in the malignant and endothelial cells compared with the normal and premalignant cells (MCF-10A and MCF-10AT). NRP-1 expression was shown at similar levels in all cell lines. In contrast NRP-2 expression showed a 2-fold increased expression in the premalignant cells compared to the malignant and endothelial cells. The receptor kinases, VEGF-R1 and R-2 were identified only in the endothelial cells. These data suggest that as well as a role in angiogenesis, the neuropilins may play a role in the progression of breast disease from a premalignant to a malignant state.

PC8**THE RATIO OF ANGIOGENIC TO ANTI-ANGIOGENIC VEGF ISOFORMS IS INCREASED IN METASTATIC MELANOMA**

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The anti-angiogenic VEGF_{xxx}b family of VEGF-A splice variants are significantly down regulated in primary melanomas that went on to metastasize compared with those that did not (Dunn et al. (2006). *Microcirculation* 13:519). To determine whether the ratio of total VEGF:VEGF_{xxx}b differed in metastatic/non-metastatic melanomas we stained the melanomas with a VEGF_{xxx}b-specific antibody (MAB3045), a PAN VEGF antibody (MAB293, both R & D systems), or control IgG on archival tissue of 9 primary metastatic (MM) and 7 non-metastatic (NM) melanomas, matched for Breslow

thickness, with not less than 5 years clinical history (PFA-fixed, paraffin-embedded sections). Sections were scored by 3 assessors (blinded to clinical outcome) for intensity of staining (1–5) of the melanoma in vertical and horizontal growth. Data were analyzed by standardizing expression in vertical and horizontal growth phases (VGP and HGP) to that of the surrounding normal epidermis and then using the resulting ratios to compare staining of PAN VEGF:VEGF_{xxx}b antibodies for each section. The ratio was significantly greater in the MM than in the NM in both the VGP (2.66 ± 0.37 MM, 1.3 ± 0.16 NM, $p < .01$, Bonferroni test) and the HGP (2.81 ± 0.33 MM, 1.57 ± 0.12 NM, $p < .05$, Bonferroni test). MM ratios were significantly different than those of normal skin ($p < .01$, Bonferroni test) but NM ratios were not. These results indicate that metastatic melanoma has a more angiogenic VEGF isoform profile than nonmetastatic melanomas.

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PC9**PREDICTING MELANOMA METASTASIS**

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Malignant melanoma is the fastest growing cancer in the United Kingdom, causing almost 1800 deaths a year. Metastatic deposits rather than the primary lesion cause death and initial metastatic spread is through the lymphatic system. At present there is no reliable prognostic indicator for metastasis. Breslow thickness is the main determinant of prognosis, but 15% of patients with a thin melanoma, and therefore not at high risk of dissemination, still develop metastases (White et al., 2002). In 2004, Shields et al. described a new prognostic index including lymphatic vessel density, lymphatic vessel invasion and Breslow thickness (Shields et al. (2004). *Br J Cancer* 90:693). To investigate the clinical value of these indicators we compared areas under the Receiver Operating Characteristic Curves (AUC). AUCs range from zero to one, where one is obtained for an indicator that is able to discriminate perfectly between cases and controls (100% sensitivity, 100% specificity). In a retrospective cohort of 35 patients, the Shields Index achieved an AUC of one. The cohort provided evidence of the superiority of the Shields Index compared to Breslow thickness alone (AUC 0.55, $p < .01$; test from Hanley et al., 1983), to lymphatic density (AUC 0.79 $p < .05$) and to AJCC staging (AUC 0.45). The AUC for Breslow thickness in a larger population of 4500 patients in the southwest was 0.57, suggesting that it is a representative sampling. These results suggest that the Shields Index is a significantly more reliable prognostic indicator than Breslow thickness, lymphatic vessel density, or AJCC staging.

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PC10**DEVELOPMENT OF A DORSAL SKINFOLD CHAMBER (DSC) METATARSAL MODEL FOR STUDY OF MICROMETASTASES IN VIVO**K. J. Reeves,^{1,2} C. Eaton,¹ F. Hamdy,¹ N. J. Brown,²¹Academic Unit of Urology and ²Academic Unit of Surgical Oncology, Section of Oncology, University of Sheffield, Sheffield, UK

Early diagnosis of prostate cancer has resulted in the large majority of patients presenting at a stage prior to the development of widespread metastasis. Intervention to prevent metastasis would be the most significant advance for the majority of patients. Understanding the timescales and processes involved in the initiation of metastases is important if this is to be achieved. Cellular imaging in vivo will be conducted using the mouse DSC model whereby II-IV metatarsals from newborn/embryonic mice are implanted into the DSC of adult mice (25 g). Metatarsals from these animals have sufficient transparency to allow the visualization of tumor cells as they arrive and establish lesions within bones. This is being done using high-resolution in vivo microscopy in real time. We have demonstrated that metatarsals are rapidly revascularized by inosculation the host vasculature by day 5 after engraftment. We are now at a stage to introduce and track GFP-labeled prostate cancer (PC3-GFP) cells in the model. This approach will allow structural interactions between the tumor and bone marrow cell populations to be studied in detail, as well as permitting the effects of manipulation of genes/processes to be observed in vivo.

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PC11**A BIOLOGICALLY ACCURATE IN VIVO MODEL FOR EARLY DEVELOPMENT OF COLORECTAL LIVER METASTASIS**

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Globally colorectal cancer accounts for approximately one million new diagnoses and half a million deaths per year. Optimal management of disseminated disease requires therapies targeting multiple stages in colorectal metastasis development. To facilitate this, biologically accurate in vivo models are required. Using different cancer cell line–host combinations, early colonic cancer liver metastasis development was studied. Two rat strains (BDIX and SD) and two colonic cancer cell lines (human HT29 and rat DHDK12—chemically induced previously in BDIX rat) were used. Using real-time intravital microscopy, the early development of liver metastases in 4 groups ($n = 6$) (HT29-BDIX, DHDK12-BDIX, HT29-SD, and DHDK12-SD) were analyzed. Data were compared using one-way ANOVA. Summary measures were used to compare experimental parameters. The total number of tu-

mor cells visualized, adherent and extravasated tumor cells, and migration rates were significantly higher in the DHDK12-BDIX combination ($p < .05$). Maximum number of cells seen 166 cells ($p < .01$) and maximum migration rate 81% ($p < .05$) were significantly higher in the DHDK12-BDIX group. No significant differences were observed in these experimental parameters between the other 3 groups or in the hemodynamic parameters between all groups. In conclusion, cancer cell line–host selection has a significant effect on early colonic cancer liver metastasis development. DHDK12-BDIX provides a highly biologically accurate in vivo model for early analysis of liver metastasis development.

PC12**CXCR4 IS UPREGULATED IN METASTATIC MELANOMA**R. Price,¹ D. B. A. Dunn,² H. Rigby,¹ D. O. Bates²¹Department of Pathology, Frenchay Hospital, Bristol, UK;²Microvascular Research Laboratories, Bristol Heart Institute, Department of Physiology, University of Bristol, Bristol, UK

Chemokines, *chemo*-attractant *cytokines*, attract immune cells to areas of inflammation and secondary immune organs. Recent evidence suggests that they play a role in the trafficking of cancer cells in the development of metastasis. CXCL12 is a chemokine secreted by cell types where distant (blood borne) metastasis form (e.g., lung, bone, liver) in malignant melanoma and other cancers. Its receptor CXCR4 is found on macrophages, lymphocytes, and leucocytes, but not skin cells. CXCR4 activation also upregulates VEGF-A secretion, leading to increased angiogenesis and inflammation (Kakinuma et al. (2006). *J Leukoc Biol* 79: 639–651). Our aim was to determine the relationship between CXCR4 expression in primary melanomas and the development of metastasis, by immunohistochemical staining with a CXCR4-specific antibody (ab2074 Abcam) on archival tissue of 5 normal skin sections and 17 primary metastatic (MM) and 29 nonmetastatic (NM) melanomas, matched for Breslow thickness, with 5 years clinical history (PFA paraffin-embedded sections). Following hematoxylin counterstaining, sections were analyzed by 3 assessors (blinded to clinical outcome) scoring intensity of staining (0–3) of the melanoma. MM expression (1.9 ± 0.15) was significantly greater than NM (1.2 ± 0.12 , $p < .001$, unpaired t test). There was no staining in the normal skin. There was no correlation between thickness of tumor and CXCR4 expression ($p > .05$, Pearson). Upregulation of CXCR4 occurs in melanoma and may be an important marker of metastatic potential at excision of primary tumors.

PC13**RETINAL ANGIOGENESIS INDUCED BY EGF IS MEDIATED BY AUTOCRINE VEGF**

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Epidermal growth factor (EGF) plays a major role in retinal development and is expressed by several types of neurones in adulthood. EGF is pro-angiogenic in many situations but has not been studied in retinal angiogenesis (AG). As normal neurovascular relationships are essential for vascular cell survival, we studied the role of EGF in angiogenesis by retinal vascular endothelial cells (RVEC) in a 3-D duplex Matrigel culture system. RVEC from bovine retina were plated in Matrigel and formed endothelial tubular networks by 24 h. Test substances were added to a second layer of Matrigel and superimposed on the primary culture spots. Angiogenic sprouting from the primary to secondary gel layers were counted after a further 24 h and the mean calculated in 10 spots per group. EGF increased the number of angiogenic sprouts obtained with control Matrigel by +60 to +150%. As the nitric oxide synthase inhibitor L-NAME reduced EGF-induced AG to control values, the downstream signaling from eNOS was investigated with ODQ (inhibitor of soluble guanylate synthase), the stable cGMP analogue 8-bromo-cGMP, and KT5823. To test whether EGF-induced AG was mediated through autocrine VEGF, EGF, and VEGF-induced cultures were treated with an anti-VEGF neutralizing antibody or irrelevant isotype-specific IgG. EGF, VEGF, and each growth factor with irrelevant IgG increased AG to approx+100% of control Matrigel ($p < .0001$), while anti-VEGF reduced both EGF-induced and VEGF-induced AG to control levels. EGF appears to exert its angiogenic on retinal vascular endothelial cells by stimulating the autocrine secretion of VEGF. EGF secretion by retinal neurones can exert pro-angiogenic effects via stimulation of endothelium-derived VEGF.

PC14

THE REGULATOIN OF GTP CYCLOHYDROLASE (GTPCH) EXPRESSION IN ISCHEMIA-INDUCED NEOVASCULARIZATION

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Nitric oxide (NO) derived from inducible nitric oxide synthase (iNOS) has anti-angiogenic effects on retinal angiogenesis following ischemia and exacerbates intravitreal neovascularization (NV). The activity of iNOS is dependent on the presence of GTPCH, the rate limiting enzyme responsible for the production of the NOS co-factor BH4. In the immune system iNOS and GTPCH are transcriptionally co-induced by proinflammatory cytokines such as TNF α . The aim of this study was to determine how TNF α contributes to the regulation of iNOS and GTPCH levels in an animal model of oxygen induced retinopathy (OIR). iNOS $^{-/-}$ and TNF $\alpha^{-/-}$ animals were subjected to OIR by exposure of neonatal mice to 75% oxygen between postnatal days 7 and 12 (P7-P12). The animals were returned to room air at P12 and the retinas removed at P12, P13, and P17 time points. Quantitative RT-PCR was performed and data were normalized to the expression of 18S and 28S ribosomal RNA. Expression of iNOS and peroxynitrite was also analyzed by Western blotting. iNOS mRNA expression was significantly increased (12-fold) in TNF $\alpha^{-/-}$ animals at P13 and

P17; in contrast, GTPCH expression was moderately increased (2-fold) at each of these time points. iNOS immunoreactivity was negligible at P12 but increased significantly at P13 in all groups. These changes coincided with a decrease in the production of peroxynitrite in the TNF $\alpha^{-/-}$ animals compared to controls. We have shown that in TNF α -depleted animals there is a significant stimulation of iNOS expression and a moderate increase in GTPCH expression. Our results suggest that while the expression of iNOS is driven by tissue hypoxia, TNF α may play a role in stimulating maximal GTPCH expression and thus full iNOS activity in the retina.

PC15

LONGITUDINAL ASSESSMENT OF BLOOD VESSEL SPROUTING IN SKELETAL MUSCLE WOUND HEALING

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Wound healing is a dynamic and complex phenomenon, subject to tight temporal regulation. Muscle heals through a repair process that is highly dependent on successful new blood vessel sprouting from preexisting vessels. This study describes the longitudinal angiogenic response in a small healing wound. The model presented here is that of a small, heat-induced injury to the panniculus carnosus skeletal muscle. To permanently expose this tissue, the mice were subjected to a skinfold window chamber implant. This allows for the noninvasive imaging of specific regions of interest over time. By injection of high molecular weight dextran coupled to fluorescein isothiocyanate, actively growing vasculature was imaged using epifluorescence and confocal microscopy techniques in live mice over a period of vessel sprouting (5-7 days postinjury). Parameters such as vessel segment length, vascular density, and widths were measured longitudinally over this period and these measures related to the rate of wound closure over time. On day 0, when the wound is inflicted, there are no blind-ended vessels present and the average wound area is $0.45 \pm 0.22 \text{ mm}^2$ ($n = 6$). Blind-ended vessel length decreases steadily from day 5 up to day 7, as does the wound area. On day 5, long (average length of $114.48 \pm 70.12 \mu\text{m}$) and numerous (more than 25 per region of interest) blind-ended vessels with chaotic directionality are abundant. By day 7, their average length had decreased to $61.15 \pm 48.06 \mu\text{m}$ but their numbers remained fairly constant. The blind-ended vessel width does not follow a linear relationship with time and wound area.

PC16

IMMUNOHISTOCHEMICAL PROFILE OF NEUROFILIN-1 AND -2 EXPRESSION DURING HUMAN DERMAL WOUND HEALING

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Angiogenesis, the formation of new capillaries from existing vasculature, plays an essential role in tissue repair, although it is not well characterized in humans. Vascular endothelial growth factor (VEGF) directly mediates wound vessel angiogenesis through receptor kinases, including VEGF-R1 and R2. The neuropilins NRP-1 and NRP-2 are also receptors for VEGF, although their role in human wound healing has yet to be determined. Therefore, this study aims to establish the temporal profile of NRP-1 and NRP-2 expression in relation to angiogenesis in human dermal wounds. Scar biopsies were obtained under local anesthesia from patients ($n = 51$) between 1 and 104 weeks following breast surgery. Control samples were taken from breast skin peroperatively ($n = 19$). Neuropilin expression was determined by immunohistochemical staining with antibodies specific to NRP1 and NRP2 and endothelial cell expression was graded from 0 (no expression) to 3 (intense staining) and correlated with age of scar. NRP1 expression was significantly ($p < .001$) elevated compared to controls in scar endothelial cells from 6 days until 52 weeks reaching maximum levels <2 weeks after surgery. In contrast NRP2 expression was significantly elevated ($p < .002$) compared to controls in scar endothelial cells 0–4 weeks after surgery, reaching maximum levels 2–4 weeks after surgery. These data show that both neuropilins are present at elevated levels during early wound healing, when angiogenesis is known to occur, with NRP1 expression remaining elevated for up to 52 weeks following surgery, suggesting that NRP1 may play a more significant role.

PC17

THE PROTEOME OF DERMAL INTERSTITIAL FLUID AS SAMPLED BY MICRODIALYSIS

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Microdialysis is a well-established technique for sampling interstitial fluid. Using microdialysis and gel-based “shotgun” proteomics technologies, we have analyzed the protein complement of human interstitial fluid. Microdialysate was collected using a 3000-kDa molecular mass cutoff membrane inserted into the upper dermis of the volar forearm of healthy human volunteers. Probes were perfused with physiological saline at 3 μ L/min and dialysate collected at timed intervals. Pooled dialysate was concentrated 7-fold to 1.2 mg total protein prior to depletion of highly abundant proteins by affinity chromatography and protein separation by SDS-PAGE. Gel slices containing the fractionated proteins were incubated with trypsin and the resulting peptides analyzed using Reverse Phase chromatography-Tandem Mass Spectrometry (Q-TOF Global Ultima, Waters). Searches of the human NCBI protein database using MASCOT (Matrix Science) and Protein-Center (Proxeon) software assigned components originating from both extra- and intracellular compartments. Proteins were functionally divided into regulatory (22), transport (7), structural (7), or unknown (4). Several of the identified pro-

teins are involved in the wounding response, while 8 appear to be dermis-specific rather than plasma-derived. In conclusion, these technologies have yielded insights into the interstitial fluid proteome and pave the way for studies to characterize changes associated with pathological conditions.

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PC18

DETECTION OF iNOS AND eNOS FOLLOWING ALA-PDT IN COMBINATION WITH NOS INHIBITION

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PDT is a cancer treatment in which cell death occurs as a result of the interaction of light with a photosensitizing drug. It has been shown that NO may play a role in photofrin-PDT and more recently in ALA-PDT in vivo. The aim of this study was to determine if NOS inhibition prior to ALA-PDT altered tumor eNOS and iNOS expression. Murine tumor cells expressing differential NO levels, EMT6 (low) and RIF-1 (high), were injected into the cremaster muscle of Balb/c or C3H mice ($n = 6$ /group), respectively. Animals were prepared for in vivo microscopy 7–14 days later. Mice received oral ALA (200 mg/kg) 4 h before PDT. L-NAME, L-NNA, 1400 W (10 mg/kg), or PBS was administered via the jugular vein 5 min before PDT. Tumors were harvested 2 h after ALA-PDT and fixed in 10% buffered formal saline for immunohistochemistry (iNOS and eNOS). RIF-1 and EMT6 tumors treated with ALA-PDT and L-NAME or L-NNA demonstrated no eNOS expression in the tumor blood vessels when compared to controls and ALA-PDT-treated tumors. In both tumors there was minimal iNOS expression in blood vessels, with a few positive inflammatory cells in controls and ALA-PDT-treated tumors. EMT6 tumors treated with ALA-PDT and NOS inhibition demonstrated no increased in iNOS expressing inflammatory cells. In contrast, RIF-1 tumors treated with ALA-PDT and NOS inhibition demonstrated a dramatic increase in inflammatory cells expressing iNOS. This study demonstrated NOS modulation during ALA-PDT.

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PC19

VEGF_{xxx}b IN PLASMA OF NONPREGNANT AND PREGNANT WOMEN

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Vascular endothelial growth factor (VEGF) is thought to play an important role in preeclampsia, a common hypertensive disease of pregnancy causing the syndrome of hypertension, increased vascular permeability and an anti-angiogenic state.

A subset of VEGF isoforms termed VEGF_{xxx}b, inhibit conventional VEGF isoforms. In normal placentae conventional VEGF and VEGF_{xxx}b levels are positively correlated, whereas in preeclamptic placentae there is a negative correlation between VEGF and VEGF_{xxx}b (Bates, D.O., et al., (2006). Clin Sci 110:275–285). Plasma levels of VEGF_{xxx}b in pregnancy have not been described. We developed an enzyme-linked immunosorbent assay (ELISA) using a VEGF_{xxx}b specific capture antibody (RnD MAB3045) and a biotinylated pan-VEGF detection antibody. The ELISA was sensitive to 30 pg/ml VEGF₁₆₅b but did not detect VEGF₁₆₅ even at 4 ng/mL. We measured VEGF_{xxx}b in plasma from pregnant ($n = 6$) and nonpregnant women ($n = 3$). VEGF_{xxx}b levels were variable between subjects ranging from 0.030 to 8.15 ng/mL. VEGF_{xxx}b levels in plasma from healthy pregnant women were consistent within each group (coefficient of variation $17 \pm 17\%$) and increased during gestation by $50 \pm 23\%$ at 34 weeks compared with initial booking (16–28 weeks, $n = 6$, $p = .07$) at 16, 28, 34, and 38 weeks of pregnancy. VEGF_{xxx}b was present in the plasma of pregnant women at variable concentrations, but consistently increased in the third trimester of pregnancy. The proportion of total VEGF that is VEGF_{xxx}b needs to be investigated throughout normal pregnancies.

Supported by the BHF

PC21

EFFECT OF VEGF ON GROWTH AND PROLIFERATION OF UMBILICAL VEIN ENDOTHELIAL CELLS DERIVED FROM NORMAL AND TYPE 1 DIABETIC PREGNANCIES

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Increased angiogenesis is a feature of fetoplacental vessels in type-1 diabetes, with both placental and fetal macrosomia a common occurrence. VEGF has been implicated in the reported increased angiogenesis. The aim of this study was to investigate the dynamics of growth and proliferation of endothelial cells from normal or type 1 diabetic pregnancies subjected to VEGF. Freshly isolated human umbilical endothelial cells (HUVEC; 2×10^5 cells/mL) from normal ($n = 3$) and type 1 diabetic pregnancies ($n = 3$) were grown to confluence in medium (M199 + 20% fetal calf serum). Cells were serum starved (4 h, $t = 0$) and both groups were stimulated with VEGF (10 ng/mL) for 24 and 48 h. Cell number, cell height, and surface area (SA) were measured using phase and scanning confocal microscopy. Statistical significance was determined by two-way ANOVA. For both study groups, a reduction in cell numbers was observed with increased duration of VEGF ($p < .0001$). At $t = 0$, HUVEC from the type 1 group had a larger cell SA (22%). After 48 h VEGF, the SA of type 1 group were significantly larger (139% increase from $t = 0$); normal HUVEC showed a 96% increase ($p < .01$). Analyses of cell height at nuclear regions (HN) showed no significant difference in HN in cells from type 1 diabetic group ($t = 24$; $t = 48$). There was a significant decrease in HN in normal

HUVEC ($p < .01$). In conclusion, HUVEC from type 1 diabetic pregnancies have intrinsic differences in growth pattern and an heightened hypertrophic but not proliferative response to low levels of VEGF.

Supported by the ASGBI

PC22

THE EFFECT OF MILD HYPERTENSION ON SUBPODOCYTE SPACE FILTRATION BARRIER IN RAT GLOMERULAR CAPILLARIES

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Bowman's space consists of three interconnected urinary spaces forming drainage pathways for ultrafiltrate (Neal, C.R., et al. (2005). JASN 16:1223). Glomerular filtrate may enter restrictive spaces under the podocyte (subpodocyte space, SPS) before passing to other urinary spaces. We have measured SPS in 12-week-old (early) spontaneously hypertensive rats (SHR) or Wistar Kyoto (WKY) controls after fixation at physiological oncotic and hydrostatic pressures. SHR (mean arterial pressure 140 mmHg) or WKY (MAP 96 mmHg) kidneys were perfusion-fixed with glutaraldehyde solutions isototically and at matched MAP. Serial ultrathin sections of kidney were used to reconstruct regions of glomeruli. From these, filtration slit density of the glomerular capillary wall, SPS coverage of the capillaries, and the available area for filtration into the SPS were measured and compared (mean \pm SEM, unpaired t tests unless stated). The filtration slit density in non-SPS regions of the SHR and WKY was the same ($2.3 \pm 0.2/\mu\text{m}$, $2.3 \pm 0.1/\mu\text{m}$), however, filtration slit density decreased in the covered SPS regions of SHR glomerular capillaries ($1.8 \pm 0.1/\mu\text{m}$, $p < .05$) compared with the WKY ($2.1 \pm 0.1/\mu\text{m}$). The SPS covered area of the glomerular filtration barrier was similar in both ($62 \pm 14\%$ to $72 \pm 9\%$). Mild hypertension in 12-week-old SHR shows a decrease in the number of filtration slits of the filtration barrier covered by SPS. This reduction in filtration pores suggests a lower permeability in these podocyte-covered regions.

PC23

THE EFFECT OF MILD HYPERTENSION ON THE SUBPODOCYTE SPACES OF RAT GLOMERULAR CAPILLARIES

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The urinary or Bowman's space consists of three interconnected urinary spaces forming drainage pathways for ultrafiltrate (Neal, C.R., et al. (2005). JASN 16:1223). Glomerular filtrate may enter restrictive spaces under the podocyte (subpodocyte space, SPS) before passing to other urinary spaces. We have measured and reconstructed SPS in 12-week-old

(early) spontaneously hypertensive rats (SHR) or Wistar Kyoto (WKY) controls after fixation at physiological oncotic and hydrostatic pressures. Either SHR (mean arterial pressure 140 mmHg) or WKY (MAP 96 mmHg) kidneys were perfusion fixed with glutaraldehyde solutions isotonically and at matched MAP. Serial ultrathin sections of kidney were used to reconstruct ultrastructural regions of glomeruli. Reconstruct software (John C. Fiala) was used to reassemble the sections into 3D models showing the complexity of the SPS. SPS parameters were measured and compared (mean \pm SEM, unpaired *t* tests unless stated). The SPS height from the glomerular filtration barrier was the same in both WKY and SHR (0.24 ± 0.08 and 0.26 ± 0.07 , respectively). The SHR reconstructed SPS is more chaotic and dendritic and shows a more complex SPS exit pore than the WKY but is similar in diameter ($0.13 \pm 0.05 \mu\text{m}$, $0.24 \pm 0.07 \mu\text{m}$, respectively). In both cases cellular connections between the parietal and podocyte layer were observed that were not observed with previous fixation regimes. Hypertension in 12-week-old SHR shows an increase in complexity of the SPS over WKY. We are currently looking at older SHRs to see if these differences are increased.

PC24

MATHEMATICAL MODELING OF THE RESISTANCE TO FLUID FLOW THROUGH THE SUBPODOCYTE SPACE (SPS) EXIT PORE PREDICTS THE SPS TO BE A SIGNIFICANT PERMEABILITY BARRIER IN THE GLOMERULUS

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Recent ultrastructural studies have shown that a restriction to flow of fluid across the renal glomerulus is predicted in a space underneath the podocyte cell body or its processes, the SPS (Neal et al. (2005). *JASN* 16, 1223–1235). The SPS covered up to two-thirds of the glomerular filtration barrier (GFB) surface. The SPS is bounded at the downstream end by a further narrowing, termed the SPS exit pore (SEP). To determine the SEP contribution to the SPS resistance under physiological conditions, we carried out three-dimensional reconstruction of rat glomeruli perfused at physiological hydrostatic and colloid osmotic pressures. The SEP dimensions were $0.15 \pm 0.05 \mu\text{m}$ wide and $0.25 \pm 0.05 \mu\text{m}$ long, significantly narrower than previous estimates under nonphysiological flow conditions ($0.33 \pm 0.04 \mu\text{m}$ wide, $n = 6$, $p < .05$, *t* test). Mathematical modeling of this subpodocyte space exit pore based on a circular flow model predicts that the SPS exit pore is the primary site of resistance in the SPS, the SPS resistance is 2.47 times that of the GFB, and exquisitely sensitive to changes in the SEP dimensions, indicating that the SEP could be the principal regulator of the extravascular pressure in the SPS. This suggests a physiological role of the podocyte

in the regulation of glomerular fluid flux across most of the glomerular filtration barrier.

PC25

RELATIONSHIP BETWEEN WT1 AND PRO AND ANTI-ANGIOGENIC ISOFORMS OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

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The Wilms tumor suppressor gene (WT1) is mutated in 10–15% of Wilms tumors and mutations in WT1 are also associated with Denys Drash syndrome characterized by childhood nephrotic syndrome, glomerulosclerosis, end-stage renal failure, and Wilms tumors. Recent studies have suggested that WT1 may regulate VEGF transcription and pre-mRNA splicing. VEGF exists as two families of isoforms, an angiogenic family (VEGF_{xxx}) and a sister family of anti-angiogenic isoforms (VEGF_{xxx}b) resulting from distal splice site selection in exon 8. To investigate any potential link between WT1 and VEGF distal splicing control, protein was extracted from conditionally immortalized human podocytes that were phenotypically normal, podocytes derived from a DDS patient and DDS podocytes that overexpress wild-type WT1 and overall VEGF and VEGF_{xxx}b levels were determined by ELISA. Podocytes derived from a patient with Denys Drash and Denys Drash podocytes that overexpress WT1 displayed 0.52 ± 0.016 and 0.432 ± 0.093 ($p < .001$) fold expression of VEGF_{xxx}b protein compared to wild-type podocytes. However, podocytes derived from a patient of Denys Drash displayed a 1.484 ± 0.112 -fold expression of panVEGF compared to wild-type podocytes. Conversely, the DDS cell line overexpressing wild-type WT1 resulted in the restoration to 0.484 ± 0.036 ($p < .01$) fold compared with wild-type podocytes. WT1 appears to play a role in the regulation of VEGF splicing

PC26

MEASUREMENT OF REFLECTION COEFFICIENT OF SINGLE ISOLATED MOUSE GLOMERULI

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We have measured single isolated glomerular ultrafiltration coefficient (L_pA) (Salmon et al. (2006). *J Physiol* 570.1:141–156) by analyzing changes in glomerular volume (*V*), an index of fluid flux across the glomerular filtration barrier (GFB), that occur in response to a trans-GFB oncotic pressure gradient. A similar oncometric assay has been used to measure glomerular albumin reflection coefficient (σ_{alb}), revealing values of 0.92–0.94 (Savin et al. (1992). *J Am Soc*

Nephrol 3:1260–1269). Fractional micropuncture estimates of σ_{alb} in vivo are ~ 0.9994 (Tojo & Endou (1992). *Am J Physiol* 263:F601-6). We sought to refine the oncometric method of estimating σ_{alb} . Individual mouse glomeruli, isolated by flushing renal cortical tissue through graded metal sieves, were aspirated onto a micropipette within a flow-controlled observation chamber. V was assessed during incubation in a solution of bovine serum albumin [BSA: oncotic pressure (\square) 32.17 ± 0.76 cmH₂O], and subsequently in a dextran solution prepared by dialysis across a 100-kDa cutoff membrane (\square 32.53 ± 0.12 cmH₂O). The ratio $V_{\text{dex}}/V_{\text{alb}}$ describes σ_{alb} . Using continuously flowing perfusate solutions, σ_{alb} was 0.89 ± 0.03 ($n = 6$). σ_{alb} was 0.962 ± 0.024 ($v 6$) when V was assessed in static perfusate solutions. Interestingly, when BSA was exchanged for BSA, $V_{\text{alb}(1)}/V_{\text{alb}(2)}$ (static conditions) was also 0.961 ± 0.009 ($n = 6$), indicating that technical issues that limit accurate estimation of σ_{alb} remain. Further refinement of this assay may reveal a valuable tool for estimating single human glomerular albumin permeability.

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PC27

SYNTHESIS OF BASEMENT MEMBRANE BY CULTURED HUMAN GLOMERULAR CELLS

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In vivo the glomerular basement membrane is a specialized structure consisting of heparan sulfate proteoglycans (HSPG) and particular isoforms of collagen type IV and laminin. Currently, there is controversy as to whether podocytes or glomerular endothelial cells (GEnC), or a combination of both, produce this matrix. We studied basement membrane production using conditionally immortalized human GEnC and podocyte cell lines. Both whole cell lysates and isolated basement membrane were analyzed. For the latter, cells were removed from flasks or coverslips after 1 week, using 1% Triton containing 20 mM ammonium hydroxide. This left behind basement membrane, which was examined using immunofluorescence (IF) for collagen type I and IV, heparan sulfate, and binding of wheat germ agglutinin. The ability of cells to grow on matrix produced by cells was examined by light microscopy and IF for VE-cadherin. Cell and matrix lysates were further analyzed by Western blotting for collagen type IV. IF showed collagen type I and IV, and glycoproteins, including HSPG, were produced by both cell types. Collagen IV was detectable in cell and matrix lysates by Western blotting. GEnC produced more of all the matrix components when compared to podocytes. Seeding GEnC onto podocyte produced matrix resulted in the cells growing normally, and vice versa. In summary, in vitro both cell types produce components of the basement membrane on which cells will grow. Future work will focus on the specific isoforms of collagen type IV produced by these cells.

PC28

VEGF-C, A POTENTIAL PARACRINE REGULATOR OF GLOMERULAR PERMEABILITY, INCREASES GLOMERULAR ENDOTHELIAL CELL MONOLAYER INTEGRITY

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We have previously reported expression of vascular endothelial growth factor A (VEGF-A) and C by podocytes and actions of VEGF-A on glomerular endothelial cells (GEnC), which express VEGF receptor 2. Here we define the expression of VEGFR3 in GEnC, and investigate effects of its ligand, VEGF-C, on GEnC barrier properties. Renal cortex and cultured GEnC were examined by IF and confocal microscopy, and cell and glomerular lysates by Western blotting. Effects of 1 or 10 nM VEGF-C on transendothelial electrical resistance (TEER) and flux of labeled albumin across GEnC monolayers were measured. Alternatively, the mutant VEGF-C156S, a VEGFR3-specific agonist, or VEGF-A was used. Effects of VEGF-C and -A on intracellular calcium ($[Ca^{2+}]_i$) were measured by a Fura-2AM-based fluorescence technique. GEnC expressed VEGFR3 in tissue sections and culture. VEGF-C dose-dependently increased TEER, with a maximal effect of 10 nM VEGF-C at 120 min of 6.8Ω ($p < .01$). VEGF-C156S had no effect. VEGF-A reduced TEER as previously. VEGF-C reduced labeled albumin flux by 32.8% ($p < .05$). VEGF-C and VEGF-A increased $[Ca^{2+}]_i$ by 1.15- and 1.39-fold, respectively ($p < .01$). In contrast to VEGF-A, VEGF-C increases GEnC monolayer integrity as measured by TEER and albumin passage. Both VEGFs increased $[Ca^{2+}]_i$, a change usually associated with increased permeability. These observations suggest that podocytes may exert precise control over GEnC phenotype, particularly with respect to barrier properties, via VEGF-C and a combination of VEGFR2 and 3, as well as through VEGF-A.

PC29

RED BLOOD CELL TRACKING AND VELOCITY MEASUREMENT WITH A KEYHOLE MODEL OF MOVEMENT

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A tracking algorithm is proposed to measure the velocity of red blood cells (RBC) in intravital microscopy of tissue microvessels. Intravital microscopy was carried out on tumors growing in transparent dorsal skin flap "window chambers" in unanesthetized mice. Fluorescently labeled RBCs (25 μg of DiI was used per 50 μL of packed red blood cells) were injected into a cannulated tail vein for tracking. The tracking algorithm is based on a keyhole model that describes the probable movement of a segmented cell between contiguous

frames in a video sequence. When a history of movements exists, past, present, and a predicted landing positions define two regions of probability with a keyhole shape. Preprocessing segments cells from background. The keyhole is used to determine if cells in contiguous frames should be linked to form tracks and also as a postprocessing tool to join split tracks and discard links that could have been formed due to noise or uncertainty. Outliers are removed based on the distribution of the average velocities of the tracks. The algorithm presents several advantages over traditional methods such as kymographs or particle image velocimetry: manual intervention is restricted to the thresholding, many vessels can be analyzed simultaneously, the algorithm is robust to noise, and a wealth of statistical measures can be obtained. Average velocities of 2 tumors were 207 ± 155 mm/s (mean \pm STD) with a range 15–797 mm/s, and 86 ± 60 mm/s with a range 5–300 mm/s, respectively, which are consistent with the literature. Validation against a manual method is in progress.

Supported by Cancer Research UK

PC30

Ca²⁺-SENSITIVE Cl⁻ CURRENTS PLAY A KEY ROLE IN ENDOTHELIN-INDUCED Ca²⁺ RESPONSES IN MYOCYTES WITHIN RETINAL ARTERIOLES

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This study tested the role of Ca²⁺-activated Cl⁻ currents in Et-1-induced Ca²⁺ signals. Arteriole segments from male Sprague-Dawley rats were loaded with fluo-4 AM. High-speed confocal imaging (20 fps) was used to record Ca²⁺ signals from at least 3 representative cells in each of 4 repeat experiments. Et-1 (10 nM) increased the frequency of Ca²⁺ oscillations from 0.167 ± 0.028 s⁻¹ under control conditions, to 0.342 ± 0.036 s⁻¹ (mean \pm SEM; $p < .01$, nonparametric ANOVA, Dunn's multiple comparison post hoc test). Oscillation amplitude was also increased from 0.12 ± 0.01 to 0.29 ± 0.04 for control and Et-1 treatment periods, respectively ($P < .001$). Addition of the Cl⁻ channel blocker anthracene 9-carboxylate (10 mM) abolished the effects of Et-1 on oscillations. Oscillation frequency was decreased from 0.342 ± 0.036 s⁻¹ at the end of the Et-1 treatment period to 0.142 ± 0.039 s⁻¹ when Et-1 and anthracene 9-carboxylate were both present ($p < .001$). Similarly, mean oscillation amplitude was reduced from 0.29 ± 0.04 in the presence of Et-1 to 0.13 ± 0.02 when anthracene 9-carboxylate was also added ($p < .001$). There were no significant differences in oscillation frequency or amplitude between the control period and the period of treatment with both Et-1 and anthracene 9-carboxylate ($p > .05$). It appears, therefore, that activation of Ca²⁺-activated Cl⁻ currents plays a vital role in the stimulation of Ca²⁺ oscillations by Et-1.

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PC31

STATIN TREATMENT IMPROVES ENDOTHELIAL FUNCTION IN OFFSPRING OF PROTEIN-RESTRICTED RAT DAMS

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In the rat, dietary protein restriction during pregnancy results in raised blood pressure and endothelial dysfunction in the offspring (Torrens et al. (2006). *Hypertension* 47:982–987). Statins have actions beyond their lipid-lowering effects (Davignon (2004). *Circulation* 109:III39–III43) and we tested the effect of statin treatment in our model of endothelial dysfunction. Wistar rats were fed a control (C; 18% casein) or protein-restricted (PR; 9% casein) diet throughout pregnancy and returned to standard chow postpartum. At weaning a subset of the PR group were given atorvastatin (PRS, 10 mg/kg/day) in the drinking water. At 145 days of age male offspring were sacrificed by CO₂ inhalation. Small mesenteric arteries were mounted in a wire myograph. Responses curves were conducted to phenylephrine (PE), acetylcholine (ACh), and sodium nitroprusside (SNP). Responses to ACh were repeated in the presence of L-NAME (100 μ M) and indomethacin (10 μ M). Differences were assessed by one-way ANOVA. Significance was accepted at $p < .05$. Constriction to PE was similar in all groups. Responses to ACh were blunted in the PR group compared to controls ($p < .05$) but were similar between the C and PRS groups. L-NAME and indomethacin blockade blunted ACh responses in both the C and PRS groups ($p < .05$) but had no effect in the PR group. Responses to SNP were similar in all groups. These data suggest that chronic atorvastatin therapy may restore endothelial function independently of cholesterol and likely through an NO-dependent mechanism.

Supported by the BHF & Pfizer

PC32

RHO KINASE-DEPENDENT CONTRACTION OF CARDIAC CAPILLARY ENDOTHELIAL CELLS, IN SITU, IN THE PRESENCE OF CONTINUOUS FLOW

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We previously showed that ischemia induced by interrupting vascular flow and ischemia with reperfusion cause a reduction in cardiac capillary caliber resulting from a reduction in endothelial cell dimensions, which is sensitive to Rho kinase inhibition (Y27632). Here, we investigated whether a similar change could be elicited against flow through the cardiac capillary bed. Langendorff perfused hearts from rats killed by cervical dislocation were subjected to ischemia, reperfusion, and the vasoactive agents, histamine (10⁻⁴ M) and Y27632 (1 μ M). Constant flow was maintained during perfusion. Morphometry from electron micrographs was used to monitor

endothelial cell shape. Compared to control values, histamine ($n = 6$) reduced average capillary and luminal cross-sectional areas by 23.6 and 27.8%, respectively ($p = .0001$ for both). Average abluminal and luminal membrane lengths were diminished by 12.3 and 14.3%, respectively ($p = .0001$ for both). Quantification of cell injury showed that these changes occurred without endothelial injury. When capillaries were exposed to histamine and Y27632 ($n = 5$) simultaneously, these parameters did not change significantly from control values. Endothelial cross-sectional cell areas did not alter significantly in response to histamine or Y27632. Capillary endothelial cells, in situ, are able to contract against continuous flow. These changes require Rho kinase-dependent endothelial cell contraction and mimic those observed following myocardial ischemia. Targeting the actomyosin contractile system may be useful in reducing effects of ischemia on the myocardium.

PC33

RHYTHMIC OSCILLATIONS IN BLOOD VOLUME OBSERVED SIMULTANEOUSLY IN SKIN AND UNDERLYING MUSCLE

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Vasomotion is the rhythmic changes in diameter of small blood vessels and has been predominantly studied in the relative accessible microcirculation of the skin. The mechanism of these rhythmical changes in blood volume is not fully understood but different frequencies of oscillation have been proposed to result from both local (endothelial ~ 0.01 Hz) and central (sympathetic ~ 0.04 Hz) control. The aim of this study is to investigate whether similar vasomotion can also be observed in muscle tissue. Oscillations in blood volume were measured simultaneously in skin using optical reflectance spectroscopy (ORS) and in underlying muscle using near infrared spectroscopy (NIRS) in the forearm of 15 healthy supine subjects. Using Fourier analysis, spectral amplitude (area under curve) was calculated for frequency intervals relating to sympathetic activity (0.02–0.05 Hz) and endothelial activity (0.0025–0.0175 Hz) and was expressed as a percentage of the total spectral amplitude from 0.0025 to 1 Hz. At frequencies linked with endothelial and sympathetic activity, rhythmical oscillations in blood volume of the same magnitude were demonstrated in both skin and muscle, 15.3 (4.0)% skin vs. 16.3 (5.3)% muscle for endothelial frequencies, (mean (SD), t test, $p = .633$) and 10.9 (3.8)% skin and 12.4 (5.5)% muscle for sympathetic frequencies ($p = .354$). This study suggests that vasomotion observed in the microcirculation of the skin is also present in the underlying muscle.

PC34

OCULAR MICROVASCULAR ABNORMALITIES IN SLE IDENTIFIED BY QUANTITATIVE ASSESSMENT OF DOPPLER FLOW VELOCITY WAVEFORMS

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Doppler ultrasound enables noninvasive identification of ocular microvascular hemodynamics. In patients with SLE we interrogated the ocular microcirculation using quantitative analysis of the Doppler velocity flow waveforms. 60 SLE patients were compared to 32 age-sex matched controls. Pulsed Doppler velocity waveforms from the ophthalmic (OA), central retinal (CRA), and carotid artery (CAR) were analyzed using eigenvector decomposition, and the means of the first four sinusoidal components of the average waveform were compared between groups. In the OA and CRA there were significant differences in the power of the lower-frequency (f) sinusoidal components. SLE OA power (cm/s^2) median (range), f1 8.7 (1.1–34.9) vs. 6.0 (1.0–14.3); $p = .012$, f2 8.9 (1.2–36.4) vs. 5.4 (0.5–11.7); $p = .004$, f3 no significant difference, f4 1.8 (0.1–7.3) vs. 1.5 (0.1–7.5); $p < .001$. SLE CRA power (cm/s^2) median (range), f1 2.3 (0.3–8.0) vs. 1.2 (0.6–3.6); $p < .001$, f2 2.4 (0.4–9.1) vs. 1.1 (0.4–3.5); $p < .001$, f3 0.8 (0.1–3.6) vs. 0.7 (0.3–1.9); $p = .043$; f4 no significant difference. No difference was detected in the CAR waveforms. Eigenvector analysis of Doppler flow waveforms, recorded in proximity of the terminal vascular bed, identified altered ocular microvascular hemodynamics in SLE patients not apparent in waveforms recorded from a more distant site. This noninvasive technique may provide information useful in assessing disease activity and response to treatment and ultimately in predicting future vascular complications in SLE.

PC35

SEMI-AUTOMATED DETECTION AND MEASUREMENT OF SUBCELLULAR CALCIUM SIGNALING EVENTS IN RETINAL ARTERIOLES

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Local control of blood flow to the retina is principally achieved through alterations in the diameter of retinal arterioles. Changes in $[\text{Ca}^{2+}]_i$ play an essential role in mediating changes in vascular tone. Using confocal imaging techniques, we have recently described the presence of three distinct subcellular Ca^{2+} -signaling modalities in retinal vascular smooth muscle cells (VSMCs), namely “ Ca^{2+} sparks,” “global Ca^{2+} oscillations,” and “ Ca^{2+} sparks on oscillations” (Curtis et al. (2004). IOVS 45:4409–4414). Because of the complexity of this Ca^{2+} signaling, manual analysis of the data is time-consuming and automated spark detection algorithms based on “threshold” methods are unreliable. Here we describe algorithms written into user-friendly software for the semi-automated detection and analysis of subcellular Ca^{2+} transients in retinal arterioles. The locations and properties of all three types of events are automatically determined using the *a trous* wavelet transform. In addition, the user can delete false signal events or add

events not captured by the detection algorithm. A single half-day experiment takes ~ 3 –4 days to analyze manually and the software reduces this to ~ 2 h. Future work will develop the software by implementing other algorithms to facilitate fully automated Ca^{2+} event detection.

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PC36

AUTOMATED NAILFOLD CAPILLAROSCOPY ANALYSIS

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Nailfold capillaroscopy (NC), an optical imaging method, is used to aid diagnosis of rheumatological diseases such as systemic sclerosis (SSc) by identifying enlarged and distorted capillaries within the nailbed. Using frame registration software, our system allows a panoramic mosaic of the nailfold to be formed and manual width and density measurements of capillaries to be made. Newly developed software using automated image analysis suppresses noise to enhance capillaries, automates measurement of capillary width, density, tortuosity, and derangement and aims to use features to classify disease groups. The aim of this study was to (a) compare manual and automatic measurements, (b) determine whether there were quantifiable differences in features between disease groups, and (c) determine whether there was a correlation between different automated features. NC images from 46 healthy controls (HC), 21 patients with primary Raynaud's (PRP), and 49 patients with SSc were analyzed. (a) Width and density (automatic vs. manual) had correlations of $r = 0.563$ and 0.470 , respectively ($p < .001$). (b) Significant differences between groups (ANOVA [$p < .001$]) were found for all features (table). (c) Correlations were found between width vs. density ($r = 0.563$) and width vs. tortuosity ($r = 0.482$) but not width vs. derangement.

| | Automatic (Relative Units, median) | | | | Manual (RU, median) | |
|-----|------------------------------------|-----------------------|-----|------|---------------------|-----------------------|
| | Width | Density ⁻¹ | T | D | Width | Density ⁻¹ |
| HC | 13.7 | 13025 | 3.2 | 8.8 | 12.5 | 71.8 |
| PRP | 16.9 | 16914 | 3.2 | 9.2 | 13.4 | 75.0 |
| SSc | 17.9 | 29953 | 3.3 | 11.3 | 21.6 | 135.4 |

PC37

THE ASSESSMENT OF GINGIVAL CAPILLARY DENSITY VARIABILITY USING SIDESTREAM DARK FIELD IMAGING IN HEALTHY SUBJECTS

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A link between periodontal disease and cardiovascular risk (CVR), mediated via inflammation, has been proposed. Inflammation has marked effects on the microcirculation, although whether the oral microcirculation contributes to the above link is not established. Recent advances in technology may enable exploration of its involvement. Thus, the aim of this study was to demonstrate the feasibility and reproducibility of sidestream dark field (SDF) imaging in assessing the gingival capillary density in healthy subjects. The gingival capillary density of 6 healthy female subjects (22–49 years) was assessed using a handheld Microscan Video Microscope. Three specific sites were examined in each subject, the marginal gingivae of the central incisors, and between the cuspid canine and premolar on the left and right side of the upper jaw. Capillary density was calculated per mm^2 using Capiscope and the intra-/intersubject variability analyzed. Mean capillary density was $184.6 \pm 21.7/\text{mm}^2$ (mean \pm standard deviation). The intrasubject coefficient of variation across the three sites assessed was $15.7 \pm 4.0\%$ (mean \pm SD). This preliminary study demonstrates that SDF imaging of the gingival microcirculation is feasible and gives a reproducible assessment. Due to inherent variability between areas, several sites of measurement will be required to obtain a value representative of an individual's capillary density.

PC38

TISSUE OXYGENATION AND PERFUSION IN THE TONGUE AND ORAL MUCOSA OF HEALTHY SUBJECTS

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Use of lightguide spectrophotometry (LGS) to assess bowel oxygenation is a relatively new technique. In the context of a clinical study on the use of LGS and laser Doppler flowmetry (LDF) in the bowel, it was necessary to investigate the reproducibility of oxygen saturation (SO_2) and laser Doppler flux measurements by different probes. The oral mucosa was used as a model. Ten healthy volunteers were recruited for the study. SO_2 and LDF were measured on the tongue and oral mucosa at 0, 6, and 24 h by 5 different probe configurations (3 for LGS and 2 for LDF). Measurements were taken at 10 places on the tongue and mucosa by each probe on each occasion and mean values were recorded. SO_2 measurements by different probes showed significant correlation in the tongue and oral mucosa ($p < .05$). There was also good agreement of the SO_2 measurements by different probes as shown by Bland and Altman plots. The mean (coefficient of variation %) SO_2 (%) values for the tongue at 0, 6, and 24 h were 78.5 (12.8), 81.7 (9.4), and 78.9 (8.0). The mean (CV) values for the mucosa were 82.6 (8.0), 85.8 (6.3), and 84.4 (5.3). The SO_2 measurements on the tongue and mucosa at 6 h were significantly higher than at 0 and 24 h in all but one case. The LDF measurements on the tongue and mucosa by the 2 probes were correlated significantly ($p < .05$) but the measurements had large coefficients of variation ranging from 23.6 to 51.3%. SO_2

measurement by LGS using different probes are reproducible and comparable. As compared to SO_2 , LDF measurements have large variations.

PC39

ALTERED MICROVASCULAR RESPONSES TO THE L-TYPE CALCIUM CHANNEL AGONIST BAYK8644 IN SPONTANEOUSLY HYPERTENSIVE RATS IN VITRO

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Control of vascular tone within the microcirculation occurs via calcium (Ca^{2+}), which enters the cell through L-type Ca^{2+} channels located on vascular smooth muscle. Several studies suggest exaggerated function of L-type Ca^{2+} channels during hypertension. In agreement, we have previously demonstrated increased constriction of mesenteric arterioles to the Ca^{2+} channel agonist BayK8644, in hypertensive rats in vivo (Lawton et al. (2006). *FASEB J* 20:A269). We therefore hypothesized that during hypertension there is increased sensitivity of L-type Ca^{2+} channels in the microcirculation and this study aimed to confirm our in vivo findings in vitro, using isolated vessels. Third-order mesenteric arteries from age-matched (14–16 weeks) male normotensive (WKY, $n = 6$) and spontaneously hypertensive rats (SHR, $n = 6$) ($\sim 300 \mu\text{m}$ at 60 mmHg) were mounted on a pressurized myography system. Intraluminal diameters were measured in response to increasing concentrations of BayK8644 (0.1, 0.3, 1, 3, 10, 30, 100, and 300 nM). BayK8644 caused dose-dependent constriction of arteries, but this constriction was significantly exaggerated in WKY compared to SHR as EC_{50} and E_{max} values were $-135.2 \pm 10.81 \mu\text{m}$, $0.52 \times 10^{-8} \pm 0.087 \text{ M}$ and $-59.8 \pm 3.93 \mu\text{m}$, $1.74 \times 10^{-8} \pm 0.246 \text{ M}$, respectively ($p < .05$). The results indicate that L-type Ca^{2+} channels within mesenteric arteries of SHR exhibit decreased sensitivity to BayK8644. This is not in agreement with our in vivo findings and requires further investigation.

PC40

RELATIONSHIP BETWEEN MUSCLE BLOOD FLOW AND INSULIN SENSITIVITY IN METABOLIC SYNDROME

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This study investigates insulin-induced changes in muscle microvascular blood flow using a novel noninvasive laser Doppler surface probe in individuals with features of the metabolic syndrome. Twenty-one volunteers (age 37–67 y; 8 male) with two or more features of the metabolic syndrome (central obesity, dyslipidaemia, increased blood pressure, and dysglycaemia) were studied. All gave written informed consent. Blood flux in muscle and skin was measured by laser Doppler fluximetry (Moor Instruments, UK) using a 785-nm, 20-mW, 4-mm separation (muscle) and a 1-mW, 0.5-mm separation (skin)

probe placed above the anterior tibialis muscle, at rest and during a reactive hyperemia (RH) to arterial occlusion, before and during euglycemic hyperinsulinemic clamp. Insulin sensitivity was calculated as the rate of glucose disposal (M value in mg/kg/min). Resting blood flux in muscle and skin was 80 ± 3 and $9 \pm 0.3 \text{ AU}$ (arbitrary perfusion units), respectively. Insulin at high dose increased blood flow in muscle and skin at rest ($p < .04$). This was linearly associated with insulin sensitivity ($r = .45$, $p < .05$). No increase in RH response was seen during insulin infusion in either muscle (in % before: 312 ± 5 ; low dose: 411 ± 59 ; high dose: 240 ± 47) or skin (350 ± 59 ; 423 ± 96 ; $288 \pm 55\%$). We conclude that insulin's vascular action within skeletal muscle measured using noninvasive high-power laser Doppler fluximetry is attenuated in individuals with features of the metabolic syndrome and that this impaired response may contribute to the reduced insulin sensitivity and glucose handling by muscle.

PC41

THE EFFECT OF CHRONIC INSULIN EXPOSURE ON FREE FATTY ACID UPTAKE IN ENDOTHELIAL CELLS

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Insulin resistance has been shown to be associated with altered free fatty acid (FFA) utilization. Prior to their metabolism FFAs are actively transported into the cells, for example by fatty acid binding proteins (FABPs). Insulin has been shown to enhance FFA uptake, via FABPs, in a variety of cells, e.g., adipocytes and cardiac myocytes. This study aimed to investigate the effect of insulin on FFA uptake in endothelial cells, which is currently unknown. Human umbilical vein endothelial cells (HUVEC's) from three different isolations were treated for 6 h under control, physiological (1 nM), or hyperinsulinaemic (5 nM) conditions. After chronic insulin exposure FFA uptake was assessed by the uptake of radio labeled tracer ($[^3\text{H}]$ oleic acid, $2 \mu\text{Ci/mL}$) bound to albumin in the presence of unlabeled oleate (0.3 mM). After 2 min, the assays were quenched and cells were then lysed in 1 N NaOH. Samples were removed for scintillation counting and protein quantification. Data was normalized to control values. Initial analysis using Kruskal-Wallis test indicated a significant difference between the three experimental conditions ($p = .046$). Further analysis was therefore performed using the Mann-Whitney test and both 1 nM (median: 92.13% range: 69.7–96.56) and 5 nM insulin (median: 80.46% range: 55.23–86.93) significantly reduced FFA uptake compared to the control (100%). This study indicates that chronic insulin exposure inhibits the uptake of FFA in HUVEC's.

PC42

STIMULATION OF PHOSPHOLIPASE D IN ENDOTHELIAL CELLS BY HYPERINSULINEMIA IS NOT ASSOCIATED WITH TRANSLOCATION OF PKC α , RHOA, OR ARF

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Phospholipase D (PLD) is stimulated by many agonists to produce the second messenger phosphatidic acid. Cellular activators of PLD include protein kinase C α (PKC α), RhoA, and ADP ribosylation factor (ARF), which all translocate from the cytosol to the membrane (where PLD is localized) upon activation. We previously showed that insulin activates PLD in endothelial cells and that hyperinsulinemia (5 nM insulin for 6 h) increases basal PLD activation. This work examines whether the increased basal PLD activity induced by hyperinsulinaemia is associated with the enhanced activation (i.e., membrane localization) of PKC α , RhoA, and ARF. [³H]myristic acid-labeled human umbilical vein endothelial cells were incubated \pm 5 nM insulin for 6 h and then treated \pm 1 nM insulin for 20 min and PLD activity was assessed by production of ³H phosphatidylethanol. For subcellular localization of ARF, RhoA and PKC α cells were treated \pm 5 nM insulin for 6 h, and the membrane and cytosolic fractions immunoblotted with specific antibodies. Although hyperinsulinemia significantly increased baseline PLD activity to $171.67 \pm 48.32\%$ ($p = .002$, $n = 6$) vs. control 100%, this was not accompanied by an increase in either PKC α or ARF associated with the membrane fraction. Conversely, the proportion of RhoA associated with the membrane fraction appeared to decrease slightly. Increased PLD activity induced by hyperinsulinemia is not associated with translocation of the PLD activators ARF-1, RhoA, and PKC α from the membrane to the cytosolic compartment.

PC43

MICROARRAY GENE EXPRESSION PROFILING OF NORMAL AND DIABETIC RETINAL ARTERIOLES

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The changes in microvascular gene expression associated with diabetic microangiopathy are largely unknown. We have employed novel amplification techniques to facilitate the analysis of gene expression in freshly isolated retinal arterioles from nondiabetic and diabetic rats using microarray technology. Retinas were dissected on ice and first- and second-order retinal arterioles mechanically isolated and pooled from at least 3 normal or diabetic animals (streptozotocin induced: 3 months disease duration) RNA was extracted using a Qiagen RNeasy micro kit. RNA was amplified and biotin-labeled cDNA generated using the Ovation System (Nugen). This was hybridized to Affymetrix Rat 230.2 genechips and the data were analyzed using Affymetrix software and robust microarray analysis. Approximately 100 ng of intact RNA was obtained from each sample and several μ g of cDNA generated. Hybridization was variable and only one normal and one diabetic sample passed all the quality-control criteria. Analysis of absolute expression levels revealed genes highly expressed in the microvascula-

ture, such as *Vezf1*, *Pdgfra*, and *Hsd17b12*. Candidate genes potentially altered in diabetes include *Pfkfb2* & *Ednrb* (up-regulated) and *Zfp533* and *Nosip* (downregulated). We have demonstrated that it is possible to extract sufficient RNA from microvessels and perform microarray analysis to reveal both genes expressed specifically in the microvasculature and candidates for involvement in diabetic microvascular disease.

PC44

THE Y402H VARIANT OF THE COMPLEMENT FACTOR H GENE IS ASSOCIATED WITH AGE-RELATED MACULAR DEGENERATION BUT NOT WITH DIABETIC EYE DISEASE IN A POPULATION WITH TYPE 2 DIABETES: A GODARTS STUDY

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The Y402H variant of complement factor H (CFH) has been consistently associated with risk of age-related macular degeneration (ARMD). Diabetic retinal disease shares many common pathophysiological characteristics with ARMD, including complement activation. We therefore investigated the impact of this variant on retinal disease in a large cohort of patients with type 2 diabetes undergoing regular systematic eye screening. 2350 patients with type 2 diabetes were genotyped for the CFH Y402H variant. A retrospective case control study demonstrated that the HH genotype was associated with an age-adjusted odds ratio of 7.4 of ARMD ($p = 2.9 \times 10^{-11}$) compared to other genotypes. In a subsequent longitudinal study in the disease free cohort the age-adjusted hazard ratio was 2.8 ($p = 2.4 \times 10^{-7}$). The life time hazard ratio was 3.4 ($p = 2.1 \times 10^{-16}$). We found no association of the variant with the development of sight-threatening diabetic retinal disease. These data demonstrate the ability of a routine diabetic eye-screening service to discriminate between two important but distinct retinal disease entities that can also be segregated at the molecular level. This highlights the potential utility of future genetic analyses to contribute to an increased understanding of the pathophysiology of degenerative retinal diseases.

PC45

ALTERATION OF CUTANEOUS VASOMOTOR RESPONSES IN PATIENTS WITH INSULIN RESISTANCE

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The aim of this study was to evaluate the relationship between insulin resistance and cutaneous vasomotor responses (endothelial-dependent vasodilatation and peripheral

sympathetic failure: noradrenergic control of smooth muscle cells – vasoconstriction and neuropeptides induced vasodilatation) in patients with metabolic syndrome (MS). Patients with insulin resistance and MS, but without hypertension, were divided into two groups: 18 patients with type-2 diabetes mellitus (without insulin therapy and without pronounced diabetic complications) (DM) and 18 patients without DM. 18 healthy subjects were selected as controls (C). The study groups were matched for age and sex. Insulin resistance was measured by HOMA-IR method. We recorded changes in laser Doppler flux (LDF; PeriFlux 4001, Perimed) on the foot. Basal LDF (b-LDF), postocclusive hyperemia (m1-LDF), vasoconstrictor response (v-LDF) to deep inspiration on the pulp of the toe (apical skin); and heat (+44 °C; PeriTemp 4005) induced hyperemia (m2-LDF) on the dorsum of the foot (nonapical skin) were estimated using a PeriSoft for Windows program. b-LDF and local skin temperature did not differ among the study groups ($p > .05$). v-LDF was significantly less pronounced only in diabetics compared to healthy subjects (DM 31.8 ± 13.7 vs. C $52.6 \pm 8.5\%$, $p < .05$). m1-LDF was decreased in both patient groups in comparison with the controls ($p < .05$), but only in diabetics the decrease of m2-LDF was pronounced (DM 134 ± 61 vs. C 192 ± 78 PU, $p < .05$). Our findings show that MS patients with insulin resistance have significant cutaneous vasomotor dysfunction.

PC46

ADENOVIRUS DELIVERY OF FLUORESCENT CAMELEONS TO ASSESS SUBCELLULAR LOCALIZATION OF Ca^{2+} IN HUMAN MICROVASCULAR ENDOTHELIAL CELLS

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Adenovirus (AdV) vectors infect microvascular endothelial cells (MVEC) in vitro. To determine local Ca^{2+} flux within subdomains of MVECs we developed AdV-derived caveolin-targeted fluorescent cameleon Ca^{2+} indicators. A construct containing a fusion protein of caveolin, cyan fluorescent protein (CFP), calmodulin, calmodulin binding peptide, M13, and an enhanced YFP (Isshiki et al. (2002). *J Biol Chem* 277:43389–43398) was cloned into shuttle vector and the cassette co-electrotransformed with an AdV replicant-deficient backbone plasmid into *E coli* BJ5183 competent cells. Recombinant viral plasmids were transfected into Hek293 cells and packaged virus particles isolated. Caveolin constructs (Ad-CYC) or caveolin-deficient constructs (Ad-YC) were generated. The effectiveness of AdV transduction was confirmed by measurement of fluorescence resonance energy transfer (FRET) by wide-field microscopy between CFP and YFP in CHO cells. Ad-CYC and Ad-YC were transduced into human MVEC. A 20% decrease in FRET was seen (indicating decreased Ca^{2+}) after addition of 30 μ M ATP and 100 nM thapsigargin in the absence of extracellular Ca^{2+} followed by transient 30% increases in FRET when extracellular Ca^{2+} was restored. AdV-mediated infection of endothelial cells in vivo

was also observed in isolated perfused mesenteries of the 5% halothane anaesthetized rat. Thus, Ca^{2+} can be measured in subcellular domains in intact endothelial cells in vitro and potentially in vivo.

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PC47

SIMVASTATIN MODULATES RETINAL ENDOTHELIAL CELL FUNCTION

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Statins are inhibitors of cholesterol biosynthesis that are widely used to treat patients with hypercholesterolemia. Interestingly, some reports indicate that statins could protect against diabetic retinopathy, but further experimental evidence is required before speculating on the potential clinical implications of these findings. Therefore, the aim of this study was to investigate the effects of clinically relevant concentrations of Simvastatin on retinal microvascular endothelial cell (RMEC) function. The modulatory effects of Simvastatin on bovine RMECs were investigated using assays for quantifying cell proliferation, cell death, migration, sprouting, and tubulogenesis. Modulation of RMEC function by Simvastatin was dependent on the concentration used. We found that 0.01 μ M Simvastatin promoted cell migration ($p < .05$) and sprouting ($p < .001$) when compared to controls. Simvastatin at both 0.1 and 1 μ M enhanced tubulogenesis ($p < .001$). At 0.1 μ M, Simvastatin-treated cells showed higher rates of BrdU incorporation and population doublings, while concentrations higher than 1 μ M significantly inhibited cell proliferation, migration, sprouting, and tubulogenesis. High concentrations of Simvastatin also caused significant enhancement of TUNEL-positive RMECs. This study demonstrates that at low concentrations Simvastatin promotes angiogenesis and endothelial repair, while higher doses not only impair angiogenesis but induce endothelial cell death in the retinal microvasculature.

PC48

CALCITONIN TRANSPORT ACROSS C-CELL–BLOOD (CCB) BARRIER IN THE NORMAL AND GUANETHIDINE-SYMPATHECTOMIZED RAT THYROID

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Efficiency of endogenous calcitonin (CT) transport across CCB barrier is expressed as CT bioavailability (F_{CT}), i.e., that portion of CT molecules extruded by the thyroid C-cell basal pole which enters circulating blood. As F_{CT} cannot be estimated directly, the novel methodology was designed

for its estimation (Krasnoperov et al. (2006), *J Physiol Sci* 56: 281–286). The essence of this methodology is to assess the CT level before (X) and after (Y) the CCB barrier, to study these input–output CT relations, and to find the F_{CT} range allowed by the relations. In this context, the subplasmalemmal granule numerical density in C-cell basal pole (X) and CT concentration in the right ventricular blood (Y) were measured, with anesthesia having been used for the decapitation. In the normal rats (age: 52 weeks), F_{CT} values were found to lie in the range $87\% < F_{CT} < 100\%$ ($p \geq .95$). Under sympathectomy, actual F_{CT} was estimated as being in the vicinity of 50%. This F_{CT} was combined with a longer distance of interstitial CCB transport (D_{CCBI}) and signs of that the interstitial CT losses account for a significant part of the XY prediction error. Oscillatory behavior of the CCB barrier was also estimated. In the normal rats, a relatively stable CT outflow from thyroid was ensured ($CV_{out} = 5.2\%$, $CV_{in}/CV_{out} = 1.97$). Under sympathectomy, the CCB barrier was shown to enhance CT secretion oscillations ($CV_{out} = 10.7\%$, $CV_{in}/CV_{out} = 0.53$) providing it possibly by changing D_{CCBI} . Thus, guanethidine sympathectomy changes pattern of the CT transport across the CCB barrier in the rat thyroid.

PC49

THE VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) ISOFORM VEGF_{165b} BINDS VEGFR-1 BUT DOES NOT TRANSDUCE A SIGNAL IN VITRO

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We aimed to determine whether VEGF_{165b} could bind to and signal through VEGFR-1. We used BIAcore to measure the binding of VEGF_{165b} to VEGFR-1 in real time. Analysis was performed using BIAevaluation software assuming a 1:1 Langmuir-binding model that incorporated baseline drift. An association reaction constant, $k_a = 2.06 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ($n = 8$), a dissociation reaction constant, $k_d = 1.03 \times 10^{-4} \text{ s}^{-1}$, and an affinity/dissociation constant, $K_D = k_d/k_a = 50 \text{ pM}$ was observed. Chinese hamster ovary cells (CHOs) transfected with VEGFR-1 (CHO-VEGFR-1 cells) containing vector were left untreated (control) or incubated with 1 nM VEGF₁₆₅, 1 nM VEGF_{165b}, or a combination of 1 nM VEGF₁₆₅ and 1 nM VEGF_{165b} for 20 min at 37°C. Incubation with 3.4 nM of placental growth factor (PIGF)-1 (a VEGFR-1-specific ligand) for 20 min was used as a positive physiological control. Samples were analyzed by Western blot analysis with antibodies against phospho-p44/p42 MAPK and phospho-Akt. Treatment of CHO-VEGFR-1 cells with PIGF-1 significantly increased the relative levels of phospho-p44/p42 MAPK, which was not seen in cells treated with VEGF₁₆₅ or VEGF_{165b}, alone or in combination. The relative levels of phosphorylated Akt did not increase above basal levels, regardless of growth factor treatment. Therefore, although VEGF_{165b} can bind VEGFR-1, this receptor is unable to transduce a VEGF_{165b} intracellular signal when overexpressed in CHO cells.

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PC50

INVOLVEMENT OF MAPK (MITOGEN-ACTIVATED PROTEIN KINASES) AND CLK (CDC2-LIKE KINASES) IN TGF- β 1 (TRANSFORMING GROWTH FACTOR- β 1)-DEPENDENT SPLICING OF ANTI-ANGIOGENIC ISOFORMS OF VASCULAR ENDOTHELIAL GROWTH FACTOR

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VEGF, the predominant regulatory molecule in angiogenesis is differentially spliced in its terminal exon to result in pro-angiogenic VEGF_{xxx} and anti-angiogenic VEGF_{xxx}b isoforms. We have shown that TGF- β 1 specifically upregulates VEGF_{xxx}b expression in proliferating podocytes. Here, we investigated the role of the p38 and p42/44 MAPKs in TGF- β 1-stimulated VEGF_{xxx}b expression after 48 h of treatment. VEGF_{xxx}b protein was measured in cell lysates by ELISA using a biotinylated antibody specific for the c-terminus of VEGF_{xxx}b. VEGF isoforms were determined by isoform specific Western blotting and RT-PCR. VEGF_{xxx}b mRNA and protein expression were increased by TGF- β 1 (1 nM) (protein 1.55 ± 0.15 -fold), even during inhibition of p42/44 MAPK phosphorylation by PD98059 (30 μM). SB203580 (10 μM), a p38 MAPK inhibitor, reduced TGF- β 1 induced VEGF_{xxx}b expression (0.96 ± 0.11 -fold to control). TG003 (10 μM), an inhibitor of the splicing factor kinases Clk1 and 4, inhibited TGF- β 1-induced VEGF_{xxx}b mRNA and protein expression (0.78 ± 0.13 -fold). TGF- β 1 stimulates the synthesis of VEGF_{xxx}b isoforms in proliferating podocytes through p38 MAP and Clk kinases.

PC51

MOLECULAR AND FUNCTIONAL EXPRESSION OF TRP CHANNELS IN THE RETINAL MICROCIRCULATION

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The mammalian transient receptor potential (TRP) superfamily of ion channels consists of voltage-independent, nonselective cation channels with diverse physiological functions. Our objective was to identify the TRP channel subtypes expressed in retinal arterioles. Arteriole segments from male Sprague-Dawley rats were isolated and TRP channel mRNA expression was evaluated by RT-PCR. Immunofluorescence staining was used to test for cell-specific expression of TRP channels in retinal arterioles. Rat brain and kidney samples were used as positive controls. Fura-2-based Ca^{2+} microfluorimetry was used to test for functional expression of TRPV4 in cultured bovine retinal endothelial cells (BRECs). With RT-PCR, mRNA of TRPC1, TRPC3, TRPC4, TRPC7, TRPV1, TRPV2, TRPV4, TRPM1, TRPM2, TRPM3, TRPM7, TRPML1, and

TRPML3 was detected in retinal arterioles. Immunofluorescence labeling revealed a punctuate distribution of TRPV2 throughout the smooth muscle cell layer of retinal arterioles, while TRPV4 was specifically localized to the plasma membrane of endothelial cells. Consistent with the RT-PCR results, TRPC5 could not be detected in retinal arterioles. The TRPV4 agonist 4α -PDD ($1 \mu\text{M}$) induced an increase in Ca^{2+} influx in cultured BRECs and these transients were blocked by the nonspecific TRPV4 antagonist, ruthenium red ($1 \mu\text{M}$). Multiple TRP channels are expressed in retinal arterioles. It is likely that they play important roles retinal microvascular function.

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PC52

THE EFFECT OF LOW-DOSE ASPIRIN ON EXPRESSION AND FUNCTION OF INTERLEUKIN 8 IN THE HUMAN COLON

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Myofibroblasts (MF)/pericytes secrete pro-angiogenic growth factors, including interleukin 8 (IL8); the latter is increased in colorectal cancer patients. Clinical data suggest that low-dose aspirin is protective against colorectal cancer, with inhibition of tumor angiogenesis a possible mechanism. The hypothesis for this study is that low-dose aspirin alters the expression of angiogenic genes in colonic myofibroblast cells and inhibits downstream angiogenesis. Primary MF and microvascular endothelial cells (HuCE) were isolated from human colonic mucosa. The effect of aspirin (10^{-5}M) on IL8 expression and secretion was investigated using gene array and ELISA. The effect of IL8 \pm aspirin (10^{-5}M , 10^{-3}M) on angiogenesis in HuCE was investigated using the growth factor reduced matrigel angiogenesis assay. IL8 gene expression was downregulated 16-fold by 10^{-5}M aspirin in MF, while secreted IL8 was found to be reduced by 43%. Within 24 h on matrigel, untreated HuCE underwent vascular remodeling, whereby monolayers of cells exhibited circular clearings. Addition of IL8 resulted in formation of tube-like projections. This was inhibited with both supra-physiological and low-dose aspirin treatments. These data suggests that low-dose aspirin can inhibit angiogenesis via its effect on gene and protein expression of IL8 and plays a role in regulation of angiogenesis in HuCE. The relationship between myofibroblasts and colonic microvascular endothelial cells warrants further investigation using co-culture studies.

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PC53

OVEREXPRESSION OF CD105 MODULATES CELL SIGNALING

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CD105 (endoglin), a homodimeric transmembrane glycoprotein with an apparent molecular weight of 180 and 90 kDa under nonreducing conditions, plays an important role in angiogenesis. It is a marker of activated endothelium and a modulator of transforming growth factor (TGF) β signaling. It can regulate a wide range of cellular and physiological responses, including embryonic development, homeostasis, wound healing, chemotaxis, cell proliferation, differentiation, adhesion, migration, and apoptosis. As a receptor for TGF- β 1 and TGF- β 3, CD105 modulates TGF- β signaling by interacting with TGF- β receptor I (TGF- β RI) and/or TGF- β receptor II (TGF- β RII). CD105 participates in cell attachment to the extracellular matrix (ECM), modulates cell morphology, inhibits some cellular responses to TGF- β 1, and interferes with the downstream TGF- β signaling through small-mothers-against-decapentaplegic (Smad) proteins. Our gene microarray analysis found 40 genes were upregulated ≥ 2 -fold in CD105 transfectants compared with mock cells. They were confirmed by reverse-transcription-polymerase chain reaction (RT-PCR). Moreover, our protein microarray studies using a kinexus phospho-protein screen showed 35 ≥ 2 -fold differences between mock and CD105 transfectants. CD105 inhibits the phosphorylation of extracellular signal-regulated protein kinase (ERK) and c-jun n-terminal kinase (JNK) in CD105 transfectants and modulates TGF- β -induced cell signaling through a signaling pathway involving ERK-JNK expression in CD105 transfectants in vitro.

PC54

HYPOXIA AND GLUCOSE CONCENTRATION ON METABOLIC RATE OF HUMAN COLON ADENOCARCINOMA CELLS IN VITRO

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Phenotypic heterogeneity develops in tumors due to intrinsic genetic changes and extrinsic environmental variations, which may alter metabolic functions and, consequently, responses to therapy. The aim of this study is to quantify interaction between O_2 and glucose on metabolic activities of human colon adenocarcinoma HT29 cells. HT29 cells were cultured in monolayer with different concentration of glucose supplements (3, 6, and 9 g/L) and subjected to normoxic (21% O_2) and hypoxic (<2% O_2) conditions. O_2 utilization by HT29 cells was obtained using the BD biosensor system (Becton Dickinson Biosciences, Oxford, UK). Glucose concentration was measured using Infinity glucose assay reagent (Sigma Diagnostics). Lactate concentration was determined using β -NAD⁺ glycine buffer and LDH reagents (Sigma, Poole, UK). Strong interaction between the energy metabolic rate and O_2 tension was observed. Both glucose consumption rate and lactic acid production rate are >20% different between normoxic and hypoxic conditions, and both differences are statistically significant (<1%). Furthermore, O_2 and glucose consumption by HT29 cells are sensitive to glucose concentration at below 16.7 mM. Results give quantitative insight into HT29 cell metabolic switch between anabolism and catabolism, depending on O_2 and glucose supplies. Upregulation of O_2 depletion at low glucose concentration corresponds to inhibition of glycolysis, consistent with the Crabtree phenomenon.

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