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Abstracts of the 58th Meeting of the British Microcirculation Society 1–2 April 2008, King's College London, UK

Editor: Neena Kalia, PhD
Birmingham, UK

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SYMPOSIUM ON GENE THERAPY FOR ANGIOGENESIS

S1

GENE TRANSFER FOR THERAPEUTIC ANGIOGENESIS

S. Ylä-Herttuala

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Therapeutic angiogenesis involves generation of new capillaries, collateral vessels or both in ischaemic muscles using either recombinant growth factors or their genes. Most commonly used growth factors for therapeutic angiogenesis are vascular endothelial growth factors (VEGF) and fibroblast growth factors (FGF). Some other cytokines and growth factors may also have angiogenic effects *in vivo*. Improved perfusion can be achieved by angiogenesis and arteriogenesis. Angiogenesis means sprouting of new blood vessels from pre-existing ones as a result of proliferation and migration of endothelial cells. Arteriogenesis is a process caused by increased sheer stress at the arteriolar level resulting in the formation of large conduit vessels from pre-existing small vessels. Optimally, both angiogenesis and arteriogenesis should be achieved in order to improve muscle perfusion. Stem cells and endothelial progenitor cells may also be useful for therapeutic angiogenesis. However, exact cell types and mechanisms related to the potentially useful effects of cell therapy remain unclear. Ylä-Herttuala S, Alitalo K. Gene transfer as a tool to induce therapeutic vascular growth. *Nat Med* 2003; 9: 694–701.

S2

REGULATION OF THE ANGIOGENIC ENDOTHELIAL PHENOTYPE

R.H. Adams

Cancer Research UK London Research Institute and Max Planck Institute for Molecular Biomedicine, Münich, Germany

Blood vessels form a highly organised hierarchical network throughout the vertebrate body, integrate functionally into very different tissue environments, and remain remarkably adaptable to changing local requirements. The angiogenic programme controls much of the growth of the vasculature, which allows a remarkable expansion of the vascular network without compromising blood circulation or tissue access to oxygen and nutrients. Angiogenesis involves a wide range of cellular processes such as proliferation, sprouting, migration, adhesion, the formation of endothelial cell junctions or the recruitment of mural cells, i.e., pericytes (PCs), and vascular smooth muscle cells (vSMCs). The Notch signalling pathway has been recently implicated in arteriovenous differentiation and the regulation of vessel sprouting and branching. Inhibition of Notch signalling mediated by the ligand Delta-like 4 (Dll4) leads to the loss of arterial identity and increased numbers of tip cells and enhanced vessel branching. Our results show that besides Dll4, the Notch ligand Jagged1 is also expressed in the developing vasculature in a pattern that partially overlaps with Dll4. To address the role of Jagged1 in angiogenesis, we have generated endothelial cell-specific and inducible gain-of-function and loss-of-function mutants. Characterisation of the mutant vasculature in the embryonic dermis and the postnatal retina revealed that Jagged1 is a critical regulator of endothelial sprouting and branching. We conclude that the endothelial angiogenic phenotype is controlled by the balance between Dll4 and Jagged1 and that the activity of both Notch ligands is essential for normal vascular morphogenesis.

S3

STEM CELL GENE TRANSFER MODELS IN ANGIOGENESIS

L. Claesson-Welsh

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Spheroids of differentiating embryonic stem cells, denoted embryoid bodies, constitute a high-quality model for vascular development, particularly well suited for gain- and loss-of-function analysis of genes required for early embryogenesis. Establishment of endothelial precursors can be identified at day 4–6 of differentiation. It is a clear advantage that development of these precursors proceeds in a continuous context of non-endothelial cells. Two-dimensional (2D) cultures of embryoid bodies in the presence of growth factors such as vascular endothelial growth factor (VEGF) allow formation of a crude vascular plexus that undergoes pruning and reorganization to form capillary networks during day 6–8. The embryoid bodies can also be placed in a 3D matrix such as collagen I. Inclusion of VEGF in the culture medium stimulates formation of angiogenic sprouts that invade the matrix. There is an apparent unique demand for VEGF for angiogenic sprouting; other growth factors such as platelet-derived growth factor or fibroblast growth factors do not promote angiogenic sprouting. The sprouts are surrounded by supporting cells, pericytes, and an *in vivo*-like vascular basement membrane. Eventually, the sprouts develop lumen. Thus, these processes do not require blood flow, which is lacking in this model. Embryonic stem cells deficient in expression of the major VEGF receptor, VEGF receptor-2, are arrested in their vascular development. Ongoing work involves the use of lentivirus-mediated reconstitution of *vegfr2-/-* stem cells with wild type and mutant VEGF receptor-2 to determine the contribution of signal transduction pathways required for endothelial differentiation and angiogenesis.

S4

THYMOSIN β4 AND CORONARY ANGIOGENESIS

P. Riley

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Neovascularisation, an integral component of the cardiac remodelling process to restore coronary vasodilatory capacity after myocardial infarction, is limited and insufficient to preserve viable myocardium. Molecules that regulate collateral growth in the ischaemic heart also regulate coronary vasculature development during embryogenesis. By selectively knocking down Thymosin β4 (Tβ4) in the developing heart, we uncovered its essential role in coronary vasculogenesis, angiogenesis, and arteriogenesis. We demonstrated that Tβ4, secreted from the myocardium, provides a paracrine stimulus to the cells of the epicardium to promote their migration and differentiation into endothelial and smooth muscle cells to form the coronary vasculature. Translation of a vascular development role for Tβ4 to that of angiogenic therapy for coronary artery disease in the adult heart relies on the release of the adult epicardium from a quiescent state and restoration of pluripotency. Tβ4 treatment of adult cardiac explants stimulated extensive outgrowth of epicardial cells (EPDCs) which, as they migrated away from the explant, differentiated into fibroblasts and smooth muscle and endothelial cells. Subsequent *in vivo* studies, in both intact and injured adult mouse hearts, confirmed an equivalent vasculogenic potential within the adult epicardium. Tβ4- stimulated EPDCs gave rise to extensive numbers of PECAM- and SMαA-positive cells throughout the underlying myocardium which became organised into perfused vessels. Tβ4 was previously shown to minimise cardiomyocyte loss and improve cardiac function after MI; our studies present neovascularisation of the adult heart as the principal mechanism of cardioprotection by Tβ4 and could contribute towards the evolution of more efficient therapies for ischaemic heart disease.

Supported by the BHF and MRC

S5

GENE DELIVERY STRATEGIES IN ANGIOSTATIC CANCER TREATMENT

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Gene therapy has been a promise in cancer treatment for years. Effective cancer gene therapy relies on a cell population that can be easily targeted and that is important in the process of tumour growth. Endothelial cells in the tumour vasculature are essential for tumour angiogenesis and display a specific phenotype. For this reason tumor endothelial cells fulfill these requirements. Tumour angiogenesis is emerging as a target for specific anti-cancer therapy and is therefore also recognized as a promising approach in gene therapy. Recent achievements identified molecular targets on angiogenically activated endothelial cells that can be used for specific targeting and therefore for delivery of

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genes to the tumour site. Additional advantages of targeting the tumour vasculature are that endothelial cells are easily accessible and less prone to mutations, and that disturbing the vasculature by killing a few EC will result in massive tumour cell death. The key to successful gene therapy is effective delivery of the gene to the target site. Over the years, several tools have been developed to transport the therapeutic gene to the site of interest. These so-called gene transfer vehicles can be divided into viral and non-viral ones. We have used both approaches in an effort to target tumour endothelial cells. In these approaches the designer peptide anginex, a potent anti-angiogenic and anti-cancer compound, was used to target the vehicles to the tumour vasculature. Anginex has been shown to target galectin-1 on the tumour vasculature. Other targets such as alpha-v-beta-3 integrin and vimentin have been shown to be excellent targeting devices as well. Current research will determine the most efficient mechanisms for delivery of genes to the tumour vasculature, as approaches in the development of anti-cancer therapies.

S6

STEM CELLS AND THE REGENERATION OF THE AGING CARDIOVASCULAR SYSTEM

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Cardiovascular disease (CVD) predominantly affects the elderly population. Indeed, advanced age is itself a significant risk factor for CVD. This is due in part to progressive impairments in cardiovascular repair pathways with increasing age. Elucidation of mechanisms to reverse these impairments may lead to the development of novel therapeutic strategies to treat CVD. The identification of cardiovascular stem and progenitor cells in the last decade has raised the possibility of novel cell-based approaches to promote cardiovascular regeneration. *In vivo* experiments suggest that these cells, including endothelial progenitor cells, are capable of replacing damaged cardiomyocytes and vascular cell types as well as enhancing local angiogenic function. Despite this capacity, the endogenous actions of cardiovascular stem and progenitor cells appear to be insufficient to protect against CVD in older individuals. It is becoming increasingly clear that age-associated changes both in the stem and progenitor cells themselves as well as their microenvironment can lead to impairments in regenerative pathways that likely impact on cardiovascular health. Further elucidation of the mechanisms of age-associated dysregulation of stem and progenitor cell function should ultimately lead to improved strategies to treat and potentially prevent cardiovascular disease.

S7

GENE OR STEM CELL THERAPY FOR ANGIOGENESIS: WHICH IS THE FUTURE IN CLINICAL TRANSLATION?

J. Martin

British Heart Foundation, University College London, UK

Interest in stem cell regeneration of the heart began with the observation that a female heart transplanted into a man, when analysed after transplant failure, showed cells containing Y chromosomes to be incorporated into coronary arteries and myocardium. This, plus experiments in rodent models of MI, gave rise to the hypothesis that circulating multipotential cells might repair injured myocardium. Clinical studies (which were not ideally controlled) where circulating progenitor cells and bone marrow-derived progenitor cells were infused into the appropriate coronary artery after MI caused increase in ejection fraction. Importantly, there was no evidence of inflammation, cardiac damage, or arrhythmia in these patients. Gene therapy is a qualitative leap in therapeutics that will bring great benefit. However, it may be limited by (i) ideas, (ii) delivery systems, (iii) ethical problems, and (iv) the attitude of government regulators. The most beneficial concept of cardiovascular gene therapy is the local over-expression of therapeutic proteins. The most widely investigated of these has been vascular endothelial growth factor (VEGF), which has been used to induce angiogenesis in ischaemic muscle. However, the end-point of increased oxygen delivery to muscle needs to be used as a measurement of success. VEGF has also been shown to be arterioprotective, inhibiting initial features of atherosclerosis in the arterial wall. Local expression systems offer great advances in safety. Although in the laboratory many vectors have been studied it is not clear whether liposomal delivery systems will achieve sufficient transfection efficiency in a clinical situation. The first generation of adenoviruses is the vector most likely to be used in the immediate future for delivering therapeutic genes to the cardiovascu-

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lar system. Mechanical delivery devices such as catheters, which are used to deliver a gene to the luminal surface of arteries, have the disadvantage of liberating gene and vector into the systemic circulation. Delivery from the outside of the arteries using reservoirs has the advantage that systemic distribution of genes is less likely. However, surgical access is needed for these reservoirs to be applied to arteries. The development of therapeutic agents to combat cardiovascular disease has transpired in three steps: (1) small organic molecules, (2) gene therapy, and (3) stem cell therapy. Each has arisen from a different science, different organisational processes, and different clinical needs. In particular, stem cell therapy has great challenges for funding research and development. The role of VEGF as an adenoviral gene delivery system will be discussed in the treatment of arterial intimal hyperplasia. This will be compared to the use of autologous bone marrow transplantation. CD34+ cells will be delivered down the coronary artery to treat acute myocardial infarction.

ORAL COMMUNICATIONS

OC1

RHAMM AND CD44 MEDIATE DISTINCT ANGIOGENIC SIGNALLING PATHWAYS IN VASCULAR ENDOTHELIAL CELLS TREATED WITH OLIGOSACCHARIDES OF HYALURONAN

S. Matou[1], J. Gaffney[1], S. Kumar[2], J. Krupinski[3], M. Slevin[1,3]

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Hyaluronan is an important extracellular matrix glycosaminoglycan involved in angiogenesis. The stimulation of endothelial cells (EC) with oligosaccharides of hyaluronan (o-HA) triggers the activation of signalling pathways resulting in cell proliferation and migration. Using short-interfering RNA (siRNA) technology we investigated the mechanisms through which CD44 and RHAMM- mediate o-HA-induced angiogenesis in vascular EC. RHAMM knock-down decreased o-HA-induced angiogenesis characterized by a complete inhibition of EC proliferation and a significant decrease in EC migration and tube formation. Although surprisingly, knock-down of CD44 increased tube formation in un-stimulated cells, it abolished o-HA-induced EC proliferation and significantly decreased o-HA-induced tube formation. In contrast to RHAMM knock-down, CD44 knock-down did not modify o-HA-induced EC migration. To gain an insight into the mechanisms of CD44 and RHAMM-mediated angiogenesis, an analysis of o-HA-induced signaling pathways was undertaken. Inhibition of o-HA-induced phospho-extracellular signal-regulated kinase and phospho-cell division cycle 2 kinase expressions were observed by Western blotting after CD44 or RHAMM knock-down. Only CD44 knock-down abolished o-HA-induced phospho-protein kinase Cα (PKCα) and membrane cytoskeletal phospho-?-adducin (a PKC substrate) expression. Furthermore, the involvement of ?-adducin in o-HA-induced F-actin cytoskeleton rearrangement and tube formation was CD44-dependent. Only after RHAMM knock-down, o-HA-induced phospho-Src expression was notably reduced. Thus, a separate role for RHAMM and CD44 in o-HA-induced angiogenesis has been demonstrated, identifying novel signalling intermediates through RHAMM and CD44 in o-HA-mediated angiogenesis.

OC2

IN VITRO PROCESSES THAT MODEL CEREBRAL ANGIOGENESIS ARE INHIBITED BY TRAIL, STIMULATED BY FAS LIGAND, AND MODIFIED BY VASCULAR ENDOTHELIAL GROWTH FACTOR

P.-L. Chen, A.S. Easton

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The impact on cerebral angiogenesis of the tumour necrosis factor (TNF)-related ligands TRAIL (TNF-related apoptosis-inducing ligand) and Fas Ligand (FasL) was modeled *in vitro* with the human brain endothelial cell line hCMEC/D3. By flow cytometry, surface expression of the FasL receptor Fas and TRAIL receptor R2 was demonstrated. The VEGF (vascular endothelial growth factor) receptors R1 and R2 were both expressed. Endothelial proliferation was assessed from uptake of bromodeoxyuridine (BrdU). Treatment with VEGF (100ng/ml) reduced proliferation to $74.03 \pm 2.69\%$ of the BrdU incorporation in untreated cells (mean $\pm$ s.e.m., $n = 5$,

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$P<0.001$). By contrast, basic fibroblast growth factor bFGF (100ng/ml) increased proliferation to $130.22 \pm 12.15\%$ ($n=4$, $P<0.05$). TRAIL (10, 100, 1000ng/ml) induced a dose-related reduction in proliferation (mean 96.12, 60.39, 49.50%, respectively, $n=3$–6 per dose). By contrast, FasL either had no effect (1, 10ng/ml) or increased proliferation (100ng/ml, $110.47 \pm 4.75\%$, $n=3$, $P<0.05$). FasL (10ng/ml) was enhanced in combination with VEGF (100ng/ml) to $128.73 \pm 7.20\%$ ($n=6$), significantly greater ($P<0.01$) than these doses of FasL or VEGF alone. TRAIL (100ng/ml) combined with VEGF (100ng/ml) was not significantly different from VEGF alone, but the reduction with TRAIL was lessened ($n=6$, $P<0.01$). To examine tube formation, cells were seeded on Matrigel. Qualitative reductions in tube number and density were induced by TRAIL (10, 100, 500ng/ml). We present evidence that cerebral angiogenesis is inhibited by TRAIL and stimulated by FasL, with modification by VEGF.

OC3

ANGIOGENIC POTENTIAL DIFFERS BETWEEN EPOETIN DELTA AND DARBEPOETIN ALFA *IN VITRO*

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Introduction: Erythropoietin (EPO) has been shown to promote angiogenesis through activation of the EPO-receptor (EPOR) or CD131 (β-common chain) expressed on microvascular endothelial cells. Erythropoiesis-stimulating agents (ESA) might also be able to modulate angiogenesis although the differences among commercially available ESAs remain unknown. **Methods:** We investigated the angiogenic activity of human-cell-derived epoetin delta and hyperglycosylated darbepoetin alfa at similar ranges based on the conversion factor: 1 μg darbepoetin alfa = 200 IU epoetin delta). Angiogenic potential was assessed in primary isolates of human dermal microvascular endothelial cells (HDMECs) and the HMEC-1 endothelial cell line. Angiogenesis was investigated in a unique *in vitro* 3-D model. This allows quantification of new vessels from a preformed vascular network. **Results:** There was a 1.5 increase in angiogenesis with a low concentration (0.0005 μg/mL) of darbepoetin alfa in the primary cells and cell line, no change with an intermediate concentration (0.025 μg/mL), and an approximately fivefold increase at a higher concentration (0.1 μg/mL) in the cell line and 1.5-fold increase with the primary cells. Epoetin delta at 1IU caused a significant reduction in angiogenic activity when compared with vehicle-only controls ($P<0.01$–0.001) in the cell line. This was similar in the primary cells. At concentrations greater than the therapeutic range (= 20 IU/ml), both ESAs produced a significant increase in angiogenesis ($P<0.001$) although this increase was less with epoetin delta than with darbepoetin alfa in both cells. **Conclusions:** Using both HDMECs and HMEC-1 cells, epoetin delta and darbepoetin alfa differentially modulate angiogenic activity *in vitro*. Epoetin delta showed significantly less angiogenic potential than darbepoetin alfa at concentrations not exceeding the therapeutic range. This has implications for usage of these ESAs in anaemic patients who could have enhanced risk of tumour neovascularization, rheumatoid arthritis, and proliferative retinopathy.

OC4

ANGIOGENESIS IN ABDOMINAL AORTIC ANEURYSMS

C.A. Honstvet, V. Jagadesham, S. Perry, C. Hammond, D.J.A. Scott, P.F. Jones

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Abdominal aortic aneurysm (AAA) is a common condition affecting predominantly men over the age of 65. In AAA, the abdominal aorta expands over time, leading to a significant risk of rupture. Recent studies have identified that the intraluminal thrombus (ILT) plays a significant role in the expansion of the abdominal aorta. The purpose of this study is to further characterize the process of angiogenesis within the aortic wall and relate this and the ILT to the inflammatory infiltrate. Human AAA (infrarenal atherosclerotic) samples (1x5 cm strip from the anterior aortic wall) have been collected from patients undergoing elective AAA repair. Immunohistochemistry was performed using antibodies against CD31 and CD105 (markers of all endothelium and sprouting endothelium, respectively), and D2-40 and LYVE-1 (markers of lymphatic endothelium). The number of positively stained vessels was

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correlated with the presence of inflammatory infiltrate (as determined by light microscopy) and the thickness of ILT on CT. All results were analysed using non-parametric tests— Mann U Whitney and Spearman Rank Correlation. Qualitative analysis revealed neoangiogenesis in the media of the aortic wall, an area in which vessels are not normally found. Spatially, tracks of inflammatory cells and associated vasculature are seen within the wall, creating distinct bands. In addition, we have the first evidence of lymphangiogenesis within the AAA wall, confirmed by both D2-40 and LYVE-1 positive staining. There was a significant correlation ($p < 0.05$) between the number of CD31, CD105, and D2-40 positively stained vessels ($r^2 = 0.669$, 0.853 and 0.812, respectively) and the number of lymphocytes per high-powered field. The number of CD31+ vessels (median 24) and CD 105+ (median 13) were significantly different ($p < 0.05$). At present there is no statistically significant relationship between the number of vessels or lymphocytes and ILT. These results demonstrate evidence of mature and actively sprouting vessels within the AAA wall. This is closely linked to the inflammatory response and supports the concept of an immune-mediated disease process. Most excitingly, this research provides the first evidence of lymphatic vessels within the AAA wall and their relationship with the inflammatory infiltrate.

OC5

ONCOLYTIC VIRUS EXPRESSING REGULATORY ANGIOGENIC GENE THERAPY IN THE TREATMENT OF BREAST CANCER

M. Bazan-Peregrino[1], R. Sainson[2], R. Carlisle[1], R. Waters[3], A.L. Harris[2], R. Hernandez-Alcoceba[4], L.W. Seymour[1]

Clinical Pharmacology Department, [1] Weatherall Institute of Molecular Medicine,[2] and Centre for Statistics in Medicine, University of Oxford, UK[3]; Center for Applied Medical Research (CIMA), Pamplona, Spain[4]

A breast cancer therapy capable of destroying both tumour and its vasculature was designed using an oncolytic virus expressing regulatory angiogenic proteins. AdEHE2F kills cancer cells with estrogen receptor and hypoxia signalling defects and demonstrated optimal oncolytic activity and selectivity against breast cancer over five other oncolytic adenoviruses. Accordingly, this virus was engineered to express soluble Flt1 and soluble Delta like-4 (Dll4). sFlt1 is the soluble extracellular domain of VEGFR1 and binds to and sequesters VEGF-A, thereby preventing VEGFR2 stimulation which is crucial to trigger angiogenesis. sDll4 is the soluble extracellular domain of Dll4 and has been shown to block Dll4/Notch signalling which inhibits excessive VEGF-induced angiogenesis. sFlt1 and sDll4 were substituted for viral genes E3-6.7K/gp19K of AdEHE2F-X. AdEHE2F-X transgene expression was shown to be selective to breast cancer cells under estrogenic and hypoxic conditions *in vitro* and *in vivo*. sFlt1 expressed from AdEHE2F-sFlt1 inhibited endothelial cell proliferation and sprouting, whereas sDll4 expressed from AdEHE2F-sDll4 increased proliferation and branching *in vitro. In vivo*, sFlt1 expressed from AdEHE2F-Flt1 decreased the number of tumour vessels near the viral infection sites. *In vivo* AdHE2F expressing sFlt1 or sDll4 both showed superior anticancer activity compared to parental AdEHE2F, indicating at least additive efficacy between virotherapy and angiogenic approaches.

OC6

VEGF$_{121}$b IS ANTI-ANGIOGENIC IN CANCER AND EYE DISEASE

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Vascular endothelial growth factor-A (VEGF) induces angiogenesis and thereby the progression of cancer and neovascular eye disease. VEGF$_{xxx}$b is a family of splice variants of VEGF-A where xxx indicates the number of amino acids. VEGF$_{165}$b has been shown to be anti-angiogenic by inhibiting migration, proliferation, vasodilatation and angiogenesis. Normal human colonic epithelium predominantly expresses VEGF$_{121}$b and VEGF$_{165}$b, but a switch in expression occurs during the development of colon carcinoma resulting in a predominance of angiogenic VEGF isoforms in colon carcinoma. Over-expression of VEGF$_{121}$b in LS174t colon carcinoma xenografted tumours reduced tumour growth compared to empty vector (290 ± 66 mm^3

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vs 925 ± 268 mm^3 after 14 days, $p < 0.05$, unpaired t-test Welch correction). Histological analysis of the xenografted tumours revealed a decreased microvascular density in the smaller $VEGF_{121}b$ over-expressing tumours compared to empty vector tumours (1.7 ± 0.1 vs 5.0 ± 5.7% vs 3.5 ± 0.9, $p < 0.05$, unpaired t-test). The oxygen induced retinopathy mouse model causes vessel obliteration in the retina followed by a neovascularisation of the ischemic areas. Intraocular injection of $VEGF_{121}b$, reduced the ischemic area by 45% and caused a normalisation of the vasculature by 40% compared to control. *In vitro* models showed that $VEGF_{121}b$ influences endothelial cells by inhibiting VEGF-mediated migration but is cytoprotective. These results indicate that $VEGF_{121}b$ can lead to normal vessel growth by inhibiting VEGF-induced excess angiogenesis.

OC7

cAMP AND cGMP MODULATE RHOA-GTP SIGNALING AND ENDOTHELIAL RESPONSES TO COMBRETASTATIN-A-4-PHOSPHATE

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Cross talk between the endothelial microtubule network and the actin cytoskeleton, involving activation of the RhoA-GTPase pathway, plays a key role in the response of endothelial cells to the tubulin-binding vascular disrupting agent Combretastatin A-4-Phosphate (CA-4-P) [1]. cAMP and cGMP inhibit RhoA, by PKA- and PKG-mediated phosphorylation and are therefore possible modulators of CA-4-P activity. Monolayers of EA.hy926 or human umbilical vein endothelial cells were treated with either forskolin or cAMP/cGMP analogues. These agents improved basal endothelial barrier function and inhibited the CA-4-P-induced permeability rise. CA-4-P-mediated actin remodelling, disruption of cell-to-cell VE-cadherin junctions, and phosphorylation of the Rho kinase target and myosin light chain kinase, were also attenuated by pre-treatment with forskolin or the cAMP/cGMP analogues. Low doses of CA-4-P (1–10 nM) inhibited endothelial migration, and cAMP/cGMP analogues or Rho pathway inhibitors Y27632 and C3 exoenzyme significantly blocked this effect, suggesting that RhoA activation is key to the mechanism by which CA-4-P inhibits migration. Our results demonstrate that interactions between cGMP/cAMP and RhoA influence both the vascular disrupting and anti-angiogenic activities of CA-4-P and point to cAMP/cGMP as potential targets for improving the efficacy of this drug. [1] Kanthou, C.; Tozer, G. *Blood* 2002; 99:2060–2069.

OC8

INTERACTION BETWEEN BRADYKININ AND TNF-ALPHA TO INCREASE CEREBROVASCULAR PERMEABILITY

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Cerebrovascular permeability, as revealed by gadolinium-DTPA imaging, is often increased prior to, and during, multiple sclerosis relapse, and both bradykinin (Bk) and TNFα have been previously identified as candidate molecules involved in this process. We have examined the acute interaction between these molecules to investigate mechanisms that underlie a possible potentiation of their permeability increasing effects. The permeability to sulforhodamine dye (588 Da) of single small venules of anaesthetized rats (urethane i.p.) was measured as previously described [1] TNFα (0.5 nM) application for 10 minutes resulted in a 30-fold increase in sensitivity to bradykinin: the EC50 for Bk was decreased from $1.3x10^{-7}$M to $3.4x10^{-9}$M. The acute permeability response to Bk had been shown to depend on free radical formation from cyclooxygenase and lipoxygenase, so it is possible that TNFα treatment introduced an additional free radical producing pathway that may be activated by bradykinin. NADPH oxidase is one such pathway, which can be inhibited by apocynin, and we found that there was no potentiation of the Bk permeability response when TNFα was co-applied with apocynin (100 μM). In conclusion, the synergistic effects of inflammatory agents may contribute to the increased cerebrovascular permeability seen in MS through NADPH oxidase activation. [1] Sarker et al. *J. Physiol.* 2000; 528:177–187.

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OC9

CERAMIDE PLATFORMS REGULATE IONIZING RADIATION (IR) SENSITIVITY IN ENDOTHELIUM

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In contrast to the conventional understanding of radiotherapy, recent evidence indicates that tumor cell microvasculature apoptosis, mediated by acid sphingomyelinase (ASMase), plays a prominent role in tumor response to IR [1]. Investigating the mechanism by which ASMase initiates apoptotic signaling in endothelium, we showed that IR induces rapid translocation of ASMase onto the outer leaflet of the plasma membrane, increasing ASMase activity and generating ceramide in bovine aortic endothelial cells (BAEC). Using standard fluorescence and confocal microscopy, we demonstrated that in response to IR BAEC generate ceramide-rich platforms (1–5 microns) on the exoplasmic leaflet of the plasma membrane. Platforms increase within 30 sec of 15 Gy, peak at 1 min and dissipate to baseline within 10 min. Genetic, pharmacologic, and biochemical approaches that disrupt IR-induced platform formation attenuate apoptosis, demonstrating that ceramide-rich platforms are obligate for radiation-induced endothelial apoptosis. We therefore hypothesized that overexpressing ASMase would radiosensitize endothelium to platform-mediated apoptosis. To this end, we developed an adenoviral vector to express ASMase under control of the endothelial-specific promoter. Cell culture studies demonstrate that ASMase overexpression results in four-fold enhanced ASMase activity, with concomitant increases in ceramide generation and apoptosis upon irradiation. [1] Garcia-Barros et al. *Science* 2003; 300 (5622):1155–9.

OC10

EFFECTS OF LAMINAR FLOW ON SIGNAL TRANSDUCTION IN HUMAN GLOMERULAR ENDOTHELIAL CELLS

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Mean glomerular shear stress *in vivo* is 10–20 dyn/cm^2, indicating that shear stress may be an important parameter in determining metabolic function of glomerular endothelial cells. Here we determine the effects of varying levels of laminar shear stress on the phosphorylation of eNOS, Akt, and AMPK in human glomerular endothelial cells (hGEnCs). hGEnCs were exposed to either 0 or 10, 15 or 20 dyn/cm^2 of steady shear stress for 24 h on an orbital rotator. Protein was extracted and subjected to western blot analysis. In hGEnCs, exposure to laminar shear stress for 24 hours increased the serine 1177 phosphorylation of eNOS by 1.7-fold, when compared to the static control. The phosphorylation of eNOS at Ser-1177 was not dependent on the degree of shear stress. Similarly, hGEnCs exhibited an increase in Akt phosphorylation, at Ser-473, following exposure to laminar shear stress, when compared to the static controls. In contrast, AMPKα was de-phosphorylated, at Thr-172, in hGEnC exposed to 24 hours of laminar shear stress. Again, the de-phosphorylation of AMPK was not dependent on the degree of shear stress. In summary, we have shown that both eNOS and Akt are phosphorylated, whilst AMPKα was de-phosphorylated, in response to 24 hour exposure to laminar shear stress, indicating that shear stress can be an important factor in the metabolic function of glomerular endothelial cells.

Supported by Kidney Research UK

OC11

ANGIOPOIETIN-1 ALTERS MICROVASCULAR PERMEABILITY COEFFICIENTS BY MODIFYING ENDOTHELIAL GLYCOCALYX

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We examined Ang1 effects on microvascular hydraulic conductivity (L_P) and reflection coefficient to albumin (s) in MS222-anaesthetised frogs using a modified Landis-Michel technique before and after 200 ng.ml^{-1} Ang1 perfusion. Glomerular filtration coefficient (L_PA) and s were measured oncometrically *ex vivo* [1,2,3]. L_PA in Wistar rat glomeruli

and s in individual mouse glomeruli were measured after incubation in either vehicle or 200 ng.ml^{-1} Ang1.

	Baseline	Ang1	n =	p =	test
Mesenteric L_P	2.0 ± 0.9	1.2 ± 0.9	11	< 0.05	Wilcoxon
Mesenteric ?s	0.78 ± 0.03	0.91 ± 0.03	10	< 0.05	Paired t-test
Glomerular L_PA	1.00 ± 0.09	0.76 ± 0.06	30	< 0.05	Unpaired t-test
Glomerular s	0.9725	0.9804	15	< 0.05	Paired t-test

We hypothesised that endothelial glycocalyx modification induced these diverse changes. Ang1-treated human microvascular endothelial cell supernatant decreased 488nm light absorbance by Alcian Blue [0.13 ± 0.02 (n = 6); vehicle: 0.05 ± 0.02 (n = 6); p < 0.05, unpaired t-test], indicating increased glycosaminoglycan content. Ang1 prevented the pronase-induced mesenteric microvessel L_P fold increase (pronase: 2.6 ± 0.4; Ang1 + pronase: 1.2 ± 0.3; p < 0.05, unpaired t-test) previously attributed to glycocalyx loss. Ang1-induced endothelial glycocalyx modification may explain the Ang1-induced microvascular permeability coefficient changes. [1] Salmon et al. *J Physiol* 2006; 570:141–56 [2] Sage et al. *Microcirc* 2007; 14:635–65 (abstract) [3] RH Adamson *J Physiol* 1990; 428:1–13.

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OC12

IDENTIFICATION OF THE PRO-ANGIOGENIC ACTIVITY OF NEUROTROPHIN-3 (NT-3)

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Neurotrophins (NTs) regulate neuronal survival and differentiation. Non-neural functions of NTs include angiogenesis promotion by NGF and BDNF and involvement of NT-3, via its trkC receptor, in heart development. The possibility that NT-3 promotes angiogenesis has not been evaluated yet. We investigated the pro-angiogenic potential of NT-3 in a mouse model of limb ischemia. We preliminarily found that endothelial cells (ECs) of capillaries and arterioles in skeletal muscles are responsive to NT-3 since they express trkC. Unilateral limb ischemia was induced in anesthetised CD1 mice by femoral artery ligation and an adenoviral vector carrying rat NT-3 gene (Ad.NT-3, 10^8pfu) was immediately injected in ischemic adductors. Controls received *Ad.Null.* Capillary and arteriole densities and blood flow (BF) recovery were evaluated by immunohistochemistry (IHC) and color laser Doppler , respectively, on 14d post-ischemia. To study the effect of *Ad.NT-3* on ECs proliferation, IHC for BrdU and Proliferating Cell Nuclear Antigen was performed on adductors harvested after 3d from the surgery. Activation of trkC, Akt, and eNOS in ischemic muscles was also analyzed by western blot and VEGF-A expression was measured by RT-PCR and ELISA. *Ad.NT-3* improved BF to the ischemic foot (P < 0.05 vs. *Ad.Null*), enhanced ECs proliferation (P < 0.05) and increased capillary (P < 0.01) and arteriole (P < 0.05) densities in ischemic adductors. *Ad.NT-3* induced phosphorylation of trkC, Akt, and eNOS without changing VEGF-A levels. Our data provide evidence that local NT-3 delivery may offer a new strategy for inducing therapeutic neovascularization in response to ischemic vascular disease.

OC13

PODOCYTE INDUCIBLE ANGIOPOIETIN-1 OVEREXPRESSION RESULTS IN ALBUMINURIA AND PODOCYTE FOOT PROCESS ABNORMALITIES

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The Angiopoietin (Ang)-1/Ang-2 system is important in vessel injury and repair. In the normal glomerulus Ang-1 is expressed, while Ang-2 is absent. In glomerular disease it is often noted an upregulation of both Ang-1 and Ang-2 expression with the latter being of a greater degree. To obtain insights into the role of Ang-1 in glomerular pathophysiology we generated mice with inducible podocyte-specific Ang-1 overexpression. When the transgene was induced in adults for up to 10 weeks, mice had significant increases in albuminuria, paralleled by podocyte foot process fusion and reduction in glomerular endothelial fenestrations. We did not observe any change in the expression of Ang-2 in renal cortex lysate while we detected a downregulation of their cognate receptor, Tie-2. Vascular endothelial growth factor-A was upregulated in Ang-1 overexpressing mice. The expression of nephrin, one of the most important components of the podocyte slit diaphragm, was similar within all the groups of animals studied. The observed phenotype was paralleled by nonsignificant changes of systemic blood pressure, creatinine clearance, or kidney weight/ macroscopic appearance. This study highlights the complexity of the molecular mechanisms regulating the normal function of the glomerular filtration barrier, and how Ang-1 leads to alteration of the glomerular filtration barrier and albuminuria.

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OC14

ANGIOPOIETIN-1 MODULATES SEPSIS-INDUCED MICROVASCULAR LEAK *IN VIVO*

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Loss of endothelial integrity contributes to oedema and hypotension during sepsis. The angiogenic factor angiopoietin-1 (Ang-1) has vessel stabilising effects, improving blood pressure and reducing mortality during lipopolysaccharide (LPS) exposure in mice[1]. The current study investigated whether a novel Ang-1 variant with improved solubility and activity (MAT-Ang1), prevented loss of microvascular endothelial integrity in a murine model of sepsis[2]. A titanium chamber was chronically implanted into the dorsal skinfold of male C3H/HEN mice ($n = 12$, 7–10 weeks) to visualise 4th order striated muscle venules (<25um). Mice were sedated with ketamine/ atropine/ xylazine (60/6/6mg.kg^{-1} i.p.) during 0–4 and 20–24hrs of LPS administration (1mg.kg^{-1} LPS at 0hrs and 19hrs) with and without MAT-Ang1 (1.2mg.kg^{-1}, i.v. at 19 hrs). Fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA, 200µl.100g^{-1}, 20hrs i.v.) quantified macromolecular leak with computerised image analysis (IPPROPLUS). LPS induced macromolecular leak (mean +sem) (40.7% at 22 hrs$\pm$2.3 and 64.0% at 24hrs$\pm$2.0, $n = 6$) which was reduced by coadministration of MAT-Ang1 (29.9%$\pm$3.1 and 46.1%$\pm$1.7 , respectively, $p = < 0.05$, $n = 6$). Hence, MAT-Ang-1 appears to reduce microvascular endothelial damage and oedema during sepsis, which may contribute to maintenance of blood pressure and organ perfusion. [1] Witzenbichler, B., et al. *Circ* 2005; 111 97–105. [2] Cho et al. *Proc Nat Acad Sci* 2004; 101, 5553–5558.

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OC15

MOLECULAR MECHANISMS INVOLVED IN HIGH GLUCOSE-INDUCED HO-1 EXPRESSION IN BOVINE AORTIC ENDOTHELIAL CELLS

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Hyperglycaemia and diabetes are associated with enhanced oxidative stress, leading to the progression of diabetic vascular pathologies.[1] The redox sensitive transcription factor Nrf2 mediates induction of protective genes, such as heme oxygenase-1 (HO-1), via activation of an antioxidant response element (ARE).[2] HO-1 metabolises pro-oxidant heme to the antioxidants biliverdin and bilirubin.[3] The present study has examined the signalling pathways involved in high glucose enhanced superoxide generation, Nrf2 activation, and HO-1 induction in bovine aortic endothelial cells (BAEC). Confluent monolayers were treated for 0–24 h with DMEM containing 25mM D-glucose, in the absence or presence of the superoxide ($O_2^{\bullet -}$) scavengers SOD (200 Uml-1) and Tiron (10µM), inhibitors of NADPH oxidase (apocynin, 100 µM), flavoproteins (DPI, 10µM), eNOS (L-NAME, 50µM), or MAPK (SB203580, 10µM; SP600125, 20µM; U0126,

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10μM). HO-1, nuclear Nrf2, and phosphorylated MAP kinases levels were determined by immunoblotting, Nrf2 translocation by immunofluorescence, and $O_2^{\bullet -}$ by L-012 chemiluminescence. Treatment of BAEC with 25mM D-glucose (0–24h), but not D-mannitol, elicited concentration- and time-dependent increases in $O_2^{\bullet -}$, Nrf2 translocation and HO-1 expression. Increased HO-1 expression induced by high glucose was attenuated by Tiron and L-NAME, but not by extracellular SOD or NADPH oxidase inhibitors. Although high glucose induced rapid phosphorylation of $p38^{MAPK}$ and JNK, only inhibition of JNK abrogated high glucose-induced HO-1 expression, implicating the JNK signaling pathway in high glucose-induced activation of the Nrf2-ARE pathway and HO-1 expression..[1] Goldin et al. *Circulation* 2006;114: 597–605. [2] Mann et al. *Cardiovasc. Res.* 2007;75:261–274. Siow et al. *Redox Rep.* 2007;12:11–15.

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OC16

DIABETES INDUCES STRUCTURAL AND FUNCTIONAL ALTERATIONS OF BONE MARROW MICROVASCULATURE

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Background: Dysfunction of vascular endothelial cells (EC) and endothelial progenitor cells (EPC) contributes to cardiovascular complications in diabetes (D). We propose the novel hypothesis that D impairs EPC function both directly and by altering the bone marrow (BM) vascular niche, which normally supports stem cell maturation and mobilization. **Methods:** Eight-week-old mice were made diabetic by streptozotocin and sacrificed 8 or 26 weeks thereafter, together with age-matched non-diabetic controls (C), for IHC of BM microvasculature and flow cytometry of BM progenitor cells. Blood flow was determined by fluorescent microspheres. Trans-migration of murine EPC (labeled with PKH67) through murine BM EC was studied under basal conditions and using SDF-1 as a stimulus. **Results:** In D mice, capillary rarefaction developed from week 8 (19.9 ± 4.6 vs 23.4 ± 2.3 cap/mm^2 in C, P = N.S.) to week 26 (11.7 ± 2.1 vs 22.5 ± 2.8 cap/mm^2 in C, $P < 0.02$), which was paralleled by doubling of TUNEL-positive capillaries ($P < 0.05$). Blood flow was significantly reduced in BM of D mice ($P < 0.01$ vs controls). Mononuclear cells (MNC) and Lin-cKit$^+$Sca1$^+$, Lin-Sca$^+$CD31$^+$, and Lin-Flk1$^+$CD31$^+$ progenitor cells were similarly abundant in BM of D and C mice. However, D BM MNC and progenitor cells expressed Annexin V ^{high} with higher frequency (MNC, 5.4 ± 1.2 vs $2.3 \pm 0.1\%$ in C; Lin-cKit$^+$Sca1$^+$, 11.1 ± 3.8 v. $2.3 \pm 1.0\%$ in C, $P < 0.01$). Limb ischemia resulted in reduction of BM capillary density and triggered cell necrosis in ipsilateral BM of D mice (7-aminoactinomycin-D staining, $P < 0.05$ vs C). Spontaneous trans-migration of murine BM EPC through BM EC was increased and the migratory response to SDF-1 abolished by D. **Conclusions:** Results newly demonstrate the presence of BM microangiopathy in D mice. This microvascular pathology may have detrimental effects on the mobilization of stem cells and thereby negatively influence vasculogenesis

OC17

MECHANISMS OF HYPOXIC PULMONARY VASOCONSTRICTION IN ISOLATED RAT INTRAPULMONARY ARTERIES

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Hypoxic pulmonary vasoconstriction (HPV) maintains the ventilation-perfusion ratio during alveolar hypoxia. We studied the mechanisms contributing to HPV using intrapulmonary arteries (500 – 800 μm dia.) mounted in a myograph. Drugs were applied 20 min before hypoxia, which was imposed by gassing the chamber with 95% N_2/5%CO_2 (pO_2 = 15–20 Torr). The L-type Ca^{2+} antagonists diltiazem (10 μM, n = 6) and nifedipine (3 μM, n = 5), and the store-operated Ca^{2+} channel antagonist La^{3+} (100 μM, n = 4) had no significant effect on HPV. HPV persisted in Ca^{2+}-free Krebs containing EGTA (200 μM; n = 4; n.s.). Both ryanodine (100 μM; n = 4; $P < 0.05$) and the lysosomal H^+ -ATPase inhibitor concanamycin A (1 μM; n = 8; $P < 0.05$) strongly suppressed HPV, but neither the cyclic ADP-ribose antagonist 8-bromo-cADPR (300 μM; n = 3) nor the the AMP-activated protein kinase (AMPK) inhibitor compound C (10 μM; n = 4) had a significant effect. The rho-kinase inhibitor Y-27632 (1 μM) also markedly attenuated HPV (n = 5; $P < 0.05$), and hypoxia caused a biphasic increase in the phosphorylation of the myosin phosphatase targeting subunit MYPT1. These results are consistent

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with a model in which HPV in these arteries does not require AMPK activation or the influx of extracellular Ca^{2+}, but involves Ca^{2+}-sensitisation and intracellular Ca^{2+} release from concanamycin and ryanodine sensitive compartments.

OC18

COLLAGEN RECRUITS PLATELETS VIA GPIb-IX-V TO MUCOSAL VILLI FOLLOWING MURINE INTESTINAL ISCHAEMIA — REPERFUSION (IR) INJURY *IN VIVO*

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The small intestinal (SI) mucosal villi are very susceptible to IR injury. Previous studies suggest platelets contribute to injury following recruitment by intact endothelium. However, IR injury can lead to endothelial denudation and subsequent collagen exposure. We therefore hypothesise platelet-collagen interactions lead to platelet recruitment following I/R injury. Therefore, roles for platelet collagen glycoprotein receptors, GPVI and integrin $\alpha_2\beta_1$, and the indirect collagen receptor GPIb-IX-V (via VWF), were determined intravitally. Intestinal IR injury was induced for 30min in anaesthetised (ketamine/xylazine) C57BL/6 (WT) or FcR?-chain$^{-/-}$ (also lack GPVI) mice. Some WT mice were perfused with function blocking mAb to $\alpha_2\beta_1$ (SAM G4) or GPIbα (Xia-B2). Donor and endogenous platelets were fluorescently labelled to quantitate singular and aggregated platelets , respectively. Large microthrombi and diminished blood flow were observed in jejunal villous microvessels upon IR injury. In contrast, single adherent platelets, as well as fewer/smaller aggregates and maintained blood flow were observed in the ileum. Platelet recruitment was not significantly different in FcR?-chain$^{-/-}$ mice or those pre-treated with $\alpha_2\beta_1$ mAb. In contrast, significantly smaller ($p < 0.001$) and less fluorescently intense ($p < 0.001$) microthrombi with improved blood flow were observed jejunally in animals pre-treated with the GPIb mAb. Identifying therapeutic avenues targeting GPIb-IX-V may prove beneficial in improving clinical morbidity associated with intestinal I/R injury.

Supported by the British Heart Foundation

OC19

MECHANOSENSITIVE HYALURONAN SECRETION; ROLE OF TRANSCRIPTION-TRANSLATION *IN VIVO*

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Hyaluronan (HA) is a major determinant of interstitial hydraulic permeability and fluid retention in joints. We assessed the coupling of synovial HA secretion to movement and the role of protein synthesis. Endogenous HA was washed out from cannulated knee joints of anaesthetised rabbits and newly secreted HA recovered after 5h for HPLC analysis. Joints were cycled at varying frequencies (0, 0.17, 0.5, 1.5Hz) and durations (0, 1, 3, 9min per 15min) during the 5h. Protein synthesis was blocked in some moved joints and in some phorbol ester (PMA) stimulated static joints by intra-articular actinomycin-D and puromycin (AD-PM) or cycloheximide (CX). Golgi transport was inhibited by brefeldin A (BFA). Results are normalised as secreted μg HA/hour per 100 μg endogenous HA (%qHA).%qHA showed a graded, curvilinear response to movement frequency and duration ($p = 0.0001$, ANOVA). CX reduced movement-stimulated HA secretion (MSHA) by $\sim 18\%$ ($p = 0.05$, $n = 10$, paired t test) and AM-PM reduced MSHA by $\sim 30\%$ ($p = 0.03$, $n = 9$). A 19% reduction of MSHA by BFA did not reach significance ($p = 0.2$, $n = 9$). CX and AD-PM also reduced PMA-stimulated static secretion, by 35% ($p = 0.03$, $n = 5$) and 30% , respectively ($p = 0.005$, $n = 8$, paired t test). Neither agent reduced the unstimulated static secretion rate. The results show for the first time a graded stimulation of HA secretion by movement that is partly dependent on protein synthesis. Mechanosensitive secretion may protect tissues from HA depletion and underpin exercise therapy for osteoarthritis.

OC20

MICROVASCULAR FUNCTION IS IMPAIRED IN PEOPLE WITH SYMPTOMATIC CORONARY ARTERY DISEASE INDEPENDENT OF ATHEROSCLEROTIC LOAD

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Atherosclerotic coronary artery disease (CAD) is the biggest killer in the UK; however, there is a poor correlation between coronary angiographic stenosis and symptoms. CT coronary artery calcification (CAC) scores, a non-interventional quantitative measure of coronary atherosclerotic load, and skin laser Doppler fluximetry in response to heating and ischaemia, assessing microvascular function, were measured in 83 men with angiographically confirmed atherosclerotic CAD and 84 age-matched healthy controls. Men with symptomatic angiographic CAD had poorer microvascular hyperaemic response and reactive hyperaemia than asymptomatic controls (114 (95% CI 106–122) vs 143 (134–153) arbitrary units (au) $p < 0.001$ and 42(38–46) vs 53(48–58)au; $p = 0.001$). This was unaccounted for by conventional cardiovascular risk factors ($p = 0.02$ and 0.009 after adjustment). Of the asymptomatic group, 32 men (38%) had elevated CAC scores (CAC scores > 40). This group had a similar risk profile (e.g., weight, lipids) and CAC score as the symptomatic CAD group (312 (193–506) Agatston Units (AU) vs. 298 (208–428) AU , respectively; $p = 0.8$) but better maximum and reactive hyperaemia (147(131–166)au vs. 114(106–122)au: $p = 0.0003$ and 55(46–65) vs. 42(38–46)au: $p = 0.005$, respectively). Angiographic symptomatic CAD is associated with microvascular dysfunction independent of coronary artery calcification. Individuals with elevated CAC scores but no symptoms had better microvascular function.

OC21

HAEMATOPOIETIC STEM CELL RECRUITMENT TO HEPATIC MICROCIRCULATION FOLLOWING ISCHAEMIA-REPERFUSION INJURY IS DEPENDENT ON THE $\alpha_4\beta_1$ INTEGRIN

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Hematopoietic stem cells (HSCs) migrate to injured liver and may aid tissue repair. However, no studies have identified the molecular mechanisms underlying HSC recruitment. We utilised fluorescent intravital microscopy (IVM) to determine the roles of $\alpha_4\beta_1$, CD31, CD44, and CD18 in promoting HSC recruitment after hepatic ischaemia-reperfusion (IR) injury *in vivo*. An *in vitro* flow-based adhesion assay was used to monitor kinetics and molecular events governing recruitment *in vitro*. Hepatic ischemia was induced for 90 min in anaesthetised C57BL/6 mice (ketamine/xylazime). CFSE-labelled HSCs ($1x10^6$ HPC-7s; ia) were administered post-reperfusion and IVM observations were made for 60 min. Some HPC-7s were pre-treated with function blocking antibodies to the above stated adhesion molecules. IR injury significantly increased HPC-7 adhesion (e.g., 13.3 ± 1.7 vs 3 ± 1.0 at 30 mins; $p < 0.05$). HPC-7 pre-treatment with an $\alpha_4\beta_1$ blocking antibody significantly reduced adhesion compared to IgG control pre-treated cells (5.5 ± 1.5 vs 15.5 ± 1.9 at 55 mins; $p < 0.001$). However, pretreatment with CD31, CD44, and CD18 antibodies did not reduce adhesion at any time point. The critical role of $\alpha_4\beta_1$ integrin was further confirmed *in vitro* since firm adhesion and migration were significantly ($p < 0.001$) reduced, but rolling increased, on murine cardiac endothelial cells. Our novel data indicate a central role for $\alpha_4\beta_1$ in mediating HSC recruitment, indicating similar adhesion molecules to those used by mature leukocytes are also used by HSCs.

Funded by the Medical Research Council

OC22

NANO-OPTICAL-MECHANICAL MANIPULATION OF STEM CELLS IN PHYSIOLOGICAL FLOWS: DESIGN PRINCIPLES FOR DELIVERING STEM CELL THERAPY

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A growing body of research is investigating the control and maintenance of stem cell phenotype for application in a broad range of clinical therapies. However, little is known about the ability for the cells to maintain phenotype through vascular delivery which can often involve subjecting cells to physiological flows. We have developed a novel optical-mechanical technique which enables the manipulation of cells in an amiable manner and quantitatively correlates the flow-induced nanome-

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chanical forces with the deformation behaviours and ultimately gene expression in cells. This method uses optical tweezers to trap and move the microbead-attached mesenchymal stem cells (MSCs) in a microfabricated channel at various speeds for mimicking physiological flows. This significantly minimizes laser heating and photon-induced stress for normal operation with laser-trapped cells. Computational fluid dynamics (CFD) has been applied to simulate flow-induced shear stress over the cell's membrane and to quantitatively correlate the forces with the cell deformations. Ultimately, the gene expression of the cells examined by bioassay will be correlated to the forces they experienced. More importantly, the cells can be manipulated in a natural status by this approach, which may open an avenue to design principles for the next generation of stem cell sorting and delivery.

OC23

LONGITUDINAL ANALYSES OF VESSEL PERMEABILITY DURING WOUND HEALING INDUCED ANGIOGENESIS *IN VIVO*

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Regeneration of injured tissue is a dynamic process that is critically dependent on the formation of new blood vessels and restructuring of the angiogenic plexus. Intravital microscopic analysis of vascular restructuring in response to injury within the mouse dorsal skinfold chamber permits the monitoring of structural and functional changes within the plexus of any individual wound over time (approximately 2 weeks). Using Fluorescence Recovery after Photobleaching (FRAP) *in vivo*, three distinct functional regions, viz "normal" vasculature adjacent to the wound, angiogenic plexus, and blind-ended vessels (BEVs) are compared from days 5 to 7 post wounding (the period of most intense angiogenesis). Thinner and shorter BEVs, found in the leading edge of the newly developing vascular plexus, tend to exclude erythrocytes and FRAP measures do not recover to baseline fluorescence intensity levels. From days 5 to 7, angiogenic plexi feeding the nascent vessels become far less leaky. FRAP measures within the normal plexus recover instantly and display a cyclical pattern of fluorescence intensity with a periodicity of around 20 seconds. Further *ex vivo* whole-mount *in situ* dual immunostaining of tissue recovered following the last FRAP measurement was performed for the endothelial (GS isolectin B4) and pericyte specific (α-SMA) markers. Although the angiogenic plexus is generally sparsely covered by pericytes, some sprouts have a perycyte coating close to the inlet, never at the tip. We hypothesize that there is a correlation between flux of the plasma marker across the vessel wall with the maturity and surface area of the vessel, which would link structure with function.

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OC24

ADAM 15 IS ESSENTIAL FOR REGULATED ANGIOGENESIS

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Angiogenesis is a complex process whereby neocapillaries sprout from pre-existing vessels in response to hypoxia. ADAM 15 (metargidin) is a member of the mammalian disintegrin-metalloprotease family that is highly expressed in vascular cells and is known to bind to integrins expressed on endothelial cells. However, the biological function of ADAM 15 remains unclear as null mice show no discernible developmental abnormalities but appear to display impaired pathological angiogenesis [1]. In the present study we show that ADAM 15 is essential for regulated angiogenesis. Impairing ADAM 15 function with novel site-directed antibodies in the murine retina promoted the development of an abnormal vascular plexus comprised of vessels displaying reduced perfusion, patency, and branching. Under these conditions, VEGF-induced endothelial cell survival, proliferation, and Akt activation were severely impaired whereas cell migration, associated urokinase activity, and uPAR antigen levels were elevated. In contrast, stable expression of ADAM 15 in monocytic U937 cells, a high uPAR expressing cell line, promoted severe loss of surface uPAR antigen levels and urokinase

activity. In addition, recombinant ADAM 15 but not ADAM 12 metalloprotease domain cleaved recombinant uPAR in a purified *in vitro* assay. These studies establish a role for ADAM 15 in regulating angiogenesis through mechanisms promoting VEGF signalling to Akt and via the negative regulation of the plasminogen activation system through the proteolytic processing of uPAR. [1] Horiuchi K, et al. *Mol Cell Biol* 2003; 23(16):5614–24.

OC25

A SINGLE NUCLEOTIDE POLYMORPHISM IN THE HUMAN TISSUE KALLIKREIN GENE LEADS TO REDUCED ANGIOGENESIS *INVIVO*

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Human Tissue Kallikrein (hTK) is known to play an important role in the reparative angiogenic response to ischaemic insult[1]. A recently identified loss of function polymorphism (R53H) in the hTK gene leads to a 50% reduction in urinary enzymatic activity[2]. With this in mind, wild type (WT) and mutant (R53H) hTK were overexpressed in the adult rat mesentery, and intravital and confocal imaging was used to assess the impact of the R53H polymorphism on *in vivo* angiogenesis. The R53H mutant showed a significant reduction in the hTK induced increase in functional vessel area ($202 \pm 49\%$ vs $480 \pm 110\%$, $p < 0.05$) and also led to a reduction in vessel density (372 ± 46 vs $624 \pm 53 mm^{-2}$, $p < 0.01$). Surprisingly, however, R53H induced vessels maintained specific characteristics of WT vessels when compared to control, including increased pericyte coverage ($31 \pm 3.7\%$ vs $48 \pm 4.7\%$ vs $58 \pm 5.3\%$, $p < 0.05$) and reduced sprout point density (10.9 ± 3.1 vs 8.2 ± 2.3 vs 8.5 ± 2 mm^{-2}). These data provide evidence for a functional effect on angiogenesis that may have clinical implications for recovery in ischaemic tissue. With R53H heterozygotes representing around 5–7% of the white population, large-scale epidemiological investigation is required to determine the effect of this SNP on cardiovascular outcome. Data presented are mean $\pm$ SEM, (eGFP vs R53H vs WT) with $n = 6$. [1] Emanueli et al. *ATVB* 2000; 20:2379–85. [2] Azizi et al. *JCI* 2005; 115:780–787).

OC26

THE VITREOUS HUMOUR GLYCOPROTEIN OPTICIN IS AN ENDOGENOUS INHIBITOR OF ANGIOGENESIS

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Purpose: To determine whether opticin inhibits experimentally induced preretinal neovascularisation.

Materials and Methods: Opticin null mice were generated and bred onto a C57/BL6 background. The opticin null mice or wild-type controls were placed in 75% oxygen from P7-12 and then brought back to normoxia. At day P17 the eyes were fixed, wax embedded, and sections were stained with haematoxylin and eosin. The number of neovascular nuclei on the vitreal side of the ILM per section was measured. In further experiments wild-type mice were exposed to 75% oxygen between P7-12 and then at P14 one eye was injected intravitreally with recombinant opticin (2.5 μg) and the contralateral eye with carrier buffer alone. The eyes were analysed at P17 as above. **Results:** No morphological abnormalities were observed in the eyes of opticin null mice not exposed to hyperoxia. The opticin null mice that were exposed to high oxygen between P7-12 had an increased number of preretinal neovascular nuclei per cross section (109 ± 5.6 S.E) compared to wild-type controls (73 ± 2.9) ($P < 0.001$). The opticin injected wild-type eyes showed a decrease in preretinal neovascular nuclei per cross-section (38 ± 3.1) compared to eyes injected with PBS alone (76 ± 4.4) ($P < 0.001$). **Conclusion:** Opticin inhibits preretinal neovascularisation in a concentration-dependent manner. This complements *in vitro* data using endothelial cells showing that opticin interacts with the $\alpha_2\beta_1$ integrin and disrupts focal adhesion and actin stress fibre formation.

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POSTER COMMUNICATIONS:

PC1

THE ROLE OF ANGIOSTATIN (AS) IN THE PATHOPHYSIOLOGY OF FETAL GROWTH RESTRICTION (FGR)

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Activation of inappropriate haemostasis in the placenta and fibrin deposition is associated with pregnancy complications and generation of a range of pro/anti-angiogenic proteins at the cell surface. Levels of free plasminogen, a key haemostatic cascade pro-enzyme that is pro-angiogenic, increase in normal pregnancy and may contribute to the growth and maintenance of the vasculature. Angiostatin$_{4.5}$ (AS$_{4.5}$: an anti-angiogenic 52kDa proteolytic cleavage product of plasminogen), is produced at the cell surface by tethering of plasminogen to β-actin and its subsequent cleavage by uPAR. We hypothesised that AS is capable of inducing human FGR and that the mechanism is related to trophoblast turnover and generation of AS on the villous surface. *In vitro* studies reveal a dose-dependent effect of AS$_{4.5}$ on apoptosis and the rate of wound healing in human trophoblast cell lines (SGHPL-4 and HTR8/SVneo), with no such effect in non-trophoblast cell lines. Preliminary results of dual immunostaining have revealed an increased level of co-immunoreactivity for β-actin and uPAR within human FGR samples. The proportion of cells which are immunoreactive for cleaved Caspase-3 (an effector caspase in apoptosis) is increased within human FGR placentas. These results provide evidence that angiostatins are implicated in the pathogenesis of this condition.

PC2

IDENTIFICATION OF THE PROSURVIVAL AND ANGIOGENIC ACTIVITY OF NERVE GROWTH FACTOR ON CARDIAC MYOCYTES

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Recent research showed that the potential of neurotrophins (NTs) expands beyond the nervous system to include cardiovascular actions. This study investigated the novel hypothesis that the neurotrophin nerve growth factor (NGF) is a pro-survival factor for the heart and explored underlying mechanisms of this action. We worked on cultured rat neonatal cardiomyocytes (RNCMs) and on a murine model of myocardial infarct. We demonstrated that RNCMs express NGF and its trkA receptor. RNCMs given a neutralizing antibody for NGF or the trkA inhibitor K252a underwent spontaneous apoptosis. Stimulation of RNCMs with NGF induced trkA phosphorylation, followed by Ser473-phosphorylation and nuclear translocation of protein kinase B (Akt). In response to Akt activation, Forkhead transcription factors Foxos were phosphorylated and excluded from the nucleus. RNCMs subjected to hypoxia/reperfusion or angiotensin II stimulation underwent apoptosis, which was prevented by adenovirus (*Ad*)-mediated *NGF* over-expression. Inhibitory approaches using K252a, LY294002 (a pan-PI3K inhibitor), an adenovirus carrying a dominant negative mutant form of Akt (*Ad.DN.Akt*), or an adenovirus carrying an Akt-resistant Foxo-3a (Ad.AAA-Foxo-3a) demonstrated that the pathway encompassing trkA, PI3K-Akt, and Foxo is essential for the pro-survival effect of *NGF* to take place. Finally, direct intra-myocardial *NGF* gene transfer inhibited cardiomyocyte apoptosis and promoted neoangiogenesis in a murine model of myocardial infarct. Our study is the first to demonstrate that NGF is an endogenous pro-survival and pro-angiogenic factoring heart. These findings significantly add to the understanding of the cardiovascular properties of NTs.

PC3

TIME-COURSE OF ANGIOGENESIS STIMULATION, AND CAPILLARY REGRESSION AFTER CESSATION OF STIMULUS

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Elevated shear stress results in angiogenesis via capillary splitting, [1] where extent of angiogenesis is correlated with femoral blood flow (FBF), with a maximal response by 28 d. [2] An increase in capillary to fibre ratio (C:F) precedes that of FBF,

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suggesting an arteriogenic and angiogenic component. We examined vascular remodelling after cessation of vasodilator stimulus, which has not been studied in this angiogenic phenotype. Male C57BL10 mice received prazosin (50mg/L) *ad libitum* for 28 d; groups (n = 4) were sampled at 2, 4, 7, 14, and 28 d as well as 7, 14, and 42 d of recovery. FBF increased from control levels (0.40 ml/min) to 0.61 ml/min at 28 d of treatment, dropped within 1 wk of recovery (0.54 ml/min; $P < 0.05$), and returned to 0.40 ml/min at 6 weeks post-treatment. Tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were sampled; alpha smooth muscle actin and lectin immunohistochemistry staining were used to estimate arteriolar density (AD) and C:F, respectively, as an indication of arteriogenesis and angiogenesis. C:F of EDL and TA showed regression began within 1 wk of treatment cessation, and was not significantly different from control by 2 wk. A greater density of αSMA-positive capillaries at 4 weeks prazosin treatment indicated that arteriogenesis had occurred, but by 1 wk after cessation of treatment was no different from control values. These data indicate a direct correlation between flow and angiogenesis, revealing an arteriogenic component contributing to the shear stress signal, and regression on withdrawal of angiogenic stimulus. [1] Egginton et al. *Cardiovasc Res* 2001; 49:634–46; [2] Williams et al. *J Physiol* 2006; 570:445–54

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PC4

EVALUATION OF THE NEOVASCULARISATION OF A NOVEL FIBRIN-BASED DERMAL SCAFFOLD

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We have shown that endothelial cell migration *in vitro* is more rapid and extensive in 3-D gels of fibrin compared to collagen. A fibrin-based dermal scaffold (Smart Matrix) has been developed to exploit this potential pro-angiogenic property for reconstruction of full-thickness skin loss wounds. We aimed to compare the integration of Smart Matrix with a collagenous scaffold (Integra). Cell ingrowth and neodermal vascularisation was evaluated using a porcine full-thickness wound chamber model. Punch biopsies of scaffolds were taken for histological and immuno-cytochemical analysis (CD31, vWF, VE-cadherin) at days 3, 7, 14, and 21. Neovascularisation was assessed by examination, and neovessel profile counts per field at day 7. Vessel stability was inferred by the extent of associated pericyte (α-SM-actin +ve) staining. Cellular ingress from the wound bed occurred more rapidly into Smart Matrix than Integra. However, ingress into Smart Matrix, but not Integra was associated with a variable neutrophil infiltration. This was mainly dependent on the amount of calcium used for scaffold manufacture, being significant at = 12mM. The rate and extent of neovascularisation between Integra and Smart Matrix was significantly different. Whereas capillary ingress into Integra was via angiogenesis, and the vascular density at day 21 remained relatively low, Smart Matrix displayed evidence of rapid vasculogenesis by differentiation of granulation tissue, from the profile of endothelial differentiation marker expression. Thus, fibrin-based matrices may offer a more favourable environment for accelerated tissue regrowth in critical skin loss wounds than conventional collagen-based scaffolds.

PC5

AN *IN VITRO* ANGIOGENESIS ASSAY BASED UPON THE OVARIAN FOLLICLE

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Within the ovary, there are multiple follicles at various stages of development. Each follicle develops its own microvasculature as it grows, so we hypothesized that the growth of individual follicles *in vitro* would lead to the development of vascular outgrowths, which could be quantified, after incubation with putative pro- or anti-angiogenic agents. In order to obtain the maximal number of suitable follicles, pre- or early antral stage follicles were isolated from ovaries of immature rats. To enhance reproducibility and maintain follicle health, follicles were incubated with serum-free medium containing various supplements (n = 6 per treatment). The follicles were surrounded by Matrigel and different media treatments were added as appropriate and incubated at 37°C and 5% CO_2. Follicles were photographed and measured every 2 days and

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were analysed using Image ProPlus to quantify blood vessel outgrowths. At the end of the 6-day culture period follicles were fixed, sectioned, and stained for activated caspase-3 to assess follicular apoptosis. The results showed that follicles developed vascular outgrowths during the 6-day culture period. In addition, follicles cultured with media supplemented with ascorbic acid, L-glutamine, penicillin, streptomycin, bovine serum albumin, insulin, selenium, and transferrin had the greatest average increase in size ($P < 0.02$) and in endothelial outgrowths, maintained normal morphology and had less apoptosis than follicles cultured with other media supplements. In conclusion, we have developed a culture system that promotes follicular growth and angiogenesis, which should elucidate the role of specific factors in ovarian angiogenesis.

PC6

ASSESSING GENE EXPRESSION IN COLD-INDUCED ANGIOGENESIS BY A NOVEL COMPUTATIONAL METHOD

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Many animals are subjected to annual cycles of environmental cooling, and one way of overcoming limitations to aerobic activity is to increase tissue capillary supply. Although the underlying process is unclear, cold-induced angiogenesis may be due to altered hormonal levels or changes in the mechanical environment of EC [1]. For the first time, we used an *in silico* approach to determine whether orthologs between fish and human genes that are associated with angiogenesis could be identified, tested if these were differentially expressed on cold exposure, and identified what proportion of responding genes were endothelial-specific. Common carp were acclimated to 30°C, gradually cooled, then maintained at 10°C for 22 days. Fish were sequentially sampled, RNA isolated and hybridised to a microarray constructed from 13,349 PCR-amplified cDNA clones spotted onto glass slides [2]. Following array normalisation the list of common response genes was extracted, and identified by comparing expression in control and cooled animals. cDNA sequences were clustered to create overlapping contigs of the same carp genes, a translated BLAST analysis was carried out with all EST clusters and singletons against the human Refseq peptide database, and gene expression profiles compared to endothelial-specific genes recently identified [3]. Thirty muscle and 20 heart genes were differentially expressed in cold carp. We anticipate this approach would be useful in other non-model species for which a genome sequence is not yet available. [1] S Egginton *Comp Biochem Physiol* 2002;132A:773–87; [2] Gracey et al.. *PNAS* 2004;101:16970–5; [3] Herbert et al. *BMC Genomics* 2008, in press.

PC7

ALPHASTATIN AND BETASTATIN: NOVEL INHIBITORS OF ACTIVATED ENDOTHELIAL CELLS

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We recently identified alphastatin, a 24 amino acid peptide derived from the amino-terminus of the α-chain of human fibrinogen as having anti-angiogenic properties *in vitro*. Studies of alternative peptides from the central portion of fibrinogen have revealed betastatin, a 20 aminoacid peptide, derived from the α-chain, which also inhibits endothelial cells. The aim of the current study therefore is to compare the activity of these two fibrinogen-derived peptides on endothelial cell activity *in vitro*. Alphastatin and betastatin were tested for their ability to inhibit HuDMEC tubule formation (Matrigel assay), migration (Boyden chamber), proliferation (BrdU assay), and adhesion to collagen IV, fibronectin, or vitronectin. Both peptides inhibited tubule formation as shown by a decrease in tubule area and length and number of tubules. Interestingly, although both peptides inhibited tubule formation in response to VEGF to a similar extent (28–33%), betastatin inhibited tubule formation to bFGF and EGF less than alphastatin (16–17% vs 25–32%). Moreover, endothelial cell migration in response to VEGF was inhibited by alphastatin ($28 \pm 2\%$) but not by betastatin. In contrast, betastatin prevented endothelial cell adhesion to collagen IV, fibronectin, and vitronectin by 55–63% whereas alphastatin only inhibited adhesion to collagen IV by 45%. These data indicate that these two peptides derived from fibrinogen both

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demonstrate anti-angiogenic activity *in vitro*, but have differential effects, suggesting that they are likely to mediate their activity by binding to different endothelial cell receptors.

PC8

ARTERIOVENOUS MALFORMATIONS IN THE LUNG: SIGNIFICANCE OF ENDOGLIN AND ALK1 EXPRESSION

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Background: Arteriovenous malformations (AVMs) are direct connections between arteries and veins associated with loss of the intervening capillary bed. In the lungs, P-(pulmonary) AVMs can result in severe cyanosis and dyspnea, but the mechanisms underlying AVM formation are poorly understood. One important clue comes from the fact that PAVMs frequently occur in the familial disease hereditary haemorrhagic telangiectasia (HHT). Most HHT patients have mutations in one of two receptors involved in TGFbeta family signalling — either endoglin (ENG) or activin receptor-like kinase 1 (ALK1). Moreover, PAVMs occur more frequently in ENG than ALK1 patients, and are usually found in the distal rather than the proximal vasculature. **Methods:** To elucidate the potential link between endoglin or Alk1 deficiency and AVM formation, we performed a comprehensive study of Alk1 and Eng expression in wild type and Eng$\pm$mouse lungs using a combination of immunohistochemistry and rtPCR from laser-microdissected arteries, veins, and capillaries. **Results and Conclusion**: The data show that Eng and Alk1 have distinct expression profiles in the pulmonary vasculature that point to veins and pre-capillary arterioles being important regions in PAVM development in HHT.

PC9

COLD-INDUCED ANGIOGENESIS IN FISH SKELETAL MUSCLE IS INHIBITED BY NITRIC OXIDE SYNTHASE BLOCKADE

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Angiogenesis, the formation of new capillaries from a pre-existing capillary network, may occur in at least two ways: longitudinal division of capillaries is induced by luminal stimuli, while capillary sprouting requires perivascular stimuli. In mammals, angiogenesis may result from a mismatch in microvascular supply and metabolic demand. However, it has also been observed in oxidative (red) skeletal muscle of fishes acclimated to low temperatures. As metabolism is reduced but oxygen dissolved in water increases at lower temperatures, oxygen supply is unlikely to be limiting. We are investigating the cause of this counter-intuitive increase in capillarity, and the mechanisms involved. Rainbow trout (140–260g) were acclimated to cold over a period of 8 weeks, where cooled blood will become more viscous and impose a greater shear stress on vessel walls. As fish are non-model species, the development of a practical and robust method for capillary identification was required, with alkaline phosphatase being the most practical. This study confirmed that angiogenesis occurs in cold-acclimated trout; the capillary to fibre (C:F) ratio significantly increased within a week when the environmental temperature was reduced from 11°C (1.01 ± 0.04; $n = 3$) to 4°C (1.23 ± 0.04; $n = 3$; $P < 0.05$). Reducing shear stress by nitric oxide synthase (NOS) blockade with L-NNA significantly reduced C:F from 1.23 ± 0.04; $n = 3$ to below control values (0.82 ± 0.04; $n = 3$), so NOS inhibition may induce capillary regression. This suggests that elevated shear stress induces angiogenesis, is important in maintaining vessel integrity, and that metabolic and mechanical stimuli of angiogenesis may not always be coupled.

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PC10

THE EFFECT OF ASPIRIN ON SERUM LEVELS OF ANGIOGENIC AND ANTI-ANGIOGENIC FACTORS IN HUMANS

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Aspirin and/or non-steroidal anti-inflammatory drugs are postulated to prevent some types of cancer, although the underlying mechanism is unknown. These drugs inhibit cyclooxygenase (COX)

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isoenzyme activity and thus prostaglandin production, and consequently proangiogenic factors might be blocked. The purpose of this study was to determine whether aspirin (300 mg/day for 20 days) affects the serum levels of angiogenic (vascular endothelial growth factor (VEGF) or antiangiogenic (endostatin, sFlt-1) factors in healthy human subjects (n = 40), as assessed by ELISA. The volunteers were 40.2 ± 0.6 years (n = 30) and normal body mass index (25.5 ± 0.5 kg.m^{-2}). Statistical analysis used a paired Student t test. Aspirin significantly reduced serum VEGF (from 419 ± 36 pg/ml to 364 ± 33 pg/ml, $P < 0.01$) and sFlt-1 (from 589 ± 20 pg/ml to 526 ± 13 pg/ml, $P < 0.01$), but had no effect on serum endostatin levels (90 ± 4 ng/ml vs 89 ± 4 ng/ml). In conclusion, this preliminary study suggests that aspirin may have a differential effect on pro- and anti-angiogenic factors. Depression of the serum levels of VEGF may be due to blocking of COX activity, while endostatin appears not to be related to VEGF levels. Serum levels of sFlt-1 were regulated in parallel with VEGF, suggesting feedback control. These results indicate that the postulated beneficial effects of aspirin for some cancer types might be due to its effect of reducing serum level of VEGF and maintained endostatin levels.

PC11

CO-REGULATION OF ENDOTHELIAL CELL GENE EXPRESSION AND MIGRATION BY SHEAR STRESS AND VEGF

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Angiogenesis is a multistep process involving endothelial cell (EC) migration and proliferation, tightly regulated by a complex system of promoting and inhibiting factors. Stimulation of angiogenesis by increased shear stress *in vivo* is associated with an increase in production of vascular endothelial growth factor (VEGF) in skeletal muscle [1]. We aim to investigate the hypothesis that shear stress and VEGF interact to modulate behaviour of EC, and hence control angiogenesis. Using real time PCR, changes in expression of genes known to play a role in angiogenesis (VEGF, VEGF receptor (R) 1 and 2, eNOS, angiopoietin (ANG) 1 and 2, neuropilin (NP) 1 and 2 and β_3-integrin) were investigated in human umbilical vein EC (HUVEC) cultured under stasis or shear stress (1.5 Pa) for 24h, ± VEGF in the final 4h. To assess migration, confluent HUVEC were wounded and then exposed to stasis or shear stress (± VEGF); wound recovery was measured at 0, 8, and 24h. Gene expression analysis showed that shear alone increased eNOS, VEGFR2, and NP2 expression, but decreased expression of ANG 1 and 2 and NP1. These broadly "pro-angiogenic" shear-induced changes were all maintained in the presence of VEGF. Interestingly, the only significant change requiring combination of shear and VEGF was an increase in VEGFR1 expression. Initial wound-recovery studies indicated that VEGF and shear stress could each independently enhance endothelial motility. These results start to identify the separate and inter-linked responses to shear stress and VEGF that may modulate the integrated angiogenic response observed *in vivo*. [1] Williams et al. *Clinical Science* 2006; 110: 587–595

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PC12

INVESTIGATING HEPARAN SULPHATE MODIFYING ENZYMES, 6-O ENDOSULFATASES IN VASCULAR DEVELOPMENT

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The presence of heparan sulphate (HS) proteoglycans (PGs) on the cell surface and in the extracellular environment is critical to many physiological processes including angiogenesis. It is mainly through the complex sulphated sugar chains that they can bind to a variety of protein ligands, including growth factors which are critical to the angiogenic balance. Variations in the sulphation of HSPGs have been shown to be important in this interaction, for example the important role of 6-O sulphation on vascular development has been conferred by the knockdown of the 6-OSTs in zebrafish embryos [1]. Our study has focused on elucidating the role of the sulf enzymes, in angiogenesis, that act to fine tune heparan sulphate's interactions with growth factors such as VEGF by removing 6-O-sulphation from HS at the cell surface. Our results show all three sulfs transcripts to be temporally expressed at the time points around angiogenesis. The spatial expression patterns of these transcripts

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were found to be distributed in the developing brain, pronephric ducts, and tail caudal plexus. Following knockdown of individual sulf enzymes in fli1-gata fish, confocal imaging of the 48hpf Sulf1c morphants revealed oedema in the brain and defects in the caudal artery formation, leading to occlusion of the distal aorta. By day 3, 40% of these embryos display haemorrhages. Furthermore, data show that combined knockdown of both sulf2 enzymes displays defects in intersegmental vessel migration alluding to a role of sulfs in vasculature integrity and patterning. [1] Chen et al. *Dev Biol* 2005;284:364–376.

PC13

VEGF-REGULATED TRANSCRIPTION FACTORS Egr3 AND Nur77 MEDIATE ANGIOGENESIS

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Early growth response 3 (Egr3) and Nur77 are zinc finger transcription factors which we previously found to be strongly upregulated by Vascular Endothelial Growth Factor-A (VEGF) in an oligonucleotide microarray screen of endothelial cells [1]. In the present study we show that Egr3 is the predominant Egr family member upregulated by VEGF in endothelial cells at 45 minutes, and that VEGF induced a rapid increase in Egr-dependent transcriptional activation mediated via its major signalling receptor, VEGFR2/KDR and the protein kinase C (PKC) pathway. VEGF-induced Egr3 gene expression was also mediated in part via a PKC-dependent activation of Protein Kinase D1 (PKD1), whereas VEGF-induced Nur77 gene expression was mediated via PKC-dependent activation of PKD1 and PKD2. Inhibition of Egr3 and Nur77 gene expression by RNA interference was effective in inhibiting basal and VEGF-induced Egr3 and Nur77 gene expression, and also inhibited VEGF-mediated endothelial cell proliferation, migration and tubulogenesis. These findings indicate that Egr3 and Nur77 have an essential downstream role in VEGF mediated endothelial functions leading to angiogenesis, and may have particular relevance for adult angiogenic processes involved in vascular repair and neovascular disease. [1] Liu et al. *ATVB* 2003; 23:2002–2007.

PC14

$VEGF_{165}$b INHIBITS STRUCTURE EXTENSION OF HUMAN MICROVASCULAR ENDOTHELIAL CELLS *IN VITRO*

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Blood vessel growth is regulated by various isoforms of the VEGF protein, including both pro-angiogenic $VEGF_{165}$ and anti-angiogenic $VEGF_{165}$b. To determine the effects of $VEGF_{165}$b on structure formation in an *in vitro* model of angiogenesis, the inhibitory effect of $VEGF_{165}$b was investigated. HMVE cells were serum starved for three hours and then exposed to a gradient of 1nM $VEGF_{165}$, $VEGF_{165}$b, and equimolar $VEGF_{165}$/$VEGF_{165}$b in the gel on a culture slide. Structure formation over a period of six hours was observed under a bright field microscope. Cells were fixed and visualized with phalloidin Alexa 488 for F-actin. An extensive tubular structure network was observed after challenge with $VEGF_{165}$ but cells showed poor, spidery, and isolated (non connected) structures when treated either with $VEGF_{165}$b or a combination of the two splice forms. In result, the number of structural elements was reduced by $VEGF_{165}$b treatment, such as branch points (by 75%), sprouts (by 70%), and closed polygons (by 90%) compared with $VEGF_{165}$. A similar 2D experiment on glass showed that $VEGF_{165}$b impairs the spreading of the HMVEC on the surface, leaving them small and round with no defined cytoskeleton visible compared to control cells. They also lacked the typical ruffles and lamellipodia seen in control cells. These results indicate that $VEGF_{165}$b affects downstream signaling pathways regulating cell motility.

Supported by NERC and Richard Bright VEGF Research Trust

PC15

EXTRACELLULAR MATRIX-GROWTH FACTOR SIGNALLING PARADIGMS IN ENDOTHELIAL CELLS and ANGIOGENESIS

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Recent studies have brought to light the important role of the extracellular matrix (ECM) in regulating angiogenesis. One of the important functions of the ECM is the retention of growth factors (GF) which generates important gradients that drive cellular chemotaxis during processes such as vessel patterning and maturation. Recent work from our laboratories have identified and mapped VEGF and HGF (Scatter factor) binding domains on the ECM components fibronectin (Fn) and vitronectin [1–3] that promote cooperative signalling between their cognate receptor tyrosine kinases and integrins. ECM-GF complexes promote signal amplification to downstream effectors coupled to cell migration and proliferation responses as a consequence of receptor cross-linking. In the absence of growth ECM-GF complex formation, signal coordination between integrins and receptor tyrosine kinases induced by co-administration of non-complexed ECM and GF engenders activation of effectors and cell responses that does not occur when either component is administered in isolation. The implications of these signalling paradigms in angiogenesis will be discussed. [1] Wijelath et al. *Circ. Res* 2002; 91:25–31; [2] Rahman et al. *BMC Cell Biology* 2005; 6:8; [3] Wijelath et al. *Circ Res* 2006; 99(8):853–60.

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PC16

INVESTIGATING THE ROLE OF RhoB IN ANGIOGENESIS

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Neovascularization can take place via angiogenesis or vasculogenesis. Angiogenesis is mediated by the proliferation and migration of endothelial cells (EC) while vasculogenesis requires the recruitment of endothelial progenitor cells (EPC). RhoB is a member of the small GTPase family and RhoB null mice show a delay in vascular development in the retina [1]. The aim of this study is to investigate further how RhoB plays a role in angiogenesis. Initially, *in vitro* assays were undertaken on EC following the silencing of RhoB expression. RhoB did not appear to affect EC during proliferation, migration, or adhesion. Several signalling pathways that are activated by vascular endothelial growth factor (VEGF) also appear not to be affected by the silencing of RhoB. An *in vitro* angiogenesis assay, which allows EC to form tubes in the presence of fibroblasts, suggests that when RhoB is silenced in EC tube density decreases. A RhoB knockout mouse is being used to further study this observation *in vivo*. Interestingly, an *ex vivo* sprouting angiogenesis assay showed no difference in the number of sprouts on aortic rings between control and RhoB null mice, suggesting that the role of RhoB might be in vasculogenesis. Furthermore, a unilateral limb ischemia study on the RhoB knockout mice revealed a delay during recovery. Additional unilateral limb ischemia and hypoxic *in vitro* experiments are currently underway. Our data suggest that RhoB plays a role in vasculogenesis, thus revealing its importance in neovascularization [1]. Adini, I.; Rabinovitz et al. *Genes Dev* 2003; 17:2721.

PC17

THE SPLICING FACTOR, SRp55, IS REQUIRED FOR PROTEIN EXPRESSION OF ANTI-ANGIOGENIC ISOFORMS OF VEGF

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We have previously demonstrated that over-expression of the splicing factor, SRp55, can affect the differential splicing of VEGF, favouring production of the anti-angiogenic isoform, $VEGF_{xxx}b$ [1]. The aim of the present study was to determine whether knock-down of SRp55 can affect $VEGF_{xxx}b$ expression in cells known to express both pro- and anti-angiogenic isoforms of VEGF. Retinal pigmented epithelial (RPE) cells were grown in six-well plates and subsequently transfected with SRp55, or two plasmids containing different short hairpin interfering RNA sequences (shRNAi 1 and shRNAi2) against SRp55. Transfection efficiency was 76% using nucleofector. Protein extracts were subjected

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to Western blotting and probed with either an SRp55-specific antibody, or a $VEGF_{xxx}$b-specific antibody. Blots probed with anti-SRp55 antibody demonstrated that SRp55-transfected cells increased band intensity, whereas shRNAi 1 and 2 both significantly decreased band intensity, compared to untransfected cells. Blots probed with $VEGF_{xxx}$b-specific antibody showed that cells transfected with SRp55 increased $VEGF_{165}$b protein expression, whereas knock-down of SRp55 with shRNAi dramatically reduced $VEGF_{165}$b expression. The results demonstrate that SRp55 is involved in the regulation of $VEGF_{xxx}$b. The switch from anti- to pro-angiogenesis is a fundamental control mechanism in a number of vascular diseases. These results place SRp55 as a central target in regulation of the angiogenic activity of $VEGF_{165}$b expression. [1] Woolard et al. *Faseb J* 2006; 20: A539

PC18

ENDOGLIN (CD105) REPRESENTS A POTENTIAL ANTI-ANGIOGENESIS THERAPEUTIC TARGET

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Background: Endoglin, a TGFbeta family receptor, has been shown to be essential for angiogenesis in early development. We sought to test whether this property could be utilised to inhibit angiogenesis in adult mice and whether endoglin represented a potentially useful therapeutic target to inhibit angiogenesis in a clinical setting. **Methods:** Angiogenesis in adult mice was tested using a small subcutaneous matrigel plug supplemented with FGF and VEGF to promote neovessel formation. Endoglin was depleted in the experimental mice by combining our recently derived floxed endoglin mouse and an inducible vascular specific Cre mouse. Cre was activated using tamoxifen, and one group each of two control groups of mice were tamoxifen treated and untreated. The efficiency of endoglin gene deletion and the formation of new vessels in the matrigel implant were monitored using immunohistochemistry. **Results:** The efficiency of endoglin gene deletion was close to 100% in the skin. The number of new vessels invading the matrigel plug was significantly less in endoglin-deficient adult mice compared with control mice ($p = 0.0163$). **Conclusions**: Endoglin is required for new blood vessel formation and supports the idea that endoglin represents a potentially valuable target for anti-angiogenesis therapy.

PC19

DOSE-RESPONSE OF $VEGF_{165}$B TREATMENT IN MURINE OXYGEN-INDUCED RETINOPATHY (OIR)

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We investigated the therapeutic effects and dose-response of recombinant $VEGF_{165}$b in a murine OIR model. Twenty-five C57Bl/6 pups were incubated in 75% oxygen on 7th postnatal day (d7) for five days. Twenty-four hours after the animals returned to the room air, 0.01, 0.1, 1, or 10ng of $VEGF_{165}$b in HBSS was injected into the left eye in each group of five mice, HBSS was injected as the control. The mice were sacrificed on d17 and retina whole mounts dissected and stained with isolectin B4. Areas of central avascular region, pathological pre-retinal proliferation region and peripheral normal vascularised region were measured and quantified. 0.1–10ng/eye single injections demonstrated a significant inhibition of pathological pre-retinal proliferation (0.1ng/eye: $14.7 \pm 9.9\%$; 1ng/eye: $19.2 \pm 9.7\%$; 10ng/eye: $18.4 \pm 13.9\%$), compared with control ($40 \pm 6\%$). $VEGF_{165}$b inhibited neovascularisation with an IC50 of 1.46×10^{-11}g (14.6pg) per eye. Physiological vascularisation of peripheral retina in the OIR mice was increased after a single dose injection with 0.1ng–10ng $VEGF_{165}$b (0.1ng/eye: 75.7 ± 12.7; 1ng/eye: $75.5 \pm 12.7\%$; 10ng/eye: $72.3 \pm 17.8\%$) compared with control ($45 \pm 8.6\%$). Thus, whilst $VEGF_{165}$b effectively reduced pathological pre-retinal proliferation, the physiological revascularisation in the central retina was promoted. These results indicate that $VEGF_{165}$b is potentially a preferential candidate for ocular neovascularization in an ischemic setting such as in proliferative diabetic retinopathy.

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PC20

EFFECTS OF HYPERGLYCEMIA ON VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) AND VASCULAR ENDOTHELIAL-CADHERIN EXPRESSION IN A PLACENTAL VILLOUS EXPLANT CULTURE

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Hyperglycemia in diabetes often requires tighter control in pregnancy. Throughout gestation, the placenta is subjected to periods of hyperglycaemic insult. The effect of this on placental endothelial integrity is not known and was the aim of this study.

A chorionic villous explant culture system was used to measure effects of different duration of hyperglycaemic insult. The explants maintain the 3-D architecture and allow cross talk between the endothelium and adjacent trophoblast cells. Explants were taken from normal term placenta ($n = 3$) obtained by elective Caesarean section. They were incubated for 4 and 24 h in oxygenated Medium 199 (with 5% fetal calf serum and 100U/ml penicillin, 100μg/ml streptomycin) at 37°C, with or without the addition of 10mM glucose (to give a final concentration of 15mM glucose). 10mM mannitol was used in the osmotic control group. Vessels were counted on an intensity scale (1–3), with VE-cadherin intensity 1 being diffuse non-junctional staining while intensity 3 was exclusive to paracellular junctions. A contingency table analysis (Chi-squared) revealed a significant difference ($p < 0.001$) in VE-cadherin immunoreactivity between 5mM and 15mM glucose samples at 4h, however, this was shown to be an osmotic effect. At 24h, the 5mM glucose showed a statistically significant reduction in vascular profiles with intensity 1 pattern, compared to hyperglycaemia ($p < 0.001$) but not mannitol. For VEGF, there were no significant differences between the two groups at 4h; however, by 24h there was a significant decrease of VEGF in euglycemia compared to hyperglycemia ($p < 0.001$), which was in part (but not wholly) caused by the higher osmolarity. These data suggest that in explant culture, blood vessels recover from initial wounding or excision of villi by 24h in euglycemic but not hyperglycemic conditions; high glucose causes increased VEGF production and delays return of VE-cadherin to junctions.

PC21

INTRAPERITONEAL ADMINISTRATION OF RECOMBINANT HUMAN $VEGF_{165}$B INHIBITS DISSEMINATION OF METASTATIC MELANOMA CELLS *IN VIVO*

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Vascular endothelial growth factor is differentially spliced to form pro-angiogenic ($VEGF_{xxx}$) and anti-angiogenic isoforms ($VEGF_{xxx}$b where xxx is the number of amino acids). We have previously shown that $VEGF_{xxx}$b isoforms are downregulated in metastatic but not non-metastatic melanoma. [1] To determine whether this was causal or an effect of metastasis, the effect of recombinant human $VEGF_{165}$b administration to metastatic melanomas grown intraperitoneally was investigated. Injection of $1x10^6$ B16-Luc metastatic melanoma cells over-expressing luciferase in CD1 mice resulted in large intraperitoneal tumours after 8 days. Bio-luminescence imaging revealed a significant reduction in viable tumour in these immunocompetent mice in both the animals treated with 100μg $VEGF_{165}$b bi-weekly and control mice over the following two weeks. Upon killing of the mice, and removal of the large primary tumour from the abdominal cavity, the remaining disseminated tumours were imaged by bioluminescence. Surprisingly, there were significantly more multiple separate viable tumours remaining in the control treated mice (6.5 ± 0.5 lesions per mouse) compared with the $VEGF_{165}$b treated mice (1.6 ± 0.8 lesions per mouse, $p < 0.01$,Mann Whitney U test). These results indicate that $VEGF_{165}$b is an anti-metastatic agent, and that the reduction in expression of $VEGF_{165}$b in metastatic melanomas in humans may be a contributory factor to their spread. It also suggests that $VEGF_{165}$b therapy may be effective in preventing metastasis of malignant melanoma. [1] Pritchard, Jones et al. *Br J Cancer* 2007; 97(2):223–30.

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PC22

TIA-1 AN RNA BINDING PROTEIN, UPREGULATES THE ANTI-ANGIOGENIC ISOFORM OF VEGF IN COLON CANCER CELLS

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TIA-1, (T-cell intracellular antigen 1) modulates RNA splicing, apoptosis, or translational control, depending on the binding locus within RNA transcripts. Deficient TIA-1 RNA binding was observed in colon carcinoma cells that overexpressed cyclooxygenase 2 (COX-2) mRNA and protein [1]. Colonic tumorigenic cells with high COX-2 mRNA and protein expression expressed higher levels of $VEGF_{165}$ (pro-angiogenic isoform, 0.089 ± 0.014 pg/mg protein) than their parental cells, AAC1 (0.031 ± 0.016 pg/mg protein). Sequence analysis of TIA-1 in these cells showed a 60 bp insertion in the RRM2 (RNA Recognition Motif) of TIA-1, which may bind the COX-2 and VEGF pre-mRNAs. This insertion was not seen in the parental or other adenoma colon cell lines. In this cell line, RNA imunoprecipitation followed by RT-PCR-mediated detection and electrophoretic mobility shift assay showed defective TIA-1 binding to COX-2 RNA. Transfection of these cells with wild type TIA-1 reduced COX-2 protein expression as detected by Western blotting and switched splicing from anti-angiogenic ($VEGF_{165}b$) to pro-angiogenic isoforms of VEGF as detected by RT-PCR. Wild type TIA-1 overexpression in a second colonic carcinoma cell line with a TIA-1 insertion (LS174T) also switched splicing from $VEGF_{165}$ to $VEGF_{165}b$. These findings suggest that TIA-1 activation may be a potential anti-angiogenic therapeutic strategy either by silencing COX-2 or switching splicing towards anti-angiogenic isoforms of VEGF. [1] Dixon DA. et al. *JEM* 2003; 198:475–481.

PC23

CHRONIC TREATMENT OF TUMOURS EXPRESSING SINGLE ISOFORMS OF VEGF-A WITH A VEGFR INHIBITOR ALTERS ANGIOGENESIS AND RESPONSIVENESS TO THE VASCULAR DISRUPTING AGENT COMBRETASTATIN A-4-P

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Mouse fibrosarcomas expressing single vascular endothelial growth factor (VEGF)-A isoforms (120, 164, or 188), or all isoforms (WT) under endogenous promoter control have been developed. Subcutaneous $VEGF_{120}$ tumours transplanted into mice are highly vascularised, with poor vessel development, and are susceptible to vessel breakdown by CA-4-P treatment. Tumour growth is also retarded by CA-4-P treatment. $VEGF_{188}$ tumours are less vascularised, with mature vessel wall development and are less responsive to CA-4-P, demonstrating that $VEGF_{188}$ influences the therapeutic outcome after CA-4-P treatment, as well as vascular effects. We tested the effect of chronic treatment with a VEGF receptor inhibitor, SU5416, on this differential tumour vascular response to CA-4-P. SU5416 did not affect growth rates in any tumour line. Expression of α-smooth muscle actin in SU5416-treated $VEGF_{120}$ tumours was higher than in controls, implying greater vascular maturity. All VEGF-isoform tumours treated with SU5416 exhibited lower total vessel length than controls. Chronic treatment with SU5416 protected $VEGF_{120}$ tumours against vessel breakdown by CA-4-P, possibly by facilitating the development of more stable and mature blood vessels.

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PC24

BLOCKING EXPRESSION OF PRO-ANGIOGENIC ISOFORMS OF VEGF USING MORPOHOLINO ANTISENSE OLIGONUCLEOTIDES

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VEGF, the predominant regulatory molecule in angiogenesis is differentially spliced in its terminal exon to result in pro-angiogenic $VEGF_{xxx,}$ using a proximal splice site (PSS), and anti-angiogenic $VEGF_{xxx}$b isoforms using a distal splice site (DSS). To determine whether inhibition of mRNA splicing sites could alter VEGF splice site choice, morpholino antisense oligonucleotides (MO) were designed to target the 3' proximal (MO-PSS) and distal (MO-DSS) splice sites in exon 8. HEK293-T cells were grown in six-well plates, and subsequently transfected with a MO control with fluorescein or different concentrations of MO-PSS and MO-DSS (1 µM, 5 µM, 10µM) using the transfection reagent Endo-Porter for 48 hours. The transfection efficiency was 72%. $VEGF_{xxx}$b and $VEGF_{xxx}$ expression was determined by competitive semi-quantitative RT-PCR of both isoforms as previously described [1].At 10 µM PSS the upper band corresponding to $VEGF_{xxx}$ disappeared. The DSS resulted in a dose-dependent reduction in the intensity of the $VEGF_{xxx}$b band relative to the intensity of band corresponding to $VEGF_{xxx}$. These morpholinos therefore appear to be able to inhibit splice site specific splicing in HEK cells. This leads to a potential application in the manipulation of splice sites using antisense oligonucleotides to switch splicing from either pro-angiogenic. [1] Bates et al. *Cancer R*es 2002;62: 4123–4131).

PC25

BEVACIZUMAB RESISTANCE AND NEUROPILINS

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Bevacizumab (Bz), a humanised antibody to VEGF, prevents VEGF binding to VEGF-R1/2, and increased progression-free survival in breast cancer phase III clinical trials in combination with chemotherapy. However, eventually the tumours evade treatment control, possibly because Bz does not prevent VEGF from binding to its alternative receptors, the neuropilins (Nrp1 and 2). Therefore this study will test the hypothesis that simultaneously blocking VEGF-neuropilin binding will enhance the efficacy of Bz treatment. Western blot analysis of MDA-MB-436, MCF-7, and T47D breast cancer cell lines and endothelial cells (HuDMEC and HUVEC) indicated that Nrp1 and Nrp2 were expressed by all cell lines, whereas VEGF-R2 was only expressed by the endothelial cells and VEGF-R1 by MCF-7 and endothelial cells. These data were confirmed by flow cytometry. Immunohistochemistry revealed Nrp1 and Nrp2 staining in some vessels in normal breast tissue, DCIS and invasive breast cancer. HuDMECs stimulated by VEGF formed cord-like structures on Matrigel *in vitro*, representing early tubule formation and the number of branch points/field of view were counted in three wells/experiment. Both Bz and anti-Nrp1 antibody significantly ($P < 0.001$) inhibited VEGF stimulated cord formation (VEGF: 43 ± 6; Bz: 25 ± 3; anti-Nrp1; 26 ± 4), whereas the combination of Bz and anti-Nrp1 caused a further significant ($P < 0.003$) reduction (17 ± 3). These data show that neuropilins are expressed in both breast cancer and endothelial cells and that treatment with anti-Nrp1 enhances Bz inhibition of endothelial cord formation, suggesting that a neuropilin blocking agent may enhance efficacy of Bz treatment for breast cancer.

PC26

LOW PLASMA $VEGF_{165}$b IS A FIRST TRIMESTER MARKER FOR PRE-ECLAMPSIA

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Pre-eclampsia is a pregnancy-related condition of hypertension, proteinuria, and endothelial dysfunction. $VEGF_{165}$b, formed by alternative splicing of VEGF inhibits $VEGF_{165}$-mediated vasodilatation

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and angiogenesis, but has not been quantified in pregnancy. Enzyme-linked immunoassays (ELISA) were used to measure mean ± SEM plasma $VEGF_{165}b$, soluble endoglin (sEng), and soluble Flt1 (sFlt1). At 12 weeks gestation the plasma $VEGF_{165}b$ concentration was significantly lower in patients who later developed pre-eclampsia (467pg/ml ± 209) compared with plasma from normotensive pregnancies (4898pg/ml ± 1664, $p < 0.01$ Mann Whitney U test). In contrast, at term there was no difference in plasma $VEGF_{165}b$ concentrations between pre-eclamptic (3754pg/ml ± 2243) and normal pregnancies (10580pg/ml ± 3736). Thus, pre-eclampsia was associated with an 8 ± 1.8 fold increase in plasma $VEGF_{165}b$ from first trimester to pre-delivery, compared with a 2 ± 0.3 fold increase in normotensive plasma ($p < 0.0012$). Patients with a lower than median plasma $VEGF_{165}b$ at 12 weeks had elevated soluble fms-like tyrosine kinase receptor 1 (sFlt1) and soluble endoglin (sEng) pre-delivery. Concentrations of sFlt1 (1.20 ± 0.07 ng/ml and 1.27 ± 0.18ng/ml) and sEng (4.4 ± 0.18 vs 4.1 ± 0.5) were similar at 12 weeks gestation in the normotensive and pre-eclamptic groups, respectively. Low $VEGF_{165}b$ may therefore be a clinically useful first trimester plasma marker for increased pre-eclampsia risk.

PC27

THE FORMATION OF NASAL POLYPS IS INDEPENDENT OF ANGIOGENESIS

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Nasal polyposis is a common disease, the aetiology of which is currently unknown. Several mechanisms have been proposed, and recent studies have shown up-regulation of several pro-angiogenic factors [1,2]. The aim of this study was to assess and quantify the degree of angiogenesis in nasal polyps, and therefore whether angiogenesis is important in initiating polyposis. Biopsies of (1) polyp tissue and (2) nasal mucosa were taken from patients undergoing polypectomy, and compared with (3) nasal mucosal samples from control patients ($n = 5$). Biopsies were either stained with fluorescent lectins for confocal microscopy or snap-frozen and sectioned for histology for the examination of multiple measures of angiogenesis. No significant differences in capillary density, capillary-associated proliferation, capillary surface density, or capillary volume density were seen among the three study groups. The regression of surface density vs volume density described the same linear relationship in all three groups, indicating no gross differences in architecture of the capillary bed. Polyp samples showed increased capillary diameter and interstitial proliferation. These results show no active angiogenesis or changes in capillary bed architecture in the polyp, although polyp capillaries appear more oedematous. As the capillary supply increases in line with the physiological needs of growing polyps, we conclude that angiogenesis is not a driving force in the aetiology of nasal polyposis, but is recruited in a feedback manner to support tissue expansion. [1] Coste A et al. *Eur Resp J* 2000; 15:367–372; [2] Norlander T et al. *Rhinology* 2001; 29:88–92.

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PC28

SCALING OF CAPILLARY SUPPLY IN HUMAN MYOPATHIES

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In skeletal muscle both ontogenetic growth and adaptive remodelling, e.g., in response to exercise, result in a capillary supply that is scaled according to the size of muscle fibres [1,2]. We examined whether this scaling also exists in human skeletal muscle when remodelling is pathological. Muscle biopsies of the medial gastrocnemius or lateral quadriceps were taken from patients with idiopathic clinical muscle atrophy or muscle fibre hypertrophy, and control samples from patients undergoing varicose vein surgery. All patients gave informed consent. Biopsies were snap-frozen and serial cryosections stained for capillaries and muscle fibre types using *Ulex europeaus* lectin and myosin ATPase activity. Images were digitised and capillary density (CD) and capillary to fibre ratio (C:F) calculated. There was a 2-fold range in mean fibre area among groups (~2800, 4500 and 6300 μm^2, respectively). The CD of hypertrophic myopathies showed no significant difference from controls, but

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with an increase in C:F in response to the increased fibre size (2.05 vs 1.25), suggesting scaling effects similar to non-pathogenic stimuli (e.g., some strength training). Atrophic myopathies showed no difference in C:F from controls, but an increase in CD (430 vs. 310 mm^{-2}) suggesting maintenance of the existing vasculature that is independent of intramuscular scaling. These data are consistent with larger fibres being more susceptible to metabolic error signals, leading to reduced intracellular PO2 acting as an angiogenic signal [3]. [1] Egginton S. *Adv Comp Environ Physiol* 1990; 6:73–141; [2] Ahmed et al. *Exp Physiol* 1997 ; 82:231–4; [3] Deveci et al.. *Am J Physiol* 2001; 281:H241–H252.

PC29

EXPLORING THE TUMOUR ENDOTHELIAL MARKER ROBO4 AS A CANCER VACCINE

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Robo4 is a novel roundabout receptor expressed exclusively in endothelium. *In situ* analysis has shown Robo4 to be absent from adult tissues, but strongly expressed at sites of active angiogenesis, including tumour vessels. Expression of Robo4 only on the tumour vasculature makes it a potential target for immunotherapy (e.g., vaccination). Recent studies have shown that immunisation against antigens over-expressed on the tumour vasculature, for example VEGF receptors, holds promise as an anti-cancer strategy. The aim of this study is to investigate the immunogenicity of the Robo4 protein in humans. A sensitive T cell assay (EliSPOT assay of human IFN-? release) was used to investigate CD8+ T cell responses to peptide epitopes derived from Robo4 predicted to bind with high affinity to the common HLA class I alleles HLA-A0201, HLA-2401, HLA-B2702/05, and HLAB4402/03. Six normal donors and six patients tested so far have not responded to the peptides, showing that an immune response to the Robo4 self-antigen is normally weak or absent. We are now evaluating antigen-specific T-cell responses to Robo4 by measuring IFN-? production from PBMC cultures nucleofected with plasmid DNA-encoding Robo4 protein. Finally, we have produced a Robo4 extracellular domain-his tagged protein and are at present developing an ELISA using this antigen in order to determine antibody responses in plasma samples from patients with a variety of tumours and in normal controls. We will present preliminary results from the above experiments that pertain to the immunogenicity of Robo4.

PC30

THE ANGIOGENIC SWITCH IN HUMAN BREAST CANCER OCCURS AT THE ONSET OF DYSPLASIC TRANSFORMATION IN THE MAMMARY MILK DUCT

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The precise timing of the angiogenic switch and the role of angiogenesis in the development of breast malignancy are currently unknown. Therefore we quantified the expression of CD31 (pan endothelial cells), endoglin (actively proliferating endothelial cells), hypoxia inducible factor-1 (HIF-1α), and VEGF in 187 serial sections of normal human breast (n = 12), benign (n = 35) and pre-malignant (n = 31) hyperplastic tissue, pre-invasive (n = 66), and invasive (n = 43) breast cancer specimens. Significant increases in MVD were seen between normal and hyperplastic/pre-invasive breast cancer tissue ($P < 0.005$) and between pre-invasive and invasive carcinomas ($P < 0.0005$) and were associated with significant increases in nuclear HIF-1α and cytoplasmic VEGF expression in breast epithelial ($P < 0.01$) and tumour cells ($P < 0.005$) , respectively. Furthermore, HIF-1α was not observed in normal breast, but was expressed in the nuclei of ductal epithelial cells in 60–75% of hyperplastic breast/pre-invasive cancers and over 90% of invasive cancers. Significant increases in proliferating endothelial cells were observed in pre-invasive and invasive carcinomas and were associated with significant increases in HIF-1α and VEGF expression in pre-invasive ($P < 0.005$) and invasive tumour cells ($P < 0.0005$) , respectively. These data indicate

that hypoxia exists in hyperplastic breast lesions, stimulating the expression of VEGF and angiogenesis in the earliest stages of dysplasia.

Supported by Yorkshire Cancer Research

PC31

A NOVEL METHOD OF DIFFERENTIAL GENE EXPRESSION ANALYSIS USING MULTIPLE CDNA LIBRARIES APPLIED TO THE IDENTIFICATION OF TUMOUR ENDOTHELIAL GENES

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In this study, differential gene expression analysis using complementary DNA (cDNA) libraries has been improved by the introduction of an accurate method of assigning Expressed Sequence Tags (ESTs) to genes and a novel likelihood ratio statistical scoring of differential gene expression between two pools of cDNA libraries. These methods were applied to the latest available cell line and bulk tissue cDNA libraries in a two-step screen to predict novel tumour endothelial markers. Initially, endothelial cell lines were in silico subtracted from non-endothelial cell lines to identify endothelial genes. Subsequently, a second bulk tumour versus normal tissue subtraction was employed to predict tumour endothelial markers. **Results:** From an endothelial cDNA library analysis, 431 genes were significantly up regulated in endothelial cells with a False Discovery Rate adjusted q-value of 0.01 or less, and 104 of these were expressed only in endothelial cells. Combining the cDNA library data with the latest serial analysis of gene expression (SAGE) library data derived a complete list of 459 genes preferentially expressed in endothelium. Twenty-seven genes were predicted tumour endothelial markers in multiple tissues based on the second bulk tissue screen. **Conclusions:** This approach represents a significant advance on earlier work in its ability to accurately assign an EST to a gene, statistically measure differential expression between two pools of cDNA libraries, and predict putative tumour endothelial markers.

www.compbio.ox.ac.uk/data/diffex.html

PC32

A DORSAL SKINFOLD CHAMBER (DSC) METATARSAL MODEL FOR STUDY OF MICROMETASTASES *IN VIVO*

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Academic Unit of Urology, University of Sheffield, UK[1;] Surgical Oncology, University of Sheffield, Sheffield, UK[2]; Department of Urology, Leiden University Medical Center, The Netherlands[3]; Urology Research Laboratory, University of Bern, Bern, Switzerland[4]

Micrometastasis is an impediment to the development of effective cancer therapies. Despite its prevalence, the mechanisms involved in the development of prostate metastases in bone remain unclear. The mechanisms of micrometastatic dissemination and growth are not well characterised due to an inability to follow this process adequately *in vivo*. Therefore, the aim of this work is to establish a model of prostate cancer metastasis using the DSC model. We have demonstrated that metatarsals are rapidly re-vascularised by inosculation with the host vasculature by day 5–7, following which fluorescently labelled prostate (PC3-GFP), breast (MDA-231-B02-GFP), and oral (SCC4-GFP) cancer cells (1×10^5) are injected via the heart (i.c.) to simulate micrometastatic spread. Recordings of the DSC containing the metatarsal are made at 24 hr intervals for up to 2 weeks. Tumour cells (10 ± 7) have been detected in the metatarsal area within 5 minutes of i.c. injection, remaining for up to 14 days but not appearing to proliferate. These cells are thought to be initially adhering to microvascular endothelium and/or extravasating to the metatarsal matrix within 7 days of injection. This model will allow homing interactions and extravasation between the tumour and bone marrow cell populations to be studied in detail. In addition, key tumour-derived genes can be manipulated to determine the effects on micrometastatic spread.

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PC33

STIMULATION OF VEGF-MEDIATED ANGIOGENESIS AND TUMOUR GROWTH BY LOW CONCENTRATIONS OF ANTI-ANGIOGENIC INTEGRIN INHIBITORS

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The integrins αvβ3 and αvβ5 are adhesion molecules expressed by tumour blood vessels. Inhibitors of αvβ3 and αvβ5 integrins have entered clinical trials as anti-angiogenic agents for cancer treatment. However, their performance in the clinic has been disappointing thus far. Here, we present *in vivo* evidence for a mechanism that could seriously compromise the efficacy of integrin inhibitors. We show that nanomolar concentrations of αvβ3/αvβ5-inhibitors actually can stimulate VEGF-mediated angiogenesis, tumor growth, and tumor angiogenesis. Low concentrations of αvβ3/αvβ5-inhibitors promote the expression of VEGFR2 *in vitro* and *in vivo*. Moreover, in human endothelial cells, αvβ3/αvβ5-inhibitors (a) enhance the Rab4A-mediated recycling of VEGFR2, and (b) suppress the degradation of VEGFR2 in a Rab4A-dependent fashion. These data provide evidence for a novel mechanism via which angiogenesis inhibitors could actually promote angiogenesis. By enhancing the recycling of VEGFR2, low concentrations of αvβ3/αvβ5-inhibitors prevent VEGFR2 degradation. The resulting elevated expression of VEGFR2 in endothelial cells then gives rise to increased angiogenesis. In conclusion, we show that low concentrations of an anti-angiogenic agent could actually promote angiogenesis. This may have important consequences for the design of anti-angiogenic cancer therapy regimes.

PC34

ROLE OF MACROPHAGES IN TUMOUR RECOVERY FOLLOWING CA-4-P TREATMENT *IN VIVO*

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Viable blood vessels have been identified in the periphery of tumours following treatment with CA-4-P, an anti-cancer vascular disrupting agent (VDA). It is hypothesized that tumour-associated macrophages (TAMs) infiltrate areas of vascular damage and promote tumour angiogenesis from residual vessels. A positive correlation exists between tumour progression and TAM density in the Polyoma Middle T (PyMT) spontaneous murine breast cancer model, indicating a pro-angiogenic role for TAMs. Immunohistochemistry revealed vascular endothelial growth factor (VEGF) to be of stromal origin in this model, representing of a minority of human breast cancers in which tumour cells do not express VEGF [1]. VEGF mRNA was localised in the PyMT model using in situ-hybridization (ISH). Formalin-fixed sections obtained from late-stage PyMT tumours were stained using anti-sense human VEGF and β-actin probes labelled with autoradiographic silver grains. Autoradiography was performed at 4°C; slides were developed at 8 days and counterstained with Giemsa. Preliminary ISH studies indicate both tumour cell and TAM derived VEGF mRNA are present in the PyMT tumour model. Continued studies are required to elucidate whether the expression of VEGF increases following treatment with CA-4-P. [1] Leek et al. *J Path* 2000; 90:430–6.

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PC35

THE CONTRIBUTION OF THE RHO PROTEIN PATHWAY TO THE TUMOUR VASCULAR DISRUPTING ACTION OF CA-4-P

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The vascular disrupting agent, combretastatin A-4-phosphate (CA-4-P), activates GTPase Rho A and Rho kinase in endothelial cells *in vitro*. The aim was to investigate whether this pathway contributes to vascular shutdown by CA-4-P *in vivo*. Colorectal carcinoma SW1222 cells were subcutaneously implanted into SCID mice and treated i.p. with CA-4-P (30 or 100 mg/kg) alone or in combination with

Y-27632 (50mg/kg), a Rho kinase inhibitor. One or 3 hours post-treatment, mice were injected with fluorescein labelled lectin (i.v.) for endothelial labelling of perfused vessels, and tumours removed 5 minutes later for subsequent staining for endothelial CD31. Control tumour blood vessels had 38.5% effective perfusion (percentage of lectin relative to CD31 staining). CA-4-P (100mg/kg) significantly reduced perfusion at 3 hours to 11.8%, and this was elevated to 20.5% with Y-27632 pre-treatment. These data suggest that Rho kinase is involved in the mechanism of action of CA-4-P. Another inhibitor, dibutyryl cAMP (dbcAMP), which phosphorylates and inactivates Rho via activating protein kinase A, was also used. *In vitro*, dbcAMP blocked CA-4-P action in human umbilical endothelial cells. When used *in vivo*, dbcAMP alone significantly increased perfusion of tumour blood vessels relative to controls. Further studies using laser doppler flowmetry are in progress to confirm this response. The data taken together imply that the Rho protein pathway is involved in the relative perfusion of tumour vessels, and this pathway may contribute to the vascular disrupting action of CA-4-P.

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PC36

SIMULATION OF BLOOD PERFUSION IN INTRAVASCULAR-INTERSTITIAL SPACES BASED ON 3D TUMOUR ANGIOGENIC MICROVASCULATURE

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A coupled mathematical model of tumour blood perfusion based on 3D angiogenic vascular networks is developed, providing the link between microvasculature and interstitial space perfusion and combining the intravascular-interstitial flow by vascular leaky terms. The novelties of the study are: (1) 3D model of tumour angiogenesis with an arteriole-venule system developed, to generate an intact microvasculature for blood circulation; branching generations with different vessel diameters are considered; (2) coupled model of tumour intravascular-interstitial fluid flow is developed, vascular permeability, vessel compliance, and blood rheology are taken into account; (3) a specific coupling procedure is built, based on the various iteratively numerical simulation techniques of different iteration loops. The results present the basic features and characteristics of tumour microcirculation, such as nearly uniform intravascular pressure, slow blood flow, low haematocrit, elevated interstitial pressure, and extremely low interstitial flow inside the tumour, which consists with the corresponding experimental observations reported. They also predict an intimate relationship between the tumour intravascular and interstitial flow, among which the vascular leakiness is the key factor on governing systemic flowing pattern, influencing tumour internal environment and contributing to metastasis of tumour cells.

PC37

TARGET IGF-I AS AN ANTI-ANGIOGENESIS THERAPY TO TREAT COLORECTAL CANCER

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It has been well established that angiogenesis plays an important role in the growth, progression, and metastatic spread of solid tumour. Colorectal cancer is highly angiogenic and is the first tumour type to exhibit significant responses to anti-angiogenic therapy. Angiogenesis is a complex of endothelial cell proliferation and differentiation with interactions between different growth factors and their receptors. It has been reported that insulin-like growth factor I (IGF-I) stimulates the expression of VEGF. There are many studies on the functions and regulations of VEGF in angiogenesis. Few studies have defined the functions of IGF-I on regulating VEGF and angiogenesis. Using a gene therapy approach, an IGF-I inhibitory binding protein, IGFBP-4, was over-expressed on a colorectal cancer animal model. The over-expressed IGFBP-4 presumably inhibited IGF-I actions *in situ* of cancer which resulted in a significant decrease in densities of microvessels around the tumour tissue. Using an *in vitro* system, the effects of IGF-I on proliferation and differentiation of human microvascular endothelial cells (HMEC-1) were investigated. It was found that

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prevention of IGF-I binding to its receptors, by a novel IGF-I antagonist peptide, significantly decreased HMEC-1 cell proliferation and stopped them from forming microvessels. Upon further investigation of the effect of IGF-I on the production of VEGF in colorectal cancer cells, it was found that the same novel IGF-I antagonist significantly decreased VEGF production in colorectal cancer cells. This study demonstrated that IGF-I plays an important role in colorectal cancer angiogenesis and a gene therapy approach or an IGF-I antagonist may have the potential to be developed into an anti-angiogenesis therapy for colorectal cancers in the future.

PC38

COMPARISON OF DIFFERENT METHODS OF LYMPHATIC VESSEL DENSITY COUNTING TO PREDICT MELANOMA

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Malignant melanoma results in over 1800 deaths a year, through metastatic spread of the disease. At present the most accurate single prognostic indicator of metastasis is Breslow thickness, yet 15% of those patients predicted to be safe from further metastases still die. Several novel prognostic tests have recently been proposed for use in melanoma metastasis and we have previously demonstrated that the most accurate is the Shields Index [1]. However, this is a time-consuming technique and the clinical value of a test depends not only upon its accuracy but its utilisation of limited resources. The most rate-limiting step in Shields index sampling is calculation of the total epi-tumoural lymphatic vessel density (LVD), which is a relatively labour-intensive technique. To determine whether this could be improved by calculating LVD through hot spot analysis rather than total vessel counts both techniques were compared in 14 archival melanoma samples. Hot spot analysis was a much faster method (5.5 ± 0.6 min per sample compared with 19.0 ± 1.1 min per sample for Shields Index) and resulted in an LVD that correlated with total LVD ($p < 0.0001$, Pearson). When hot spot calculated LVD was used as part of the Shields Index, there was still a significant difference in mean Shields Index value between the metastatic compared to non-metastatic patients ($p < 0.05$, t test), suggesting that it also resulted in prediction of metastasis. The use of hot spot analysis for sampling LVD as part of routine Shields Index analysis may therefore provide a pragmatic solution to reducing the time needed to perform Shields Index analysis, thus increasing its clinical value. [1] Emmett et al. *Microcirculation* 2007; 14: 648.

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PC39

INHIBITION OF CHEMOKINE SIGNALLING BLOCKS TRACKING OF MELANOMA CELLS TO LYMPHATICS *IN VIVO*

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Malignant melanoma metastasises through the lymphatic microvasculature, using a mechanism that is not yet clear. We have previously shown that metastatic melanoma cells can recognise lymphatic secreted chemokines and tumours formed from such melanoma cells migrate towards depots of lymphatic endothelial cells, which express chemokines such as CCL21. Here we investigate whether inhibiting such chemokines can prevent tracking of melanoma cells towards lymph nodes *in vivo*, B16-Luc metastatic mouse melanoma cells over-expressing firefly luciferase were transfected with either Chemotrap1 or Chemotrap189. These are Fc-fusion proteins of the chemokine binding domain of THAP proteins, whereby TRAP1 binds chemokines including CCL21 and CCL19, and TRAP189 has no chemokine binding activity (courtesy of JP Girard, Toulouse). 1×10^6 cells were injected subcutaneously and tumours allowed to grow in CD1 mice. Bioluminescent imaging of tumours growing from cells expressing TRAP1, grew in a more circular mode than tumours expressing TRAP189, which grew in a more elongated fashion. Quantification revealed that the ratio of the major to minor axis of the TRAP 189 cells was 3.9 ± 0.53, whereas in the TRAP1 tumours it was 2.57 ± 0.23 ($p < 0.05$, paired t test, $N = 6$). Upon dissection it was clear that TRAP189 tumours had tracked towards the lymph nodes, whereas tracking in TRAP1 cells was rare. These results show that inhibition of chemokine activity in metastatic melanoma cells can prevent their tracking towards lymph nodes, and provides further evidence

that chemokine inhibition is a potential therapeutic strategy to prevent metastasis.

PC40

SUBSET OF HIGH-FILTERING WOMEN AT INCREASED RISK OF LYMPHOEDEMA AFTER BREAST CANCER TREATMENT?

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In breast cancer-related lymphoedema of the arm (BCRL), subcutis and muscle lymph drainage are reduced (Modi et al. Lymph Res Biol 2007; 5:183–202). Here we investigated lymph flow during the time between surgery and onset of BCRL. Ethics approval and informed consent were obtained. In 36 breast cancer patients without BCRL treated by axillary surgery 7 months (m) previously, ^{99m}Tc-human IgG (^{99m}Tc-HIG, 0.2ml, 0.5MBq) was injected into the subcutis of each forearm. The removal rate constant *k* (lymph flow/distribution volume) was measured over 3h by quantitative lymphoscintigraphy. The study was repeated using intramuscular injections 7 days later. Both studies were repeated at 30m post-surgery, when 7 women had developed BCRL. k_{muscle} was 2–3x $k_{subcutis}$ at 7m, also at 30m in non-BCRL women, showing that muscle contributes the greater lymph load. At 7m there were no between-arm differences in k, even in the group destined to develop BCRL, so there was no pre-BCRL lymphatic failure. Surprisingly, both k_{muscle} and $k_{subcutis}$ were 22–50% higher in both arms of the pre-BCRL group at 7m than in the women who did not develop BCRL ($P < 0.01$, 2-way ANOVA). Subsequent to BCRL onset, $k_{subcutis}$ fell in both arms but k_{muscle} did not. $k_{subcutis}$ and k_{muscle} both rose from 7–30m in the BCRL-spared women. The results indicate that women destined to develop BCRL have elevated lymph formation rates, and so, presumably, high capillary filtration rates. This is the first evidence for a subgroup constitutively liable to secondary lymphoedema.

PC41

DECREASED MICROVASCULAR FUNCTIONAL VASODILATORY RESERVE AND FEATURES OF THE METABOLIC SYNDROME

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Metabolic syndrome is a cluster of risk factors predisposing to cardiovascular disease. Increasing evidence suggests that the muscle microvasculature is impaired in individuals with metabolic syndrome. We have investigated insulin-induced changes in microvascular conductance in individuals with features of the metabolic syndrome. Thirty-three volunteers [52 ± 9 years (mean SD); $n = 15$ men)] were studied. All gave written informed consent. Blood flux (LDF) was measured using laser Doppler fluximetry and a novel 785 nm, 20 mW, 4 mm separation fluximeter probe (Moor Instruments Ltd, UK), placed above the anterior tibialis muscle of the non-dominant lower limb, and insulin sensitivity by hyperinsulinaemic euglycaemic clamp. Measurements were made at rest and during reactive hyperaemia (RH) to arterial occlusion, before and during the clamp. Vascular conductance (VC) calculated as LDF/MABP in arbitrary units, at baseline and during RH, decreased with $>/=3$ features of the metabolic syndrome ($p = 0.01$) and calculated overall CVD risk ($p = 0.01$). Baseline VC was significantly increased at high dose insulin (0.8 ± 0.4 vs 1.1 ± 0.6, $p = 0.001$), but paradoxically reduced during peak RH (before insulin: 413 ± 32 vs high dose insulin: 363 ± 35%baseline, $p = 0.01$). The impaired RH response (peak VC and rate of recovery from peak) was associated with plasma total cholesterol ($r^2 = 0.51$, $p = 0.01$) and LDL cholesterol concentrations ($r^2 = 0.49$, $p = 0.05$). Features of the metabolic syndrome and increased CVD risk are associated with a reduced functional vasodilatory reserve and that this impaired response may contribute to decreased insulin sensitivity and glucose uptake by muscle.

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PC42

VISIBLE LIGHT SPECTROPHOTOMETRY – A NOVEL TECHNIQUE FOR CLINICAL ASSESSMENT OF COLON MICROCIRCULATION

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Assessment of colon circulation, in suspected ischaemic conditions, is a challenging task. Mesenteric angiography, which is considered as a gold standard for assessment of circulation, Laser Doppler flowmetry and tonometry have their own limitations. The aim of this study is to investigate the applicability of visible light spectrophotometry (VLS) in assessing mucosal tissue oxygenation (SO_2) in normal colon. **Methods:** Ten patients undergoing elective colonoscopy were recruited for this study. All of them had normal looking mucosa without any macroscopic evidences of inflammation or bleeding at the time of the procedure. The colonoscopies were done under sedation and patients were given oxygen at the rate of 2L/minute by nasal catheter during the procedures. Tissue oxygenation (SO_2) was measured in the different parts of the large bowel with VLS. The measurements were done by means of a catheter probe introduced via the biopsy channel of the colonoscope. **Results:** The median age of the patients was 57 (22–79). The results showed that there was no significant difference in the SO_2 between the different parts of the colon. The mean SO_2 ($\pm$SD) in the colon mucosa was 81% ($\pm$8.5). There were no procedure-related complications. **Conclusion:** This study shows that VLS is a reliable and safe technique for assessing the microcirculation in the colon through the measurement of tissue SO_2. It thus has the potential for clinical use in suspected colon ischaemia.

PC43

ANALYSIS OF CAPILLARY STRUCTURE AND BLOOD FLOW AT THE NAILFOLD

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In microvascular disease both the structure and function of the microvessels undergo changes that are most easily monitored in the skin. Structurally, these changes can be observed with optical microscopy of the nailfold (nailfold capillaroscopy). Functionally, they can be measured by high resolution laser speckle contrast imaging. The aim of this study was to investigate the relationship between capillary morphology and blood flow, and to determine how best to classify patients with systemic sclerosis (SSc), primary Raynaud's phenomenon (PRP), and healthy controls (HC). Measurements of capillary morphology and blood flow at the nailbed were made in 16 patients with SSc, 14 with PRP, and in 16 HC. Significant differences were found between groups (ANOVA [$p < 0.001$]) for capillary structure parameters but not for blood flow (means [in relative units] shown in table). No correlation was found between blood flow and capillary structure. Capillaroscopy data allowed 89% of SSc patient data to be correctly classified (via logistic regression) versus PRP patients and controls. Inclusion of blood flow data did not increase specificity or sensitivity to disease category.

Group	Width	Derangement	Tortuosity	Capillary distance	Blood flow
HC	12.6	8.8	3.2	81.5	205.2
PRP	14.6	10.0	3.2	87.2	233.6
SSc	15.8	10.8	3.3	168.8	230.7

Capillary morphology is more suitable than blood flow as a discriminator among SSc, PRP, and HC.

PC44

ALTERATION IN BLOOD VESSEL DENSITY IN MULTIPLE SCLEROSIS LESIONS IMPLIES A ROLE IN BRAIN SCARRING

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Background: Multiple sclerosis (MS) is an inflammatory, demyelinating, and neurodegenerative disorder of the central nervous system characterised by a relapsing-remitting clinical progression, i.e., cycles of damage and repair, which results in the development of lesions and chronic glial scarring. During normal wound healing, angiogenesis (new blood

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vessel formation from existing vessels) occurs to restore vascular integrity in areas undergoing repair. However, in MS where damage and repair are ongoing, erratic angiogenesis might influence scar deposition. **Aim:** To examine whether blood vessel density is altered in the cerebral white matter of individuals with MS. **Methods:** Blood vessels were quantified in sections of post-mortem cerebral white matter from normal control (NC), and MS cases by triple label immunohistochemistry for collagen IV, von Willibrand factor, and myelin. MS tissue was classified as normal-appearing white matter (NAWM), and acute, subacute, and chronic lesions. Total vessels in three random fields from each sample were counted and the results analysed statistically by Mann-Whitney test. **Results:** Total blood vessel numbers were significantly increased in all MS tissues compared with control; 43.9 ± 8.5 blood vessels were observed in control tissue compared to NAWM (65.9 ± 20.7; $p = 0.01$, $n = 10$), acute (57 ± 13.3; $p = 0.043$, $n = 9$), subacute (84.2 ± 13.3; $p = 0.001$, $n = 8$) and chronic lesions (77.5 ± 23.2; $p = 0.002$, $n = 12$), from MS tissue. **Conclusions:** Blood vessel density is increased in the cerebral white matter of individuals with MS. This may indicate that angiogenesis is an important factor in scarring in MS.

PC45

ANGIOGENIC GENES ARE DIFFERENTIALLY EXPRESSED IN THE INFRARENAL ATHEROSCLEROTIC AORTA

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Aim: Neovascularization of the vessel wall has been implicated as an important process in atherosclerosis which is evident in both aortic occlusive (AOD) and aneurysmal disease (AAA). The aim of the study was to compare expression of angiogenic genes in AAA and AOD with normal aortic wall (NA). **Methods:** Complementary DNA was prepared from full-thickness aortic wall tissues of transplant, AAA, and AOD patients ($n = 5$ each). cDNA was hybridised to HU133 Plus 2.0 micro array interrogating the whole human genome. Data were analysed using GeneSpring software. A p-value < 0.05 after multiple correction testing was considered significant. **Results:** Of the genes regulating angiogenesis, 20 were differentially expressed AOD vs NA (10 upregulated and 10 downregulated); 15 genes were differentially expressed AAA vs NA (5 upregulated and 10 downregulated); 13 genes were common to AOD and AAA. IL-8 showed the highest fold difference in both groups (20-fold and 9-fold, respectively). FGF was decreased in both groups. VEGF-A was equally expressed in all 3 groups while VEGF-C was upregulated and VEGF-D downregulated in AAA and AOD. **Conclusion:** Genes have been identified that are specific to atherosclerosis in the infra-renal aorta. The pattern of expression may be important in disease progression.

PC46

PLASMA LOW-DENSITY LIPOPROTEIN-CHOLESTEROL (LDL-C) WITHIN THE NORMAL RANGE PREDICTS LARGE ARTERY COMPLIANCE

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We tested the hypothesis that plasma LDL-C concentration below that considered clinically "high" (< 4.1 mmol/L; NCEP guidelines) was a significant predictor of large artery compliance (CC; via carotid ultrasound and tonometry) in a cross-section of 115 carefully screened, healthy, non-smoking, un-medicated men with a broad age range. LDL-C was significantly associated with CC ($r = -0.36$, $p < 0.001$). We then used regression and part-correlation (part-r) to determine the association of LDL-C with CC independently of age, aerobic fitness (maximal oxygen uptake), and waist circumference. Both independent variables predicted CC in models with LDL-C and age (part-r $= -0.21$, $p = 0.02$ and part-r $= -0.36$, $p < 0.001$, respectively), LDL-C, and aerobic fitness (part-r $= -0.21$, $p = 0.02$, and part-r $= 0.42$, $p = 0.001$), but only LDL-C predicted CC in the model with LDL-C and waist circumference (part-r $= -0.24$, $p = 0.02$). In a combined model with all four independent variables, LDL-C remained a significant predictor of CC (part-r $= -0.18$, $p = 0.04$). Our analyses indicate that LDL-C below that considered clinically high predicts directly measured CC, and that this is independent of age, aerobic fitness, and waist circumference. The

possibility that LDL-C within this range affects vascular health warrants further investigation. This could have relevance to the primary prevention of diseases associated with large artery stiffening.

PC47

CAPILLARY RAREFACTION IN NORMOTENSIVE ASIAN INDIVIDUALS: A PREDICTOR FOR FUTURE CARDIOVASCULAR DISEASE?

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Cardiovascular disease (CVD) is the main cause of mortality and morbidity in the developed and the developing world. Standard CVD risk factors such as hyperlipidemia and diabetes mellitus are more common in South Asians and yet this does not entirely explain the increased risk of CVD in these individuals. Hypertension is increasingly recognized as a disease of the microcirculation. Many microcirculatory abnormalities including capillary rarefaction have been implicated in the pathogenesis of hypertension and cardiovascular disease. The aim of this study was to compare skin capillary density (SCD) in healthy South Asian individuals and in matched Caucasian subjects. We studied 33 healthy normotensive Asian students (Group 1) mean age 22 ± 1.4 years, and 12 Caucasian subjects (Group 2) mean age 26 ± 4 years. We used intravital videomicroscopy to measure SCD on the dorsum of the left middle finger. Subjects were seen after a 2-hour fast in a temperature-controlled laboratory (22 ± 2ºC) after 20 minutes of rest for acclimatization. Sitting and standing BP were measured with a semiautomatic Omron 907C. Four fields on the dorsum of the middle phalynx were examined continuously for 5 minutes. Venous congestion was used to maxim. Sitting BP was 110/66 ± 10/8 vs 110/65 ± 11/7 mmHg, respectively (P = 0.54). BMI was 22.4 ± 3.6 vs 23.3 ± 3.9 (p = 0.49), respectively. Baseline SCD was 45 ± 8 vs 58 ± 8 capillaries/0.68 mm^2 ($p < 0.001$) and maximal SCD were 49 ± 9 vs 64 ± 11 capillaries/0.68 mm^2 ($p < 0.001$) in Group I and Group II, respectively. Statistical analysis was done using Statview 5 and unpaired t-test was used. Healthy normotensive individuals of South Asian origin have significant skin capillary rarefaction compared to Caucasian individuals. Capillary rarefaction may prove to be a novel independent risk factor for cardiovascular disease. Improving microvascular rarefaction may provide an innovative way to reduce CVD risk.

PC48

MEASURING WHOLE BODY ENDOTHELIAL GLYCOCALYX VOLUME: SOME QUESTIONS OF METHOD

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Measurements of the volume of the luminal endothelial glycocalyx (EGL) in conscious human subjects could be of great value in investigating vascular disease. Recent estimates by Nieuwdorp et al. [1] are based on the difference between the initial volume of distribution of Dextran 40 (D40), which enters the EGL, and that of labelled red cells, which are excluded from it. While the general idea seems reasonable, some questions need to be answered before the method is valid in principle: 1) Are the concentrations of D40 in plasma and the fluid within the EGL equal at equilibrium (i.e., does the partition coefficient, ?, equal one)? If ? < 1, EGL volume is underestimated. If ? > 1, EGL volume is overestimated; 2) Is the D40 used in this study a single molecular species? Commercially available Rheomacrodex has been used and this is a mixture of dextrans with a mean molecular weight of 40 KD. The presence of a significant proportion of small dextran molecules, which can leave the circulation before initial mixing is complete, and the first blood sample taken compromises the assumption of mass conservation at zero time that underlies this method; and. 3) Why use labelled red cells rather than plasma markers such as labelled albumin or fibrinogen? Both these molecules are initially excluded from the EGL and unaffected by rheological factors that determine the axial distribution of red cells in microvessels. The presentation will consider possible answers and their implications.

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PC49

WHAT IS THE CLINICAL SIGNIFICANCE OF SATURATION ($S_{mb}O_2$), THE MEAN OXYGEN CONTENT OF BLOOD?

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Increasingly, we are monitoring the distribution of oxygen through the microcirculation, using optical techniques such as optical reflectance spectroscopy (ORS) and near infrared spectroscopy (NIRS). Mean blood saturation ($S_{mb}O_2$) and tissue oxygen index (TOI), derived by ORS and NIRS, respectively, evoke a concept that we can measure oxygen delivery to the tissue. The aim of this study is to establish whether ($S_{mb}O_2$) is an appropriate indicator of tissue oxygenation. Mean blood saturation ($S_{mb}O_2$) was measured at rest in the skin microcirculation of forearm or index finger in 30 healthy subjects (15 males, age 21–42 years). Wavelet analysis was applied to the spontaneous fluctuations in $S_{mb}O_2$ measured by ORS as changes in concentration of oxyhaemoglobin (HbO2) and deoxyhaemoglobin (Hb). Two distinctly different spontaneous falls in ($S_{mb}O_2$) were observed and identified as v-swings and f-swings. V-swings induced by fluctuations in arterial blood volume resulted from the effects of respiration, endothelial, sympathetic and myogenic activity. There was no apparent change in [Hb]. In contrast, f-swings resulted from a fall in [HbO_2] accompanied by a rise in [Hb] and were only induced by endothelial and sympathetic activity.Thus the same fall in ($S_{mb}O_2$) can be induced by two distinct mechanisms. A v-swing does not suggest an inadequacy in oxygen delivery whilst the f-swing may indicate a change in oxygen delivery to tissue. Blood oxygen saturation cannot therefore be accepted as a definitive marker of tissue oxygenation.

PC50

RELATION BETWEEN VOLUME AND APPLIED ONCOTIC PRESSURE OF ISOLATED MOUSE GLOMERULI *EX VIVO*

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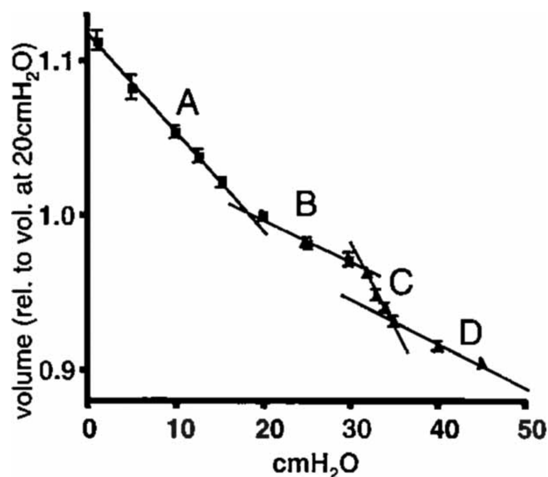
Glomerular reflection coefficient to albumin (s_{alb}) can be estimated by applying an oncotic pressure (p) gradient across the glomerular filtration barrier to drive fluid flux, resulting in changes in glomerular volume (V). To characterize the relation between V and p we perfused single isolated



glomeruli with successive BSA solutions having a range of oncotic pressures. Glomeruli were isolated, aspirated onto a micropipette within a flow-controlled chamber, then perfused with the first p of BSA and examined under static conditions. Perfusate was then switched and observation repeated with subsequent BSA solutions of increasing p. V during each perfusate incubation period was recorded on video and calculated using ImageJ and Adobe Photoshop software. The V/p relationship appeared to have four different slopes (compliances) from 0–20 (A), 20–32 (B), 32–35 (C) and 35–50cmH_2O (D). The steepest slope (i.e.,, the most sensitive region for volume changes for a set pressure — the highest compliance region) was from 32–35cmH_2O. These results indicate that reflection coefficient can be measured effectively as long as the pressure at which it is done is within a specific linear range, and is most sensitive from 32–35cmH_2O

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PC51

VEGF-A_{165}b REDUCED THE GLOMERULAR ULTRAFILTRATION CO-EFFICIENT (LpA) WITHOUT CHANGING GLOMERULAR CAPILLARY SURFACE AREA

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Dysregulation of renal VEGF-A expression has been documented in multiple glomerular diseases, but its pathophysiological significance remains unclear. Differential splicing of the VEGF-A gene forms two families of isoforms: the pro-angiogenic family (VEGF-A_{xxx}), and the anti-angiogenic families (VEGF-A_{xxx}b). To determine the effect of VEGF-A_{165}b on renal function transgenic mice overexpressing VEGF-A_{165}b under nephrin promoter (confined to podocytes) were established. At 18 months mice appeared normal and were not proteinuric (protein:creatinine, VEGF-$A_{165}b^{+/-}$ 21.80 ± 3.16, n = 8; controls: 19.32 ± 2.82, n = 7; unpaired t-test:p = 0.5730). We have previously shown the mice had reduced L_pA (VEGF-$A_{165}b^{+/-}$: 1.0 ± 0.18min-1mmHg^{-1}, n = 18; controls: 1.93 ± 0.32min-1mmHg^{-1},n = 8; unpaired t-test:p = 0.002). Morphometric studies on high magnification light micrographs showed that the change in L_pA was not due to a reduction in glomerular volume (VEGF-$A_{165}b^{+/-}$: 0.31 ± 0.062nl n = 3; controls: 0.24 ± 0.012nl; unpaired t-test p = 0.2825), or glomerular capillary surface area (VEGF-$A_{165}b^{+/-}$: 4.4 ± 0.43 x 104 μm^2 n = 3; controls 4.9 ± 0.67 x 104 μm^2 n = 4 p = 0.5695). Podocyte over-expression of VEGF-A_{165}b thus caused a 48% reduction in the value of L_pA without causing detectable changes in glomerular structure.

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PC52

THE SUBPODOCYTE SPACE IN HUMAN RENAL GLOMERULI

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The urinary or Bowman's space consists of three interconnected urinary spaces forming drainage pathways for ultrafiltrate [1,2]. Glomerular filtrate may enter one of these restrictive spaces under the podocyte (subpodocyte space, SPS) before passing to other urinary spaces. We have measured and reconstructed SPS in rats, and the presence of similar SPS has been observed in pigs, frogs, mice, and fish. Here we report on SPS in normal human glomeruli and compare with rat SPS. We have analysed the SPS from 4 glomeruli of normal human kidney (with ethical committee approval). Tissues were processed using standard procedures and serial ultrathin sections of kidney were used to reconstruct glomerular regions. Reconstruct software was used to make 3D models from the sections showing the complexity of the SPS. SPS parameters were measured and compared (Mean ± SEM, unpaired t-tests). The SPS height from the glomerular filtration barrier was higher in human than in Wistar rats (0.54 ± 0.07μm, 0.24 ± 0.08μm, respectively, $p < 0.05$), however, the outflow through the SPS exit pore width is the same in human and rat (0.25 ± 0.08μm, 0.24 ± 0.07μm, respectively). The foot processes anchoring the podocyte to the filtration barrier in the human SPS obscure 20 ± 3% of the SPS filtration barrier (25 ± 3% in rat). Human SPS is similar to rat and similarly provides a further resistance to urinary outflow away from the human glomerular filtration barrier. [1] Neal et al. *JASN* 2005; 16:1223; [2] Neal et al. *Am J Physiol* 2007; 293:F1787.

PC53

EVIDENCE FOR THE INVOLVEMENT OF TWEETY 3 IN THE Ca^{2+}-ACTIVATED Cl- CURRENT IN RETINAL ARTERIOLAR SMOOTH MUSCLE

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Ca^{2+}-activated Cl^- currents have been recorded in macro- and microvascular smooth muscle cells, but little is known about the molecular make-up of the channels underlying this conductance. In the present study we have investigated the molecular identity of the Ca^{2+}-activated Cl^- channels in native rat retinal arteriolar smooth muscle cells using patch-clamp, RT-PCR, immunohistochemistry, and neutralizing

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antibody studies. Application of 10mM caffeine evoked large transient Ca^{2+}-activated Cl^- currents which reversed close to ECl and were completely abolished by 100μM tetracaine or low Cl^- Hanks' solution containing the Cl^- channel inhibitor, 9-AC (1 mM). Transcripts encoding the large conductance Ca^{2+}-activated Cl^- channel, Tweety 3 were identified in freshly isolated retinal arterioles. Immunofluorescence labelling revealed a punctuate distribution of Tweety 3 localized to the plasma membrane of retinal arteriolar smooth muscle cells. Anti-Tweety 3 antibody applied intracellularly inhibited caffeine-evoked Ca^{2+}-activated Cl^- currents by ~70% (peak current densities at −40 mV were 39.82 ± 10.04 and -12.88 ± 6.30pA/pF for antibody-free and anti-Tweety 3 solutions, respectively; n = 10–11; $p < 0.005$, Student's t-test), whereas a control antibody (Anti-Tweety 1) had no effect (-40.33 ± 9.20pA/pF n = 11; p = 0.97 versus antibody-free solution). These data suggest that Tweety-3 may represent a major component underlying the Ca^{2+}-activated Cl^- current in retinal arteriolar myocytes

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PC54

MODULATION OF HEMORHEOLOGICAL PARAMETERS BY THE ERYTHROCYTE REDOX THIOL STATUS

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There is growing knowledge about the association between hemorheological blood disorders and compromised microcirculation in erythrocyte abnormalities. Effects of the non-neuronal cholinergic elements, especially acetylcholinesterase, on the erythrocyte hemorheological behaviour were characterized in the past. Little has yet been done to assess the erythrocyte hemorheological behaviour under the influence of the cellular redox thiol status, which constituted the aim of this study. Aliquots of venous blood from 10 healthy male subjects were incubated *in vitro* with increasing concentrations of a thiol reducer agent (dithiothreitol 1, 10, 50 μM) in the presence and absence of acetylcholinesterase substrate (acetylcholine, ACh) or inhibitor (velnacrine maleate, VM). The following parameters were determined: erythrocyte aggregation, erythrocyte deformability, and lipid membrane fluidity. Dithiothreitol neither induces significant changes in the hemorheological behaviour of human red cells (aggregation and deformability) nor on the erythrocyte lipid membrane fluidity. Upon cellular thiol stimulation, AChE modulation (by either ACh or VM) decreases erythrocyte aggregation and elongation indexes. The addition of DTT to blood samples aliquots, contributing to the redox thiol status, is not directly involved in the modulation of erythrocyte rheological properties. However, upon acetylcholinesterase modulation changes on the erythrocyte, hemorheological parameters are triggered by cellular-activated thiols. The lower aggregation tendency detected may become increasingly important in the clinical setting.

PC55

SYMPATHETIC ACTIVATION AND FACIAL BLOOD FLOW SYMMETRY

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The human forebrain displays lateralization for control of the autonomic nervous system, sympathetic activation during arousal, and emotional states being associated with the right hemisphere. Resting forehead blood flow was recently reported to be higher on the right than left side of the face with a higher amplitude oscillatory activity [1]. To study asymmetry in forehead flow responses to sympathetic activation, skin perfusion was measured by Laser Doppler flux (LDF) from the right and left forehead in 14 healthy subjects, 7 men, 7 women, aged 18–22 years, during exercise (EX, isometric sustained handgrip at 40% maximal voluntary contraction) performed for 2 min with either left (L) or right (R) hand, and during a cold pressor test (CPT) by immersion of either L or R foot into iced water for 2 min. Blood pressure, heart rate (ECG), and electrodermal activity (EDA) were recorded before and during each intervention. Mean arterial pressure increases were similar for L and R hand exercise (L 18.6 ± 3.1, R 17.8 ± 2.1 mmHg) and L and R foot CPT (L 8.7 ± 2.6, R 8.6 ± 1.7 mmHg), as were heart rate (exercise L 8 ± 1, R 8 ± 2 bpm; CPT L 4 ± 2, R 3 ± 1 bpm) and EDA (data not shown). Relative increases in skin vascular conductance, calculated from LDF perfusion and mean arterial pressure, were significant ($p < 0.05$) and not different in the left (L EX $130 \pm 13\%$, R EX $112 \pm 6\%$)

and right (L EX 131 ± 16%, R EX 123 ± 10%) forehead. Skin conductance changes to CPT were not significant in the left (L CPT 110 ± 6%, R CPT 107 ± 5%) or right (L CPT 111 ± 9%, R CPT 111 ± 8%) forehead. There does not appear to be laterality in regulation of forehead flow. [1] Benedicic et al. *Microvasc Res* 2007; 74:45–50.

PC56

DILTIAZEM PRETREATMENT ALTERS THE RESPONSE TO PROPOFOL ANAESTHESIA AND BAYK8644 IN SPONTANEOUSLY HYPERTENSIVE RATS *IN VIVO*

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Spontaneously hypertensive rats (SHR) demonstrate exaggerated increases in blood pressure and vasoconstriction of the mesenteric microcirculation to the L-type Ca^{2+} channel agonist (LTCC) BayK8644 [1]. This may contribute to the observed hypotension and vasodilation in hypertensives during propofol anaesthesia. The aim of the study was to investigate the effect of chronic antihypertensive therapy for SHRs on the altered cardiovascular response to propofol and BayK8644. Age-matched male SHR (n = 8, 310 ± 10.21g) were pretreated with the LTCC antagonist diltiazem (100mg/kg/day) or placebo for 14 days. On day 15 animals were anaesthetised with propofol (6mg/kg, 20–60mg/kg/hr, intravenously). The mesentery was prepared for intravital microscopy. A dose response to Bay K8644 (0.1–10.0μg/kg, 0.1ml/100g) was performed and mesenteric microvascular variables measured. At the end of the experiment there was no significant change compared to baseline in systolic arterial pressure (SAP) in the diltiazem-treated group, however, in the placebo group SAP reduced significantly (170 ± 8.46mmHg vs 115 ± 5.77mmHg, $p < 0.05$). The dose-dependent constriction of mesenteric arterioles in response to 5.0, 10.0μg/kg BayK8644 was attenuated by pretreatment with diltiazem ($p < 0.05$). Diltiazem treatment has partially blocked the LTCCs located on mesenteric arterioles. The decrease in MAP observed during propofol anaesthesia is inhibited with diltiazem pretreatment, indicating that propofol can act on LTCC to reduce MAP. [1] Lawton et al. *FASEB J* 2005; 20:A269).

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PC57

CHRONIC SHEAR STRESS REDUCES PERMEABILITY OF PIG AORTIC ENDOTHELIAL CELLS TO RHODAMINE ALBUMIN

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Transport of macromolecules across vascular endothelium is essential for the normal function of underlying tissues and may also be important in disease. We have studied factors that modify transport *in vitro*. Pig aortic endothelial cells (PAEC) at passage two were seeded onto Transwell filters coated with fibronectin and cultured for 9 days in DMEM containing serum (20%), glutamine (5 mM), and endothelial cell growth factor (0.5 $\mu g.ml^{-1}$) at 37°C and 5% CO_2. Cells were treated with 1% BSA for 60 min before adding rhodamine-labelled albumin (1 $mg.ml^{-1}$) to the top compartment. Samples were taken from the bottom compartment at 60-min intervals and the concentration of rhodamine albumin determined using a fluorimeter in order to calculate permeability. Exposure to shear stress for 8 days, using an orbital shaker, significantly lowered permeability to rhodamine albumin compared to cells cultured under static conditions (1.5 ± 0.2 cf. 2.7 ± 0.1 x 10^{-6} $cm.s^{-1}$ at 60 min, mean ± SEM, $P < 0.01$, 6 wells from 2 independent experiments). The direction of the effect was opposite to that obtained in previous studies of shear stress and permeability *in vitro*, where acute application of shear has generally been observed to increase permeability [1]. The results may have relevance to atherosclerosis, in which shear-dependent alteration of endothelial permeability to low-density lipoprotein is thought to account for the localised occurrence of lesions in areas of arterial curvature and branching. [1] Jo et al. *Am J Physiol* 1991; 260:H1992–1996.

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PC58

THE EFFECT OF FETAL HYPERINSULEMIA ON HUMAN PLACENTAL VASCULAR FUNCTION: PERFUSION OF FETAL MICROVASCULAR BED WITH HIGH INSULIN RESULTS IN INCREASED VASCULAR LEAKAGE AND LOSS OF JUNCTIONAL BETA-CATENIN

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In diabetic pregnancies, fetal hyperinsulinaemia occurs as a result of increased insulin production by the fetal pancreas in response to augmented transplacental glucose flux. The effect of this on placental vasculature is not known. This study is the first to examine whether high insulin, administered from the fetal side, can cause alterations in junctional occupancy of the key adherens junction molecule ß-catenin and vascular leakage. Microvascular beds of normal human term placentae were perfused using an independent maternal and fetal dual perfusion method. Human recombinant insulin (25mU/L, the median value of insulin in cord blood serum from Type1 diabetic pregnancies at term) was added to the fetal circuit of the experimental group(n = 3), but not to control perfusions (n = 3).After 20 min perfusion, a 76 M_r dextran tracer (0.5mg/ml) was introduced to the fetal circuit for a further 10 min. After fixation and processing, selective random sampling was used to analyse extent of endothelial junctional β-catenin and associated tracer leakage. The insulin-perfused group displayed a significant ($p < 0.05$) loss of junctional β-catenin, with 47% of vascular profiles exhibiting complete loss of β-catenin, in comparison to 8% in the normal placentae. In the insulin-perfused placentae the mean percentage of vessels with tracer leakage was 28%, significantly ($p < 0.05$) higher than the 9% seen in control perfusions. The data suggest that fetal hyperinsulinaemia may contribute to junctional disruption, increased placental vascular leakage, and altered beta-catenin signalling. Beyond implications in placental barrier function, the results raise the possibility that high insulin may cause alterations in the vasculature of the fetus, with implied potentially long-lasting effects on the infant.

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PC59

THE EFFECTS OF RHO INHIBITION ON LPS-INDUCED MICROVASCULAR CHANGES *IN VIVO*

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Sepsis may be modelled *in vivo* using lipopolysaccharide (LPS) to induce microvascular disturbances such as leucocyte-endothelial interactions and macromolecular leak (ML). There is evidence to suggest that such effects may be due to the small GTPase protein Rho and this study aimed to investigate this. Male Wistar rats (258 ± 8.67g) were administered with the Rho inhibitor fasudil (3 mg/kg, n = 6 or 10 mg/kg, n = 6) immediately prior to administration of a low-dose continuous infusion of LPS (150µg/kg/hr) (n = 20) or an equivalent volume of saline (1 ml/kg/hr) (n = 15) (i.v.) under thiopental anaesthesia (100 mg/kg/hr, i.v.). FITC-BSA (25mg/100g, i.v.) was administered and intravital microscopy used to observe the mesenteric microcirculation and determine leucocyte-endothelial interactions and ML from post-capillary venules every 15 min between 0 and 240 min. LPS increased the number of leucocytes adhering to the endothelial cell surface at all time points (7.29 ± 1.15, $P < 0.001$). Leucocyte adhesion decreased with a 10 mg/kg dose of fasudil after 60 min of LPS, an effect maintained at 240 min (60 min: 3.00 ± 0.89, $P < 0.05$; 240 min: 1.50 ± 0.80, $P < 0.01$). 3 mg/kg fasudil was only effective in preventing leucocyte adhesion at 240 min (1.75 ± 0.25, $P < 0.05$). LPS increased ML over the 240 min period in comparison to the saline group (11.80 ± 4.06, $P < 0.01$). Fasudil did not significantly alter ML at either dose. This study suggests that Rho inhibition is effective in reducing leucocyte adhesion during the early period of sepsis, but does not appear to be involved in ML induced by LPS.

Supported by theMRC

PC60

PHOSPHODIESTERASE INHIBITORS (PDES) REDUCE SPONGE-INDUCED INTRA-PERITONEAL ADHESION IN MICE

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The most typical lesion that develops after surgical procedures in the abdominal cavity is formation of adhesions constituting major postoperative morbidity and mortality. A class of compounds that has emerged as potential inhibitor of these lesions belongs to the phosphodiesterase (PDE) family, because of their wide range of actions in tissue proliferation activities. In this study, three PDE

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compounds, pentoxifylline (PTX, 400 mg/kg) cilostazol (CTZ,500 mg/kg), and dipyridamole (DPM, 100 mg/kg) given orally were used to investigate their effects on adhesion components induced by sponge matrix implanted intraperitoneally (i.p.) in anaesthetized Balb/c mice. Angiogenesis was assessed by hemoglobin and VEGF content, and inflammation was determined by TNF-α levels and by myeloperoxidase (MPO) and n-acetylglucosaminidase (NAG) activity. Collagen and TGF-β1 contents were used as markers for fibrosis. These compounds reduced adhesion wet weight/mg (Control:197 ± 3; PTX 144 ± 6; TCZ:144 ± 3; DPM:159 ± 12; n = 8/group), caused inhibition of angiogenesis (about 45%), and decreased fibrosis (TGFβ1 and collagen levels, 30% and 85%, respectively). Interestingly, although the intraimplant levels of TNF-α were inhibited by the compounds they had no effect on the activity of the enzymes (MPO or NAG). These results show that PDE inhibitors exhibit potent anti-angiogenic/anti-fibrogenic activities in this model of peritoneal adhesion through modulation of growth factors

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PC61

ROLE OF ENDOTHELIN-1 IN THE RESPONSE OF SPLENIC VEINS TO LIPOPOLYSACCHARIDE

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Sepsis leads to numerous conditions, including hypovolaemia. Levels of endothelin-1 (ET-1), a powerful vasoconstrictor, are increased during the onset of sepsis. Exposure to lipopolysaccharide (LPS), a model of sepsis, triggers ET-1-mediated constriction in splenic veins [1]. This could result in fluid loss via the spleen due to increased capillary pressure, and may mediate the onset of hypovolaemia during sepsis. ET-1 receptors have a number of subtypes, namely ET_A and ET_{B2} receptors located on the vascular smooth muscle and ET_{B1} receptors on endothelium. The role of these receptor subtypes in LPS-induced constriction is unclear. Thus, in this study, we investigated the effect of ET_A and ET_B selective antagonists ABT-627 and A-192621, respectively, on LPS-induced constriction of intact and denuded splenic veins. Intact (n = 5; diameter 552.2 ± 83.5μm) and denuded (n = 6; 473.0 ± 42.0 μm) splenic veins from male Wistar rats were mounted in a pressurized myography system. Intraluminal diameters were measured during exposure to LPS (240 mins). Further veins were exposed to LPS in the presence of ABT-627 (n = 5; diameter 469.0 ± 37.7 μm) and A-192621 (n = 5; 439.0 ± 25.1 μm). Significant constriction occurred at 70 min post LPS incubation in both intact veins (41.6 ± 10.0%) and denuded veins (39.8 ± 13.4%). This constriction was attenuated by both the ET_A or ET_B antagonists. The results indicate that constriction is endothelium-independent and therefore not via ET_{B1} receptors. However, ET_A and ET_{B2} receptors on the vascular smooth muscle both appear to be involved in the LPS-mediated constriction of splenic veins, but the precise contribution of each requires further investigation. [1] Mansart et al., preliminary work.

PC62

ARE THE BENEFICIAL EFFECTS OF STATINS IN SEPSIS MEDIATED VIA NITRIC OXIDE SYNTHASE (NOS) *IN VIVO?*

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Sepsis may be modelled *in vivo* using lipopolysaccharide (LPS) to induce microvascular disturbances such as leucocyte-endothelial interactions and macromolecular leak (ML). Such effects may be caused by altered levels of eNOS and iNOS and there is evidence to suggest that statins may be protective in sepsis by restoration of their physiological balance. Male Wistar rats (270 ± 7.71g) were injected with atorvastatin (AV) (200 μg/kg) or an equivalent volume of 0.1% ethanol (20 ml/kg) (s.c.) 18 and 3 hrs prior to administration of a low-dose continuous infusion of LPS (150μg/kg/hr) or saline (1 ml/kg/hr) (i.v.), under thiopental anaesthesia (100 mg/kg/hr, i.v.). A continuous infusion of L-NAME (10 μg/kg/min) or 1400W (20 μg/kg/min) (i.v.) was given concurrently with LPS administration. FITC-BSA (25mg/100g, i.v.) was administered and intravital microscopy used to determine leucocyte-endothelial interactions and ML every 15 minutes (min) between 0 and 240 min. LPS increased leucocyte adhesion (7.29 ± 1.15, P < 0.001), an effect which

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was reversed by AV (1.83 ± 0.98, $P < 0.001$) and L-NAME + AV (1.33 ± 0.84, $P < 0.01$) at all time points. 1400W + AV and 1400W alone gave a partial reduction but no effect was observed with L-NAME alone. LPS increased ML (11.80 ± 4.06, $P < 0.01$), which was significantly decreased by 1400W (1.52 ± 1.59, $P < 0.01$) and AV + 1400W (0.33 ± 1.02, $P < 0.01$) but not by L-NAME. This study suggests that ML may be caused at least in part by iNOS. Statin-mediated inhibition of leucocyte adhesion may be due to a NOS-independent mechanism.

Supported by the MRC

PC63

NITRIC OXIDE AND FREE RADICALS DEPENDENT CEREBRAL MICROVASCULAR PERMEABILITY INCREASE IN THE ANAESTHETIZED RAT BY LYSOPHOSPHATIDIC ACID (LPA)

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LPA is a single-chain fatty acid secreted from platelets, which activates a number of G protein families including the G_i, G_q,and G_{12}. It increases the permeability of brain endothelium in culture and in single pial venules, but the signaling pathways have yet to be elucidated. The permeability of pial venular capillaries of rats (anaesthetized with pentobarbitone: 60 $mg.kg^{-1}$ i.p.) to sulforhodamine dye (P_R; 574 da) was measured in single vessel occlusion experiments.

LPA increased P_R rapidly and reversibly and dose-dependently when applied acutely to the brain-side of vessel, and also when applied to the lumen. After 10 min. application the response diminished to about 30%, and the acute response remained suppressed for between 15 to 25 min. The acute response was unaffected by the histamine receptor antagonist cimetidine (5μM; which did block the permeability increase induced by des-Arg^9bradykinin), but was blocked by the LPA-1 receptor antagonist KI16426 (10 μM). The response to 1 μM LPA (2.4 ± 0.3 $cm.s^{-1}x10^{-6}$) was not significantly affected by the NOS inhibitor L-NMMA (1.5 ± 0.7), nor free radical scavenging by a combination of superoxide dismutase and catalase (2.0 ± 0.7), but when these were given in combination the response was abolished (0.1 ± 0.1). This is consistent with the LPA-1 receptor activating $G\alpha_q$ and G_{io}.

PC64

NADPH OXIDASE ACTIVATION IN RAPID INTERLEUKIN-1β POTENTIATION OF BRADYKININ-INDUCED PERMEABILITY INCREASE

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The permeability response of venules to acutely applied bradykinin (Bk) depends on the generation of reactive oxygen species (Ros). We have recently shown that IL-1β rapidly potentiates the response to Bk by increasing the ROS production from an additional source, which is NADPH oxidase assembly and activation. This process requires PKC activation. IL-1β activates sphingomyelinase to produce ceramide which in turn can activate PKC, and here we have tested whether ceramide application also rapidly potentiates the permeability response to Bk. The isolated perfused cremaster muscle preparation of a freshly killed rat was used. The muscle was superfused with Krebs buffer at 37°C and perfused with Krebs-albumin (10 $mg.ml^{-1}$) and FITC-albumin (5 $mg.ml^{-1}$). Permeability was calculated from the rate of decrease in fluorescent signal gradient across a venule when flow was stopped. Dose response curves to brief Bk topical application were compared with those obtained following ceramide application for 10 min. The ACE inhibitor captopril (1 μM) was applied throughout to increase the potency of Bk. The response to Bk was dose-dependent ($logEC_{50}$ -6.3 ± 0.3) with maximal response 7.7 ± 1.9 x10 $^{6}cm.s^{-1}$. Pretreatment with ceramide (1 μM) moved the dose-response curve to the left ($logEC_{50}$ -7.3 ± 0.3) with a maximal response 8.5 ± 1.4 x10 $^{6}cm.s^{-1}$ ($n = 30$ vessels from 4 animals; $p < 0.01$ analysis of co-variance). In conclusion, ceramide is a possible mediator of the IL-1β rapid potentiation of the response to Bk. [1] Sarker MH. *J Physiol* 2000; 540:209).

PC65

A POSSIBLE ROLE FOR ENDOTHELIAL PROGENITOR CELLS (EPC) IN VASCULARISATION OF FIBRIN-BASED SCAFFOLDS?

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Fibrinogen may be a valuable protein to exploit for tissue engineering scaffolds with enhanced bio-functionality, especially vascularisation. Fibrinogen was previously identified as pro-angiogenic compared to collagen in a 3-D gel migration assay. Endothelial cells interact in a tertiary-structure-dependent adhesion with fibrinogen or fibrin-E degradation fragments via RGD/αv integrins. *In vivo*, fibrin-based scaffolds show rapid cell ingress, and neovascularisation by within-scaffold vasculogenesis, whereas collagen-based scaffold is populated slowly and vascularised by wound bed angiogenesis. The role of specific cell types in neovascularisation could be critical, however, since endothelial progenitor cells (EPC) have been found to accelerate dermal wound healing. Initial results showed fibrinogen or fibrin-E covalently coated surfaces supported attachment and accelerated expansion of EPC clones from human umbilical cord blood, in comparison to gelatin. Native collagen did not support attachment. EPC isolated from fibrinogen-based surfaces had a greater expression of MMP9, which is required for migratory activity. This is the first demonstration of specific effects of fibrinogen molecules on EPC recruitment. These data tentatively suggest a mechanism for EPC involvement in the rapid vasculogenesis within fibrinogen-based scaffolds, supplemental to or orchestrating endothelial cell stimulation. Understanding this role of EPC could lead to refinements of scaffold design for wound healing and tissue engineering.

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PC66

THE EFFECT OF TRANSFORMING GROWTH FACTOR-β ON ENDOTHELIAL PROGENITOR CELLS

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Background: Bone-marrow-derived endothelial progenitor cells (EPCs) have been used as a therapeutic agent in heart disease patients with modest success. Transforming growth factor-β (TGFβ) is known to control the balance of cell proliferation and differentiation responses in a number of cell types. For example, TGFβ signalling through ALK(activin like kinase)-1 and endoglin receptors leads to endothelial cell activation, whereas signalling through the ALK5 receptor leads to endothelial cell quiescence [1]. We investigated whether TGFβ signalling regulated a similar balance between proliferation and differentiation responses in EPCs. **Methods:** Murine bone marrow mononuclear cells were isolated and cultured for 7 days prior to identifying EPCs by double immuno-fluorescent staining for endothelial markers. **Results:** An ALK5 inhibitor (SB-431542) caused a 25% increase in EPC proliferation compared with untreated cells ($p < 0.05$). In contrast, endoglin-deficient cultures showed a 25% reduction in EPC number ($p < 0.05$) which was rescued by the ALK5 inhibitor. **Conclusions**: These results suggest that TGFβ signalling through ALK1, ALK5, and endoglin is active in EPCs and maintains a balance between differentiation and proliferation. Furthermore we propose that ex vivo expansion of EPCs with an ALK5 inhibitor, prior to administration to patients, could have therapeutic value. [1] Lebrin et al. *Cardiovascular Research* 2005; 65: 599–608.

PC67

PI3Kγ REGULATES THE SURVIVAL OF BONE MARROW-DERIVED ENDOTHELIAL PROGENITOR CELLS

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Phosphatidylinositol 3-kinase gamma (PI3K?) acts as a transducer of chemokine signaling through G protein-coupled receptors in leukocytes and endothelial cells, but its relevance for the survival of bone marrow-derived endothelial progenitor cells (BM-EPC) remains unknown. We therefore studied the functional contribution of PI3K? to the activation of the Akt/eNOS/FOXO signaling pathway, which plays a key role in the regulation of EPC survival. BM-EPC were obtained from mice lacking PI3K? (PI3K? $^{-/-}$) or expressing a catalytically inactive mutant PI3K? (PI3K? $^{KD/KD}$) and wild type controls (WT). Exposure to hypoxia resulted in the upregulation of PI3K? and phosphorylation of Akt and eNOS in WT BM-EPC. Conversely, hypoxia-induced phosphorylation of Akt and eNOS was attenuated in both PI3K? $^{-/-}$ and PI3K? $^{KD/KD}$ BM-EPC. PI3K? $^{-/-}$ BM-EPC showed higher nuclear retention of the pro-

apoptotic transcription factor FOXO1 and higher expression of the apoptotic marker activated Caspase-3, as compared with WT or PI3K? $^{KD/KD}$. Moreover, production of the survival factor nitric oxide (NO) was significantly reduced in PI3K? $^{-/-}$ BM-EPC only. These findings indicate that PI3K? regulates the survival of BM-EPC through kinase-independent mechanisms. Thus, PI3K? may represent a new target to strengthen the resistance of BM-EPC to apoptotic cell death.

PC68

DEVELOPMENT OF A METHOD TO ISOLATE HUMAN OMENTAL MICROVASCULAR ENDOTHELIAL CELLS (OMEC)

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Background: Ovarian cancer frequently metastasizes to other organs in the abdomen. Most ovarian tumours form on the outside of the ovary and spread by the transcoelomic route. A major site of metastasis is the omentum. Metastasis development involves complex interactions between the ovarian tumour cells and the omental microvasculature. The omental microvascular endothelial cells (OMECs) are thought to become activated and thus create a microenvironment that favors angiogenesis — a process vital to metastasis growth. However, there is no available data on the specific cellular changes occurring in OMECs in response to the ovarian cancer cells. **Aim:** To develop a method to isolate OMECs in order to examine the interactions between OMECs and ovarian cancer cells. **Methods:** OMECs were isolated from omental adipose tissue in a sequential process involving mechanical release of microvascular fragments, enzymic digestion, filtration, and immunoselection. Cultures were maintained at 37°C in a humidified incubator with 5% CO_2 and characterised with the endothelial specific markers PECAM-1 and von Willibrand Factor. Mesothelial cell contamination was determined by immunocytochemical staining for cytokeratins 8 and 18. **Results:** Pure cultures of OMECs were obtained by this method as confirmed by immunocytochemical staining and phenotypical observations. **Conclusion:** We have developed an isolation procedure for obtaining homogenous populations of OMECs.

PC69

HUMAN ADULT VEIN CONTAINS VASCULOGENIC STEM CELLS ABLE TO GIVE RISE TO PERICYTE-LIKE CELLS

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Stem cells (SC) resident in peripheral tissues ensure local repair. Previously, $CD34^{+}CD31^{-}$ cells have been shown to reside in vessel wall and participate in sprouting [1]. We aimed to isolate them in view of autologous transplantation. Saphenous veins derived from patient undergoing cardiac surgery ($n = 30$, age 61 ± 10 years) were digested and cells cultured in NeuroCult medium (Stem Cell Technologies, London, UK). Flow cytometry of freshly digested veins showed that $39.0 \pm 23.2\%$ of cells were $CD34^{+}$. Within the $CD34^{+}$ population $17.0 \pm 12.4\%$ co-express CD31, $2.2 \pm 1.8\%$ CD133, and $1.7 \pm 1.5\%$ KDR. Culture of the total population led to the formation of spheroids growing in suspension (VSP) and selectively supported the survival of $CD34^{+}$ cells ($55.7 \pm 15.6\%$). VSP were shown to express KDR ($42.4 \pm 0.7\%$) and low levels of CD133 ($1.8 \pm 0.9\%$) and to release an array of angiogenic factors and chemokines in the medium. Culture of VSP with EGM2-containing serum led to adhesion and a high proliferation rate. Adherent cells expressed markers of mesenchymal/pericyte lineage, as confirmed by staining for vimentin, NG2, and α-SMA while they were negative for endothelial markers (Lectin/DiL and CD31). **Conclusions:** Our data newly demonstrate that SC are present in veins of elderly patients. We also show the feasibility of isolation and culture of these progenitor cells in the perspective of future applications of regenerative medicine. [1] Zengin et al. *Development* 2006; 133: 1543–1551

PC70

KININ-MEDIATED RECRUITMENT OF PROGENITOR CELLS IS ALTERED IN DIABETES

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Reduced recruitment of vasculogenic (VPC) and endothelial progenitor cells (EPC) is associated with an increased incidence of ischaemic complications in diabetic patients. Previously, we and others demonstrated an implication of kinins in reparative angiogenesis, mediated by mature endothelial cells (EC). Here we investigate the hypothesis that bradykinin (BK) serves as a chemoattractant for VPC and EPC and that BK-mediated recruitment of VPC is impaired in diabetic patients. EPC were cultured from peripheral blood-mononuclear cells (PB-MNC) of non-diabetic (C) and diabetic donors (D). $CD133^+$ and $CD34^+$ VPC were isolated from PB-MNC by magnetic sorting. Expression of BK receptors and migration of VPC and EPC towards BK were studied. BK-responsive and non-responsive cells were isolated from PB-MNC and checked for antigen expression and their ability to give rise to EC. The constitutive kinin B2 receptor (B2R) was expressed on $CD133^+$ and $CD34^+$ VPC as well as on cultured EPC. $CD133^+$ VPC and EPC migrated dose-dependently towards BK. BK-induced migration was significantly reduced in the presence of B2R antagonist HOE140 ($43.3 \pm 9.9\%$ vs BK, $p = 0.002$) and PI3-kinase inhibitor LY294002 ($62.9 \pm 14.8\%$ vs BK, $p = 0.03$). BK-responsive MNC harboured a 3.9-fold higher number of $CD133^+$ and a 2-fold higher number of $CD34^+$ VPC and gave rise to 2.5-fold more EC as compared to the non-BK-responsive subpopulation ($p < 0.05$ for all). In diabetic subjects, the abundance of $CD34^+$ and $CD133^+$ VPC among $B2R^+$ cells was decreased as compared to non-diabetic subjects. BK-induced migration of VPC and EPC represents a novel alternative signalling pathway mediating circulating progenitor cell recruitment to facilitate vasculogenesis. A relative reduction of VPC/EPC among the whole spectrum of cells recruited by kinins might impede vascularisation and wound healing in ischaemic limbs in diabetes.

PC71

FACTORS CONTRIBUTING TO HEMATOPOIETIC STEM CELL RECRUITMENT TO MURINE CREMASTER MICROCIRCULATION

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Hematopoietic stem cells (HSCs) may be utilised therapeutically for a number of degenerative diseases. However, it is not known whether adhesion molecules and cytokines required for HSC homing to non-bone marrow tissue, and indeed whether the kinetics of recruitment are similar to those described for mature leukocytes. This was investigated in injured and cytokine-stimulated cremaster muscle intravitally. The cremaster muscle underwent ischemia-reperfusion (IR) injury or was stimulated with IL-1β (4 hrs; 12.5ng/200μl) in anaesthetised (ketamine/xylazime) C57BL/6 mice. CFSE-labelled HSCs ($1x10^6$ HPC-7 cells; ia) were administered 30, 60, 90, 120 mins post-reperfusion or post-IL-1β. Some HPC-7s were pre-treated with a function-blocking CD18 mAb (GAME-18). HPC-7 rolling and static adhesion was significantly increased in post-capillary venules (PCVs) in both IR injured ($p < 0.05$ at 60 and 90 mins) and IL-1β stimulated ($p < 0.05$ at all time points) animals compared to appropriate controls. Significant ($p < 0.05$) capillary recruitment was also observed with IL-1β. The CD18 mAb significantly inhibited both IR injury and IL-1β induced PCV adhesion and IL-1β induced capillary adhesion. Rolling was not prevented by CD18 mAb in any animals. FACS demonstrated that HPC-7 surface expression of CD18 was not increased in response to IL-1β incubation. Our novel results illustrate that HSCs can indeed roll and firmly adhere to injured and cytokine-stimulated tissue in a manner similar to mature leukocytes. Furthermore, this phenomenon also occurs predominantly in PCVs and shares similar adhesion molecules to mature leukocytes.

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PC72

FACTORS RELEASED FROM LUNG TUMOUR CELLS ALTER ADHESION MOLECULE EXPRESSION IN HUMAN BRAIN ENDOTHELIAL CELLS

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Background: Lung tumours frequently metastasise to the brain. During cerebral metastasis formation the tumour cells must adhere to, and migrate through, the endothelial cell (EC) layer that lines

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the cerebral blood vessels. It is possible that adhesion molecules on the surface of both the endothelial cells and the metastatic cells may mediate this interaction. However, it is not known if lung tumour cells influence endothelial adhesion molecule expression.

Purpose: To examine whether factors released from lung tumour cells alter adhesion molecule expression in cerebral brain ECs. **Methods:** Two different lung tumour cell lines A549 (adenocarcinoma) and SK-MES (squamous cell carcinoma) were cultured in a defined basal medium (DMEM-BS) and the factors released by the tumour cells were collected (conditioned medium (CM)). Human brain microvascular endothelial cells (HCMEC-D3) were seeded on 96 well plates, treated with CM or DMEM-BS for 4hrs and 24hrs and adhesion molecule expression (ICAM-1, VCAM-1, and E-selectin) analysed by ELISA. **Results:** Compared to control levels (100%) SK-MES CM significantly increased ICAM-1 expression after 24hrs to $122.5 \pm 6.8\%$ ($p = 0.037$, $n = 3$) and VCAM-1 expression after 4hrs and 24hrs to $169.99 \pm 91.35\%$ and $142.11 \pm 44.04\%$, respectively ($p = 0.037$, $n = 3$). E-selectin expression was increased after 4hrs activation with A549 and SK-MES CM to $120.99 \pm 11.26\%$ and $117.25 \pm 10.74\%$, respectively ($p = 0.002$, $n = 6$). **Conclusions:** Proteins/factors released from the two different lung tumour cell lines alter adhesion molecule expression on cerebral ECs.

PC73

HUMAN BRAIN ENDOTHELIAL CELLS EXPRESS HIGH LEVELS OF GLUT1 AND SUPPORT INFECTION BY HTLV-1

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Human T-cell leukemia virus type I (HTLV-1) is associated with a variety of clinical disorders, among them tropical spastic paraparesis or HTLV-1-associated myelopathy (TSP/HAM). Viral detection in the CNS of TSP/HAM patients demonstrates the ability of HTLV-1 to cross the blood-brain barrier and infect CNS resident cells. Here we report data showing that the HTLV entry receptor, the glucose transporter GLUT1, is expressed both *in vitro* and *in vivo* by human brain endothelial cells (BEC) and astrocytes. GLUT1 expression was detected in white and grey matter capillaries and in grey matter glial cells of the spinal cord in control individuals as well as in a HAM/TSP patient. Expression of Glut-1 was increased in the neuropil surrounding lymphocyte infiltrates in the white matter of the thoracic spinal cord of a TSP/HAM patient. We investigated whether a human brain capillary endothelial cell line, hCMEC/D3, supported HTLV-1 infection *in vitro* when exposed to human lymphocytes chronically infected by HTLV-1 (HUT102). Our study demonstrates that cell-cell fusion occurs between HTLV-1-infected lymphocytes and human BEC, the latter being susceptible to transient HTLV-1 infection. Indeed, it was estimated that up to 10% of human BEC expressed the viral structural core protein p24 following 72 h incubation with HUT102 lymphocytic cells. Transmission electron microscopy combined with immunogold techniques confirmed expression of p24 by human BEC. We also used LacZ retroviral vectors pseudotyped with HTLV-2 envelope to demonstrate infection of human BEC by HTLV. In these assays, HTLV infection was completely reversed by pre-incubation with serum from a seropositive patient but not with that of a control patient. These aspects may aid in the understanding of the pathogenic mechanisms associated with neurological diseases induced by HTLV-1 infection.

PC74

TARGETING ADENOVIRAL GENE DELIVERY TO TNF-α-ACTIVATED ENDOTHELIAL CELLS *IN VITRO* WITH A MOUSE ANTI-HUMAN E-SELECTIN ANTIBODY

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Targeted adenoviral gene delivery using physiological ligands including peptides, growth factors, and monoclonal antibodies is a promising strategy for achieving efficient, safe, and selective transduction of disease target tissues. Gene therapy of metastatic cancer requires systemic intravenous delivery and tumour-associated endothelium represents an attractive cellular target because of its ease of access from the bloodstream. We have investigated the possibility of targeting adenovirus type 5 expressing

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luciferase (Ad-Luc) to angiogenic-activated endothelial cells *in vitro* using human umbilical vein endothelial cells (HUVEC). Ad-Luc was first coated with an amino-reactive multivalent hydrophilic polymer based on poly[N-(2-hydroxypropyl)methacrylamide] [pHPMA-gly-gly-TT] to detarget natural virus tropism. The polymer-coated virus was then retargeted with a mouse anti-human E-selectin mAb (MES), purified from the H18/7 hybridoma cell line, to transduce TNF-α-activated HUVEC cells expressing E-selectin. The MES-retargeted virus showed 35-times higher transfection efficiency in TNF-α-activated HUVEC relative to non-retargeted polymer-coated virus, and infected E-selectin positive (TNF-α-activated) HUVEC 18-fold more efficiently than non-activated HUVEC cells. Receptor specificity was also shown by competition with free MES antibody resulting in a 4.2-fold reduction, with no effect on the activity of the unmodified virus. Furthermore, increasing TNF-α concentrations was found to correlate with E-selectin expression levels as well as gene expression levels from MES-retargeted Ad-Luc virus. Future work will be to explore the usefulness of this retargeting system in tumour-associated endothelium *in vivo*, using a rat anti-mouse E-selectin antibody.

PC75

ENDOTHELIAL CELL POLARITY IN CAPILLARIES

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Cell polarity is important in a diverse range of events including cell migration, differentiation, and morphogenesis. The process of angiogenesis is complex, involving migration of endothelial cells and vast changes in cell shape. Thus, it seems likely that cell polarity may play an integral role in the formation of new capillaries — a hypothesis which is supported by data showing that genes for establishing and maintaining polarity are upregulated in endothelial cells in an *in vitro* model of tubulogenesis [1]. In addition, we have found evidence of stable microtubules in endothelial cells in the *in vitro* model of angiogenesis we use, where human umbilical vein endothelial cells are co-cultured with human fibroblasts, resulting in the spontaneous formation of capillary-like structures. Microtubules are thought to be involved in establishing and maintaining polarity. We wish to use immunofluorescence microscopy to observe the locations of structures which are often reorientated during cell polarisation, such as the microtubule-organising centre and the Golgi. In addition, we will use siRNA knock-downs to identify key signalling pathways which drive polarity and are essential for *in vitro* angiogenesis. The Rho GTPase cdc42 has been shown to be important in establishing polarity, and thus we intend to study proteins downstream of this signalling protein. [1] Glesne et al. *Cancer Res* 2006; 66: 4030–4040.

PC76

INFLUENCE OF LUMINAL PRESSURE ON Ca^{2+} PUFFS AND WAVES IN RAT CREMASTER ARTERY ENDOTHELIAL CELLS

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Rat cremaster arteries develop spontaneous myogenic tone when exposed to luminal pressures above 30 mmHg [1]. This tone is associated with depolarization of smooth muscle cells [1] and increases in smooth muscle cell $[Ca^{2+}]_i$ [2], and is sensitive to inhibitors of voltage-gated Ca^{2+} channels [1]. This study established the effect of luminal pressure on endothelial cell $[Ca^{2+}]_i$. Arteries (1A) were isolated from rat cremaster muscle, and Oregon Green 488 BAPTA-1 selectively loaded into endothelial cells [3]. Spontaneous Ca^{2+} events were observed at 5 mmHg (2.2 ± 0.4 /cell/min, n = 6) and were reduced at 80 mmHg (0.9 ± 0.2 /cell/min, n = 6). These events were abolished by an inhibitor of phospholipase C (U-73122, 3 μM, n = 3), supporting a role for inositol trisphosphate. The activator of ATP-sensitive K+-channels, levcromakalim (3 μM) increased Ca^{2+} events at 5 mmHg (3.6 ± 1.1 /cell/min, n = 5) and 80 mmHg (2.5 ± 0.4 /cell/min, n = 4). Addition of 45 mM KCl also increased the frequency of Ca^{2+} events (5 mmHg: 6.4 ± 1.5 /cell/min, n = 4; 80 mmHg: 10.8 ± 0.6 /cell/min, n = 3). Overall these data show that spontaneous Ca^{2+} puffs and waves in endothelial cells are inhibited by rises in intraluminal pressure by a process not simply dependent

on smooth muscle cell membrane potential or Ca^{2+}.

[1] Kotecha and Hill. *Am J Physiol* 2005; 289:H1326–H1334; [2] Zou et al. *Am J Physiol* 1995; 269:H1590–H1596; [3] Kansui et al. *Cell Calcium* 2008-In Press.

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PC77

HYPERBARIC OXYGEN DOWN REGULATES THE ENDOTHELIAL ADHESION MOLECULES ICAM-1, VCAM-1, AND E-SELECTIN IN AN *IN VITRO* CHRONIC WOUND MODEL

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Hyperbaric oxygen (HBO) therapy involves the intermittent inhalation of 100% oxygen, whilst inside a treatment chamber at a pressure greater than 1 atmosphere absolute (ATA). It is effective in the treatment of chronic wounds, the pathology of which includes excessive neutrophil accumulation that contributes to the persistent inflammation observed. Neutrophil recruitment is regulated by endothelial cell (EC) adhesion molecules including ICAM-1, VCAM-1, and E-selectin. The aim of this study was to examine whether HBO reduces EC adhesion molecule expression. This could aid wound healing by reducing neutrophil accumulation. An *in vitro* chronic wound model was established for human umbilical vein ECs. Cells were treated with 0.5 μg/ml lipopolysaccharide and 1 ng/ml tumour necrosis factor-α at 2% O_2 (i.e.,, hypoxia), which stimulates adhesion molecule expression. After HBO treatment for 90 min at 2.4 ATA (patient treatment protocol) adhesion molecule expression or release was measured by ELISA at 0, 5, and 24 h post-treatment.

At 24 h post-treatment, cells treated with HBO expressed only 53.4 ± 12.8% ($p = 0.038$, $n = 16$) of ICAM-1 and 88.1 ± 29.4% of VCAM-1 ($p = 0.017$, $n = 18$) compared with control cells (100%). HBO-treated cells also released 0.85 ± 0.17 ng/ml E-selectin compared with just 0.66 ± 0.18 ng/ml in control cells ($p = 0.041$, $n = 6$). Protein assays confirmed these results were not due to cell loss. In conclusion, HBO treatment significantly reduces ICAM-1 and VCAM-1 expression and increases E-selectin release in human ECs.

PC78

INTERCONNECTION OF HEMORHEOLOGICAL DISORDERS AND ENDOTHELIAL DYSFUNCTION IN RHEUMATOID ARTHRITIS

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Objective: To discover the role of hyperviscosity in development of endothelial dysfunction (ED) in patients with rheumatoid arthritis (RA). **Methods:** We observed 90 RA patients aged 18 to 59 years. We assessed plasma (PV) and blood viscosity (BV); tissue oxygen supply ratio (OSR); L-arginine, NOx levels; endothelium-dependent (EDVD) and endothelium-independent (EIVD) vasodilatation on brachial artery (BA); and BA shear stress (SS) value. RA inflammatory activity was set by disease activity score (DAS28). **Results:** Hyperviscosity is typical for RA patients and connected with inflammatory activity and L-arginine-NO disbalance: PV and BV correlated to DAS28 ($r = 0{,}48$, $p = 0{,}006$ and $r = 0{,}56$, $p = 0{,}001$, respectively), PV correlated to Rhaynolds phenomenon ($r = 0{,}4$, $p = 0{,}01$); NOx plasma level correlated to BV ($r = 0.47$, $P = 0.03$) and OSR ($r = 0.58$, $P = 0.006$); DAS28 had negative correlation with OSR ($r = -0.54$, $P = 0.003$). We found also correlations of L-arginine and PV with EDVD ($r = 0.43$, $P = 0{,}005$; $r = -0.53$, $P = 0.005$, respectively) and EIVD ($r = 0.32$, $P = 0.04$; $r = -0.42$, $P = 0.03$, respectively). SS correlated to PV ($r = 0.39$, $P = 0.03$), L-arginine ($r = 0.6$, $P = 0.001$) and EDVD ($r = 0.45$, $P = 0.02$), negatively to OSR ($r = -0.42$, $P = 0.02$). **Conclusions:** Hyperviscosity in RA is accompanied by SS increase and L-arginine-NO disbalance that both result in ED development. Thus, hyperviscosity, ED, and L-arginine-NO disbalance in RA patients are all associated with RA activity and interconnected.

PC79

EFFECTS OF OXIDATIVE STRESS AND SERUM FROM PATIENTS WITH SCLERODERMA ON ENDOTHELIAL CELL CYTOKINE GENE EXPRESSION

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Vascular damage is a primary event in the pathogenesis of scleroderma (SSc). It is thought to be triggered by a number of genetic and environmental stimuli including oxidative stress and anti-endothelial cell antibodies (AECA) present in patient sera. In this study we examined the influence of oxidative stress induced by hydrogen peroxide (H_2O_2) and a nitric oxide donor, DETA-NONOate (DETA-NO) *in vitro* and compared this to the effects of serum samples from SSc patients containing a high AECA titre. Human umbilical vein endothelial cells (HUVEC) were treated with H_2O_2 [50 or 100 μM], DETA-NO [1mM], or patient/control serum [20%] for 6 and 24 hours. Transcript levels of a group of pre-selected cytokine genes, known to be dysregulated in patients with SSc, were measured by real time PCR. We observed a different pattern of gene regulation in response to oxidative stress in endothelial cells treated with H_2O_2 and DETA-NO. Members of the interleukin family (IL1β, IL6, and IL8) were up-regulated by H_2O_2, whilst DETA-NO showed no effect on these genes. Monocyte chemoattractant proteins (MCP-1 and MCP-3) were down-regulated by DETA-NO whereas only MCP-1 showed a small reduction with H_2O_2 at 24 hours. Serum from patients with SSc did not result in similiar changes in gene expression observed with either H_2O_2 or DETA-NO but instead caused a down-regulation in all measured genes. In particular, RANTES, which did not change in response to H_2O_2 or DETA-NO, was considerably reduced after treatment with patient sera. Hemoxygenase 1 (HMOX1), an anti-oxidant response gene, was significantly increased in cells exposed to either H_2O_2 or DETA-NO, as expected. In contrast HMOX-1 levels decreased in cells incubated with patient sera. Our results show that changes in endothelial gene expression on exposure to H_2O_2 or NO do not mimic the effects of SSc sera, suggesting that oxidative stress is not a major component of the altered signalling induced by SSc serum.

PC80

SIGNALLING PATHWAYS THAT STIMULATE HYALURONAN SECRETION *IN VIVO*

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Hyaluronan (HA) dominates interstitial matrix permeability, but the regulation of its secretion *in vivo* is little studied. *In vitro* it can be stimulated via a Ca^{2+}-PKCα-pERK pathway [1]. We examined the role of this path in movement-stimulated HA secretion (MSHA) *in vivo*. Endogenous HA was first removed from cannulated knee joints of anaesthetised rabbits. In paired, passively cycled joints one side received drug and the other vehicle. Newly secreted HA was harvested after 5h for analysis. Paired studies were also conducted on static and phorbol ester (PMA) stimulated static joints. Movement almost doubled HA secretion ($p < 0.001$, $n = 20$, paired t test). PLC inhibitor U73122 nearly halved the MSHA ($p < 0.001$, $n = 9$). But neither the PKC inhibitor bisindolylmaleimide (BIM, $n = 16$) nor MEK-ERK inhibitor U0126 ($n = 9$) reduced MSHA significantly, though both inhibited PMA-stimulated secretion. The phosphatase inhibitors calyculin and vanadate increased static secretion by 20–25%. The Ca^{2+} ionophore ionomycin increased it by 106% ($p = 0.02$, $n = 5$). The findings indicate that intracellular Ca^{2+}, PKC, and ERK are each capable of stimulating HA secretion *in vivo*. Nevertheless, MSHA is not dependent on the PKC-ERK pathway, unlike the stretch response *in vitro*. MSHA is mediated partly by PLC activation, however. The results also indicate that constitutive kinase/phosphatase activity regulates HA secretion by static synovium. Further elucidation of the pathways may open avenues for the therapeutic manipulation of HA secretion. [1] Momberger et al. *Matrix Biol* 2006; 25:306.

PC81

RESPONSES TO SHEAR STRESS AND TO CYTOKINES BY ENDOTHELIAL CELLS FROM DIFFERENT VESSELS

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The local stromal and haemodynamic microenvironment is believed to determine the phenotype of endothelial cells (EC). We aim to understand how these factors regulate leukocyte recruitment in response to cytokines such as tumour necrosis factor α (TNF) and interleukin-β (IL-1). Early-passage EC from umbilical veins (HUVEC) or arteries (HUAEC), or from coronary arteries (HCAEC; Clonetics, San Diego, CA, USA), were compared. Initial studies using various culture media showed

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that TNF-treated HUVEC and HUAEC recruited flowing neutrophils in a dose-dependent manner, with responses that depended on the medium, and tended to be slightly greater for HUVEC. In medium recommended for HCAEC, HUVEC and HCAEC showed nearly identical response to TNF. All cells responded robustly to IL-1 in all media. Responses of the different EC to shear-conditioning were tested initially in their own "optimal" media. After exposure to shear stress (2.0Pa for 24h), response of HUVEC to TNF (but not IL-1) was much reduced, judged by neutrophil recruitment. However, HUAEC and HCAEC showed impaired responses to both cytokines after conditioning. When HUVEC were sheared in "HUAEC medium," response to IL-1 was down-regulated, but this was not the case in "HCAEC medium." Human fibroblast growth factor (FGFb) was not present in HUVEC medium but was present in others. After its removal from HUAEC medium, the HUVEC response to IL-1 was again insensitive to shear. These studies show that enviromental factors, such as shear stress and growth factors, can greatly modify responses in an inter-linked manner. In addition, behaviour of cultured EC from different sites may depend as much, or more, on the culture conditions as on their origins.

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