Report: SMR Angiogenesis Symposium

By Irene Francois

Angiogenesis is a complex process by which new blood vessels are formed from pre-existing capillaries. In normal physiology this process is under stringent control and occurs only during embryonic development, endometrial regulation and wound repair. However, many pathological conditions such as ischaemic heart disease, atherosclerosis, psoriasis, rheumatoid arthritis, solid tumour growth, diabetic retinopathy and age-related macular degeneration appear to be driven by persistent upregulation of the angiogenic process. It is universally accepted that angiosuppressive agents will have numerous clinical applications and a number of drug candidates are now in development.

On 9th July 1999, the Society held a one-day meeting on Angiogenesis at the National Heart and Lung Institute, London. The speakers covered many aspects of this fundamental process, both in normal development and in a variety of angiogenic dependent diseases.

The meeting opened with an excellent overview by **Professor Helmut Augustin** (University of Gottingen, Germany). He began by defining the three processes involved in vascular morphogenesis. *Angiogenesis* is the sprouting of new capillaries from pre-existing ones; *vasculogenesis* is the *in situ* differentiation of a primary capillary plexus from haemangioblastic stem cells; *intusseption* is the branching of a capillary by longitudinal splitting. The only organ site with physiological angiogenesis, in the adult, is the female reproductive system. During ovulation there is dramatic growth of the *corpus luteum* due to an increase in angiogenesis. At the end of the cycle there is a regression in sprouting and the growth of blood vessels. Professor Augustin's research team have compared the cyclic angiogenic processes in the ovarian vasculature that regulate the ''life-cycle'' of the transient *corpus luteum* with the pathological angiogenesis that is associated with tumour growth.

Angiogenesis is under multiple control and there are now between 10 and 20 different factors, and several genetic mechanisms, known to elicit neovascularisation. Hypoxia and acidosis in the microenvironment of a tumour stimulate specific angiogenic growth factors such as VEGFs-A,B,C,D and E and PIGF. Local angiogenic stimulators of pleiotropic nature include bFGF, aFGF, TGFα, TGFβ, and PD-ECGF. Autocrine and

paracrine processes in angiogenesis are associated with endothelial cell activity involving cytokines, adhesion molcules and chemokines. Secondary enhancement leads to recruitment of monocytes and leukocytes whilst the capillary organisation involves angiopoietins.

There are positive and negative regulators of angiogenesis. Positive regulators include VEGF, PIGF, TGFs, angiogenin, IL-8, HGF, GCSF and PDGF. The endogenous negative regulators of angiogenesis that have been identified include thrombospondin, angiostatin and glioma-derived angiogenesis inhibitor factor. The angiopoietins (Ang 1,2,3,4) are antagonistic and control the maturation of new vessels. The Ang2/Ang1 ratio is important. The ratio is 1 in the the resting and new growth state of the c*orpus rubrum* and c*orpus luteum* but Ang2 is upregulated during vessel regression

Tumours with greatest vasculature are generally associated with poorer prognosis and increased metastasis and a prognostic marker is definitely needed. Vessel density counts reflect the vascular status of a tissue but this is not the best measurement for angiogenic status. Measuring panendothelial cell surrogate markers, such as von Willebrand factor (VWF), CD31, CD34, are better; however these are cell markers and do not measure angiogenesis *per se.* Several techniques, including dual labelling of proliferating endothelial cells have been developed to identify specific markers for angiogenesis. The *proliferating capillary index* is used to compare tumour angiogenesis with physiological angiogenesis in the ovary. Generally, tumour angiogenesis is much less than that observed in the ovary with some tumours being more angiogenic than others: glioblastoma>renal>colon mammary>lung>prostate. Therefore, some patients are more likely to benefit from anti-angiogenic intervention than others.

The faster the growth of the tumour, i.e. the more angiogenesis, the less mature is the vessel bed. This is an important point because it is not just the number of vessels, but their maturity that has an impact on their efficiency and capabilities. One can measure the *microvessel maturation index* which is different in normal physiology than in a tumour, where the vasculature is very immature.

Another marker, monocyte chemoattractant protein-1 (MCP-1) is regulated by inflammatory cytokines. It is a more potent inducer of

angiogenesis than either VEGF or bFGF, for example, in corneal angiogenesis, induction of angiogenesis by MCP-1 is concomitant with a massive inflammatory response and macrophage recruitment. With VEGF an increase in angiogenesis occurred but there was an absence of macrophage recruitment. There are quite distinct qualitative differences in the contribution of an inflammatory response to the induction of angiogenesis, which differentiates physiological and pathological angiogenesis.

Rheumatoid arthritis affects ~2% of western populations, and osteoarthritis of one or more joints is almost inevitable by the age of 70. Current treatments for these conditions are palliative and not cures. Since angiogenesis is important in the underlying causes of arthritis (inflammation, joint damage), tackling angiogenesis in arthritis offers a potentially better treatment for the disease. These underlying causes, reasons for its persistence, severity of inflammation and joint damage were all topics covered by the next speaker **Dr David Walsh** (City Hospital, Nottingham). Neovascularisation of inflammatory synovium is a hallmark of the disease. Whilst cartilage is avascular, the synovium has one of the densest vasculatures in the body. Many stimulators of angiogenesis are expressed in synovial tissue e.g. VEGF and integrin $\alpha_v\beta_3$, but it is not clear which are the most important inhibitors. Foci with upregulation of angiogenic factors were also those with proliferating endothelial cells. There is no real increase in the total vasculature therefore proliferation must be balanced by an increase in cell death (*via* apoptosis) and redistribution of vessels.

Rheumatoid synovium has focal hypoxia and a defective vasoregulatory system i.e. immature vessels are not able to be regulated efficiently and consequently do not oxygenate/remove metabolites effectively. There is also a focal absence of nerves. New vessels are laid down inappropriately, and the pannus begins to invade the cartilage and bone. Innervation occurs very slowly. In turn, hypertrophic chondrocytes and osteoblasts generate angiogenic factors which stimulate ossification.

A carageenan synovitis model of angiogenesis with an endpoint of joint swellings and macrophage infiltration, showed that there was a peak in cell proliferation occurring well before vascular density changes. The model also showed that both endothelial cell proliferation and cell death are biphasic, persistant after 28 days and concomitant with an increase in macrophage infiltration. Angiogenesis on day 3 appears to predict persistent

macrophage infiltration at day 28. Consequently, it may be possible to knockout early stage synovial angiogenesis, thus preventing persistence. Integrin $\alpha_v\beta_3$ antagonists have been shown to inhibit antigen/bFGF arthritis (Storgard et al., *J. Clin. Invest.*, 1999, 103, 47-54). This and other studies confirm that pharmacological angiogenesis inhibition is a viable therapeutic strategy for rheumatoid arthritis.

Dr Margaret Rees (John Radcliffe Hospital, Oxford) described the key factors associated endometrial angiogenesis. One in five menopausal women, by the age of 55, undergo hysterectomy for excess bleeding, which results in 50,000 hysterectomies being performed each year. Menstrual bleeding is a phenomenon restricted to humans and sub-human primates which have coiled blood vessels or spiral arterioles in their endometrium. These undergo growth or angiogenesis throughout the menstrual cycle and are under the control of endogenous and exogenous steroids; oestradiol and progesterone.

Many angiogenic factors are present in the endometrium. These include: EGF/EGFR, TGFα, aFGF, bFGF, VEGF-A, pleiotrophin, midkine, thymidine phosphorylase and adrenomedullin. Inhibitors include: TGF-βs, IL-1, TNF-α, PL-4 and thrombospondin. The angiopoietin/Tie system has recently been identified in mouse uterus. A number of endometrium models have been developed by culturing epithelium from this tissue (hysterectomy specimens). Also, endometrial stromal cells have been used. The addition of endothelial growth supplement to the culture medium is essential for active growth. A range of characteristics were used to show the epithelial cells and to determine the oestrogen/progesterone receptors. Most carcinoma cell lines do not contain these receptors. In sections of stromal and epithelial cells there are differences in steroid levels. Isolated human decidual endothelium from first trimester decidual tissue was used to study the effect of angiogenic markers. It was found that the cells were uniquely responsive to VEGF and that VEGF-A was upregulated by steroids e.g. oestradiol. It is noteworthy that only M293 monoclonal antibody provided specific staining for characterisation. Strong induction of the angiogenic enzyme thymidine phosphorylase was observed using a combination of IFNγ and TNFα. There was no effect on expression using oestrogen or progesterone but the latter in combination with TGFβ caused an increase in enzyme levels. Adrenomodullin (52 amino acid peptide, first isolated from human phaeochromocytoma) is a novel growth factor for endothelial cells

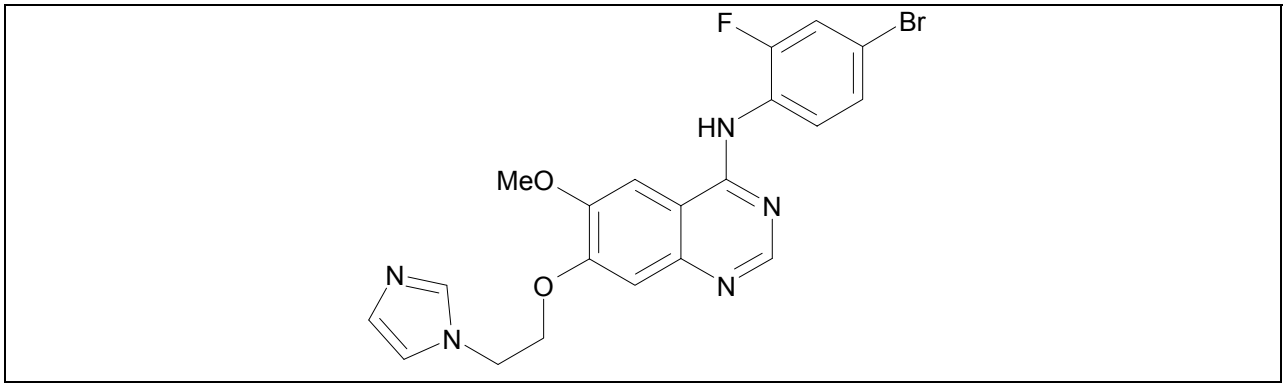
which shows potent angiogenic activity *in vivo* in the chick chorioallantoic membrane assay. These studies give credence to the belief that the three major players in endometrial angiogenesis are VEGF, thymidine phosphorylase and adrenomedullin.

The role of CXC chemokines in the regulation of angiogenesis in several different animal models was presented by **Dr Douglas Arenberg** (University of Michigan, US). Net neovascularisation is determined by the balance of angiogenic and angiostatic stimuli. CXC chemokines are a unique class of cytokines which are capable of either increasing or decreasing angiogenesis. Those containing an ELR motif (ELR+) immediately preceding the CXC motif, e.g. IL-8, Gro $\alpha/\beta/\gamma$, ENA-78, are capable of stimulating angiogenesis. Those which do not contain the ELR motif (ELR-), e.g. IP10, MIG, PF4, are angiostatic. PF4 inhibits angiogenesis induced by ELR+ chemokines. Tumour progression is associated with over expression of ELR-CXC and under expression of non-ELR-CXC; in tumour regression this scenario is reversed.

In non small cell lung cancer, IL-8 and ENA-78 were increased compared to normal. In initial experiments to characterise tumourogenesis using SCID mice injected with A549 adenocarcinoma cells, it was observed that the rate of tumour growth was directly proportional to IL-8 levels. Anti-IL-8 antibody decreased tumour growth. The rate of proliferation was not altered by IL-8 or ENA-78 antibody but induced an increase in apoptosis. Similar results were obtained with a squamous cell carcinoma which is slower growing. In this carcinoma, higher levels of an endogenous inhibitor of angiogenesis, IP10, were found compared to A549. If the A549 adenocarcinoma was treated with IP-10, the tumour growth slowed down and the number of metastases were reduced.

In a model system of idiopathic pulmonary fibrosis (IPF), which causes respiratory obstruction with 50% mortality, the lungs were 'primed' for angiogenesis. Removal of IL-8 decreased angiogenesis. In contrast, a decrease in IP-10 increased angiogenesis. Mutagenesis studies revealed that the arginine of the ELR motif in CXC was most important for receptor binding and that the whole chemokine molecule is required for activity. The ELR peptide is not sufficient on its own to have an effect (compare RGD mimetics).

Dr David Ogilvie (Cancer and Infection Research, AstraZeneca) focused on the inhibition of VEGF signal transduction in solid tumour disease, particularly in non-hormone dependent cancers. There is strong evidence that VEGF contributes to tumour growth through the promotion of both angiogenesis and vascular permeability (*Cancer and Metastases Review,*1993, 12, 303). Its sesquestration has been shown to reduce tumour growth in animal models (Kim et al., *Nature,* 1993, 362, 841). A VEGF antibody is in Phase 2 clinical trials. AstraZeneca scientists have designed a number of inhibitors of VEGF receptor associated tyrosine kinase (RTK) Flt. The compounds are competitive with respect to ATP. These compounds, which arose from a EGFR programme, have a different profile of inhibition on the two receptors showing selectivity is possible. Moreover these differences translated into different *in vivo* profiles in xenograft tumour models, and at doses unlikely to effect tumour cells directly.

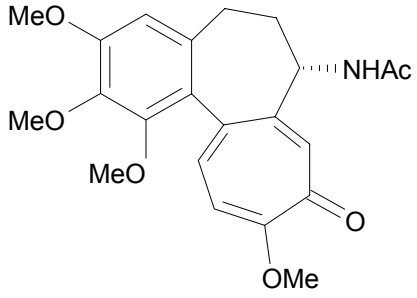
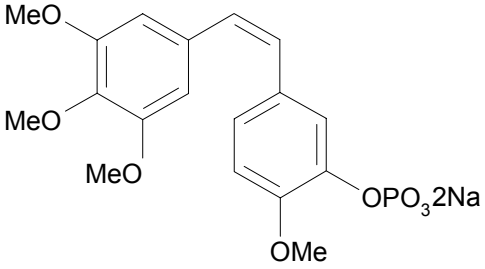


ZD4190

In an acute rat hypotension model, ZD4190 significantly inhibited hypotension induced by VEGF, but not that induced by bFGF or acetylcholine responses. When tested in models using established tumours the compound was still able to suppress tumour growth, e.g. it sustained inhibition of PC-3 tumour growth over 20 days. ZD4190 was well tolerated on repeat dosing; cessation of treatment resulted in renewed tumour growth. Using dynamic contrast medium-enhanced MRI, acute therapeutic doses of ZD4190 have also been shown to reduce vascular permeability in tumour xenographs. In summary, ZD4190, an orally active inhibitor of VEGF signal transduction, displays a non-cytotoxic, pan-carcinoma anti-tumour profile, and activities consistent with the proposed mode of action.

Next, **Dr David Chaplin** (Rhone-Poulenc Rorer, Vitry, France) discussed the potential for agents that can alter the function of already formed vasculature, on the premise that, if the tumour vasculature was disrupted, the tumour would regress due to hypoxia and increased metabolites. There are several advantages to this approach: damage to a single vessel can kill thousands of tumour cells; it is not necessary to kill endothelial cells; endothelium is adjacent to the blood stream; endothelial cells are non-malignant so mutants are unlikely to emerge; in close proximity to tumour cells; low intravascular oxygen tensions; hypoxia is a common feature in cells and in the vasculature.

There have been a number of antibody based and gene therapy approaches to vascular targetting. Drug based approaches have included flavanoids, xanthenones, serotonin agonists, and tubulin binding agents, e.g. colchicine, which cause haemorrhagic necrosis in tumours. These compounds work in all tumours but only at their maximum tolerated dose. Using an *in vivo* model as a primary screen, a novel class of tubulin inhibitors has been identified, called combretastatins, which come from the bark of the African Bush Willow.

	
Colchicine	CA4P

One of the most effective vascular targetting agents is combretastatin CA4P, a soluble form of combretastatin CA4, which inhibits tumour growth at $1/10^{th}$ of its maximum tolerated dose. It was found to be most effective on immature vessels and differs from other agents such as taxotere, a tubulin stabilising agent, which has no effect on blood flow and vasculature at the maximum tolerated dose. CA4P induces an increase in blood flow resistance going into the tumour, but not into the rest of the vasculature. The effect was immediate and observed within 20 minutes. The hypothesis is that new blood vessel cells are more sensitive to the tubulin modifying agent due to their more 'plastic' nature. These effects are ascribed to the compounds ability to alter endothelial cell shape causing new cells, at the

junction between the old and the new vessels, to occlude blood flow into new vessels/tumour. The inside of the tumour quickly dies due to apoptosis. However, the outside of the tumour, supplied by the mature vessels, remains visible and quickly grows again. This therapy must therefore be combined with other treatments such as radiology which kills tumour cells in the tumour periphery. In xenograph models this has resulted in 50% of the animals being 'cured' in a multiple dosing regimen. These studies confirm the potential of CA4P as a neovasculature targetting agent and have highlighted that endothelial cell shape changes are a key element in its mode of action.

Dr Wen Jiang (University of Wales College of Medicine, Cardiff) discussed the regulation of tumour and angiogenic factor induced angiogenesis and its implication in wound healing. In cancer, tumour and stromal cells release a range of angiogenic factors which stimulate the proliferation, migration and morphogenesis of endothelial cells. A factor that is critically involved is hepatocyte growth factor/scatter factor (HGF/SF) produced by stromal fibroblasts. The human HGF/SF gene spans 70kb, is composed of 18 exons giving an mRNA of 6kb. The protein has 4 unit kringle domains (K1-K4) with a N-terminal hairpin (N), and is produced as a pre-protein which is processed into a α and β subunits held together by a disulphide bridge. It is mediated by the C-Met receptor protein. The α subunit (N-K1) is responsible for receptor binding. HGF/SF is found to increase motility, integrin expression and invasion in tumour cells. In endothelial cells, HGF/SF increased motility and CD44 expression, and decreased the gap juctions.

HGF/SF was produced in a soluble form, NK4, and used as an antagonist of HGF/SF in an *in vitro* tube forming assay. NK4 was shown to reduce cell motility and hence tube length. The fatty acid gamma linolenic acid (GLA) can directly reduce the level of vascular endothelial (VE) cadherin on VE cells, through which tube formatyion is suppressed. Fibroblasts, which are a good supplier of HGF/SF and matrix embedded fibroblasts, have been shown to enhance the tubule formation of the endothelial cells and new vessel formation from tissues of acute and chronic wounds. It is also possible that angiogenic factors, such as HGF/SF, are matrix-bound and are released upon stimulation in a wound environment. This indicates a possible role in the intervention of wound healing.

Proffesor Eva Kohner (St. Thomas's Hospital, London) discussed the pathogenesis, treatment and possible prevention of new vessel formation in the diabetic retina. The two most critical factors in diabetic retinopathy are maculopathy (leakage) and new vessel formation, which are the result of ischaemia/hypoxia, and indirectly, high glucose. Abnormalities in blood components are seen, such as reduced red cell deformability, increased platelet aggregation, decreased white cell deformability, and increased white cell adhesion.

Recent studies suggest that upregulation of cor-2 enzyme by high glucose in the larger white cells, alters glycoproteins in the cell membrane causing increased adherence to the vessel wall *via* I-CAM and selectin. This results in capillary occlusion. Hyperglycaemia also causes dilation of the capillaries, resulting in increased blood flow that causes sheer-stress and damage to the endothelium and to the pericytes, which control endothelial function and proliferation. An increase in the number of pericytes is the earliest abnormality observed in diabetes. They are damaged primarily by non-enzymatic glycation and extra- and intracellular advanced glycation end-products (AGE) formation. Ischaemia results in the liberation of a host of vasoproliferative factors, the most important being VEGF. Protein kinase-C (PKC) β_2 enzyme is necessary for increased VEGF expression and is activated by hypoxia and ischaemia. New vessels tend to grow into the posterior of the vitreous which contracts, pulling on the retinal vessels resulting in haemorrhage and retinal detachment, ultimately leading to visual loss.

The only treatments are: (a) photocoagulation (laser) which destroys the ischaemic retina and allows the accumulation of angiostatic substances. New blood vessels close, shrink and disappear; and (b) the control of glucose levels to minimise glucose toxicity and the control of high blood pressure.

A novel PKC β_2 inhibitor is under clinical trial at present. The role of growth hormone (GH) in ischaemia-associated retinal vascularisation has been studied suggesting that GH receptor antagonists may also have therapeutic potential. It is hoped that these new therapies will prevent severe visual loss in the future.