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Endothelium, Blood Vessels and Angiogenesis – A Workshop Report

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INTRODUCTION

The aim of this workshop was to discuss in the broadest terms the role of blood vessels in establishing and maintaining a healthy pregnancy. It focused on the vessels at the feto/maternal interface namely the vessels of chorionic villi and the maternal spiral arteries. Cells of these vessels interact closely with trophoblast cells but with very different outcomes. In the chorionic villi this interaction must work to promote and maintain vessel integrity whilst the interactions between the extravillous trophoblasts and the cells of the maternal spiral artery lead in early pregnancy to vessel instability and remodelling and later vessel stabilisation. Knowledge of the mechanisms behind these paradoxical events may allow new therapeutic approaches to pregnancy complications such as placental insufficiency. The specific questions addressed by organisers and participants included the nature of the interaction between the developing chorionic vascular tree and the chorionic villous trophoblast stem cells, the nature of the interaction between extravillous trophoblasts and the cells of the maternal spiral artery specifically whether apoptosis plays a role in the remodelling of maternal spiral arteries. The role of traditional growth factors such as vascular endothelial growth factor (VEGF) in regulation of maternal and fetal vascular growth and vascular tone was also addressed.

HETEROGENEITY OF ENDOTHELIAL GENE EXPRESSION

The heterogeneity of fetal endothelial cells per se was addressed by Bill Kalionis (Australia) in his talk entitled 'Homeobox gene expression in placental microvascular endothelial cells'. Using a variation of the perfusion-based method described by Lang et al. [1] placental endothelial cells (PLEC) were isolated by perfusion of the fetal placental vasculature with proteolytic enzymes and enrichment of the cells by Percoll gradient centrifugation. Yields of $5x10^4$ to $6x10^5$ cells per cotyledon, with 93-98% purity and 66.3-100% viability, were obtained by this technique. Using RT-PCR analysis of uncultured PLEC, the homeobox genes DLX3, DLX4, MSX2, GAX and HLX1 were found to be expressed in microvascular endothelial cells. Furthermore, one of these homeobox genes, HLX1, was not expressed at high levels in human umbilical vein (HUVEC) cells indicating that the macrovascular HUVEC and microvascular PLEC display significant gene expression differences. Microarray analysis on enriched placental endothelial cells also detected expression of the above homeobox genes but revealed six additional homeobox genes [2] whose expression in PLEC were confirmed by RT-PCR. One of these homeobox genes, HEX, is an important regulator of endothelial cells in the cardiovascular system but had not been previously identified in the placenta. The remaining five homeobox genes, again had not previously been identified in the placenta, or in endothelial cells, but were shown to be expressed in embryonic cell types and tissues. Kalionis concluded that it was important to study gene expression on enriched cell populations, rather than whole placental tissue, since this approach has broadened the repertoire of potentially important genes in the placental vasculature.

VILLOUS TROPHOBLAST - ENDOTHELIAL INTERACTIONS

Josette Badet (France) addressed the important issue of vasculogenesis within the chorionic villi and whether adjacent cells may influence this. During human placental development, the chorionic villi grow in coordination with the establishment of a large capillary network that occurs by means of both vasculogenesis from *in situ* differentiating endothelial cells and angiogenesis by sprouting capillaries from pre-existing vessels [3]. Endothelial cells differentiate into blood vessels as a result of signals from adjacent cells. In early placenta, vasculogenesis might be controlled by villous cytotrophoblast [4]. However, in different models, endothelial cells have been

shown to provide developmental signals to the growing organ throughout development [5] and might induce essential steps in organ formation, prior to blood vessel function [6]. Badet et al. showed that there was immunoreactivity to angiogenin, a potent inducer of blood vessel formation, in key points in the developing early chorionic villi. It has been previously shown by her group to be expressed in the human term placenta [7]. At this workshop, angiogenin was shown to be associated with actively dividing endothelial and trophoblast cells which might reflect cordinated morphological development. Cells and the cellular extensions that bridge the cytotrophoblast layer to the nascent vessels expressed endothelial markers and were strongly labelled for angiogenin as well as the trophoblasts facing them. Given the biological activities of angiogenin *in vitro* and the induction of expression in response to hypoxia recently shown by Rajashekhar and collaborators in placental tissue culture [8], these data suggest that angiogenin might play a role in placental organogenesis in concert with other regulators, and opens the discussion on the possibility for endothelial cells to driving or signaling placental growth and morphogenesis.

REGULATION OF MATERNAL AND FETAL VASCULATURE BY PLACENTAL VEGF

Vascular endothelial growth factor (VEGF) is a well known angiogenic growth factor and permeability factor. Paul Brownbill (UK) in his talk entitled ‘Placental secretion of VEGF into the fetal land maternal circulations’ introduced the novel concept of the placenta as a major regulatory organ of maternal VEGF endocrinology, as well as being involved as a vasodilator of fetal vessels [9] and in fetal angiogenesis [10, 11]. Data were presented on the measurement of (i) the summated concentrations of total VEGF 121 & 165 isoforms (tVEGF, RIA), (ii) the summated concentrations of all free isoforms of VEGF-A (fVEGF, sandwich ELISA) and (iii) the total concentrations of the soluble VEGFR-1 receptor (sVEGFR-1, sandwich ELISA), in fetal- and maternal-side venous perfusate samples from term healthy *in vitro*, open-circuit, superoxic-perfused placental lobules, and also in cord sera. tVEGF was secreted into both the fetal and maternal circulations during perfusion, with levels in both circulations plateauing after 60 min. When all measured ligands were considered within all groups of perfusate and serum samples, there was found to be a common inverse relationship between

sVEGFR-1:tVEGF ratio and fVEGF levels. This relationship is in agreement with other studies on maternal serum [12,13].

Measurements of tVEGF in the fetal cord arterial and venous sera were around 2.5 times that measured in the perfused placental lobule. (The lower fetal-side release *in vitro* might be attributable to the superoxic nature of the model, as currently employed). The release of all ligands into the fetal circulation was low, but tVEGF release exceeded sVEGFR-1 release, so that there were measurable, but very low levels of fVEGF in fetal venous perfusate. However, arterial cord sera levels of fVEGF were much higher than this, suggesting that the placenta has a relatively unimportant fetal endocrine role with regards to the determination of fVEGF sera levels. An fVEGF venous serum concentration of around 39 pM, based on the m/w of the VEGF-165 isoform, closely approximates to the EC_{50} of 45 pM found for the vasodilatory response of fetoplacental vasculature found in the *in vitro* perfused term healthy lobule [12]. VEGF thus is a physiologically significant regulator of fetoplacental tone *in vivo*.

Interestingly, in these experiments, very high level of sVEGFR-1 was detected in the maternal-side venous perfusate, which exceeded that of high tVEGF levels, causing complete sequestration of VEGF, leading Brownbill et al. to conclude that the placenta may be a major regulatory organ of maternal VEGF endocrinology. Immeasurable levels of fVEGF in the maternal venous perfusate contrasts with the small but significant level of fVEGF in the peripheral maternal circulation [12]. This imbalance must be addressed *in vivo* by further net secretions of VEGF from other maternal compartments to permit bio-activity of VEGF within the maternal peripheral circulation and to assist in the appropriate regulation of systemic maternal vascular function.

TROPHOBLAST – MATERNAL VESSEL INTERACTIONS

The second part of the workshop was dedicated to talks on the invading fetal trophoblast cells with cells of the maternal vessels. Rosemary Keogh (UK) and Lynda Harris (UK) addressed the issues of ‘Vascular remodelling in pregnancy’. Keogh, gave an elegant summary of the main features of this interaction. Vascular remodelling of resistance arteries typically involves medial thickening through hyperplasia of smooth muscle cells (SMC) and deposition of extra-cellular matrix.

The result is a reduction in lumen diameter and increased resistance. In human pregnancy the opposite occurs when the uterine spiral arteries are remodelled. There is loss of both endothelium and SMC resulting in medial ablation, luminal expansion and a decrease in resistance. The factors that influence vessel remodelling can be classified as cellular, micro-environmental or genetic. In pregnancy, the cellular effect, namely the presence of the trophoblast, is the most important factor that determines the outcome of remodelling. Due to the scarcity of appropriate tissue and models, little work has been done to understand the basics of spiral artery remodelling in human pregnancy. Recent work on pregnant rats has identified features that suggest they could be a useful model for future investigations into the fundamental mechanisms of remodelling in human pregnancy [14].

Shallow or incomplete trophoblast invasion with limited vessel remodelling have been associated with complications of human pregnancy such as pre-eclampsia. Work on spiral artery remodelling has focussed on defects that may lead to this outcome, however, little is known about the basis of remodelling in normal pregnancy. Data were presented by Keogh and co-workers demonstrating that primary cytotrophoblast can initiate SMC apoptosis in spiral arteries via the Fas/Fas ligand pathway [15,16]. A trophoblast-dependent apoptotic mechanism could thus contribute to SMC loss during remodelling. Apoptosis is a slow and gradual event, typically involving single cells which can take upwards of 24 hours to die following an initial apoptotic signal [17]. Apoptosis thus proceeds in an asynchronous manner leading to a cumulative effect. This is in keeping with the time course of spiral artery remodelling *in vivo* which is a gradual process occurring over a period of weeks.

Many factors will affect the interactions between trophoblast and SMC. Secreted chemokines may attract trophoblast to SMC and recent work has identified a role for the eph/ephrin family in stimulating trophoblast invasion [18,19]. Placental hormones such as human placental lactogen and human chorionic gonadotropin (hCG) may act to positively or negatively regulate SMC apoptosis. hCG has been reported to both protect cells from apoptosis [20] and to increase apoptosis [21]. Cytokines found in the maternal-fetal environment can alter expression of apoptotic ligands such as TNF-related apoptosis-inducing ligand (TRAIL) which is produced by trophoblast and is up-regulated by TNFα and IFNγ stimulation [22,23]. Spiral artery remodelling in pregnancy thus relies on a complex coordination of micro-environmental factors, cell-cell interactions and apoptotic events over an extended time frame that maintains

vessel integrity. The end result is a unique transformation of maternal vessels which is essential for the maintenance of a successful pregnancy.

L. Harris (UK) provided evidence that indeed a soluble factor released by first trimester trophoblast initiates apoptosis of vascular smooth muscle, in the absence of direct cell contact. Human arterial SMC incubated with primary cytotrophoblast (CTB)-conditioned culture medium undergo apoptosis, and this phenomenon is inhibited by a Fas ligand-blocking antibody. These findings were replicated in a 3-dimensional model of endovascular trophoblast invasion. Spiral artery segments denuded of endothelium were perfused with primary CTB-conditioned culture medium, in the presence or absence of the Fas ligand-blocking antibody. After 24h in culture, SMC apoptosis was assessed by TUNEL. CTB-conditioned medium pre-treated with NOK-2 induced significantly less vascular SMC apoptosis than CTB-conditioned medium pre-treated with a control IgG ($P<0.001$, repeated measures ANOVA). In addition, immunohistochemistry demonstrated the presence of Fas ligand in vesicle-like structures within the cytoplasm of first trimester CTB. Loss of SMC during arterial remodelling in pregnancy involves in part, cell death triggered by soluble Fas ligand released by invading CTB.

Timing of remodelling events is crucial and luminal cells are not all exposed to apoptotic stimuli at the same time. Interactions between CTB and SMC involve only a few cells in a localised microenvironment at one time. This allows remodelling to take place over a period of weeks, thus maintaining vessel integrity and function. Remodelling events may also be mediated by trophoblast-derived proteases that degrade the extracellular matrix. Data were presented providing evidence of first trimester CTB engulfing and digesting elastin fibres. The use of three-dimensional models of endovascular trophoblast invasion appears to be a useful advance in studies into how the extracellular matrix and vessel structure may influence remodelling events.

PHENOTYPIC PLASTICITY

As concluding remarks, Lopa Leach (UK) cautioned that although micro-heterogeneity may be a feature of endothelial cells in vivo, this was not necessarily due to intrinsic and unchangeable properties of the cell. The environment itself, dictates the phenotype. Data were presented showing that when human umbilical

vein cells (HUVEC) are grown in a three-dimensional model (trilayer with HUVEC, amnion and retinal pigment epithelial cells) to mimic the outer retinal barrier, HUVEC cells do not show fidelity of origin but demonstrate a fenestrated rather than continuous endothelial phenotype, resembling the chorio-capillaris [24]. Localisation of VEGF in the trophoblast of first trimester placenta (i.e. developmental stage) and in the terminal villous trophoblast of third trimester placenta (i.e. functionality: conduit versus exchange) correlates with a dynamic junctional phenotype of angiogenic and exchange microvessels; endothelial junctions here do not possess the tight junctional adhesion molecule occludin or plakoglobin, the adherens junctional molecule [11,25]. In other vascular beds, such as the rat blood-brain barrier, induced transitory loss of astrocytes results in reversible loss of occludin from tight junctional complexes and loss of barrier function [26]. Thus the cross- talk between epithelial and endothelial cells is important for phenotypic determination and functioning, both in vivo and in vitro.

CONCLUSIONS AND FUTURE DIRECTIONS

This workshop highlighted the need for multi-dimensional human models where different cell types, and their paracrine secretions are allowed to contribute to the final outcome of the experimental stimulus. Rather than reductionist methods alone, such as that provided by cell monocultures, the use of more complex ex vivo and in vitro models such as the extra-corporeally perfused human placenta, the co-cultures and trilayers may well lead the way in this complex and important field of development. These models will allow one to reveal how the fetal trophoblast influences the fate of endothelial cells – angiogenesis and stability for fetal vessels whilst exerting regionally defined apoptosis for maternal vessels. Greater collaboration and communication between scientists working on maternal spiral artery remodelling and chorionic villous remodelling is essential to address some of the important questions about trophoblast-endothelial interactions which allow seeming paradoxes to exist in early development of maternal and fetal circulation of the human placenta.

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