Significance of changes in fetal perfusion pressure to factors controlling angiogenesis in the human term placenta

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The objective of the study was to determine the effects of mechanical factors on endothelial proliferation in the human placental villous vasculature. Individual fetal lobules were perfused with tissue culture medium at two different standard pressures (40 and 100 mm Hg). The perfused area was then removed and diced into small blocks which were quench frozen in liquid nitrogen. Cryostat sections were obtained and fixed in acetone at 4°C. Proliferating cell nuclear antigen was then identified using Ki67 antibody as a marker. Proliferating nuclei were scored, using a light microscope, and a comparison made between the two pressures used. More proliferating endothelial nuclei were found at 100 mm Hg than at 40 mm Hg ($P < 0.05$). It is therefore concluded that mechanical factors may play a role in villous angiogenesis and the formation of terminal villi.

Introduction

Angiogenesis can occur under both physiological and pathological conditions, and in all situations new vessel formation starts from the venous end of pre-existing capillaries (for example Ausprunk and Folkman, 1977; Folkman *et al.*, 1981). The basement membrane is broken down by proteinases released from endothelial cells which convert plasminogen to plasmin. The plasmin in turn causes proteolysis of laminin and fibronectin, both components of the basement membrane. Endothelial cells protrude through the gaps in the basement membrane and migrate into the perivascular space where mitosis finally takes place (Glaser *et al.*, 1983).

Various stimuli have been implicated in the regulation of angiogenesis. These include mechanical factors, such as increased transmural pressure, shear stress and interactions of erythrocytes with vascular endothelium (Myrhage and Hudlická, 1978; Hudlická and Tyler, 1986), and chemical factors such as changes in pH and hypoxia (Gospodarowicz *et al.*, 1979; Knighton *et al.*, 1981). It is difficult to determine which of these is the most important, and it is likely that under most conditions a combination of factors operate together (Hudlická and Tyler, 1986).

In the human placenta, angiogenesis is thought to play a role not only in the development of the villous vasculature but also in the formation of terminal villi. The development of terminal villi is believed to be controlled by the relative rates of longitudinal growth of mature intermediate villi and their contained capillaries (Kaufmann *et al.*, 1985). As soon as growth of the capillaries exceeds that of the mature intermediate villi, the capillaries become coiled and form loops (Kaufmann *et al.*, 1985; Benirschke and Kaufmann, 1990). The greater the disparity in growth rates, the more the capillaries become coiled. The capillary coils then obtrude from the surface of the mature

intermediate villi, elevating domes of trophoblast and forming new terminal villi.

Along the course of capillaries within the terminal villi, focal dilatations called sinusoids occur. In some areas, the sinusoids are particularly close to the overlying trophoblast so that the villous membrane may be as little as 1–2 µm in thickness (Amstutz, 1960; Boyd and Hamilton, 1970; Ludwig, 1972; Fox and Agrofojo-Blanco, 1974). These areas, called vasculosyncytial membranes are well suited for gaseous exchange between mother and fetus (Boyd and Hamilton, 1970; Ludwig, 1972). As gestation advances, the vasculosyncytial membranes become more conspicuous and their surface area increases as a consequence of further enlargement of the sinusoids. It is believed that sinusoids may be the result of local remodelling or proliferation of endothelial cells on the outer wall of the capillary loops (Burton and Tham, 1992; Karimu and Burton, 1994).

Through the formation of terminal villi and vasculosyncytial membranes, the various factors affecting angiogenesis play crucial roles in determining the capacity of the placenta for diffusional exchange. However, the extent to which the different factors operate in the villous vasculature is not known. The present study was undertaken to determine the effects of mechanical factors on endothelial proliferation in the villous vasculature. Experiments were carried out in which individual placental lobules were perfused with tissue culture medium at two different standard pressures. Activation of endothelial proliferation was demonstrated by labelling with Ki67 antibody which binds to cyclin and proliferating cell nuclear antigen in actively dividing cells. It is a quicker alternative to conventional autoradiographic methods.

Materials and Methods

Placental material

The approval of the local Ethical Committee and consent of the patients were obtained for the use of placental material. The

placental material was obtained from ten normal singleton pregnancies delivered by Caesarian section at 38–40 weeks gestation, for cephalopelvic disproportion with no complicating maternal pathology such as hypertension or diabetes mellitus. All sections resulted in live births. The umbilical cords were usually clamped within 1 min of delivery.

After inspecting the placenta to rule out areas of obvious pathology such as infarction, an artery and its accompanying veins supplying an area of the placental disc close to the periphery was chosen. The vessels were cannulated and the placental lobe placed in a bath of physiological saline.

Compressed air (95% air and 5% CO₂) from a cylinder, controlled by a needle valve, provided the pressure required to perfuse the fetal circulation. In the first five placentae, a pressure of 40 mm Hg was applied, while in the second five placentae, a pressure of 100 mm Hg was applied. A bottle containing 1 l of Medium 199 (Sigma, Poole), 10% (v/v) calf serum (Sera-Lab, Sussex), 0.1 g glutamine (Flow Laboratory, Costa Mesa, CA) and 10 ml of antimycotic/antibiotic (Sigma) pH 7.2–7.4 was fed into the cannula to perfuse the placental lobe. The perfusate emerging from the venous cannula was collected into another bottle and recirculated through the placental lobe with the aid of a peristaltic pump. A continuous circulation was therefore maintained over 6–8 h. The whole system was maintained at 37°C in a water bath. The perfused area was then removed and diced into five small blocks, cryoprotected and quench frozen in melting propane cooled in liquid nitrogen. Cryostat sections (4 µm thick) were obtained and floated on formol–gelatine coated slides. The sections were air dried briefly and fixed for 5–10 min with acetone at 4°C.

Immunohistochemistry

Tissue sections were rehydrated and incubated with blocking serum 2% (w/v) BSA and 10% (v/v) normal goat serum diluted with PBS for 20 min. This was followed by incubation with Ki67 antibody (1:25 dilution; Dako Ltd, High Wycombe) for 30 min at room temperature. The second stage antibody was an anti-IgG/IgM, biotinylated antibody used at a dilution of 1:500, in which sections were incubated for 30 min at 4°C. This was followed by incubation with peroxidase conjugated streptavidin (Dako Ltd; diluted according to manufacturer's instructions) for 30 min at 4°C. Violet coloured immunoperoxidase substrate from Vector Laboratories (Peterborough) was used as a chromogen. Each step was followed by a wash in PBS. The sections were counterstained with haematoxylin for 30 s. The primary antibody was replaced with PBS in the control group, but all other steps were similar.

Scoring of Ki67 labelled nuclei

Scoring of proliferating nuclei was performed using a light microscope at a final magnification of $\times 1250$. All nuclei exhibiting staining were counted. Starting from a random point and then moving systematically in the x–y direction, the number of both positively labelled cytotrophoblastic and endothelial nuclei per villous profile were counted. A total of

Fig. 1. Proliferating villous capillary endothelial nucleus (arrow) stained with Ki67 antibody. Scale bar represents 9 µm.

100 villous profiles per pressure group was counted for each placenta.

Statistical analysis

The results of the two pressure groups were compared using Student's *t* test.

Results

The quality of tissue preparation was excellent, as shown by the villous morphology, including distinct identification of the different villous components (trophoblasts, stroma and capillaries).

Nuclear staining was observed with both pressures used. The positive nuclei ranged from light to deep purple, with minimal background staining of the cytoplasm. The non-proliferating nuclei (trophoblastic and endothelial) were counterstained blue by haematoxylin.

The positively stained nuclei were mainly endothelial, although a few cytotrophoblastic nuclei were also observed (Fig. 1).

There were more positively stained endothelial nuclei at 100 mm Hg (1.96 ± 0.18) than at 40 mm Hg (0.86 ± 0.11) and this difference was statistically significant ($P < 0.05$). However, there was no significant difference in the number of labelled cytotrophoblastic nuclei between the pressure groups (0.27 ± 0.06 and 0.25 ± 0.06 , respectively). This provided a positive control that the labelling efficiency of the primary antibody was similar in the two series.

Discussion

Proliferating cell nuclear antigen (PCNA) is a 36 kDa auxiliary protein to DNA polymerase delta (Matthews *et al.*, 1984). It is found in various concentrations within the cell throughout the cell cycle and in greatest quantity during S-phase (Celis and Celis, 1985). The use of Ki67 antibody labelling on frozen sections as an indication of cell proliferation is well established (e.g. Kamel *et al.*, 1991). There is a

gradation of labelling, and cells in S-phase stain very deeply while those not in S phase (for example at G2 phase and mitosis) exhibit only weak staining (Battersby and Anderson, 1990; Foley *et al.*, 1991).

The results of the present study show that the number of proliferating endothelial cell nuclei increases with increasing perfusion pressure. Various mechanical factors, such as increased transmural pressure, and chemical factors, such as a change in pH, are involved in angiogenesis. Chemical factors seem to play a more crucial role under pathological conditions, while mechanical factors are more important during development and in normal tissues (Hudlická and Tyler, 1986). A combination of both factors is probably operating under most conditions.

Increased intravascular pressure is an important factor stimulating the growth of new vessels (Bevan, 1976; Berry, 1978). Bevan (1976) found good correlation between the increased incorporation of [³H]thymidine into arterial smooth muscle cells and arterial pressure. Increased blood flow associated with increased vascular pressure has been observed to induce capillary growth in all contracting muscles (Kasalicky *et al.*, 1977). It is probable, however, that the various components of increased flow, such as increased velocity, wall tension, shear stress and interaction of endothelial cells with erythrocytes, play synergistic roles in angiogenesis (Hudlická and Tyler, 1986).

The findings of the present study demonstrate that increased perfusion pressure plays a significant role in stimulating villous angiogenesis. This in turn may control the formation of terminal villi and their vasculosyncytial membranes. The capillaries within the terminal villi follow a tortuous course and, at points of bends, display sinusoidal dilatation (Boyd and Hamilton, 1970; Kaufmann *et al.*, 1985). It has been proposed that these dilatations result from a combination of high transmural pressure and shear stress exerted on the outer surface of the bend (Burton and Tham, 1992) which ultimately promote local endothelial proliferation in the outer wall of the bend (Hudlická and Tyler, 1986). Consequently the capillary tends to balloon outwards towards the overlying syncytiotrophoblast, reducing the intervening stromal distance and forming a vasculosyncytial membrane.

Karimu and Burton (1994) demonstrated that the placental capillaries are compliant. The capillary volume fraction and ratio of capillary to villous surface area increases with increasing fetal perfusion pressure, as a result of preferentially orientated dilatation of capillary sinusoids in the region of vasculosyncytial membranes. If the fetal perfusion pressure remains high for a long time, local endothelial cell proliferation will ensue. Through this mechanism, placental differentiation may be matched to the respiratory demands of the fetus (Burton and Feneley, 1992). There is, however, an underlying assumption that the peripheral placental vasculature is exposed to forces which reflect the dynamics of fetal circulation *in vivo*. As placental circulation is of low resistance and operates at maximal dilatation under normal conditions (Rankin, 1989; Boura and Walter, 1991), this probably occurs. The results of this study therefore provide strong evidence in support of a role of mechanical factors in the regulation of villous angiogenesis.

A combination of mechanical and chemical factors are probably involved *in vivo*. During fetal hypoxia, peripheral vasoconstriction results in increased fetal blood pressure and diversion of flow within the descending aorta to the umbilical circulation (Mott and Walker, 1983). At the same time there is an accumulation of metabolites such as ADP and lactic acid, which are known to be angiogenic (D'Amore and Shepro, 1977; Fraser and Simpson, 1983). Moreover, growth promoting factors such as vascular endothelial derived growth factor, platelet derived growth factor and epidermal growth factor are also released under these conditions (Ross *et al.*, 1974; Karnovsky, 1981). Chronic hypoxia may therefore stimulate angiogenesis by a combination of mechanical and chemical factors, the result of which is structural adaptation to improve fetal oxygenation.

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