STEREOLOGICAL STUDIES ON FETAL VASCULAR DEVELOPMENT IN HUMAN PLACENTAL VILLI

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ABSTRACT

In human pregnancy, fetal well-being depends on the development of placental villi and the creation and maintenance of fetal microvessels within them. The aim of this study was to define stereological measures of the growth, capillarization and maturation of villi and of fetoplacental angiogenesis and capillary remodelling. Placentas were collected at 12-41 weeks of gestation and assigned to six age groups spanning equal age ranges. Tissue samples were randomised for position and orientation. Overall growth of peripheral (intermediate and terminal) villi and their capillaries was evaluated using total volumes, surface areas and lengths. Measures of villous capillarization comprised capillary volume, surface and length densities and capillary:villus surface and length ratios. Size and shape remodelling of villi and capillaries was assessed using mean cross-sectional areas, perimeters and shape coefficients (perimeter^2/area). Group comparisons were drawn by analysis of variance. Villous and capillary volumes, surfaces and lengths increased significantly throughout gestation. Villous maturation involved phasic (capillary:villus surface and length ratios) or progressive (volume, surface and length densities) increases in indices of villous capillarization. It also involved isomorphic thinning (cross-sectional areas and perimeters declined but shape coefficients did not alter). In contrast, growth of capillaries did not involve changes in luminal areas or perimeters. The results show that villous growth and fetal angiogenesis involve increases in overall length rather than calibre and that villous differentiation involves increased capillarization. Although they do not distinguish between increases in the lengths versus numbers of capillary segments, other studies have shown that capillaries switch from branching to non-branching angiogenesis during gestation. Combined with maintenance of capillary calibres, these processes will contribute to the reduced fetal vascular impedances observed during pregnancy.

Keywords: angiogenesis, capillary remodelling, fetal capillaries, placenta, villi, villous capillarization.

INTRODUCTION

Fetal well-being depends on the creation and maintenance of maternal (uteroplacental) and fetal (fetoplacental) vasculatures and efficient transplacental exchanges between the two circulations. The fact that increases in total oxygen diffusive conductances are commensurate with changes in fetal weight (Mayhew *et al*., 1993) suggests that these processes operate effectively. They also imply that there is coordinated growth between the two vasculatures and the intervening tissue compartments of the placental villi. Indices of resistance to blood flow determined at different stages of gestation have demonstrated that the fetoplacental vasculature exhibits low impedance and that this resistance to flow gradually declines as pregnancy advances (Coppens *et al*., 1996; Kurmanavicius *et al*., 1997). Since vascular anatomy is an important determinant of resistance, this implies that changes in vascular anatomy may occur throughout gestation.

Vascular anatomy may affect resistance to flow via changes in the spatial arrangements and/or dimensions of vessels or vessel segments. Alterations of vessel dimensions may involve their calibre and/or their total length or segment length but calibre expressed in terms of mean cross-sectional area is the most influential determinant of resistance. However, other things being equal, the most effective angioarchitecture for reducing resistance to flow is a spatial arrangement in which vessel segments are linked in parallel rather than in series. As in other tissues (e.g. Nyengaard, 1993), there is evidence that both anatomical strategies are adopted during fetoplacental vascular development and that they make a substantial impact on the development of placental villi (Benirschke and Kaufmann, 2000; Mayhew, 2002a).

Primitive (mesenchymal) villi are common during the first trimester and eventually give rise to different topological regions which are reminiscent of the trunks, branches and twigs of a tree (Castellucci *et al*., 1990; Benirschke and Kaufmann, 2000). Stem villi represent the trunks and terminal villi the twigs but, in between, there are immature and mature intermediate villi which represent the larger and smaller branches of the tree. Because of their numbers, extensive surfaces and minimal materno-fetal intervascular distances (influenced by the degrees of villous capillarization), terminal villi are most influential in transplacental exchange, especially by passive diffusion (Jackson *et al*., 1992; Mayhew *et al*., 1993; Benirschke and Kaufmann, 2000). Consequently, growth of villus trees involves a switch of emphasis during gestation from early production of stem and immature intermediate villi towards production of mature intermediate villi and terminal villi. Mid-gestation is an important watershed period in this transition.

After mid-gestation, there is a dramatic acceleration of the growth of peripheral (mature intermediate and, especially, terminal) villi and their capillaries (Mayhew, 2002a). The switch is probably influenced by changes in intervillous oxygen tensions which, in turn, trigger changes in angiogenic growth factors and receptors (Kingdom and Kaufmann, 1997; Ahmed *et al*., 2000; Dunk and Ahmed, 2001; Geva *et al*., 2002). Amongst these factors are vascular endothelial growth factor (VEGF), placenta growth factor (PlGF) and the angiopoietins (Ang-1 and Ang-2). Altered levels of these factors and their receptors are also linked to differences in angiogenic patterns, with an early phase of branching angiogenesis gradually shifting towards a dominance of non-branching angiogenesis (Benirschke and Kaufmann, 2000). Branching angiogenesis will help to reduce vascular resistance to flow but a shift towards greater non-branching angiogenesis would be expected to increase resistance unless compensated by increases in vessel cross-sectional areas and/or changes in physiological properties (e.g. perfusion pressure or blood viscosity). Since differential capillary growth contributes to formation of new terminal villi, these angiogenic events also affect villous architecture (Kingdom and Kaufmann, 1997; Benirschke and Kaufmann, 2000).

In this study, structural aspects of fetoplacental angiogenesis and villous development have been quantified between 12 and 41 weeks of gestation using stereological methods. Various indices of angiogenesis were calculated (Mayhew *et al*., 2003a). Overall growth of villi and fetal capillaries was monitored by estimating volumes, surface areas and lengths. Villous capillarization was assessed using various estimators, namely, capillary volume, surface and length densities within villi and capillary:villus surface and length ratios. To monitor villous maturation and capillary remodelling, estimates of mean cross-sectional areas, perimeters and shapes were obtained.

MATERIALS AND METHODS

PROVENANCE OF PLACENTAL MATERIAL

Ninety placentas were collected from uncomplicated pregnancies between 12 and 41 gestational weeks postmenstruation (Boyd, 1984; Jackson *et al*., 1992). Gestational ages were checked by ultrasound and clinical examinations. Placentas at 12-24 weeks were from spontaneous abortions (due to cervical incompetence) or pregnancies terminated for social reasons with no fetal abnormalities. Placentas at 25-36 weeks were from previously uncomplicated pregnancies ending in premature labour without hypertension, diabetes or pre-eclampsia and no fetal abnormality apart from immaturity. Remaining placentas were collected at 37-41 weeks after uncomplicated pregnancies ending in spontaneous labour with no neonatal problems or malformations. Immediately after delivery, umbilical cords were clamped and fresh placental weights determined after removing blood clots and attached membranes. Fresh volumes were determined by fluid displacement before immersion-fixation in buffered formalin.

SAMPLING AND STEREOLOGICAL TOOLS

Random sampling for position and orientation was undertaken in order to derive design-based stereological estimates of volumes, surfaces and lengths (Howard and Reed, 1998). Full-depth tissue pieces were sampled systematically, diced, re-immersed in formalin and embedded haphazardly in paraffin wax. Sections (3-5 μm in thickness) were stained by connective tissue procedures. Light microscopical fields of view were selected by systematic random sampling (Gundersen and Jensen, 1987; Mayhew, 1997) and prepared as photomicrographs (magnification x250) and slide transparencies (x2000).

To obtain measures of overall growth of the fetal vascular bed (the nett outcome of the processes of angiogenesis and vessel pruning), total volumes, surfaces and lengths per placenta were calculated by multiplying appropriate component densities by

placental volumes. The same estimators were used to monitor overall growth of the peripheral villi. Component densities were derived by counting test points, test line intersections and villous and vessel transections. Criteria for recognising peripheral villi were mostly those described previously (Benirschke and Kaufmann, 2000). Where appropriate, these were corrected for tissue shrinkage distortions using the diameters of maternal erythrocytes as internal standards (Mayhew and Burton, 1988). Previously, these adjustments have been shown to produce structural quantities similar to those found in glutaraldehyde-fixed, resin-embedded sections but, in any event, tissue distortions were similar at different gestational ages and so the gestational comparisons retain their validity.

Several alternative indices of villous capillarization were adopted. Apart from component densities (capillary volume, surface and length densities within villi), we included estimates of capillary:villus surface and length ratios. The surface ratio relates the adluminal surface of vascular endothelium to the maternal surface of villous trophoblast. The length ratio is related to the number of capillary loops per villus.

Volumes and lengths allowed calculation of villous/ capillary cross-sectional areas and interpretation of volume change in terms of growth in length and/or calibre. Similarly, surfaces and lengths allowed calculation of cross-sectional perimeters. Finally, a dimensionless 'shape' coefficient, related to shape as viewed in cross-section, was obtained for villi and capillaries using $perimeter^2/area$. This coefficient offers a convenient index of shape remodelling.

STATISTICAL ANALYSES

Means and coefficients of variation (CV = standard deviation expressed as a percentage of the corresponding mean) were calculated for each of six gestational age groups: 12-16, 17-21, 22-26, 27-31, 32-36 and 37-41 weeks respectively. Values of CV were chosen so as to provide suitable measures of the observed inter-individual variation at each gestational stage. Comparisons between groups were undertaken by one-way analyses of variance (Sokal and Rohlf, 1981). Null hypotheses were rejected if the probability level, P, was less than 0.05.

RESULTS

GROWTH OF VILLI AND CAPILLARIES

Changes in absolute volumes, surfaces and lengths are summarised in Table 1. The volume of peripheral villi increased during gestation from a mean (CV) of 31.8 cm^3 (30%) at 12-16 weeks to 226 cm^3 (22%) at 37-41 weeks (variance ratio, F = 62.9; degrees of freedom, df = 5.84; P < 0.001). Over the same period, villous surface areas increased from 0.86 m^2 (29%) to 11.3 m^2 (21%) (F = 85.2; P < 0.001) and total length from 3.96 km (35%) to 82.7 km (20%) (F = 80.6; P < 0.001).

Corresponding changes in capillary volumes were 2.25 cm^3 (54%) at 12-16 weeks to 49.2 cm^3 (38%) at term (F = 39.5; P < 0.001). Capillary luminal surface areas increased from 0.44 m^2 (63%) to 11.1 m^2 (41%) (F = 40.7; P < 0.001) and lengths from 12.8 km (64%) to 325 km (36%) (F = 50.5; P < 0.001).

These results suggest that inter-individual variability tends to be greatest between 17-26 weeks of gestation and that capillary dimensions exhibit greater inter-individual variation than those of villi.

Table 1. *Indices of the overall growth of villi and capillaries during gestation. Values are group means (CV%).*

Variable	12-16 wks n=11	17-21 wks n=8	22-26 wks n=11	27-31 wks n=17	32-36 wks n=23	37-41 wks n=20
Peripheral Villi						
*Volume, cm^3	31.8 (30%)	37.2 (32%)	106 (39%)	127 (24%)	186 (21%)	226 (22%)
*Surface, m^2	0.86 (29%)	1.09 (30%)	4.17 (43%)	5.52 (26%)	8.68 (20%)	11.3 (21%)
*Length, km	3.96 (35%)	5.30 (33%)	25.8 (50%)	38.6 (30%)	64.2 (26%)	82.7 (20%)
Fetal Capillaries						
*Volume, cm^3	2.25 (54%)	3.73 (48%)	13.4 (47%)	20.6 (40%)	38.1 (30%)	49.2 (38%)
*Surface, m^2	0.44 (63%)	0.79 (36%)	2.93 (50%)	4.31 (44%)	8.21 (24%)	11.1 (41%)
*Length, km	12.8 (64%)	23.0 (52%)	76.2 (56%)	114 (44%)	228 (23%)	325 (36%)

Key: * denotes significant differences between gestational groups

VILLOUS CAPILLARIZATION

Alternative measures of villous capillarization are provided in Table 2. At 12-16 weeks of gestation, the volume density of capillaries in peripheral villi amounted to 7.0% (37%) and this figure rose to 21.4% (21%) at term (F = 26.1; P < 0.001). The surface and length densities of capillaries also increased significantly (F = 34.2 and 34.9 respectively; P < 0.001 in both cases). Significant group differences in capillary:villus surface and length ratios (F = 10.1 and 3.35; P < 0.001 and P < 0.01 respectively) were also detected but these differences appeared to result from phasic rather than progressive changes. As with absolute measures, CV values tended to be greater at earlier stages of gestation (in this case slightly earlier, at 12-21 weeks of gestation).

REMODELLING OF VILLI AND CAPILLARIES

Cross-sectional sizes and shape coefficients are shown in Table 3. The increases in total volume and length of villi were accompanied by a gradual reduction in mean cross-sectional area (F = 55.0; P < 0.001). Similarly, the increases in surfaces and lengths were accompanied by reduced perimeters (F = 39.5; P < 0.001). The corresponding shape coefficients did not alter significantly across gestational age groups and so the changes in villous areas and perimeters were isomorphic.

In contrast to villi, no significant between-group differences were detected for mean cross-sectional areas, perimeters or shape coefficients of capillaries. Coupled with the marked increases in total volumes and lengths (Table 1), these findings indicate that capillary growth is longitudinal rather than transverse.

Both capillary and villous sizes and shapes tended to show greater CV values at earlier stages of gestation and variability also tended to be greater in capillaries than in villi.

Table 2. *Indices of villous capillarization. Values are group means (CV%).*

Variable	12-16 wks n=11	17-21 wks n=8	22-26 wks n=11	27-31 wks n=17	32-36 wks n=23	37-41 wks n=20
Component Densities						
*Volume density, %	7.03 (37%)	10.3 (51%)	12.6 (32%)	15.8 (23%)	20.5 (23%)	21.4 (21%)
*Surface density, cm²/cm³	139 (50%)	215 (23%)	273 (35%)	332 (25%)	446 (18%)	482 (22%)
*Length density, m/cm³	403 (50%)	619 (47%)	698 (36%)	883 (30%)	1240 (17%)	1420 (20%)
Capillary:Villus Ratios						
*Surface ratio, m²/m²	0.50 (45%)	0.75 (31%)	0.70 (31%)	0.76 (20%)	0.96 (22%)	0.97 (24%)
*Length ratio km/km	3.3 (49%)	4.6 (54%)	3.0 (27%)	2.9 (24%)	3.7 (22%)	3.9 (27%)

Key: * denotes significant differences between gestational groups

Table 3. *Indices of villous and capillary remodelling (area, perimeter and shape in cross-section). Values are group means (CV%).*

Variable	12-16 wks n=11	17-21 wks n=8	22-26 wks n=11	27-31 wks n=17	32-36 wks n=23	37-41 wks n=20
Villi						
*Area, μm²	8520 (24%)	7350 (31%)	4530 (29%)	3390 (19%)	2960 (19%)	2760 (14%)
*Perimeter, μm	227 (17%)	209 (13%)	169 (15%)	146 (12%)	137 (12%)	137 (10%)
Shape factor, μm²/μm³	6.1 (17%)	6.1 (10%)	6.5 (9%)	6.3 (13%)	6.5 (15%)	6.8 (13%)
Capillaries						
Area, μm²	192 (28%)	172 (28%)	190 (23%)	186 (19%)	169 (27%)	153 (15%)
Perimeter, μm	34.6 (8%)	38.3 (29%)	39.7 (16%)	38.4 (13%)	36.6 (20%)	34.0 (9%)
Shape factor, μm²/μm³	6.6 (27%)	9.8 (70%)	8.6 (24%)	8.0 (14%)	8.0 (21%)	7.6 (13%)

Key: * denotes significant differences between gestational groups

DISCUSSION

This investigation has shown that villous volume and surface area expand during gestation due to linear growth accompanied by thinning. In light of known mechanisms of villous development, this is consistent with proliferation of more slender mature intermediate and terminal villous branches. Within these peripheral villi, maturation involves differential growth of the fetal capillaries, the component densities of which increase roughly 3-fold between 12-16 weeks and term. Capillary growth is also linear but, unlike villi, does not involve a change in calibre. However, like villi, capillaries maintain an isomorphic cross-sectional outline.

With reference to fetal capillaries, the term linear growth is not intended to imply a particular pattern of angiogenesis (i.e. non-branching rather than branching angiogenesis). It merely refers to an increase in the total length of capillaries unaccompanied by changes in vessel mean cross-sectional area. To monitor branching and non-branching, it would be sensible to include stereological estimators of the numbers and lengths of individual capillary segments (e.g. Nyengaard, 1993; Gambino et al., 2002). However, it is clear that the longitudinal growth of capillaries is not entirely commensurate with that of villi because capillary: villus surface and length ratios vary during gestation. In particular, the length ratios appear to peak around mid-gestation before declining and re-attaining peak values at term. These ratios (3-4 km/km) are consistent with earlier estimates (e.g. Burton et al., 1996; Mayhew, 2002b) and with an average of about two capillary loops per villus (Benirschke and Kaufmann, 2000).

Villous development during gestation involves the phased production of different villous types (stem, intermediate, terminal). Early in gestation, effort is invested in producing the trunks (stem villi) and main branches (immature intermediate villi). Later effort is directed to favour the finer branches (mature intermediate and terminal villi) which are more effective in transplacental transport processes (Castellucci et al., 1990; Kingdom and Kaufmann, 1997; Benirschke and Kaufmann, 2000). The transition occurs around mid-gestation and has been linked to changes in oxygen status and fetoplacental angiogenesis.

Fetoplacental angiogenesis is associated with the expression of various oxygen-regulated growth factor ligands and their receptors and antagonists (Ahmed et al., 2000; Dunk and Ahmed, 2000; Geva et al., 2002). Early in gestation, oxygen tensions are low and VEGF levels high. During this phase, branching angiogenesis predominates and leads to formation of multiple capillary loops. Later, oxygen tensions rise (Jauniaux et al., 2000) and tissue levels of VEGF and PlGF alter. This phase is associated with greater non-branching angiogenesis and the growth of capillaries from existing capillaries (Kingdom and Kaufmann, 1997; Benirschke and Kaufmann, 2000). Both phases of capillary growth involve proliferation of vascular endothelial cells (Simpson et al., 1992; Mayhew et al., 1994; Mayhew, 2002a). However, whilst early proliferation is probably stimulated by VEGF, later proliferation may be promoted by the combined effects of VEGF and PlGF rather than the isolated effects of PlGF (Park et al., 1994; Dull et al., 2001).

The process of forming new capillary sprouts seems to be associated with a breakdown of periendothelial basal lamina. This may weaken the interactions between endothelial cells and perivascular cells. Endothelial cells no longer associated with pericytes may differ in proliferative activity and sensitivity to VEGF (Hellström et al., 2001). Moreover, pericytes produce angiopoietins which are also regulated by local oxygen tensions and, together with endothelial cells, produce endostatin, an anti-angiogenic factor down-regulated by hypoxia (Wu et al., 2001). Low tensions transcriptionally activate Ang-2 but degrade Ang-1 and the ratio between these angiopoietin messenger RNAs alters during gestation to favour Ang-1 mRNA and affect vessel stabilisation or maturation. The declining levels of Ang-2 may allow vessels to retain their plasticity and facilitate a transition from branching to non-branching angiogenesis (Geva et al., 2002).

Present findings are consistent with the reported mechanisms of villous development and fetoplacental angiogenesis but do not provide quantitative evidence of a change in capillary branching patterns. Nevertheless, they confirm earlier stereological findings (Mayhew, 2002a) that the morphological changes are biphasic with important events occurring at a common inflection point located soon after mid-gestation. They are also consistent with production of greater lengths of more uniformly slender terminal villi (Sen et al., 1979; Mayhew, 2002a). The gradual reduction in mean calibre of intermediate villi may be due, in part, to transformation of immature into mature intermediate villi and that of terminal villi probably reflects the continuous production of increasing numbers of these finest branches (Benirschke and Kaufmann, 2000). As peripheral villi mature, the become better capillarized (Aherne and Dunnill, 1966; Jackson et al., 1992; Kadyrov et al., 1998; Benirschke and Kaufmann, 2000; Mayhew, 2002a).

The preferential increases in relative and total capillary volumes, and the increases in other indices of villous capillarization, also fit the notion that capillary growth stimulates formation of new terminal villi from mature intermediate villi (Benirschke and Kaufmann, 2000).

These sequences of events may be reflected in the patterns of inter-individual variability (expressed as CV values) observed in present datasets. Values of CV tended to be greater at earlier periods of gestation and stabilise towards term. This is consistent with the period around 20 weeks of gestation being one of great placental remodelling and accelerated growth. CV values also appeared to be greater for capillaries than villi and this is consistent with the notion that capillary changes help to sculpt villous development. Indeed, growth of capillaries contributes to the formation of new terminal villi (Benirschke and Kaufmann, 2000) and may help to explain the larger CV values for indices of villous capillarization seen at earlier periods of gestation (12-21 weeks).

The present study has not found any alteration in the mean cross-sectional area of capillaries but previous studies on the same material have detected decreases in capillary mean diameter (Jackson *et al.*, 1992). Decreases in capillary diameters have also been noted in other investigations or may be deduced from data contained therein (Aherne and Dunnill, 1966; Teasdale, 1980; Kadyrov *et al.*, 1998). Failure to detect differences in cross-sectional areas may be due to the greater variability of area versus diameter estimates or to differences in vessel calibre growth in terminal and intermediate villi (Jackson *et al.*, 1992; Benirschke and Kaufmann, 2000; Mayhew, 2002a). However, regardless of whether capillary diameter is maintained or decreases, it is clear that mean calibre does not increase or, therefore, contribute to the increase in total capillary volume.

It is pertinent to ask how fetal vascular resistances to flow can continue to decline during gestation (Coppens, 1996; Kurmanavicius *et al.*, 1997) in the face of greater non-branching angiogenesis (i.e. elongation of capillary segments), elevated fetal haematocrits (a determinant of blood viscosity, e.g. Nava *et al.*, 1996) but no increase in capillary mean cross-sectional area. It appears that the incidence of focal dilations of capillaries (sinusoids) increases during gestation and especially near villous tips (Benirschke and Kaufmann, 2000). This being so, retaining a constant capillary mean calibre implies compensatory thinning elsewhere

along the capillary bed. Alternatively, the relative incidence of sinusoids along capillaries may be so low that changes in mean cross-sectional area are difficult to detect. Whatever the explanation, the presence of sinusoids, coupled with increasing blood pressures in the fetal circulation, are the factors likely to compensate for the higher impedances which would be expected following vessel segment elongation (inherent to non-branching angiogenesis) and the progressive elevation of fetal haematocrits.

The increasing volumes and surfaces of capillaries and villi, together with reduced diffusion distances (Jackson *et al.*, 1992) and expanding intervillous volumes (Mayhew *et al.*, 1993), contribute to the increases in total placental diffusive conductances. These increases match the gains in fetal weight so that, for example, the specific diffusive conductances for oxygen are constant from at least the end of the first trimester to full term (Mayhew *et al.*, 1993). Finally, the measurements of vascular growth, villous capillarization and vascular remodelling employed here not only provide data for uncomplicated pregnancies but also a baseline to which results from complicated pregnancies may be referred. In recent publications, for example, we have shown that fetoplacental angiogenesis is stimulated in pregestational diabetes but not in gestational diabetes (Babawale *et al.*, 2000; Mayhew, 2002b) and that the enhanced growth involves changes in total capillary length but not in luminal calibre or villous capillarization. In contrast, capillary growth is compromised in placentas from women who smoke during pregnancy and this is associated with inconsistent changes in indices of villous capillarization (Bush *et al.*, 2000). Capillary growth is also stunted in high-altitude pregnancies but, here, there is a reduction in capillary mean calibre coupled with greater irregularity in the cross-sectional shape of capillaries and enhanced villous capillarization (Mayhew, 2003). Recent studies also show that fetoplacental angiogenesis is diminished in intrauterine growth restriction but not in pre-eclampsia (Mayhew *et al.*, 2003b).

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