ANATOMICAL STUDIES OF THE FETOPLACENTAL CIRCULATION IN PREGNANCIES COMPLICATED BY GROWTH RESTRICTION

by

LENA MARY MACARA

MB ChB MRCOG

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to the University of Glasgow

Department of Obstetrics and Gynaecology
Glasgow Royal Infirmary
10 Alexandra Parade
Glasgow G31 2ER

UNIVERSITY OF GLASGOW

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ABSTRACT

Severe intrauterine growth restriction (IUGR) is recognised to be a major cause of perinatal morbidity and mortality. Identifying the fetus that is truly growth restricted and therefore compromised, from the one that is simply genetically small and not at risk is difficult. Recent work using umbilical artery (U_A) Doppler waveform studies have shown that a reduction in the diastolic component of the U_A Doppler waveform correlates strongly with poor perinatal outcome. Since this technique can be used to reliably distinguish the truly growth restricted fetus at risk, it is imperative, if we are to understand the pathophysiology of severe IUGR, that the mechanisms by which abnormal Doppler waveforms arise is established.

The elaboration pattern of blood vessels within stem villi from IUGR and gestationally age-matched control placentas, was first evaluated by measuring the diameter of 600 vessel profiles, identified by the antibody anti-α smooth muscle actin, within stem villi from randomly chosen areas of placental tissue . The vessels diameters, ranging from 10-160μM, were plotted in 15μM bands and the two groups compared. There was no significant difference in the mean vessel diameter or in the distribution of vessel diameters in the IUGR and gestational age-matched control placentas. Simultaneously, the mean volume of all classes of villous tissue was calculated. There was significantly less villous tissue in the IUGR placentas than in the matched control placentas. This was the result of a selective reduction in the volume of peripheral (intermediate and terminal) villi in the IUGR placentas.

Having identified a quantitative difference in the volume of peripheral villi, scanning electron microscopy (SEM) was used to examine the three-dimensional structure of these villi and the blood vessels within them using vascular and villous tissue casts from pregnancies complicated by severe IUGR and normal preterm controls. The peripheral vascular network of capillaries was noted to be distinctly abnormal in the IUGR placentas. Fewer vessels were identified and when present, these resembled elongated drainpipes. This was in contrast to the normal preterm control vascular casts which were characterised by multiple capillary loops comprised of short, coiled, highly branched, networks with sinusoidal dilatations. Similarily, the peripheral villi of the IUGR placentas were abnormal. Terminal villous "buds", which provide a large syncytial surface area for materno-fetal exchange, were rarely identified. In contrast, the terminal villi of the IUGR placentas, like the vessels within them, resembled narrow tubes.

The ultrastastructure of these abnormal terminal villi was then determined using transmission electron microscopy (TEM) and compared with the ultrastructure of normal gestational age-

matched teminal villi. The terminal villi from the IUGR placentas were significantly smaller in diameter than their preterm counterparts. In addition, there was an increased number of syncytial nuclei, fewer cytotrophoblast nuclei, thickening of the basement membranes and an increase in the number of collagen fibres and basal lamina like material within the villous stroma.

To verify these findings and establish if the reduction in cytotrophoblast cell nuclei number was due to increased turnover or depleted proliferation, the samples were examined with the proliferation marker MIB-1. After correcting for the volume of villous tissue present, this confirmed that there were significantly fewer proliferating cytotrophoblast cells in the IUGR placentas than in normal preterm controls. Likewise using antibodies directed against individual collagen types I, II, III, IV, V, laminin and fibronectin the increase in stromal collagen in the IUGR placental villi was confirmed and recognised to result from increased staining for collagens I, III, IV and laminin.

Together these data indicate that primary vascular and villous development is abnormal in placentas from pregnancies complicated by IUGR and abnormal Doppler. As a result, though the total number of stem villous vessels may be reduced, the pattern of their distribution is identical to that seen in normal preterm controls. However, quantitative and qualitative differences were seen in the peripheral villi of the IUGR placentas, the major site of gas and nutrient exchange. The alterations observed may explain both the metabolic adaptation of the IUGR fetus and the Doppler U_A abnormalities seen in affected pregnancies. The regulation and expression of angiogenic and villous growth factors in placental development therefore requires urgent attention.

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ABBREVIATIONS

AC	abdominal circumference
AEC	3-amino-9-ethylcarbazole
AEDFV	absent end-diastolic flow velocity
AFI	amniotic fluid index
ANP	atrial natriuretic peptide
APAAP	alkaline phosphatase:anti-alkaline phosphatase
APES	aminopropyltriethoxysilane
BSA	bovine serum albumin
CN	cytotrophoblast nuclei
DAB	3,3'-diamino-benzidine
DDSA	dodecenyl succinic anhydride
DHO	disodium hydrogen orthophophate
DMP 30	tri-dimethylaminomethylphenol
DSM	digital scanning microscope
EDRF	endothelium-derived growth factor
EGF	endothelial derived growth factor
et al.	and others
ET-1	endothelin 1
FVW	flow velocity waveform
FGF	fibroblastic growth factor
g	grammes
Hz	hertz
Hg	mercury
ie.	that is
IHC	immunohistochemistry
IUGR	intrauterine growth restriction (retardation)
im	intramuscular
iu	international units
m	meters
M	molar
mg	milligrammes
mls	millilitres
mm	milimeters
nm	nanometers
NOS	nitric oxide synthase
µM	micrometres
PAP	peroxidase:anti-peroxidase
PBS	phosphate buffered saline
RI	resistance index
RNA	ribonucleic acid
sol.	solution
SD	standard deviation
SEM	scanning electron microscopy
SGA	Small for gestational age
SN	syncytial nuclei
TEM	transmission electron microscopy
TV	terminal villi
U_A	umbilical artery
U_V	umbilical vein
vs.	versus

PUBLICATIONS AND PRESENTATIONS

Macara LM, Kingdom JCP, Kaufmann P. Control of the fetoplacental circulation. Fetal and Mat Med Review 1993; 5:167-179

Kingdom JCP, Macara LM, Whittle MJ. Fetoplacental circulation in health and disease. Arch Dis Childhood 1994; 70:F161-F165

Macara LM, Kingdom JCP, Hair J, More I, Kaufmann P, Lyall F, Greer IA. Ultrastructural findings in placental villi from pregnancies complicated by severe intrauterine growth retardation and abnormal umbilical artery Doppler waveforms. Society for Gynecological Investigation. 41st Annual Meeting; Chicago, Illinois (abstract P134)

Macara LM, Kingdom JCP, Hair J, More I, Kaufmann P, Lyall F, Greer IA. Structural analysis of placental terminal villi in pregnancies complicated by intrauterine growth retardation: failure of oxygen transport from the intervillous space to the fetus. Obstet Gynecol, December 1994 (submitted for publication)

Macara LM, Kingdom JCP, Kohnen G, Bowman AW, Greer IA, Kaufmann P. Branching of the fetal vascular tree in pregnancies complicated by intrauterine growth retardation: lack of evidence to support an obliterative process. Br J Obstet Gynaecol; December 1994 (in press)

The ultrastructure of terminal villi in pregnancies complicated by severe IUGR and abnormal umbilical artery Doppler waveforms. Medical Chirurgical Society of Glasgow, Research Registrars Prize Night; November 1993

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CHAPTER 1

INTRODUCTION

1.1 HISTORICAL PERSPECTIVE

It is now more than 250 years since William Hunter reported the existence of two distinct circulations within the human placenta, establishing that fetal and maternal blood remained isolated from each other throughout pregnancy;

"Though the placenta be completely filled with any injection thrown in the uterine vessels, none of the wax finds its way into any of the umbilical vessels; and in the same manner fluids injected into the umbilical vessels can never be pushed into the uterine except by rupture or transudation"

(William Hunter; Anatomy of the Gravid Uterus 1774)

As most obstetricians were occupied in dealing with the overwhelming problems of puerperal sepsis and maternal death the placenta remained an organ of "respect" - a passive structure which occasionally elicited fear and anxiety if implanted either too low or too deep but which otherwise appeared of little clinical relevance and merited little investigation. More recently, our understanding of perinatal physiology, blood flow regulation, and the accumulating epidemiological data linking prenatal events with adult disease (Barker 1992; Rona, Gulliford & Chinn 1993) has created a renewed interest in the function of the placenta and its role in determining both fetal development and adult health.

One of the major challenges facing obstetrics today is the problem of restricted fetal growth, attributed to "utero-placental insufficiency". Despite significant advances in neonatal care over the last two decades, there has been little improvement in the outcome of this group of pregnancies. Perinatal mortality is 4-8 times higher in very low birth weight babies and perinatal morbidity is significant with evidence of neurodevelopmental delay (Taylor & Howie 1989; Todd et al. 1992; Mari & Russell 1992) learning difficulties, behavioural problems (Scottish Low Birthweight Study I & II) and cerebral palsy (Perkins 1987; Marlow, Hunt & Chiswick 1988). Learning and social difficulties often persist into later childhood and are particularly prominent amongst children born prematurely (<33 weeks gestation) with severe intrauterine growth restriction (Semedler et al. 1992). These findings have large implications for health and education resource management.

Programmes directed towards the prevention and/or treatment of this condition and its long term consequences, can only be initiated if, by understanding the pathological processes which inhibit normal fetal growth and development, we are able to detect affected pregnancies antenatally

1.2 INTRAUTERINE GROWTH RETARDATION

Even in developed countries 5-10% of pregnancies result in the delivery of a small neonate (Ebrahim 1984). The majority of these are simply statistically small for their gestational age (SGA); they have grown at a constant velocity, achieving their pre-programmed growth potential and are otherwise healthy. A minority however, are born small as a result of growth restriction *in-utero*. This may occur as a result of many pathological processes - congenital infections, structural malformations, karyotypic abnormalities (particularly triploidy and trisomy 18) - but in the majority of cases while there is neonatal evidence of intrauterine starvation - dry, wrinkled skin, loss of adipose tissue and muscle wasting - no specific underlying aetiology can be identified (Snijders et al. 1993a). Since fetal growth and development are dependent on an adequate supply of nutrients and oxygen from the mother, transported to the fetus across the placenta, these cases of "unexplained" intrauterine growth restriction (IUGR) have been attributed to 'utero-placental insufficiency'.

1.3 CLINICAL FEATURES OF IUGR PREGNANCIES

1.3.1. FETAL METABOLISM

The ability to obtain pure samples of fetal blood, usually from the umbilical vein at the cord root (Daffos et al. 1983), has revealed a great deal of information regarding the fetal environment. The growth restricted fetus, in contrast to its normally grown counterpart, is hypoxic (Soothill et al. 1987; Nicolini et al. 1990), presumably chronically so, since plasma erythropoietin is elevated (Snidjers et al. 1993b), has a metabolic acidosis (Nicolaides et al. 1989) and shows biochemical evidence of intrauterine starvation. Not only do these fetuses have reduced circulating concentrations of certain amino acids (Economides, Crook & Nicolaides 1991; Cetin et al. 1990; Pardi et al. 1993) and glucose (Nicolini et al. 1989) but also elevated levels of cortisol, reduced insulin concentrations and increased serum triglycerides (Economides & Nicolaides 1989). The latter may be interpreted as an anaerobic state, triglycerides being required more urgently to provide a vital energy source for the fetus.

Increased concentrations of erythropoietin, are also present (Snijders et al. 1993b) and erythropoiesis stimulated. In contrast to adult tissue hypoxia in which elevated erythropoietin generates erythroblastosis (Zanzani & Ascendo 1989), fetal erythropoiesis in growth retardation would appear to be mediated by release of premature erythroblasts rather than actual increased erythrocyte production (Abbas et al. 1994).

Together these biochemical data would confirm the neonatal evidence which suggests that intrauterine starvation is a major contributing factor to poor fetal growth. By implication

therefore, in affected pregnancies, either the maternal substrate supply is deficient, or placental substrate transfer significantly impaired during intrauterine life. Since the feto-maternal ratios of amino acids (Bernardini et al. 1991) and glucose (Economides & Nicolaides 1989) are significantly reduced, despite increased or normal maternal concentrations, it would appear that impaired placental transport, is the primary factor effecting fetal substrate deprivation.

1.3.2. UTEROPLACENTAL BLOOD FLOW

Early histological studies in pregnancies complicated by IUGR demonstrated impaired trophoblast invasion and transformation of maternal spiral arteries (Sheppard & Bonnar 1981). In conjunction with this, *in-vivo* blood flow studies using radioactive isotopes confirmed that placental blood flow was significantly reduced in IUGR pregnancies (Chatfield et al. 1975; Lunell et al. 1979). More recently, Doppler ultrasound studies of the uterine artery in pregnancies which are later complicated by IUGR have indicated that this lack of trophoblast invasion is reflected by an increase in uterine artery Doppler resistance during the second trimester (Campbell et al. 1986; Bewley, Cooper & Campbell 1991). An isolated reduction of uterine blood flow in normal pregnancies does not alter fetal umbilical artery blood flow. However, in a growth restricted pregnancy, progressive embolisation of the ovine uterine circulation results in abnormal umbilical artery blood flow patterns, suggesting that some feature of the 'growth restricted' placenta prevents the normal adaptation to reduced uterine blood flow (Clapp et al. 1980).

1.3.3. FETAL BLOOD FLOW DISTRIBUTION IN IUGR FETUSES

In combination with these changes in fetal metabolism, fetal blood flow is also altered in growth restricted pregnancies. Aortic blood flow is significantly depleted (Eik-Nes, Marsal & Kristofferson 1984; Griffin et al. 1984, 1985; Wladimiroff, Tonge & Stewart 1986) and as a consequence, renal perfusion diminished and urine output reduced (Arduini & Rizzo 1991). These adaptations can have longer term effects. Growth restricted neonates are particularly susceptible to necrotising enterocolitis, a condition associated with mesenteric hypoxia, and often demonstrate impaired renal function in the early neonatal period resulting in oliguria (Hackett et al. 1987).

While lower aortic blood flow is diminished in growth restricted fetuses, blood flow to the brain and coronary circulation is relatively increased, a phenomenon known as 'brain-sparing' and recognised for some time in animal models (Peeters et al. 1979). This may be due to a shift from right to left ventricular dominance, whereby the majority of left ventricular outflow is pre-ductal (Weiner et al. 1994). Normal or increased flow to cerebral tissue is sustained by a fall in cerebral resistance, thought to occur in response to hypoxia (Vyas et al. 1990). As hypoxia progresses however, maximal cerebral vasodilatation is reached,

preventing any further compensation. Beyond this nadir, further hypoxic insult results only in cardiac decompensation and cerebral vasoconstriction. These changes are recognised to be pre-terminal events which may occur up to two weeks before actual fetal demise. However, by this stage significant antenatal brain damage may already have occurred.

1.4 CONSEQUENCES OF INTRAUTERINE GROWTH RETARDATION

It is now clear that the effects of intrauterine growth restriction may also persist into adult life. Death rates from cardiovascular disease progressively rise as birthweight falls (Barker 1992; Osmond et al. 1993). Hypertension is also related to low birthweight; systolic and diastolic blood pressure in adult life rising with falling birthweight (Gennser, Rymark & Isberg 1988; Barker et al. 1989). Moreover, serum cholesterol and low density lipoprotein concentrations, both additional cardiovascular risk factors, are elevated in adults who were low birthweight babies (Barker et al. 1993). Other organ systems are also affected. Reduced numbers of renal nephrons have been noted in fetuses and infants with severe intrauterine growth restriction (Hinchliffe et al. 1992). As renal function is intimately related with cardiovascular physiology the implications of these findings warrants further investigation. Adult lung function is also impaired in those who were low birth weight babies, even after correction for premature gestational age (Barker 1992; Rona, Gulliford & Chinn 1993).

In combination, these data suggest that there are critical periods of organ development during intrauterine life which determine future structure and function. Adverse influences, such as nutrient deprivation, during critical periods of fetal life may impair normal development and permanently alter the physiology of these organ systems; thus "programming" the fetus for adult disease. This phenomenon is well recognised in animal models (Hahn 1984; Lucas 1991). As an example, chronic fetal hypoxia may activate the human fetal renin-angiotensin system (Graham et al. 1992). Since angiotensin is a vascular growth factor (Lever 1986) it may induce vascular hypertrophy, which has been demonstrated in placental vessels from IUGR pregnancies (Fok et al. 1990), and so alter the mechanical properties of vessels, increasing vascular resistance (Bertrand et al. 1993). In the spontaneously hypertensive rat, inhibition of angiotensin II production during early neonatal life can restore blood pressure recordings to "normal" physiological parameters, thus also demonstrating that early correction may prevent permanent damage and even restore normal function (Martin et al 1991).

1.5 IDENTIFICATION OF THE GROWTH RESTRICTED FETUS

Ultrasound derived measurements of fetal size, employing head circumference (HC), abdominal circumference (AC) and femur length (FL) can identify the small fetus, though generally, the estimates will be accurate to within 10-15% of the actual birthweight (Neilson 1990). However, reliably distinguishing the fetus which is also growth restricted *in-utero* is more difficult (Seeds 1984). Recent studies evaluating perinatal outcome have highlighted two additional ultrasound features which differentiate the small fetus which is also compromised and therefore at risk of perinatal death from one which is healthy; these are reduced amniotic fluid volume and abnormal umbilical artery Doppler waveforms

1.5.1 AMNIOTIC FLUID VOLUME

The origins of amniotic fluid are still poorly understood. It is clear that during the second half of pregnancy, most amniotic fluid is produced from the fetal kidneys. It is then swallowed by the fetus, absorbed from the fetal gut into the fetal blood stream and then circulated through the renal glomeruli to produce fetal urine, excreted into the amniotic fluid (Brace 1994). Agenesis and maldevelopment of the fetal renal tract (Kaffe et al. 1977) or impaired fetal swallowing result in reduced or excessive amounts of liquor respectively, confirming this circulation pathway (Brace 1991)

In the absence of structural abnormalities of the renal tract, reduced volumes of amniotic fluid correlate significantly with poor fetal outcome (Manning et al. 1981; Chamberlain et al. 1984; Rutherford et al. 1987; Moore et al. 1989). The reduction in amniotic fluid is thought to reflect impaired renal perfusion (Arduini & Rizzo 1991), a consequence of fetal hypoxia, when blood is redirected from non-vital organs such as muscle, skin and kidney to vital organs such as the heart and brain (Block et al. 1984; Al-Ghazali et al. 1989). Amniotic fluid volumes are therefore an indirect marker of fetal hypoxia, particularly in the preterm fetus (Philipson et al. 1983; Chamberlain et al. 1984).

1.5.2 UMBILICAL ARTERY DOPPLER WAVEFORMS

1.5.2.1 PRINCIPLES OF DOPPLER ULTRASOUND

Doppler ultrasound permits non-invasive, *in-vivo,* evaluation of fetal blood flow by utilising the Doppler principle which states that a sound wave reflected from a moving target i.e. blood cells returns at different frequency from the incident soundwave. The change in frequency, proportional to the velocity of the moving object, is known as the frequency shift or Doppler shift (Trudinger 1994).

In arterial vessels, blood flow is more rapid during systole than diastole while in veins, blood flow velocity is generally constant. Blood also flows more rapidly in the centre of a vessel

when compared to blood flow near the vessel wall. Thus at any one point in a vessel a spectrum of frequency shifts (proportional to the velocity of blood) are obtained. These can be averaged by computer and plotted against time to produce a smoothed waveform known as the flow velocity waveform (Figure 1.1).

The technique was first applied to the fetal umbilical circulation in 1977 (Fitzgerald & Drumm 1977). Using ultrasound estimates of vessel diameter and fetal weight, umbilical artery flow rates may be calculated from the flow velocity waveform (FVW) (Griffin, Cohen-Overbeck & Campbell 1983; Cohen-Overbeck , Pearce & Campbell 1985). These estimates are however, subject to large margins of error and therefore of limited value (Eik-Nes, Marsal & Kristofferson 1984). A qualitative assessment of placental blood flow may be derived from the diastolic component of the FVW which reflects downstream placental impedance (Skidmore & Woodcock 1980) and is independent of these other variables.

1.5.2.2 **FETAL UMBILICAL ARTERY DOPPLER STUDIES**

During normal pregnancy placental vascular impedance falls with advancing gestational age (Pearce et al. 1988; Hendricks et al. 1989) and is not significantly altered by factors such as maternal exercise (Morrow, Ritchie & Bull 1989) and fetal blood viscosity (Giles & Trudinger 1986; Fairlie et al. 1989). Several studies have shown however, that profound reductions in fetal heart rate may prolong the cardiac cycle sufficiently that absent end diastolic flow velocity is seen (Mires et al. 1987; Van den Wijngaard, van Eyck & Wladimiroff 1988).

Studies in selected high risk pregnancies noted to be small for dates, have now demonstrated that in severe cases the diastolic component of the FVW is reduced, absent or even reversed (Jouppila et al. 1984; Laurin et al. 1987; Fairlie et al. 1991), indicating increased placental vascular impedance (Adamson et al. 1990). This finding is significantly correlated with high perinatal morbidity and increased perinatal mortality rates of 50-90% (Brar & Platt 1988; Al-Ghazali et al. 1990; Woo et al. 1987). Seven randomised controlled trials have now demonstrated the clinical value of abnormal umbilical artery Doppler waveforms (Neilson & Grant 1989) and shown that by intensive monitoring and appropriate intervention, the perinatal loss in pregnancies with IUGR may be reduced (Divon et al. 1989).

In view of these results, umbilical artery Doppler studies have also been employed as a screening test for IUGR in unselected populations. Used as a single measurement, most studies have found Doppler to be of little use (Dempster et al. 1989; Low 1991); ultrasound estimated fetal weight measurements proving to be a better predictor (Divon et al. 1988). Furthermore, serial measurements in large, unselected antenatal populations at 28, 32

(Sijmons et al. 1989) and 38 weeks (Beattie & Dornan 1989) did not improve the prediction rate of growth restricted neonates.

While umbilical artery Doppler studies appear to be of little value as a screening test, amongst high risk pregnancies it provides an excellent means by which to distinguish the small fetus which is growth restricted and at risk from the fetus which is simply small but healthy (Burke et al. 1991). Since AEDFV is so intimately associated with severe intrauterine growth restriction, it is imperative that we understand the mechanisms by which abnormal umbilical artery Doppler is generated; in so doing the mechanisms resulting in restricted fetal growth might also be elucidated.

1.6 PATHOLOGICAL BASIS OF ABNORMAL UMBILICAL DOPPLER.

1.6.1 ANATOMICAL DATA

Histological studies in placentas from human pregnancies complicated by IUGR and AEDFV have suggested a reduced number of arterial vessel profiles within small stem villi (Giles, Trudinger & Baird 1985; McGowan, Mullen & Ritchie 1987). This was thought to occur as a result of vessel "occlusion" - possibly as a result of vessel spasm or thrombosis. Since growth restricted fetuses are also known to have reduced numbers of platelets (Van den Hof & Nicolaides 1990), despite an increase in platelet turnover (Wilcox & Trudinger 1991), it was proposed that the reduction in stem arterial profile number was a consequence of vessel obliteration by platelet emboli.

Fetal sheep studies confirmed that progressive embolisation of the umbilical circulation could reduce umbilical blood flow (Trudinger et al. 1987) and reproduce similar abnormal umbilical Doppler waveforms to those seen in IUGR pregnancies (Morrow et al. 1989). Further data from the sheep model demonstrated that the inverse relationship between umbilical blood flow, and the umbilical Doppler waveform was not linear, but exponential (Copel et al. 1990; Schmidt et al. 1991). With embolisation techniques, placental vascular impedance must be increased by up to 700% to reproduce AEDFV (Morrow et al. 1989). This equates, by mathematical modelling techniques, with at least a 50% obliteration of the placental vascular bed (Todros, Guiot & Pianta 1992).

1.6.2 VASOMOTOR CONTROL OF FETOPLACENTAL BLOOD FLOW

1.6.2.1 HUMAN DATA

Vasomotor mechanisms have also been implicated in the pathophysiology of IUGR. Low dose aspirin (75mg/day) given to pregnant women at risk of IUGR has been shown to

increase birthweight and gestation at delivery when compared with controls (Wallenberg & Rotmans 1987; Trudinger et al. 1988; Uzan et al. 1991). Since aspirin crosses the human placenta into the fetoplacental circulation (Jacobson et al. 1991), it may exert a direct therapeutic effect within the villous circulation as well as preventing damage within the uteroplacental circulation. There are two mechanisms whereby aspirin could work. Firstly it may counteract the excessive release of thromboxane A2, a known vasoconstrictor, resulting from increased fetal platelet turnover (Van den Hof & Nicolaides 1990; Wilcox & Trudinger 1991). Secondly aspirin may selectively inhibit placental villous production of thromboxane A2 (Nelson & Walsh 1989). Small stem villous arterioles from patients with pre-eclampsia (a condition which is often associated with IUGR) have been shown to exhibit spontaneous oscillations (Inayatulla et al. 1993) which are inhibited by indomethacin. These data suggest abnormal local production of thromboxane A2 within the villous core; another potential site where aspirin could favourably influence fetoplacental blood flow. However, aspirin cannot restore umbilical artery Doppler waveforms to normal in pregnancies complicated by AEDFV (Trudinger et al. 1988).

Endothelial regulation of fetoplacental vascular tone may also be abnormal in pregnancies complicated by IUGR. Production of the vasodilator prostacyclin, by the umbilical artery has been shown to be reduced in pregnancies complicated by pre-eclampsia (McLaren et al. 1987) and IUGR (Stuart et al. 1981) suggesting impaired vasodilatation. However, umbilical venous levels of another vasodilator, ANP, are elevated in IUGR pregnancies (Kingdom et al. 1992). and functional (guanylate cyclase-coupled) vascular receptor expression for ANP in fetoplacental vessels is actually increased (Kingdom et al. 1993) suggesting that ANP-mediated vasodilatation is likely to be enhanced in severe IUGR. This however, may be an adaptive mechanism to compensate for excessive vasoconstriction.

In keeping with this, elevated levels of ET-1, a potent vasoconstrictor, have also been found in samples of umbilical venous blood obtained from IUGR pregnancies complicated by abnormal Doppler (Hartikainen-Sorri et al. 1991, McCarthy et al. 1993). Moreover, the villous core receptors for ET-1 in such cases appear to be functional (McQueen et al. 1993). Hypoxia increases cultured endothelial cell production of ET-1 *in-vitro* (Heida 1990) suggesting a link to abnormal umbilical Doppler pregnancies. However perfusion of the normal human placental cotyledon under hypoxic and acidotic conditions failed to demonstrate increased release of ET-1 into the fetal venous perfusate (Kingdom et al. 1993).

Systemic regulation of the fetoplacental circulation, by fetal endocrine signals, may likewise be deranged. Fetal plasma levels of renin (Weiner & Robillard 1988; Tannirandorn et al. 1990) and angiotensin II (Broughton-Pipkin et al. 1977; Kingdom et al. 1993) are elevated in cord venous blood in some cases of IUGR. This may relate to hypoxia-mediated activation

of fetal renal production of renin (Graham et al. 1992). Local, as opposed to systemic, production of angiotensin II may also be altered in pregnancies complicated by IUGR but no data is yet available from pathological placentas.

1.6.2.2 ANIMAL DATA

Additional data is available from animal models. Acute hypoxia which has been postulated to induce vasospasm in IUGR pregnancies does not increase placental vascular impedance (Parer 1980) nor does it alter the Doppler waveform in the ovine model, despite redistribution of blood flow to the fetal coronary and cerebral circulation (Morrow et al. 1990). Moreover, fetal hypoxic acidemia produced (pH 7.0-7.2) only minor, insignificant changes in the umbilical Doppler waveform until severe acidosis (pH <6.8) induced fetal bradycardia, just prior to fetal death (Morrow et al. 1990).

Despite increasing ovine fetal blood viscosity by 60% (Morrow et al. 1990), significantly less than the increase noted in human growth restricted pregnancies (Giles, Trudinger & Palmer 1986; Steel et al. 1989), no significant change in the FVW was noted. Several vasoconstrictive agents have also been evaluated but though they increased placental impedance and reduced placental blood flow, the majority have effected little change in the diastolic component of the FVW (Irion & Clark 1990; Irion, Mack & Clark 1990). The thromboxane mimetic U46619 infused continuously at high pharmacological concentrations increased impedance and reduced the diastolic FVW (Trudinger et al. 1989; Irion & Clark 1990). However, in human pregnancies, inhibition of thromboxane synthesis, produces no improvement in the FVW. Vasoconstriction alone therefore does not equate with changes in the diastolic FVW.

1.6.3 HOW ARE ABNORMAL DOPPLER WAVEFORMS GENERATED?

It can be seen from both the human and animal data presented, that there is no conclusive evidence to support a vasomotor basis for the generation of abnormal FVW. Moreover, vasoconstriction alone, despite increasing placental vascular impedance and reducing umbilical blood flow, does not necessarily result in an abnormal Doppler waveform. It is therefore unlikely that abnormal umbilical Doppler is the consequence of a primary vasomotor factor, though obviously, vasomotor agents may modify or compound other factors.

Anatomically, a reduction in stem villous arterial number has been suggested in IUGR pregnancies with AEDFV. The idea of placental embolisation is plausible and has been shown to effect appropriate changes in the ovine FVW. Areas of placental vessel obliteration are a feature of normal placentas (Bracero et al. 1989) but to date there is no evidence of excessive "emboli" in the placentas of growth restricted pregnancies. Therefore while an

anatomical basis for abnormal Doppler is realistic, the mechanisms by which it might be produced are still unproven.

The biochemical and clinical features of the growth restricted fetus (section 1.3.1) which indicate intrauterine starvation, primarily as a result of impaired placental transport (Bernardini 1989), also suggests an anatomical basis for the generation/presence of abnormal umbilical artery Doppler waveforms. Since placental transfer occurs maximally in the distal, intermediate and terminal villi, these peripheral villi are very likely to be quantitatively or at least qualitatively, abnormal in IUGR pregnancies with AEDFV.

These observations can be reconciled if the reduced numbers of arterial vessels seen in IUGR pregnancies with AEDFV arise as a consequence of abnormal or impaired placental development affecting not only proximal stem villi but also these distal "exchange" villi. To date this avenue of investigation has not been pursued.

In order to evaluate if structural changes might generate abnormal Doppler waveforms in growth restricted pregnancies, we must first appreciate how low impedance is generated and placental transport mediated in the normal developing placenta.

1.7 EARLY PLACENTAL DEVELOPMENT

Early placental development begins with adhesion and implantation of the hatched blastocyst. It is formed from the outer layer of the blastocyst, the syncytiotrophoblast, which penetrates between the endometrial cells through the basement membrane and into the endometrial stroma (Schlafke & Enders 1975). As it enlarges, the syncytiotrophoblast becomes vacuolated, forming a lacunar system, engulfing maternal capillaries in its track. Radial outgrowths of cytotrophoblast cells, the inner and proliferative cell layer of the blastocyst, invade the syncytiotrophoblast network to form primary villi. It is these cells which also reach the distal extremities of the syncytiotrophoblast and extend laterally to form the trophoblastic shell (Hertig & Rock 1945). Some of these cytotrophoblast cells differentiate and become invasive, extending the size of the placental bed. This controlled invasion of the endometrium, unlike that of malignant tumours is precisely regulated; the first trimester trophoblast increasing its expression of type IV collagenase (Puistola et al. 1989), proteases (Fisher et al. 1985) and receptors for fibronectin, laminin and collagen (Loke et al. 1989). With advancing gestation, these enzymes and receptors are down regulated, and the invasive process thus modified (Lim 1993).

By the fourteenth day following conception the extraembryonic mesenchyme, derived from the embryonic disc (Luckett & Beier 1978), invades the cytotrophoblast creating secondary villi. The latter are vascularised from within (Demir 1989) and receive blood from the developing fetal circulation as early as the 6th week of gestation. At this stage there is no formal intervillous circulation as assessed by aortography (Burchill 1967) hysteroscopy (Hustin & Schapps 1987) or colour Doppler ultrasound (Schapps & Hustin 1988), so the developing placenta is effectively a barrier between the mother and embryo. First trimester transvaginal chorionic villous biopsy specimens confirm the absence of a formal intervillous circulation since these specimens are not normally contaminated by maternal blood (Hustin & Schapps 1987). Furthermore, the early developing placenta and embryonic tissues are hypoxic relative to the mother (Rodesch et al. 1992). With subsequent perfusion of the intervillous space, once maternal spiral arterioles are breached around 10-12 weeks following fertilisation (Hustin, Schapps & Lambotte 1988), the usual haemochorial arrangement between maternal and fetal blood is established. Oxygen tension in the placenta and fetus then appears to more closely match that of maternal blood (Rodesch et al. 1992). During these early weeks of pregnancy, relative hypoxia may protect the fetus and its developing organs from the damaging effects of free radicals produced at higher oxygen tensions.

It is not clear how the developing fetus obtains maternal nutrients during these first 12 weeks of pregnancy since no formal intervillous circulation exists. However, placental villi as early as three weeks post-implantation express high concentrations of insulin receptors (Desoye et al. 1994) suggesting that even at this early stage development of the fetoplacental unit is influenced by maternal factors, perhaps through plasma filtrates or uterine gland secretions.

The precise timing and order of early placental development is of unquestionable importance. There is now evidence to suggest that "unexplained" early pregnancy failure may be related to poor trophoblast invasion (Michel et al. 1990), resulting in an incomplete trophoblastic shell (Hustin et al. 1990) and the untimely entry of oxygenated maternal blood, under relatively high pressure to the intervillous space (Schapps et al. 1988).

1.8 DEVELOPMENT OF THE FETO-PLACENTAL CIRCULATION

The early developing placenta and umbilical cord can be clearly visualised with transvaginal ultrasound (Hustin & Schapps 1988). During the first trimester, the umbilical circulation is characterised by high impedance seen as absent end-diastolic flow velocity (AEDFV) in umbilical artery Doppler studies (Kurjak et al. 1990; Jauniaux et al. 1992). At this time, impedance within the uterine circulation is also high and mean blood flow in the uterine

artery low (Thaler et al. 1990). There is then a dramatic increase in uterine artery blood flow around 12-14 weeks gestation as extravillous cytotrophoblast cells invade the maternal spiral arteries (Pijnenborg et al. 1981). This invasion results in destruction of the arterial elastic media and the formation of flaccid, low-impedance uteroplacental vessels, unresponsive to vasoactive agents which are thus able to accommodate increased uterine blood flow (Brosens, Robertson & Dixon 1967; de Wolf, Robertson & Brosens 1980). The changes occurring in spiral artery structure are reflected by an increase in uterine artery diastolic flow velocity, together with a loss of the dichrotic notch (Bewley, Cooper & Campbell 1991; Bower, Bewley & Campbell 1993). This also co-incides with the appearance of end-diastolic flow in the umbilical circulation (Jauniaux et al. 1992; Kurjak et al. 1990). By 18-20 weeks gestation, in normal pregnancy, the uterine circulation consistently demonstrates a low impedance Doppler waveform. Likewise, end-diastolic flow velocity is consistently present in the umbilical artery, indicating that even at this early stage, impedance within the placental vascular bed is falling (Hendricks et al. 1989).

The appearance of umbilical artery end-diastolic flow velocity, inferring low placental vascular impedance, is thus a physiological event in early pregnancy and necessary to accommodate the large volume of fetal blood which enters the umbilical circulation. Using isotope-labelled microspheres in the sheep, this has been calculated to be 50% of the fetal cardiac output (Rudolph & Heymann 1967) and a similar value has been obtained the human fetal circulation using Doppler ultrasound (Eik-Nes 1980; Giles et al. 1986).

Since the fetoplacental circulation is not innervated (Reilly & Russell 1977; Fox & Khong 1990), low impedance to blood flow must be conferred by the anatomy of the circulation, vasomotor influences on vascular smooth muscle, or a combination of both of these mechanisms.

1.9. VASOMOTOR CONTROL OF FETOPLACENTAL IMPEDANCE

The rami and ramuli chorii branches of the fetoplacental arterial circulation containing fetal arteries and arterioles are muscularised. Thus total impedance to blood flow may be altered through changes in the smooth muscle tone of these vessels.

1.9.1 VASODILATOR MECHANISMS

Smooth muscle contractility is influenced by substances produced within the overlying endothelial cells (Vane, Anggard & Botting 1990). Prostacyclin has both vasodilator and

platelet anti-aggregatory properties, and is synthesised in greater amounts in the umbilical artery than in subchorionic vessels (Benedetto et al. 1987). However its role in mediating local placental vasodilatation is questioned since inhibition of synthesis has no effect on perfusion pressure in the dually-perfused cotyledon (Jacobson et al. 1991) or the isolated umbilical artery (Templeton 1991).

Endothelial-derived relaxing factor (EDRF), or nitric oxide, which is produced from the conversion of L-arginine to citrulline by the enzyme nitric oxide synthase (NOS) (Palmer, Ferrige & Moncada 1987) is another local vasodilating agent. The activity of EDRF was first demonstrated in the umbilical vessels (Van der Voorde, Vanderstichele & Leusen 1987) followed by the pre-constricted dually-perfused cotyledon (Myatt, Brewer & Brockman 1991). Interestingly the vascular sensitivity to EDRF may increase in the more distal branches of this circulation (Chaudhuri et al. 1991). NOS has now been located by immunocytochemistry in both fetoplacental vascular endothelium and interestingly also in the villous syncytiotrophoblast (Myatt et al. 1993a). The regulation of EDRF production within the placenta *in-vivo* is not understood at the present time but mechanical stretch of endothelial cells, and vasoconstrictor mechanisms are likely to be important factors. EDRF probably has an important physiological role in the local regulation of villous blood flow.

Smooth muscle cells have specific receptor sites for atrial natriuretic peptide (ANP) which have been characterised in placental stem vessels (Salas et al. 1991; McQueen et al. 1991). ANP does not appear to be synthesised within the normal term placenta (Inglis et al. 1993), suggesting an endocrine action from peptide released into the circulation from the fetal heart (Wharton et al. 1988), though a more recent study suggests that umbilical vein endothelial cells may synthesise ANP (Cai et al. 1993). Umbilical cord levels of ANP are higher than corresponding maternal levels, and are in the range that would stimulate smooth muscle relaxation (Kingdom et al. 1992); a similar concentration of ANP antagonised the vasoconstrictor effects of angiotensin II in the perfused cotyledon (McQueen et al. 1991) suggesting that it has a role in promoting smooth muscle relaxation under resting conditions. ANP levels increase following intravascular transfusion suggesting that ANP-mediated vasodilatation may increase to assist the fetus to cope with blood volume expansion (Kingdom et al. 1991). Other peptides may have endocrine or paracrine actions to reduce smooth muscle contractility such as parathyroid hormone, its related peptide and calcitonin gene related peptide (Mandsager et al 1993).

1.9.2 VASOCONSTRICTOR MECHANISMS

The anatomy of the fetoplacental circulation, together with the vasodilator mechanisms described above create the conditions for a low-impedance system but there are several

mechanisms whereby vascular tone may be raised. Locally, within the placenta, the trophoblast produces thromboxane; platelets may also release both thromboxane and 5-hydroxytryptamine - each of which constricts the fetoplacental circulation *in-vitro* (Mak et al. 1984). The arterial smooth muscle layers synthesise thromboxane; production of which, is stimulated by oxygen. This mechanism may be important for closure of the umbilical arterial circulation at birth (Templeton et al. 1991). The latter mechanism may be inactive *in-utero* due to the low oxygen tension, particularly as pregnancy advances (Soothill et al. 1986). Arachidonic acid products of the lipoxygenase pathway, such as leucotriene B4, are less potent vasoconstrictors than is thromboxane (Thorp, Walsh & Brath 1988).

Endothelial cells are now known to synthesise the vasoconstrictor peptide endothelin (ET-1) (Yanagisawa et al. 1988). Synthesis of ET-1 has been demonstrated within the human placenta (Benigni et al. 1991; Hemsen et al 1991) and specific receptor sites have been demonstrated on smooth muscle cells within stem villi (Wilkes et al. 1990; Roubat et al. 1991). ET-1 is a powerful and direct vasoconstrictor of the fetoplacental circulation (Myatt et al. 1991). Shear stress is an important mechanism regulating ET-1 release (Milner et al. 1990) and it is thus likely that ET acts in a local manner to regulate villous blood flow, in conjunction with EDRF production.

The perfused placental cotyledon constricts to angiotensin II (McQueen et al. 1991) and interestingly to renin (Glance et al. 1984). Specific binding sites for these substances have been identified in the fetoplacental vasculature (Cook Craven & Symonds 1981; Tence & Petit 1989). Renin, and angiotensin II, are produced in the developing fetal kidney indicating an endocrine mechanism of action within the fetoplacental circulation (Graham et al. 1992). Since the necessary components for angiotensin II production are expressed locally (Ihara, Taii & Mori 1987; Keyurrangual et al. 1991) it is possible that local generation of angiotensin II also occurs within the stem villus.

More extensive reviews of substances which can influence fetoplacental vascular tone are available (Boura & Walters 1991; Myatt 1992). It is very likely that several factors act in concert to regulate fetoplacental blood flow, both from a regional (endocrine) perspective, such as ANP, and locally in a paracrine manner (EDRF, prostaglandins and ET-1). Establishing the effect of gestational age on the production and action of both vasodilator and vasoconstrictor agents is difficult, and complicated by the structural changes which occur during pregnancy within the placental vasculature. For example, the expression of functional ANP receptors in fetoplacental vascular smooth muscle increases with gestation (McQueen et al. 1993) which might imply greater vascular bed sensitivity to ANP as gestation advances, but these findings may merely arise from increased vascularisation of the placenta.

Such studies emphasise that though vasoactive agents may be involved in umbilical resistance, their role, even in normal pregnancy, can only be interpreted in the light of placental vascular anatomy. Moreover, under conditions of maximal vasodilatation it is likely that maximal impedance is generated in the non-muscularised mature intermediate villi and their terminal buds: smooth muscle activity must favour vasoconstriction if ramuli chorii (tertiary stem villi) are to significantly influence overall vascular impedance. In pathological pregnancies therefore, it is vital that placental anatomical structure is first established in order that further studies which locate the production, or action of autocoids *in-situ* may be reliably interpreted and the pathophysiological processes resulting in restricted fetal growth clarified.

1.10 **DEVELOPMENT OF THE MATURE PLACENTA**

New villi are all derived from buds of proliferating trophoblast, known as villous sprouts, which obtain a stromal core and are vascularised by local fetal blood vessels to form primitive mesenchymal villi. During the first and second trimester, further differentiation of these villi produces immature intermediate villi which following stromal fibrosis are transformed into stem villi. By the 20th week of gestation mature intermediate villi are formed in preference to their immature counterparts. These villi do not differentiate into stem villi and therefore few new stem villi are added beyond this gestation. When growth of mature intermediate villi is exceeded by growth of their capillary loops, outpockets of terminal villi are passively formed.

Together, these villi form the functional unit of the mature placenta known as the "placentone", a villous tree suspended in the intervillous space and perfused by a central spiral arteriole. The branching arrangement of the villi, and the vessels within, is complex but important to appreciate (Castellucci et al. 1989; Leiser et al. 1991) and is illustrated in Figures 1.2 a-c. The chorionic artery penetrates through the chorionic plate into the truncus chorii; this divides to produce about 4 divisions of rami chorii, each of which divides dichotomously to form a further 3-30 (mean 11) generations of ramuli chorii. Rami and ramuli chorii represent the stem villi which are characterised by the presence of fetal arteries and veins and/or fetal arterioles and venules. Distally, the mature intermediate villi give rise to terminal villi, the final ramification of the villous tree and the site of maximal feto-maternal exchange. By term terminal villi account for 40% of total placental volume and provide a surface area for exchange of $8m^2$ (Leiser, Kosanke & Kaufmann 1991)

There is now abundant evidence to confirm that the placenta is not merely a passive membrane separating maternal and fetal circulations but a functional organ, ensuring adequate substrate transfer from mother to fetus. This is achieved by two principle

mechanisms, facilitated diffusion and active transportation. Glucose for example, is transferred by facilitated diffusion, bound to a carrier in the micovillous membrane of the syncytiotrophoblast while amino acids are actively transported by specific carrier proteins also located in the syncytium. The development of peripheral villi with extensive expansion of the syncytial surface area is therefore crucial to meet the metabolic requirements of the growing fetus.

1.11 ANATOMICAL BASIS OF FALLING IMPEDANCE

The maturation of the villous tree is undoubtedly a major factor in the development of a low-impedance high-flow circulation on the fetal side of the placenta. The umbilical arteries may communicate both within the cord (Gupta et al. 1993) and in Hyrtl's anastomosis at the cord root (Norvendall et al. 1991). Distally they branch without any further communication until terminal villous capillaries are formed (Leiser et al. 1991), though Boe (1953) has demonstrated the presence of arterio-arterial anastomosis in more peripheral areas. Shunting through these vessels is of little significance in normal circumstances, when vascular pressure is low, but may be important in pathological situations. Resistance to blood flow within the vascular tree increases continuously towards the muscularised arterioles of the small stem villi (small ramulii). Thereafter, due to rich branching of the mature intermediate villi, blood flow resistance is reduced. Under normal term conditions, the capillaries of the mature intermediate and terminal villi are long (2000 to 4000μM), winding, branched tubes with focal sinusoidal dilatations (up to 40μM in diameter) (Kaufmann et al. 1985a) which serve to further reduce impedance to blood flow (Kaufmann 1988). It is still a matter of discussion how far these terminal structures contribute to overall feto-placental vascular impedance but in view of these findings it is likely that not only the properties of arteries and arterioles but also the dimensions and structure of the capillary bed are of importance for overall vascular impedance in the fetoplacental circulation.

As impedance in the umbilical circulation is falling, the structure of both the placental villi and the placental villous tree is also changing to expand the placental vascular bed and the villous surface area available for exchange (Jackson, Mayhew & Boyd 1992). Within each villus, both the number of capillaries, and the volume of each villus occupied by capillaries, rise dramatically with advancing gestation (Jauniaux et al. 1991; Jackson, Mayhew & Boyd 1992) thus increasing the diffusive conductance to oxygen 80 fold from 10-12 weeks until 38-41 weeks.

These changes, in association with the increase in uterine blood flow, result in a more efficient haemochorial arrangement between maternal and fetal blood reducing the materno-

fetal diffusion distance from more than 50 μM to a mean of 4 μM at term (Bernischke and Kaufmann 1990) and increasing oxygen diffusion conductance significantly (Mayhew, Jackson & Boyd 1993).

1.12 **HYPOTHESIS**

In view of these findings it is clear that as the placenta and the fetoplacental vascular network continuously develop during pregnancy, vascular impedance progressively falls, resulting in a proportionate increase in feto-placental blood flow, an increased villous surface area and therefore improved materno-fetal exchange of nutrients and oxygen sufficient to meet the demands of the growing fetus.

If this process of placental villous and vascular development is interrupted, placental vascular impedance will remain high, placental blood flow restricted and placental transport impaired resulting in poor fetal growth and the clinical picture of severe intrauterine growth restriction with AEDFV in the umbilical artery.

1.13 **AIMS OF THE THESIS**

In the placentas of pregnancies complicated by severe IUGR and abnormal umbilical artery Doppler waveforms we wish therefore;

(1) To characterise the branching pattern of stem villous vasculature (chapter 3)

(2) To study the external appearance of peripheral (intermediate and terminal) villi and the arrangement of the capillary networks within them (chapters 4 and 5).

(3) To study the internal structure of terminal villi (chapter 6, 7 and 8)

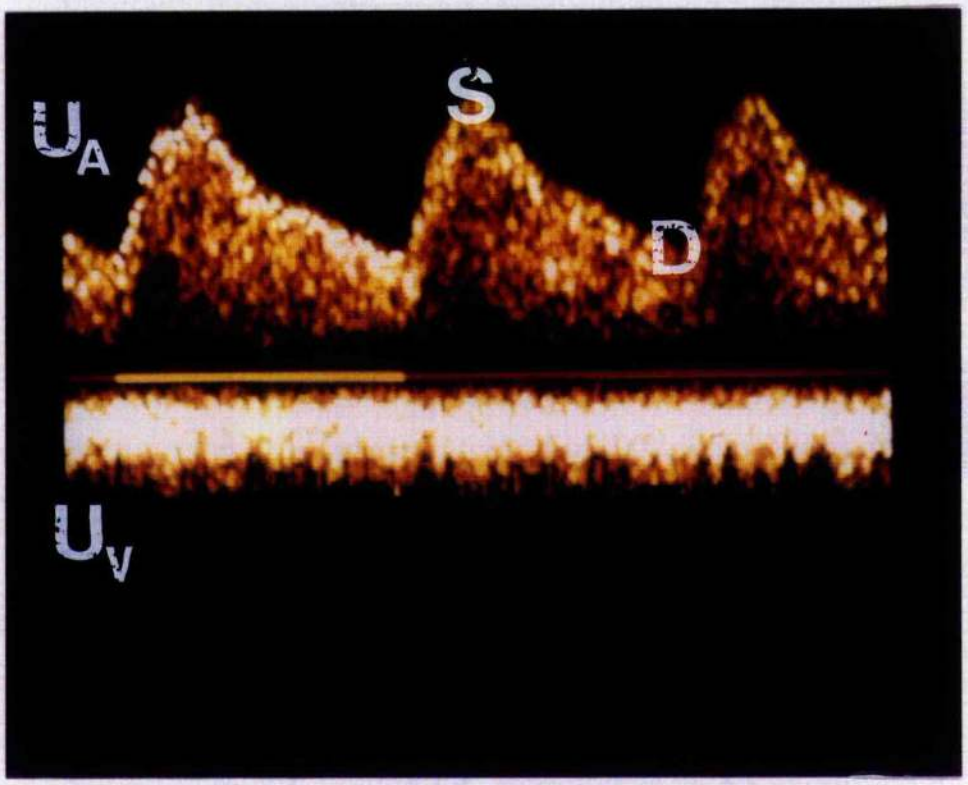

Figure 1.1 Doppler flow velocity waveform of an umbilical artery (U_A) and the umbilical vein (Uv). The peaks in the U_A correspond to systole (S) in the cardiac cycle while the troughs reflect diastole (D)

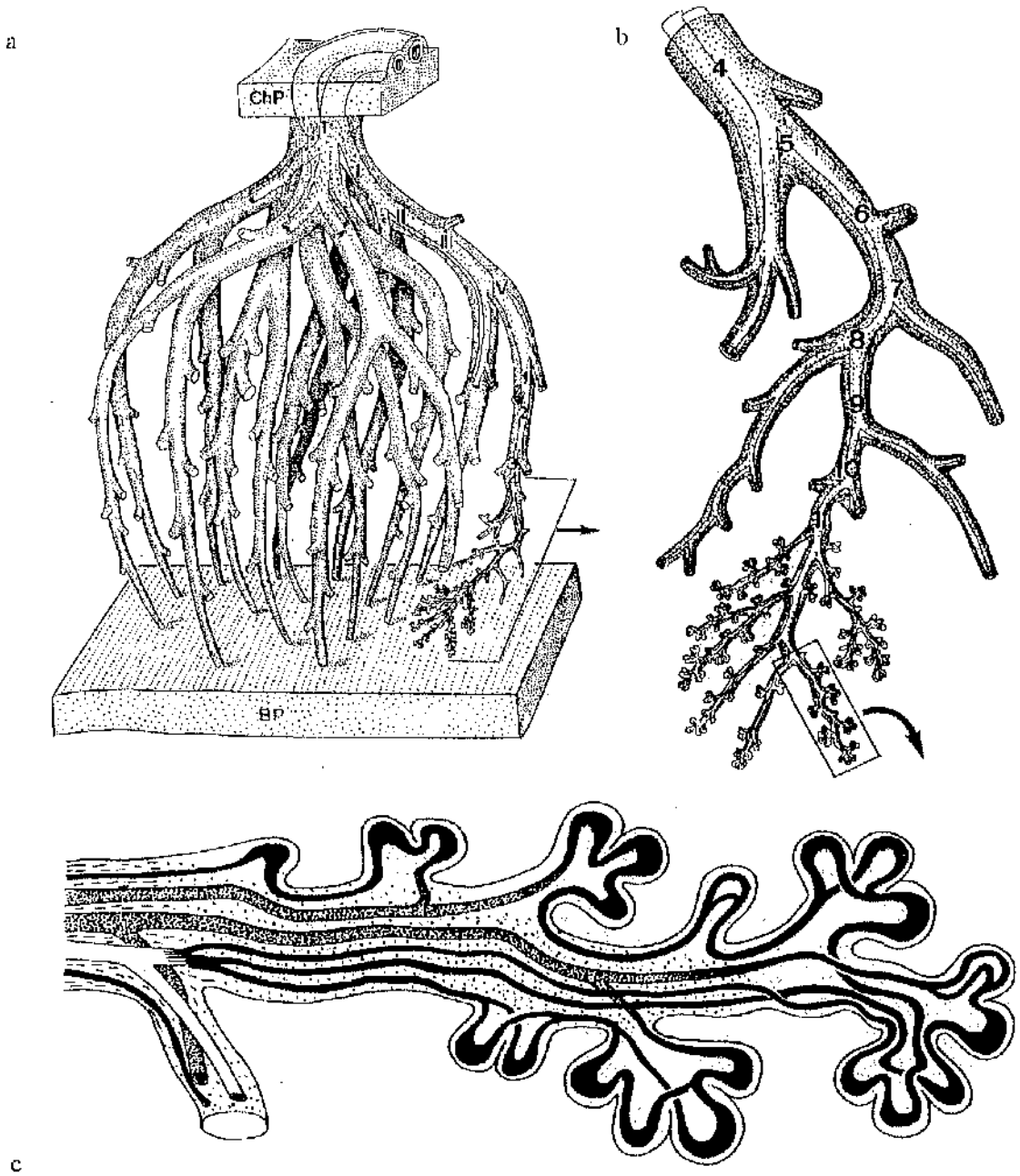

Fig 1.2 a - b.

Diagrammatic representation of the branching patterns of the villous tree. Ch P = Chorionic plate; BP = basal plate; T = truncus chorii; I-IV = for generations of rami; 1-11 = eleven generations of ramuli. The vessels are shown within the villous tree

1c.

Fetal vessel architecture of an ending stem villous of the last ramulus (line hatched) and a terminal convolute composed of one mature intermediate villus (slightly point shaded) together with its terminal villi (unshaded). Arterioles are point shaded, capillaries are black and venules are unshaded. Note the long capillary loops supplying several terminal villi in series.

CHAPTER 2

MATERIALS AND METHODS

2.1 GENERAL METHODS

2.1.1. **Transmission electron microscopy**

Even with the best optics available, light microscopy has limited resolution - approximately 2 micrometers - and is therefore unsuitable for the evaluation of cellular ultrastructure. The discovery that electron beams could be focused through a magnetic field opened the way for the development of transmission electron microscopy (TEM) by Ruska and Knoll in 1931. Though the first images obtained were limited (Knoll 1935), the technique has now been significantly refined such that modern transmission electron microscopes resolve images of approximately 1-2 nm.

The "light source" of the TEM is an electron beam generated by the passage of an electric current through a tungsten filament. The latter is enclosed in a metal case (the cathode) maintained at a higher negative potential. This ensures that the electron cloud produced by the filament is repelled from the cathode and attracted through a small defect in the cathode case to an anode plate, at zero potential, below. The wavelength (and therefore speed) of the electron beam is inversely proportional to the potential between the anode and the tungsten filament and thus can be adjusted.

The electron beam is focused onto the specimen through a system of electromagnetic coils (the lenses). Some of the electrons pass straight through the specimen while others are deflected by atoms within the specimen. The resultant emerging beam is focused by a further powerful electromagnetic lens - the objective lens - positioned just below the specimen and projected onto a fluorescent viewing screen. Undeflected electrons, which passed straight through the specimen, form visible fluorescence on the screen while the deflected electrons leave dark areas in the image. The final image seen is therefore a density map, reflecting different atomic masses within the specimen field.

While highly magnified, well focused images can be obtained, only tiny areas of tissue (< 0.5 mm^2) can be examined at any one time. This is obviously of importance in a tissue such as placenta which demonstrates significant heterogeneity within each cotyledon (Fox 1964). To ensure that an accurate representation of placental structure is obtained, multiple samples, from randomly chosen areas of the cotyledon, must therefore be examined (Habashi, Burton & Steven 1983).

Furthermore since cellular ultrastructure is sensitive to even short periods of ischaemia or anoxia (Kaufmann 1985c), the speed of tissue fixation is of utmost importance. Using vascular perfusion techniques rapid fixation of placental tissue can be achieved within

minutes. It is however, critical that identical fixation techniques are applied to all the tissue specimens obtained.

2.1.2. **Scanning electron microscopy**

In contrast to the transmission electron microscope the scanning electron microscope (SEM) evaluates three dimensional structure, utilising the electron beam to examine the external surface topography of unsectioned tissues at a magnification of up to 500 times that of optical microscopes.

As with the TEM, the electron beam is generated by passing an electric current through a tungsten filament. It is then propagated through a series of electromagnetic lenses which focus the beam to a diameter of approximately 10nm and direct it onto the specimen. A lower voltage of beam is used for SEM thus ensuring that the majority of electrons do not pass through the specimen, but rather interact with the specimen surface forming low voltage secondary electrons. These are reflected from the specimen surface and attracted to a scintillator located near the tissue which converts the electrons into an electric current. Since secondary electrons cannot travel far, few electrons will reach the scintillator from troughs in the tissue and therefore little current will be generated, while a larger number of electrons will be detected from peaks in the tissue surface. The current produced controls the light intensity of a spot on a cathode ray tube, moving in synchrony with the incident beam. Therefore, the number of secondary electrons detected, and the intensity of the light spot will reflect changes in the surface topography.

SEM is an excellent medium by which tissue and vessel topography may be appreciated. However, the images obtained are only meaningful if the tissue or vessel cast examined accurately reflects the *in-vivo* structure.

2.1.3. **Vascular cast perfusion**

Conventional methods of tissue fixation cannot demonstrate the architecture of vascular beds, buried within tissues. For this purpose, casts of the vascular bed must be prepared and examined with the SEM.

Initial placental vascular studies employed vinyl acetate (Boe et al. 1950) as a casting medium. Once infused into the placental chorionic arteries, the medium was allowed to harden prior to digestion of the villous tissue with potassium hydroxide solution. Though the cast formed demonstrated the distribution of large vessels within the placenta it was too viscous to enter smaller vessels. With the introduction of low viscosity plastics which can

perfuse capillary vessels this problem was overcome and the three-dimensional arrangement of these vessels appreciated (Habashi, Burton & Steven 1983; Leiser & Kohler 1983; Leiser 1985).

It is difficult to establish how accurately vessel casts reflect the *in-vivo* structure of blood vessels. Injection of casting medium into vessels has been reported to induce vasospasm, resulting in incomplete perfusion of the vessels and/or a reduced calibre of the vessels casted (Tompsett 1970). Initial perfusion with muscle relaxing agents inhibits vasoconstriction; while the cast formed from these vasodilated vessels is not a true reflection of the *in-vivo* vessel calibre, is still suitable for evaluating the branching pattern and number of vessels present. Simple measures such as the use of warmed perfusates also prevent induced vasospasm. However, the main focus of the current study, was the structure of capillary vessels. Since these vessels are non-muscularised they are unlikely to be subject to these artefacts arising from vasospasm.

The size of vessels in a vascular cast may also be distorted by inappropriate perfusion pressures (Hodde & Nowell 1980) particularly as it is impossible to determine the "normal" perfusion pressure for each placenta, nor to quantify the effect of intrauterine pathology such as IUGR on "normal" placental perfusion pressures. Regulating perfusion pressures by the use of a syringe pump system or preset manual infusion rates ensures standardised fixation conditions but in spite of these manoeuvres caution is required when interpreting only vessel diameters from such casts.

As with any placental study, there is always concern that the area examined is not representative of the whole organ. However, several groups (Fox 1964; Boyd, Brown & Stewart 1980; Schuhmann & Wynn 1980; Habashi, Burton & Steven 1983) have now shown that differences within placental cotyledons are more marked than those between cotyledons. If therefore, the whole cotyledon is examined, a representative picture of the vascular bed will be obtained.

2.1.4. **Immunohistochemistry**

Immunohistochemical techniques permit specific antigens to be localised *in-situ*. The method was first described in 1942 by Coon et al. who demonstrated the pnuemococcal antigen using fluroscein coupled to antibody fractions of antisera generated against the bacteria.

Modern techniques utilise the same basic principles. The antigen is first localised using immune serum generated by immunising animals with the antigen (polyclonal antibodies) or from hybridomas (monoclonal antibodies). Polyclonal serum often contains unwanted

antibodies directed against other constituents of the original human antigen and is therefore less specific than monoclonal serum. Moreover, while specificity within a batch of polyclonal serum may be well defined, there is often great variation between batches of the serum. For these reasons, with the increasing availability of monoclonal serums, polyclonal antibody serums will soon become redundant.

Bound antibody is visualised using a fluorescent marker (fluroscein, texas red or rhodamine) or an enzyme:anti-enzyme reaction which yields a stable, visible reaction product (peroxidase:anti-peroxidase [PAP]; alkaline phosphatase:anti-alkaline phosphatase [APAAP]). Since enzyme reactions can be visualised with the light microscope, these sections can be counterstained and the antigen site localised more accurately.

The markers may be attached directly to the antibody or applied indirectly using single or multiple intermediate antibody steps. Indirect methods are more sensitive and were therefore employed for all the immunohistochemical reactions described in this thesis. The intermediate antibody used, directed against the primary antibody, was labelled with the vitamin biotin which has a high affinity for the protein streptavidin (Chaiet & Wolf 1964). By complexing the peroxidase enzyme complex to streptavidin, strong indirect binding of the enzyme system to the antigen was ensured (Figure 2.1). The bound complexes were visualised by addition of a hydrogen peroxide/chromagen mixture which was catalysed by the bound peroxidase to yield a visible product; the chromagen AEC forming a red deposit and DAB a brown deposit.

The exact protocols employed for each experiment utilising methods 2.2.2., 2.2.3. and 2.2.4 are described under the methods section of chapters 3, 4, 5, 6 and 7.

2.1.5. **Pipetting**

Solution volumes in the range 0.1 to 5mls were transferred using adjustable Finnpipettes. Larger volumes were dispensed using standard laboratory glassware.

2.1.6. **Weighing**

All quantities were measured using a Mettler AT250 electronic balance.

2.1.7. **pH Measurement**

Measurements of pH were performed using a digital pH/temperature meter obtained from Electronic Instruments Ltd. (UK.). The apparatus was standardised with a solution of pH 7 prepared from buffered tablets.

2.2 CLINICAL MATERIAL

Many previous studies which have attempted to address the question of placental structure in IUGR have been hampered by studying term pregnancies which were small-for-gestational age but many of which were healthy and not truly growth restricted *in-utero*.

Though single ultrasound measurements of fetal weight will establish if the fetus is small for gestational age additional ultrasound features - oligohydramnios (Manning et al. 1981) and abnormal umbilical artery Doppler waveforms are required to select the growth restricted fetus at risk (Erskine & Ritchie 1985; Fleischer et al. 1985; Freidman et al. 1985; Hackett et al. 1987; Woo, Laing & Lo 1987; Rochelson et al. 1987)

2.2.1 **Selection criteria**

Specific criteria were therefore used to define the growth restricted study group (Gabbe 1991); an estimated fetal weight <10th centile in a structurally normal fetus, no underlying maternal or fetal condition other than pre-eclampsia, absent end-diastolic flow velocity in the umbilical artery by Doppler ultrasound and oligohydramnios ([AFI] < 5th centile) as in other studies from this department (McQueen et al. 1993; Kingdom et al. 1994)

Pre-eclampsia was defined as the development of hypertension [blood pressure persistently >140/100 mm Hg] occurring after 24 weeks gestation and significant proteinuria [>300mg in a 24 hour collection] (Davey & MacGillivary 1988).

Since placental structure varies with gestational age (Kaufmann 1982), a gestational age matched control group were also required. These pregnancies fulfilled the following criteria; normal growth parameters, with birthweight >25th centile; umbilical artery RI < 90th centile; no fetal abnormalities; no maternal disease which would affect placental/fetal development.

2.3 IDENTIFICATION OF PATIENTS

The patients included in this study were all identified and delivered at either The Royal Maternity Hospital or The Queen Mother's Hospital, Glasgow during 1992 and 1993. Since the placenta is normally discarded following delivery, formal ethical approval was not mandatory. However, all the patients in this study gave informed verbal consent for the use of the placental tissue prior to delivery. In each case placental weight and macroscopic features were recorded in the notes and a sample of placental tissue sent for conventional histological examination.

In order to exclude the possibility of dating errors, gestational age in each case was confirmed by a booking ultrasound prior to 16 weeks of gestation. Immediately preceding delivery, a further ultrasound examination was performed by the author to confirm the estimated fetal weight, measure liquor volume and assess umbilical artery perfusion with Doppler ultrasound. Pregnancies complicated by structural or chromosomal fetal abnormalities were all excluded.

2.3.1 **Estimation of fetal weight**

Fetal weight was estimated by ultrasound from the fetal abdominal circumference, as described by Campbell and Wilkin (1975), on a transverse section of the fetal abdomen, perpendicular to the fetal spine in which the umbilical vein and stomach could be identified. The values obtained were converted to fetal weights using standard tables from the British Medical Ultrasound Society (BMUS). These were plotted against gestational age and related to birthweight centiles obtained from a local database (Smalls & Forbes 1983).

2.3.2. **Amniotic fluid volume**

Though the maximum depth of a single liquor pool is often used as an indicator of total liquor volume, evidence from the pregnant sheep model would suggest that summating the maximum depth of liquor within each of four uterine quadrants (the amniotic fluid index [AFI]) more accurately reflects total amniotic fluid volume (Moore & Brace 1988). A reduced AFI is significantly correlated with fetal outcome (Manning et al. 1981).

The patients in this study were therefore evaluated as follows; the uterus was divided into four quadrants and the single deepest amniotic fluid pool in each quadrant, free of umbilical cord and fetal limbs was measured; the transducer remained parallel to the patient's sagittal plane throughout. The four measurements obtained were summated to derive the AFI. Since

amniotic fluid volume varies with gestational age, the values were plotted against a reference range (Moore 1990) and values <5th centile defined as oligohydramnios.

2.3.3 **Assessment of umbilical artery perfusion**

The principles of Doppler ultrasound have been described in section 1.5.2.1. Umbilical artery Doppler waveforms may be obtained using either a pulsed or continuous Doppler ultrasound system. Pulsed Doppler emits short pulses of ultrasound which are received by the transducer before further pulses are emitted. Signals from a specific depth can therefore be analysed separately. Pulsed Doppler is frequently used in conjunction with real time ultrasound (duplex system) so that the vessel in question, the umbilical artery, may first be visualised, the portion of umbilical cord to be assessed localised and the pulse depth set appropriately. With continuous Doppler, ultrasound is continuously emitted and received. Thus signals from all moving objects within the beam will be received and plotted, limiting the specificity of this system.

With both systems low frequency movements from the walls of blood vessels can be excluded by the use of a filter (thump filter). If set too high, however, the filter will also exclude echoes from slow moving blood cells within the vessel, giving the false impression of reduced blood flow velocity. Thump filters of 50-100 Hz will exclude vessel wall movements yet still take account of low velocity blood flow.

For this study, Doppler studies of the umbilical artery were performed using duplex pulsed Doppler (Acuson 128, Mountain View, CA), with the patient in a semi-recumbent position to ensure minimal occlusion of the vena cava. Free floating loops of the umbilical cord, near the cord root were initially identified using colour flow imaging and during a period of fetal apnoea (Indik & Reed 1990), a minimum of ten consecutive waveforms recorded. The mean RI was calculated over three waveforms. These values were plotted against a reference range (Hendricks et al. 1989) and values <90th centile considered to be normal. When umbilical artery diastolic flow velocity was absent this was recorded as AEDFV. All Doppler waveforms were recorded with the high pass filter set at 50 Hz.

2.4 PATIENT GROUPS

2.4.1 **IUGR group**

Twenty women with pregnancies complicated by severe IUGR were identified. Birthweight was confirmed to be <10th centile for gestational age, the majority being <3rd centile, in all

but one case. There were three neonatal deaths within this group; two as a result of prematurity and the third from post natal septicaemia. Post-natally all the neonates showed evidence of growth restriction, reflected by muscle wasting and dry, wrinkled skin. The clinical details of these cases are listed in Table 2.1.

2.4.2 Control group

Twenty women with gestational age-matched control pregnancies were identified. The reasons for preterm delivery were as follows; 4 women had a history of cervical incompetence and were admitted to the labour suite at full dilatation which was irreversible and delivered vaginally, 3 patients had previous uterine scar ruptures and were therefore delivered electively on this occasion between 32-34 weeks by caesarean section, 3 had unrelated medical problems which necessitated preterm delivery by elective caesarean section (bilateral pneumothorax, suspected breast carcinoma and a cerebral aneurysm which was due to be electively clipped post-natally to prevent a cerebral bleed; all were otherwise fit and well and in particular, the patient with a cerebral aneurysm showed no evidence of hypertension); the remainder were women admitted in spontaneous, unexplained preterm labour. Of those admitted in spontaneous preterm labour, 3 were delivered by caesarean section and the remainder had a spontaneous vaginal delivery. It was not always possible to perform a full ultrasound examination just prior to delivery in the latter cases. However, birthweights were all appropriate for gestational age, there was no clinical evidence to suggest intrauterine malnutrition and the neonates were both structurally and chromosomally normal. The majority of pregnancies were evaluated by ultrasound prior to delivery. In each case umbilical artery RI, AFI and estimated fetal weight were all within the normal range for gestational age. Birthweights were confirmed to be >25th centile. There were no neonatal deaths within this group of pregnancies. A summary of the clinical details are given in Table 2.2 (Case numbers 21-40)

2.5 COLLECTION OF SPECIMENS

The author was present at each of the deliveries. The umbilical cord was clamped immediately following delivery of the fetus, while cord pulsations were still present. Delivery of the placenta in both city hospitals was augmented by the use of 10 iu syntocinon im, before application of controlled cord traction to expedite delivery. The placenta, complete with membranes and a standard length of umbilical cord was then weighed and any gross pathology noted. Details of the specific methods used to prepare the placenta for each part of the project are outlined in the methods section of chapters 3, 4, 5, 6 and 7.

2.6. MATERIALS

2.6.1 **Transmission Electron Microscopy**

Agar Scientific, (Cambridge, England) supplied Araldite, 25% EM grade glutaraldehyde, Kodak D19 solution, Rapid fix solution A and B, dodecenyl succinic anhydride (DDSA), Kodak 4489 EM grade film, tri-dimethylaminomethyl phenol (DMP 30) and Kodak 4489 thick base EM film. Absolute alcohol was obtained from Hayman Ltd. (Essex, England). Lead nitrate, uranyl acetate, sodium citrate and sodium hydroxide were purchased from Sigma Chemical Company (Poole, Dorset). Osmium tetroxide, glass stripes for ultramicrotomy, disposable vials and trufs for the glass knives were bought from Leica UK Ltd (Milton Keynes, England) Disposable blades were purchased from Bayer Diagnostics (Hampshire, England).

2.6.2 **Immunohistochemistry**

Highly purified anti-goat type I and type IV collagen antibodies were obtained from Europath Ltd (Cornwall, England). Biogenesis Ltd (Bournmouth, England), provided the purified anti-rabbit type III collagen antibody and Serotec (Oxford, England) the purified anti-mouse type VII collagen. Antibodies to laminin and fibronectin were also purchased from Europath Ltd (Cornwall, England) and Dako (Buckinghamshire, England) respectively. The other reagents required were obtained from the following companies; Biotin conjugate anti-mouse and extravidin peroxidase kit (Sigma Chemical Company, Poole, Dorset), Methanol (Hayman Ltd, Essex, England), Hydrogen peroxide (BDH supplies, Poole, England), Phosphate buffer concentrate (Mercia Diagnostics, Guildford, England), Phosphate buffer tablets (Unipath Ltd, Hampshire, England), Swine anti-rabbit, rabbit anti-goat and peroxidase anti-peroxidase (Dako, Buckinghamshire, England). HA West (Clydebank, Scotland) supplied EPY 135mm film for photomicrographs.

2.6.3 **Morphometry studies**

The following reagents were obtained from Sigma Chemical Company (Poole, Dorset): aminopropyltriethothoxysilane (APES), xylene, ethyl alcohol, methanol, hydrogen peroxidase, phospate buffered saline powder (pH 7.4), citric acid and bovine serum albumin. The Histostain-SP Kit was bought from Zymed Laboratories (San Francisco, California). Primary antibodies, anti a-smooth muscle actin and MIB-1 were obtained from Sigma (Deisenhofen, Germany).

2.6.4 **Vascular casts**

Mercox ^{R} and catylyst (made by Japan Vilene Hospital) were bought from Otto Nordwald, Hamburg, Germany. BDH Supplies Ltd (Poole, England) provided methylmethacrylate and potassium hydroxide pellets. Photographic film, Ilford PanF 50, was purchased from HA West (Clydebank, Scotland).

2.6.5 **Placental tissue casts.**

Cylinder stubs and storage boxes were bought from Agar Scientific (Cambridge, England). Mounting glue for the stubs was purchased from Neubauer Chemikalien (Munster, Germany). HA West, (Clydebank, Scotland) provided Ilford Pan F150 photographic film.

2.6.6 **General materials**

Venflons and 3-port connectors were obtained from Viggo Spectramed (Helsingborg, Sweden). Baxter (Norfolk, England) provided 250ml bags of normal (0.9%) saline solution. Sutures (2/0 chromic gatgut) were bought from Ethicon (Edinburgh, Scotland). Syringes (5, 10, 30 and 60 mls) and needles (19 and 21 gauge) were purchased from Becton Dickinson (Dublin, Ireland)

2.6.7. **Apparatus used**

The following equipment was also used; Finnpipettes (Jencons Scientific Ltd., Bedfordshire, UK.; critical point drier [CPD 750] (Biorad, Microscience Division (Hertfordshire, England); Digital scanning electron microscope [DSM-940] and stereomicroscope (Zeiss, Switzerland); Edwards coating unit and plate degasser (VG Scientific, Crawley, England); light microscope (Leitz Labovert, Wetzlar, Germany); LKB ultramicrotome (Leica (UK) Ltd, Milton Keynes, England); Graticule (Graticules Ltd, Kent, England); light microscope (Olympus Optical Co. (UK) Ltd, London, UK); Gallenkamp Prelude Incubator (Scotlab, Lanarkshire, Scotland); electronic balance (Mettler AT250, Zurich, Switzerland); glassware (BDH Ltd, Poole, Dorset, England); Acuson 128 Ultrasound machine (Mountain View, California).

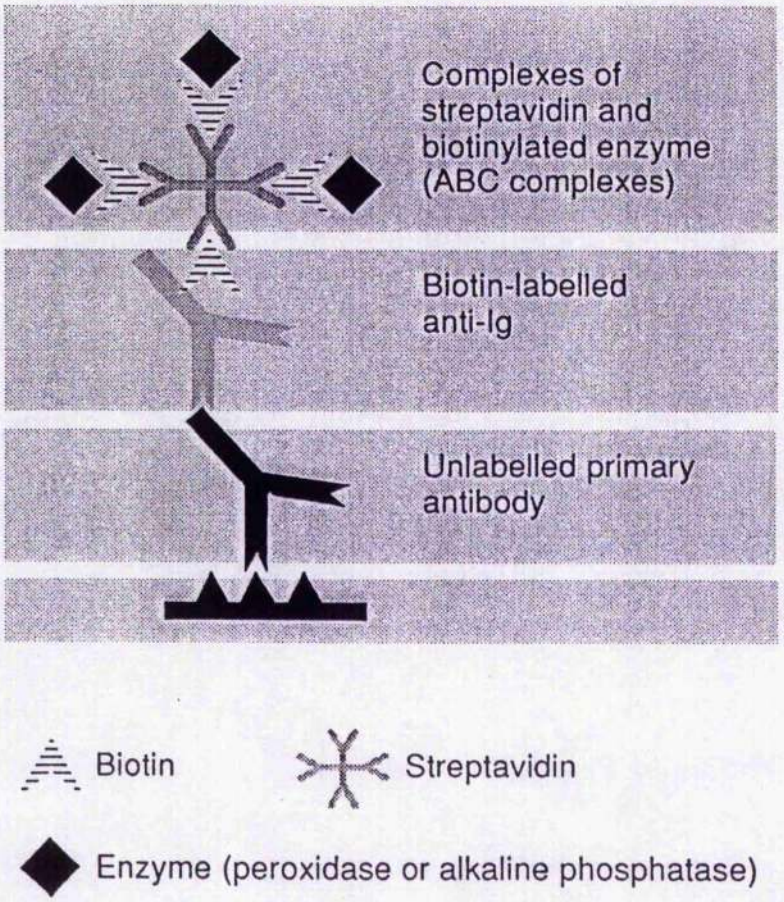

Figure 2.1. The streptavidin:biotin technique for indirect immunohistochemistry. The enzyme system, conjugated to streptavidin is pre-formed thus reducing the number of incubation steps. Though streptavidin binds strongly to its ligand, biotin, high affinity antibody binding is required at the primary and intermediate stages to ensure reliable staining.

Case No.	Gestation (weeks)	Birthweight g/[centile]	Placental weight (g)	Doppler
1	28	800 [10th]	198	AEDFV
2	34	1480 [3rd]	260	AEDFV
3 *	32	840 [<3rd]	300	AEDFV
4	30	780 [<3rd]	160	AEDFV
5	33	1280 [3rd]	242	AEDFV
6	36	1700 [3rd]	325	AEDFV
7 *	31	1200 [10th]	280	AEDFV
8 *	28	780 [5-10th]	300	AEDFV
9 *	30	1000 [10th]	270	AEDFV
10 *	34	1700 [10th]	490	AEDFV
11 *	28	940 [10-50th]	240	AEDFV
12 *	30	890 [3-5th]	200	AEDFV
13	29	700 [<3rd]	280	AEDFV
14	33	1300 [5-10th]	320	AEDFV
15	32	900 [<3rd]	200	AEDFV
16 *	28	800 [10th]	200	AEDFV
17	33	1280 [3-5th]	242	AEDFV
18 *	28	600 [<3rd]	300	AEDFV
19	34	1350 [<3rd]	300	AEDFV
20 *	27	720 [10-50th]	210	AEDFV

Table 2.1 Clinical details of the pregnancies complicated by IUGR and absent end-diastolic flow velocity (AEDFV) on umbilical artery Doppler studies. * indicates those pregnancies which were in addition complicated by pre-eclampsia.

Case No.	Gestation (weeks)	Birthweight (g)/[centile]	Placental weight (g)	Doppler	Indication for delivery
21	30	1450 [50th]	370	RI-0.79	PTL
22	32	1900 [50-90th]	430	not done	pnemothorax
23	29	1540 [50-90th]	360	RI-0.70	PTL
24	26	960 [50-90th]	405	RI-0.68	CI
25	26	840 [50-90th]	310	RI-0.74	CI
26	32	1500 [10-50th]	360	RI-0.77	scar rupture
27	34	2010 [10-50th]	380	RI-0.65	PTL
28	35	2140 [10-50th]	480	not done	PTL
29	31	2040 [50-90th]	580	not done	Breast Ca.
30	33	1890 [10-50th]	370	not done	PTL
31	32	2150 [50-90th]	500	RI-0.74	scar rupture
32	27	1140 [50-90th]	450	RI-0.78	PTL
33	34	2520 [50-90th]	498	RI-0.77	PTL
34	35	2290 [10-50th]	520	not done	aneursym
35	31	1600 [50th]	325	not done	CI
36	32	2100 [50-90th]	562	not done	PTL
37	33	1860 [10-50th]	450	not done	scar rupture
38	34	2600 [50-90th]	680	RI-0.68	PTL
39	32	1910 [50-90th]	630	RI-0.77	PTL
40	28	1120 [50th]	380	not done	CI

Table 2.2 Clinical details of the gestational-age matched preterm control pregnancies used in the study. Where possible, Doppler studies were performed and the resistance index (RI) calculated. The indications for delivery were as follows; PTL-unexplained preterm labour; CI-cervical incompetence; scar rupture-previous uterine scar rupture; aneursym- a cerebral aneursym which required intervention; pneumothorax-spontaneous unexplained bilateral pneumothorax

CHAPTER 3

VASCULAR ELABORATION IN STEM VILLI

3.1 INTRODUCTION

It is now well established that vascular impedance is dependent in part on the structure and distribution of blood vessels within the vascular bed. Great interest was therefore aroused when Giles et al. (1985) and McGowan et al. (1987) noted reduced numbers of small arteriole vessels within the tertiary stem villi of placentas from pregnancies complicated by severe IUGR and abnormal umbilical artery Doppler. This was thought to be the consequence of an obliterative process occurring within the tertiary stem villi; subsequent work in the pregnant ovine model confirmed that progressive arterial obliteration of the placenta using microspheres of 50μM diameter could induce similar Doppler changes to that seen in the IUGR fetus (Morrow et al 1989b). Having recognised that the IUGR fetus has a reduced number of circulating platelets (Van den Hof & Nicolaides 1990) possibly due to increased platelet consumption (Wilcox & Trudinger 1991), it was postulated that platelet emboli formed within the feto-placental circulation, gradually obliterate the small stem arterial vessels in IUGR pregnancies.

However, the definition of small arterial vessels in each of these studies was broad and included vessels with diameters between 20 and 90μM. It is likely that feto-placental vascular impedance will increase towards the lower end of this range since the cross-sectional area is increasing. In addition, the findings of the pathological (typically preterm) pregnancies were at times compared with gestationally unmatched (i.e. term) controls (McGowan, Mullen & Ritchie 1987; Bracero et al. 1989). Finally to date, no evidence exists to substantiate the platelet emboli theory in any portion of the vascular tree. How such an embolic process might be initiated is also unclear.

There is now also evidence to suggest that pregnancies complicated by IUGR and abnormal Doppler waveforms are characterised by a global defect in vascularisation, i.e. involving all villous classes (Jackson et al. 1995). Though the absolute number of arterial vessels would therefore be reduced, a global defect would imply that the proportion of small and large arterial vessels would be similar to that seen in control cases.

The primary aim of this study was therefore to establish if the reduction in stem arterioles was the result of primary vascular maldevelopment or a consequence of later vessel obliteration. The pattern of vessel elaboration in both IUGR and gestational age-matched control placentas was examined by evaluating specifically the relative proportions of vessel profiles within well-defined vessel diameter groups using an antibody to α-smooth muscle actin contained in contractile smooth muscle cells - this approach can therefore distinguish muscularised arterioles from large capillary vessels in the 10-20μM range. Moreover, as other vascular systems affected by increased vascular resistance demonstrate "extension" of

smooth muscle cells into tissue which is normally non-muscularised (Rabinovitch et al. 1986), our secondary aim was to evaluate whether actin positive cells, representing muscularised cells were present in normally non-muscularised intermediate and terminal villi.

3.2 METHODS

3.2.1 Clinical Details

Eight pregnancies complicated by severe IUGR were selected. All met the criteria defined in Section 2.2.1. The clinical details of these cases are given in Table 2.1 [Case numbers 5, 10-16]. Four of these pregnancies were also complicated by pre-eclampsia [Section 2.2.1]. Following delivery, birthweights, except that of case 11, were confirmed to be less than the 10th centile. All the neonates, including case 11, showed evidence of *in-utero* starvation with reduced ponderal indexes and dry, wrinkled skin.

For comparison purposes, a further 8 pregnancies with normally grown fetuses and normal umbilical artery Doppler studies were identified. These pregnancies were matched with the study group for smoking and gestational age. The clinical details of the eight cases are given in Table 2.2 [Case numbers 25, 26, 29, 30, 32-35]. Four women in each of the IUGR and control groups were smokers.

3.2.2 Collection of specimens

PREPARATION OF PHOSPHATE BUFFER: 0.02M phosphate buffer solution (PBS) was formed by dissolving 1 tablet of phosphate buffered saline (section 2.6.2) in 100 mls of double distilled water.

NEUTRAL BUFFERED FORMALIN: Prior to delivery 4% neutral buffered formalin was constituted as follows; 10.8 mls of formaldehyde (37% formalin) were added to 89.2 mls of PBS and the solution thoroughly stirred.

Within five minutes of delivery, four full-thickness sections of the placenta were excised from randomly chosen areas of the placenta, though areas with obvious gross pathology, such as infarcts, were excluded. The excised tissue was immersion fixed in the freshly constituted formalin for 8-24 hours (mean 14 hours) then transferred to 50% ethyl alcohol for a minimum of 12 hours and not longer than 24 hours. The sections were dehydrated through ascending concentrations of graded alcohol (70, 80, 90 and 100%) then transferred to acetone before embedding in paraplast. Dehydration and embedding was performed using an automated processor.

3.2.3 **Preparation of sections**

The glass slides used for immunohistochemistry were washed in ethyl alcohol, immersed for 20 seconds in 2% APES solution then washed in pure acetone (1 minute) and aqua dest (1 minute). This process improved adhesion of the paraffin embedded sections to the slides.

Serial sections (3-5 μM) of the paraffin embedded material were mounted onto the pre-treated slides and incubated at 37° C overnight. The following morning the sections were deparaffinised as follows; each section was immersed twice in xylene for 2 x 10 minutes then transferred through a graded series of alcohol (100, 95 and 90%) for 5 minutes in each grade.

3.2.4 **Immunohistochemistry**

A commercial kit (Zymed Laboratories, San Francisco) using a standardised sequence based on the streptavidin-biotin technique was employed for immunohistochemistry. The primary antibodies were all diluted in PBS with 1.5% bovine serum albumin (BSA).

Sections were first incubated with 1% hydrogen peroxide in methanol to block endogenous peroxidase activity, then washed in PBS. After a further 20 minute incubation with 10% non-immune goat serum, to prevent non-specific background staining, they were covered with primary antibody - anti α-smooth muscle actin [dilution 1:300; (Skalli et al. 1986)] for 60 minutes. Thereafter the slides were washed for 3 x 5 minutes in PBS then incubated with the second biotinylated antibody (goat anti-mouse antibody) for a further 30 minutes. After a further wash in PBS, the sections were incubated for 5 minutes with the streptavidin peroxidase conjugate (prepared according to the kit instructions). To visualise the bound antibody, the sections were washed in PBS before incubating with 3-amino-9-ethylcarbazole [AEC] for 15 minutes. The sections were rinsed in distilled water for 2 x 5 minutes, counterstained with haematoxylin for 3 minutes then washed in tap water for 10 minutes.

The sections were all mounted with coverslips using an aqueous mounting solution (Kaisers glycerol gelatine). All the incubations were performed at room temperature. Controls were generated by replacing the primary antibody by non-immune rabbit serum (10%). All the controls remained negative.

3.2.5 **Analysis**

3.2.5.1 **Analysis of stem villous vessel number**

A grid box (22 x 22 mm) containing 400 squares was formed and 100 squares marked by random allocation. The grid boxes were placed over each section using an identical orientation for each case, from chorionic to basal plate. Counting of the vessels was

performed with a MOP Videoplan Image Analysis system (Kontron, Munich, Germany) and all measurements were performed by the author.

Within the randomly chosen fields, the diameter of 600 consecutive stem arterial vessels, between 10-160μM (Kaufmann & Burton 1994) was measured in μM, using on-screen callipers. Arterial vessels were identified by the presence of a continuous ring of smooth muscle (stained by anti α-smooth muscle actin antibody) around the vessel lumen. Measurements were taken at the widest transverse diameter of the vessel, perpendicular to the axis of the vessel, and included the muscularised vessel wall (Figure 3.1). Villi which contained branching arterial vessels were also included, but in such cases measurements were taken perpendicular to the axis of the branches.

The 600 vessel diameters were then plotted in bands of 15 μM intervals from 10-160 μM diameter. The percentage of vessels within each diameter band was calculated and a mean diameter derived from the 600 counts.

3.2.5.2 **Verification of sample size**

Vessel measurements were repeated for 300, 450, 600 and 750 consecutive vessels. The mean diameter (and standard deviations) for each vessel count were as follows; 27 μM [20.8 μM]; 30 μM [28.8 μM]; 29.5 μM [28.14 μM]; 29.6 μM [20.6 μM]. Since there was no significant difference in the mean vessel diameter obtained when 450 vessels or more were examined, 600 vessels were deemed sufficient to provide a reliable sample.

To ensure reproducibility, vessel measurements were repeated on the same section at different time intervals. There was no significant difference in the mean vessel diameter or the distribution pattern of vessel diameters obtained at each count.

3.2.5.3 **Analysis of villous volume**

Since the proportion of villous tissue may differ between placental sections, the villous cross-sectional surface of each placental section was estimated using a standard 20 point counting technique (Mayhew & Burton 1988) and the proportion of stem and peripheral villi (defined as all non-muscularised intermediate and terminal villi) was calculated.

3.2.5.4. **'Extension' of smooth muscle cells**

The presence of any actin positive cells within the villous stroma of peripheral villi was evaluated during the above process.

3.2.5.5. **Statistical Analysis**

The % of vessels within each diameter category and the mean stem vessel diameter are expressed as mean and standard deviation. The villous volumes were also normally distributed and are therefore expressed mean and standard deviation. Differences between the IUGR and control groups were assessed using a Student's unpaired t-test with p values < 0.05 considered to be significant.

3.3 RESULTS

3.3.1 Comparison of the study groups

The mean gestational age at the time of delivery was similar for the two groups (IUGR group 30.75 weeks [SD 2.5] vs. control group 31.8 weeks [SD 2.4]; p=0.767). The birthweight of the IUGR group was significantly less than that of the controls (1076g [SD 320] vs. 1718g [SD 443]; p<0.05). Similarly, the IUGR placentas were significantly lighter than those of the controls (264g [SD 49.4] vs. 426g [SD 99.6]; p<0.05).

3.3.2. Villous volume

The proportion of villous tissue within each case is summarised in Table 3.1. There was significantly less villous tissue in the IUGR placentas when compared with gestational age matched controls (57.38 [SD 7.4] vs. 70.375 [SD 10.8]; p<0.05); in particular the volume of placental tissue occupied by terminal villi was significantly less in the IUGR placentas than in the control placentas (14 [SD 3.4] vs. 20 [SD 4.2]; p<0.05). There was no significant difference in the proportion of placental tissue occupied by stem villi (41 [SD 8.9] vs. 49.6 [SD 8.8]; p=0.07).

Within the IUGR group, there was no significant difference between the IUGR placentas from pregnancies with pre-eclampsia and those without in either the villous volume (62% [SD 2.9] vs 52.8 [SD 7.9]; p=0.07); the volume of stem villi (45.8 [SD 5.4] vs 36.3 [7.9]; p=0.14); or the volume of terminal villi present (13.5 [SD 4.4] vs 14.5 [SD 2.6]; p=0.7).

3.3.3. Pattern of vessel branching

A typical histogram plot for an IUGR (case 3) and preterm control (case 26) are shown in Figure 3.2. There appeared to be a smaller percentage of vessels in the 10-26μM diameter group of the IUGR placentas when compared to the preterm control cases, but this difference was not statistically significant. The mean percentage of vessels (SD) within each vessel diameter class for both groups is shown in Figure 3.3. There was no significant difference in

the mean vessel diameter (Table 3.1) of either group (31.96μM [SD 3.14] vs. 29.759μM [SD2.6]; p=0.13).

3.3.4 'Extension' of muscularised cells

Actin positive cells were consistently observed in five of the eight IUGR cases (5, 11, 12, 15 and 16). None of the preterm control cases demonstrated positive actin staining within the stroma of intermediate/terminal villi.

3.4 DISCUSSION

Light microscopic histological studies of the placenta in pregnancies complicated by IUGR have suggested a reduction in the numbers of small muscularised vessels within stem villi (Giles, Trudinger & Baird 1985; McGowan, Mullen & Ritchie 1986) which has led to the view that they are somehow obliterated by fetal platelets (Wilcox & Trudinger 1991). However, recent work has indicated that reduced vascularisation is not simply a feature of small stem arterioles (10-90μM) but of the whole vascular bed, suggesting a basic problem of placental vascular growth and development in IUGR pregnancies (Jackson et al. 1995). In order to understand the pathophysiology of intrauterine growth retardation, these two hypotheses, hypovascularisation and vessel obliteration, must be clarified.

The total proportion of villous tissue within each IUGR placental section was significantly less than that present in the control cases. Most of the data published previously on IUGR placentas has shown no reduction in total villous volume (Aherne & Dunhill 1966; Teasdale 1984; Boyd & Scott 1985), but this can probably be explained on the basis of case selection, as these studies evaluated only term SGA infants, where few babies born <10th centile will be IUGR. More recently, when true IUGR pregnancies with AEDFV were assessed, total villous volume was less (Hitschold et al. 1993; Jackson et al. 1995)

Closer evaluation of our data demonstrated that the smaller proportion of villous tissue present in IUGR placentas reflected a significant depletion in the volume of peripheral villi; there was no significant difference in the volume of stem villi between IUGR and control cases. During the evaluation of stem arterial numbers by Giles et al. and others (Giles, Trudinger & Baird 1985; McGowan, Mullen & Ritchie 1986), the total number of stem villi examined in each case was comparable, however, we have now confirmed that the total proportion of stem villi in IUGR placentas matches that of gestational age-matched control cases. A reduction in the number of stem villous vessels in IUGR placentas is not therefore secondary to a diminished population of stem villi but implies a global defect in the elaboration of the vascular tree (Kaufmann & Burton 1994). The 'reduced' stem arterial

theories do not, however, address the underlying pathology of fetal hypoxia, characteristic of the IUGR fetus as stem villous arterioles merely conduct blood into the intermediate/terminal villi where nutrient and gas exchange occurs (Macara, Kingdom & Kaufmann 1993). Since terminal villi are the site of maximal feto-maternal exchange the relative absence of peripheral villi which we noted should not be ignored. The lack of essential fetal substrates in the growth retarded fetus may well result from the depletion of these exchange villi. Previous unmatched (Aherne & Dunhill 1966; Teasdale 1984) and recent matched control studies (Hitschold et al. 1993; Jackson et al. 1995) have also made similar observations on the volume of terminal villi and therefore the structure of this portion of the placental villous and vascular tree merits further evaluation using sensitive 3-dimensional imaging and ultrastructural studies of tissue and vascular casts.

In this study, the elaboration of stem villous vessels was determined by the distribution of vessel profiles in IUGR and gestational age-matched control placentas. The number of "vessel profiles" was specifically evaluated since it is not possible to reliably distinguish arterial/arteriolar from venous/venular profiles in paraffin sections, particularly at the lower end of the vessel diameter range. However, as stem villous vessels branch dichotomously, it is reasonable to assume that the vessel profile counts obtained reflect in a qualitative way the pattern of arterial/arteriolar vessel elaboration (Leiser, Kosanke & Kaufmann 1991).

Previous studies evaluating stem villous vessel numbers have examined only small arterial vessels and failed to relate their findings to the rest of the vascular tree (Giles, Trudinger & Baird 1985; Bracero et al. 1986; McGowan, Mullen & Ritchie 1986). Stem arterial vessels follow the same pattern of dichotomous replication as stem villi themselves, and therefore, the percentage of arterial vessels of any given diameter is fairly constant for all placentas (Lucas et al. 1994). Obliteration or loss of the small stem arterioles should therefore result in a specific reduction in the percentage of small arteries, while the percentage of large vessels would remain similar or increase. Unfortunately, there is no indication in any of the three stem vessel studies mentioned previously (Giles, Trudinger & Baird 1985; McGowan, Mullen & Ritchie 1986; Bracero et al. 1987) that the number of larger stem arteries was confirmed to be normal in the IUGR placentas.

Furthermore, the definition of small arterioles used in previous studies was very broad (20-90μM), vessels at the lower end of the range having a cross-sectional vessel area almost one twentieth that of vessels at the upper limit (Jauniaux & Burton 1993). The contribution of the 20μM and 90μM vessel to placental vascular resistance would therefore vary enormously. As platelet or other emboli are most likely to occlude the vessels at the lower end of this spectrum, the methods described by Giles and others would lack the sensitivity to detect such

changes. By the use of anti α-smooth muscle actin antibody, we were able to visualise and measure vessels to 10μM, prior to becoming mature intermediate villus capillaries.

By evaluating the percentage of vessels within small well defined vessel diameter groups we were able to establish that there was no evidence of a selective reduction in the percentage of small arterioles, even amongst the smallest vessel diameters, which might indicate embolic vessel obliteration. In contrast, the proportion of vessels within each class was similar for both IUGR and control cases suggesting that the normal pattern of stem arterial development was undisturbed. Since the number of fields required for adequate sampling was calculated at the beginning of the study we can be sure our findings were not biased by sampling methods. The trend towards fewer vessels in the 10-25μM diameter group in the IUGR placentas obviously requires further investigation, using larger numbers of placentas, to establish if this does indeed reflect a genuine difference between the two groups. It is possible that these smaller vessels may be affected by a thrombotic/occlusive process. Alternatively, reduced numbers of very small arterioles may simply reflect further impairment of vascular development. Interestingly, the addition of maternal pre-eclampsia to the equation did not appear to alter these findings; suggesting that these changes were primarily related to the fetal condition.

Our findings, in conjunction with those of Jackson et al. (1995), have demonstrated that the lack of "small stem arterioles" previously reported (Giles, Trudinger & Baird 1985; McGowan, Mullen & Ritchie 1985) reflects a generalised depletion of the stem villous arterial tree. Stem villi are formed principally during the first and second trimester, at a time when EDFV is beginning to become apparent. Therefore a generalised depletion in stem villous vessel counts may reflect poor development of stem villi at this stage of gestation.

Anti α-smooth muscle actin positive cells were identified within the peripheral villous stroma of five IUGR cases but were never seen in any of the control cases. This observation was unexpected, as intermediate and terminal villi do not contain a muscularised vessel system and have been assumed to be unresponsive to vasoactive agents. The contractile filament actin is widely expressed in most birds and mammals, as one of several isoforms. α-actin isoform is specifically found in smooth muscle cells and is absent from fibroblasts (Skalli et al. 1984), though it is now also recognised to stain pericytes within the placental stroma (Skalli et al. 1989). However, the actin positive cells in the IUGR terminal villi were not found in association with any capillary vessels. The origin of these cells in the placenta is unclear, however in some pathological conditions and during wound healing fibroblasts in other tissues may differentiate into cells with contractile properties, known as myofibroblasts (Gabbiani et al. 1972; Skalli et al. 1986), which are positive for the anti α-smooth muscle actin antibody. It is interesting to speculate that terminal villous stromal fibroblasts may

under certain conditions such as impaired villous blood flow, altered oxygen tension or abnormal growth factor expression, also acquire these contractile properties.

In children with congenital heart defects and increased pulmonary artery pressure, smooth muscle cells and precursor smooth muscle cells are found to 'extend' into normally non-muscularised peripheral portions of the lung (Rabinovitch et al. 1986), changes which are similar to those found in the rat with hypoxic induced pulmonary hypertension (Meyrick & Reid 1978: Rabinovitch et al. 1979). It is clear that many severely growth retarded fetuses show evidence of increased placental vascular resistance, reflected by changes in the Doppler waveform. Though these primary Doppler changes may result from defective development of the placental vascular bed, α-actin positive cells being contractile and therefore vasoresponsive, may compound the effects and further impair blood flow within the terminal villous circulation.

We have demonstrated that obliteration of small arterial vessels is unlikely to play a significant role in the pathogenesis of abnormal Doppler waveforms in the IUGR pregnancy, since the percentage of all arterial vessel classes is similar in both IUGR and control placentas. Rather, our data would support the concept that the stem villi of IUGR pregnancies are characterised by widespread depletion of the vascular bed. These changes could contribute to the increase in placental vascular resistance and the development of abnormal Doppler waveforms in IUGR pregnancies.

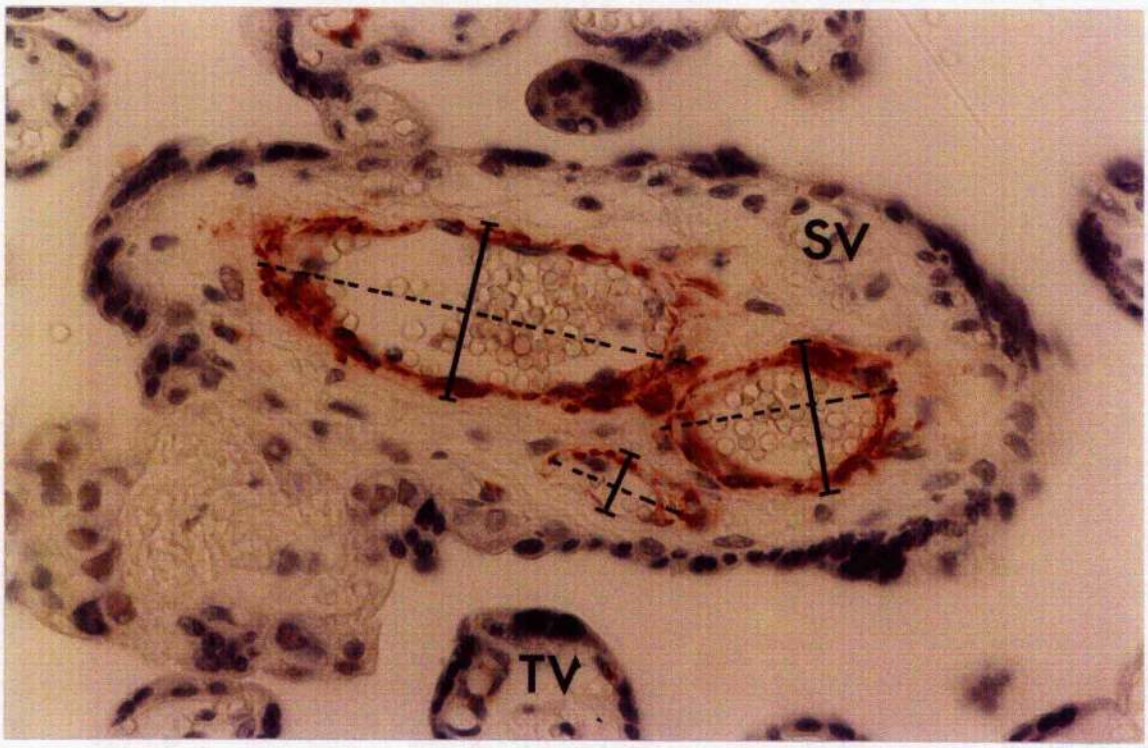

Figure 3.1 A cross-section of villous tissue stained with the antibody anti α-smooth muscle actin. Positive antibody staining is seen as a red colour. SV - stem villi; TV- terminal villi. The presumed long axis of the vessels are shown by the broken black line and the maximum diameter obtained displayed as the unbroken black line. (magnification x 100)

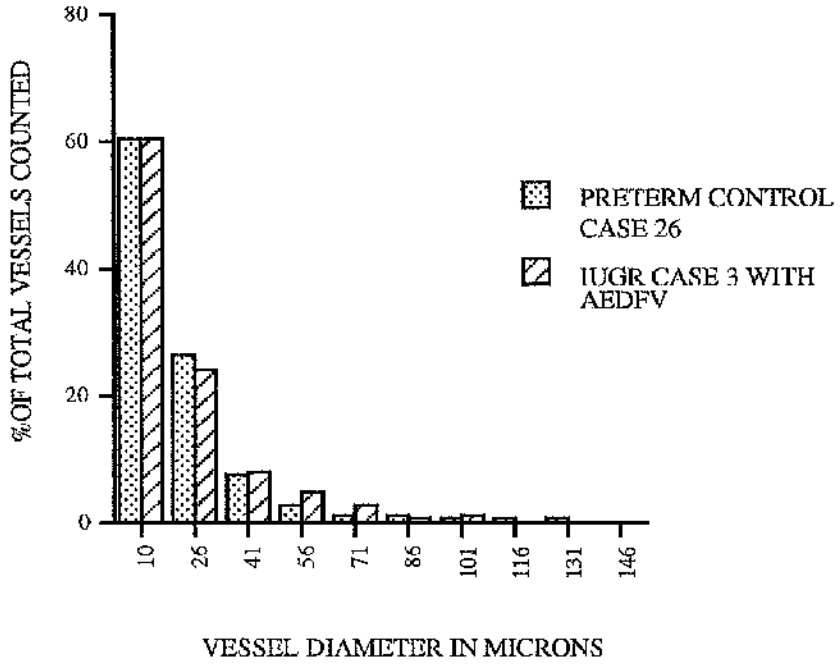

Figure 3.2 Histogram plot of the distribution of vascular profiles, identified by anti a-smooth muscle actin, within each vessel diameter group [10-25μM; 26-40μM; 41-55μM; 56-70μM; 71-85μM; 86-100μM; 101-115μM; 116-130μM; 131-145μM and 146-160μM) of an IUGR (case 3) and normal gestational age-matched (case 26) control placenta.

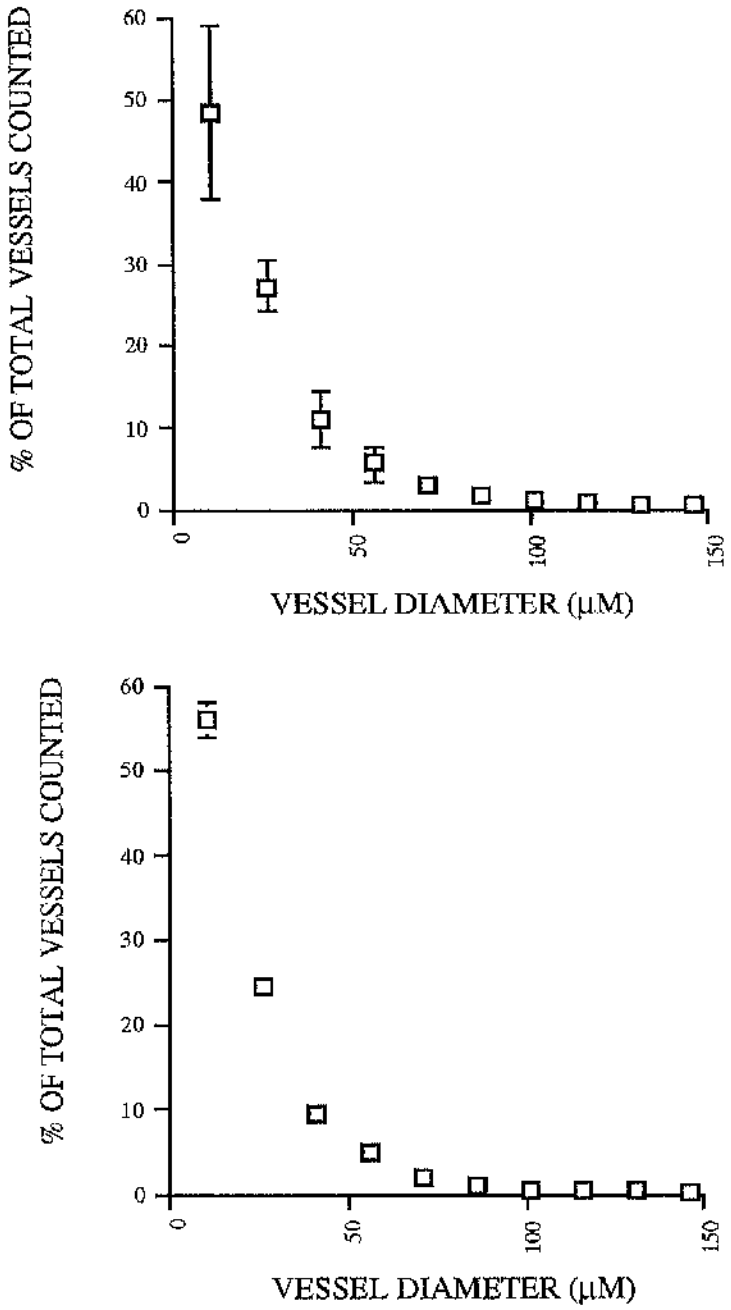

Figure 3.3 The mean (SD) percentage of vascular profiles within each diameter class (10-25, 26-40, 41-55, 56-70, 71-85, 86-100, 101-115, 116-130, 131-145, 146-160 μM) for the IUGR (upper graph) and gestational age-matched control groups (lower graph). As the SD was tightly distributed around the mean in the majority of cases, error bars can only be seen in the smaller vessel diameter groups, where the SD was larger. There was no significant difference in the distribution pattern of either group.

IUGR CASES

Patient number	% villous tissue	% stem villi	% terminal villi	MVD (mm)
5	59 %	38%	11%	29.5
10	43%	24%	11%	28.0
11	57%	40%	17%	33.9
12	61%	47%	14%	34.3
13	60%	50%	9%	34.0
14	64%	49%	15%	31.3
15	65%	46%	19%	33.0
16	50%	34%	16%	30.1

PRETERM CONTROL CASES

Patient Number	% villous tissue	% stem villi	% terminal villi	MVD (mm)
9	60%	42%	18%	27.1
10	81%	53%	18%	27.8
11	83%	62%	21%	27.9
12	78%	48%	30%	28.7
13	52%	34%	18%	27.5
14	75%	57%	18%	32.8
15	67%	48%	19%	32.9
16	67%	53%	18%	33.8

Table 3.1 The % of villous tissue and the mean vessel diameter (MVD) within each IUGR and gestation age-matched control placenta.

CHAPTER 4

THE TERMINAL VILLOUS CAPILLARY NETWORK

4.1 INTRODUCTION

Anatomical studies of the placenta from IUGR pregnancies with AEDFV have reported reduced numbers of small stem arterial vessels when compared with gestational age matched controls (Giles, Trudinger & Baird 1985; McGowan, Mullen & Ritchie 1987; Bracero et al. 1989), presumed to occur as a result of vessel obliteration by platelet emboli However, to the author's knowledge, there have been no reports demonstrating the presence of excessive platelet emboli within stem vessels of affected human cases. Moreover we have shown in chapter 3 that there is no evidence to suggest a selective reduction in the number of small arterial vessels.

Planar data such as numbers of arterial vessels may be more accurately interpreted when related to specific placental components such as placental volume, villous volume or surface area, a technique known as stereology (Mayhew & Burton 1988). Recent stereological studies of placentas from IUGR pregnancies complicated by AEDFV have demonstrated widespread depletion of the vascular network, indicating that the IUGR placenta is hypovascularised thus raising the possibility that the reduced number of arterial vessels noted previously are the result of impaired vascular development (Hitschold et al. 1993; Jackson et al. 1995). In particular the volume of peripheral (intermediate and terminal) villi was significantly depleted in the IUGR placentas when compared to gestational age-matched controls (Jackson et al. 1995). Falling placental vascular impedance with gestation is associated with the development of the distal placental vascular bed during the late second, and third trimesters of pregnancy (Kaufmann & Burton 1994). Interruption to, or modification of this stage of vascular growth could therefore, alter normal placental vascular impedance. It is interesting to note that AEDFV in the umbilical artery is observed primarily in the third trimester of pregnancy; once the stem villous arterial population is complete but before complete development of these peripheral villi has occurred.

The development of peripheral villi and their vascular network increases the trophoblast surface area available for materno-fetal exchange (Jackson, Mayhew & Boyd 1992), minimising the transfer distance between maternal and fetal blood (Mayhew et al. 1993). This process is vital to ensure that the metabolic requirements of the developing fetus are assured. Thus depletion of peripheral capillary vessels may also restrict fetal nutrition.

Though the peripheral vascular network which is central to placental vascular haemodynamics and fetal nutrition appears to be reduced in IUGR pregnancies (Hitschold et al. 1993; Jackson et al. 1995), it is unclear if this is simply due to diminished numbers of normal capillaries or maldevelopment of the capillary bed. In order to address this issue,

vascular casts from IUGR and control placentas were examined in a 3-dimensional way using scanning electron microscopy (SEM).

4.2 METHODS

4.2.1 Clinical details

Ten pregnancies complicated by severe IUGR, which met the criteria outlined in Section 2.1.1, were identified antenatally. The clinical details of these cases are given in Table 2.1 [Case numbers 1-7, 9, 10, 20]. Five of the pregnancies were normotensive and the remaining five were complicated by pre-eclampsia as defined previously in Section 2.1.1. There were no other underlying maternal or fetal conditions present. Birthweight in each case, based on a local database (Smalls and Forbes 1983), was confirmed to be less than the 10th centile for gestational age, the majority being less than the 3rd centile. All the neonates showed evidence of intrauterine starvation. There was one neonatal death in this group resulting from overwhelming septicaemia and severe respiratory distress.

For direct comparison 10 women with normally grown pregnancies, who were delivered prematurely, were also identified. The clinical details of the 10 cases are given in Table 2.2 [Case numbers 21, 22, 24, 28, and 36-40].

4.2.2 Preparation of vascular casts

Following delivery of the placenta, a chorionic branch of the umbilical artery together with its corresponding venous outflow, supplying a peripheral cotyledon were identified. The artery was cannulated with a 16 gauge cannula and secured with a single stitch (2/0 chromic catgut) to prevent backflow or extrusion. A three port polyethylene connector was attached to the cannula, permitting multiple perfusates. The venous outflow was also punctured, at a site proximal to the cord root to allow free venous drainage throughout the procedure. The vascular network of the cotyledon was initially flushed with a minimum of 100 mls of normal (0.9%) saline over a period of at least 8 minutes, at a constant rate, to remove residual fetal blood. Once the venous effluent contained only clear saline solution, the plastic polymer was constituted thus; 0.5g of catalyst was dissolved in 5 mls of methylmethacrylate. The resultant mixture was added to 20 mls of Mercox CL-2B base resin in a disposable glass container and stirred thoroughly for 20-40 seconds (Lametschwandtner, Lametschwandtner & Weigner 1990). The saline infusion was stopped and the mixed resin then injected through the second port at a constant pressure over a period of 3 minutes until the venous effluent consisted of pure plastic. The venous outflow was then ligated with a catgut suture and plastic perfusion continued until resistance to injection was felt. The arterial side was

clamped and the cotyledon was left untouched for a further 20-30 minutes to allow curing of the plastic.

Once hardened, the area of casted cotyledon was immersed in 20% potassium hydroxide at 40^0 C for a period of 6 hours, washed in running water then immersed in distilled water overnight. This process was repeated 6 times to ensure that all the surrounding placental tissue was digested, leaving the vascular cast intact and free of debris (Figure 4.1).

The casts were fractured into 3-4 parts along natural lines of cleavage under water. These portions were oven dried for 8 hours at 40^0C, mounted on stubs then sputtered with gold for 3 minutes. The coated specimens were kept in a desiccator at all times (Leiser 1985).

4.2.3 **Analysis of vascular casts**

All the casts were coded and therefore examined by scanning electron microscopy (DSM-940) blind to the clinical information. The casts were initially viewed at low power (x 100) to evaluate the general quality of preservation, the degree of plastic extravasation and the distribution of vessels within the cast, particularly to locate areas containing terminal capillaries.

All quantitative analysis was performed at higher magnification (x 500). A minimum of 20 terminal capillaries from different areas of the cast were viewed. The casts were rotated such that each terminal capillary structure was viewed in the horizontal plane. The following parameters were recorded; (1) the length of the capillary loop was measured from its base, at the arteriole junction to the tip of the loop; (2) within each capillary loop the number of visible branching points were counted; (3) the degree of capillary coiling was assessed and assigned a value from 1-3 [1=no coiling; 2=moderate coiling; 3=highly coiled]; (4) the mean diameter of each capillary loop was then estimated by measuring the capillary diameter (perpendicular to the vessel walls) at 20 μM intervals from the base to the tip of the capillary loop. Pictures were taken of each capillary loop as a permanent record. A typical normal terminal capillary network viewed by SEM (case 36) is shown in Figure 4.2 and the capillary cast from an IUGR placenta (case 3) demonstrated in Figure 4.3.

4.2.4 **Verification of methods**

To ensure that an adequate sample of terminal capillaries were analysed, the measurements obtained on four casts were repeated using measurement data from 10, 15, 20, 25, 30 and 35 terminal capillaries. There were no difference in the means and standard deviations obtained for measurements of 15 or more capillary loops and thus analysis of 20 capillary loops was deemed to be representative.

Statistical analysis of vascular casts

The data for capillary diameter and the number of capillary branches were expressed as means (standard deviation). Since terminal capillary length within each cast showed a wide distribution these data were log transformed [log (x)] before analysis and the data expressed as geometric mean and standard deviation. The number of branches, being small count data, was square root transformed prior to analysis.

From the three point scale of coiling, the proportion of capillaries within each cast which demonstrated no coiling was calculated. For each parameter, differences between the two groups were evaluated using a Chi^2 analysis.

4.3 RESULTS

4.3.1 Comparison of the study cases

The two groups were comparable in terms of the mean gestational age at the time of delivery (IUGR group 31.5 weeks [SD 2.83] vs. Control group 31.3 weeks [SD 2.87]). The birthweight of the IUGR group was significantly less than those of the controls (1150g [SD 379.5] vs. 1782g [SD 519.5]; $p<0.05$) as was the placental weight (273g [SD 90.7] vs. 487g [SD 111]; $p<0.05$). There was no difference in the placental/fetal weight ratio between the IUGR and control cases (0.245 [SD 0.58] vs. 0.285 [0.66]; $p=0.17$).

4.3.2 Vascular casts

The mean capillary lengths of the capillary loops in each cast are illustrated in Figure 4.4. Capillary loops in the IUGR casts (Figure 4.3) tended to be very sparse and were significantly longer than those in the control placentas (217.8 μM[SD 71.9] vs. 136.9 μM[SD 29.8]; $p<0.05$). The number of visible branches within each capillary loop is shown in Figure 4.5. Though longer, the IUGR capillary loops had fewer branches than the control cases (4[SD 1.9] vs. 6.1[SD 2.2]; $p<0.05$). Moreover, a significantly greater proportion of the IUGR capillary loops demonstrated no coiling (79% vs. 18%; $p<0.05$), the majority of capillaries in the control cases were very coiled. These data are illustrated in Figure 4.6. The mean capillary diameter of the IUGR capillary loops displayed in Figure 4.7 did not differ significantly from those of the control capillaries (12.7μM [SD 3.76] vs. 12.73μM [4.28]).

Within the IUGR group, casts from pregnancies which were complicated by maternal pre-eclampsia, showed no significant difference from the unaffected cases in the number of capillary branches (2.0 [SD 0.17] vs 1.99[SD 0.08]; $p=0.8$); the capillary length (2.29 [SD

0.07] vs 2.37 [SD 0.07]; p=0.11); or the percentage which demonstrated no capillary coiling (73% [SD 8.4] vs. 85% [SD 12.7]; p=0.12).

4.4 **DISCUSSION**

Until recently, the structure and function of peripheral villi in IUGR pregnancies has been largely ignored. Anatomical studies have generally concentrated on stem villi, focusing in particular on the number of arterial vessels within each villus (Giles, Trudinger & Baird 1985; McGowan, Mullen & Ritchie 1987; Bracero et al. 1989). However, stem villi account for only a small portion of the feto-placental vascular tree when compared to more distal villi. In addition, peripheral villi play a central role in metabolic transfer to and from the fetus. In view of these facts, attention has been redirected to the possible role of intermediate and terminal villi (peripheral villi) in the pathogenesis of growth restriction.

To investigate the three-dimensional structure of the distal capillary network, we used a well defined group of severely growth restricted pregnancies characterised by abnormal umbilical artery Doppler (Section 2.2.1). Moreover, since the dimensions of the peripheral vascular bed are particularly gestation dependant we were careful to ensure that an appropriate group of normal, gestational age-matched pregnancies were identified to serve as controls.

Vascular casting techniques were first described in the placenta as early as 1950 (Boe 1950) but because of the highly viscous materials available, could only perfuse and fix large placental vessels. With the development of low viscosity plastic polymers, smaller capillary vessels may be successfully perfused, thus permitting vascular casting of the microvascular placental circulation (Leiser 1985; Lee & Yeh 1986).

Vascular casts from IUGR placentas contained relatively fewer vessels and substantially fewer capillary loops when compared to gestational age-matched control casts. In addition, the IUGR capillaries were straighter, significantly longer and less branched than the control capillaries.

During the latter part of the second and throughout the third trimesters of pregnancy, as longitudinal growth of the capillaries within mature intermediate villi exceeds that of the villi themselves, the capillaries coil and form loops. These loops gradually bulge from the villous surface, eventually forming grape like outgrowths, known as terminal villi. The capillary loops form a rich network of vessels, with many intercalated branches and random, focal dilatations known as sinusoids within the longer capillary loops (Kaufmann et al. 1985a). These focal dilatations, by increasing capillary diameter, decrease the vascular impedance of

long capillary loops thus ensuring even perfusion of both long and short capillary loops. Fetal blood may pass through up to five capillary loops in series before entering a post capillary venule. From there it returns via venules and the veins of stem villi to enter the umbilical vein. Terminal capillary loops are therefore not formed by active proliferation but as the passive result of differential growth rates (Berniscke & Kaufmann 1992). The development of this vascular bed is vital to reduce placental vascular impedance and thus accommodate the significant increase in placental blood flow during the second and third trimesters of pregnancy. The finding of long, uncoiled, non-sinusoidal capillary loops in the IUGR placental casts would suggest that terminal capillary growth is impaired to such an extent that few bud-like capillary networks are not formed.

There is now increasing evidence to suggest that the mechanisms which regulate angiogenesis in the normal placenta are complex and involves the interaction of many cytokines and growth factors (Librach et al. 1994; Saito et al. 1994).

Placental hypoxia is thought to be responsible for the changes seen in the placentas of pre-eclamptic women and those living at high altitude. Such placentas are characterised by excessive numbers of terminal villous buds containing extensive networks of, highly branched capillary vessels (Kaufmann, Luckhart & Leiser 1988; Jackson et al. 1985; Jackson et al. 1988). Detailed experimental studies in the pregnant guinea-pig have confirmed the effects of placental hypoxia, demonstrating clearly that hypercapillarisation occurs (Bacon et al. 1984), with the formation of multiple, short, highly branched capillary buds. Such adaptation facilitates blood flow and expands the surface area available for materno-fetal exchange (Scheffen et al. 1988), thus increasing total oxygen diffusion to the fetus.

Isotope (Chatfield et al. 1975; Kaar et al. 1980) and more recent Doppler studies (Bewley, Cooper & Campbell 1991; Bower, Schuchter & Campbell 1993) have demonstrated that uterine blood flow is significantly reduced in growth retarded pregnancies; thought to result from defective trophoblast invasion (Brosens, Dickson & Robertson 1977; Khong et al. 1986). Since the severely growth restrictedd fetus is hypoxic (Nicolaides et al. 1988) it has been assumed that this change is the consequence of placental bed hypoxia resulting from the reduction in uterine blood flow. However, since hypoxia promotes excessive capillary growth (Bacon et al. 1984; Scheffen et al. 1988) our findings of impaired rather than proliferative capillary growth, clearly suggest that placental hypoxia does not appear to be the dominant factor regulating placental development in IUGR pregnancies complicated by AEDFV. In addition, the presence of maternal pre-eclampsia, commonly associated with "placental ischaemia", did not alter these changes. Other factors must therefore modify IUGR placental growth patterns to induce the changes we have demonstrated and additional mechanisms must therefore be responsible for the fetal hypoxia seen in growth retardation.

The mean diameter of the capillary loops did not vary between the IUGR and control placentas. However, this observation may not reflect the *in-vivo* situation, as calculations of capillary diameter are subject to several methodological limitations. It is well recognised that the diameter of cast vessels are dependant not only on the perfusion pressure applied but also on the resistance of the intervillous space (Karimu & Burton 1994) and the extracellular matrix. Moreover, there is a wide distribution of diameters in the normal placenta with narrower segments adjacent to the large sinusoidal dilatations (Kaufmann, Luckhart & Leiser 1985). Simple measurements of mean capillary diameters did not allow for the physiological variation present. The mean figures obtained however, were in keeping with the 12.2 μm calculated by others from term placental casts. This would indicate that despite the depletion in the size of the vascular bed, compensating mechanisms such as dilatation and shortening of the capillary vessels, which would reduce vascular impedance, did not occur.

Our study did not evaluate the capillary surface area *per se* but the images we obtained would support the findings of Hitschold et al. (1993) and Jackson et al. (1995), which demonstrated a reduced intravascular volume, and a diminished vascular surface area, in peripheral villi from IUGR pregnancies. Moreover we were clearly able to demonstrate that the reduction in vascular tissue was the result of abnormal capillary development and not simply the consequence of depleted numbers of normal vessels. In addition, the IUGR capillaries were largely devoid of any sinusoidal dilatations as Hitschold had previously postulated from two-dimensional analysis (Hitschold et al. 1993).

The structural changes we have demonstrated in the capillary network of IUGR placentas may affect the function of capacity of the placenta. If the peripheral placental vascular network fails to develop during pregnancy, as we have shown, placental vascular impedance which normally falls with the expansion of the capillary bed, is likely to remain elevated. Furthermore since the capillaries which form are elongated, unbranched and relatively narrow, impedance to blood flow may actually rise, producing the characteristic changes seen in the Doppler waveform of the IUGR fetus. Since placental blood flow increases with advancing gestation, irrespective of the failed vascular bed expansion, blood pressure within the placental circulation is likely to rise. Under such conditions the arterio-arterial channels (Boe 1969), which are normally closed and insignificant in the low pressure placental circulation, may become functional, shunting placental blood past the terminal villous loops, by-passing the materno-fetal exchange surface area and thus restricting fetal oxygen uptake and nutrient provision.

Even in the absence of such shunts, our images and the calculations of Hitschold (Hitschold et al. 1993) and Jackson (Jackson et al. 1995) clearly show that the capillary surface area available for perfusion, and therefore materno-fetal exchange is reduced. If the villous

surface area is likewise depleted, reducing the availability of substrate carriers, as Jackson has suggested (Jackson et al. 1995), fetal substrate uptake will be further impaired, and *in-utero* fetal starvation ensue.

Using vascular casts we have demonstrated that vascular development in IUGR pregnancies with AEDFV is abnormal and may contribute to the clinical picture of intrauterine growth restriction. Furthermore, we have shown that the pattern of vessel growth is not characteristic of placental ischaemia or hypoxia, assumed to result in the fetal hypoxia seen in IUGR pregnancies, rather the features suggest a primary developmental defect in placental angiogenesis.

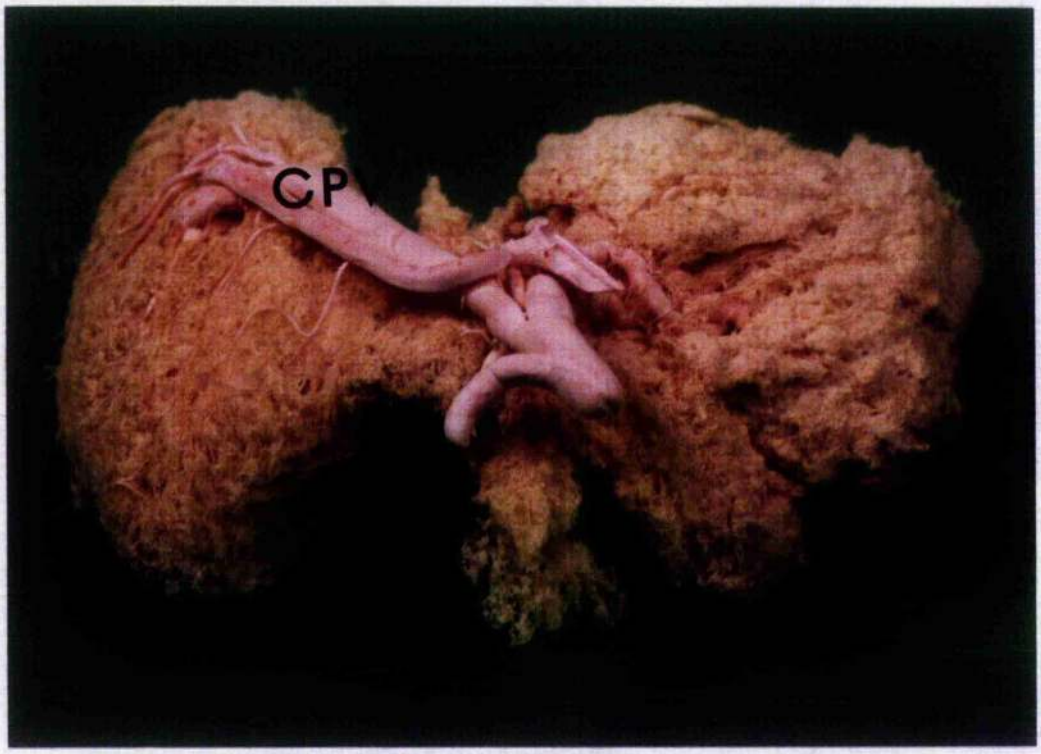

Figure 4.1 A vascular cast of a placental cotyledon following digestion of the placental tissue by potassium hydroxide. The chorionic plate vessels by which the plastic medium is introduced to the cotyledon are identified (CPV).

Figure 4.2 Scanning electron micrograph of the terminal vascular network in a normal preterm placenta. The terminal capillary "buds" (C) are formed of multiple, short, highly branched vessels.

Figure 4.3 Scanning electron micrograph of the peripheral vascular network in a placenta from a pregnancy complicated by IUGR and AEDFV. Terminal capillary "buds" are absent from these placentas and are replaced by elongated, unbranched "hose-pipe" type vessels (V).

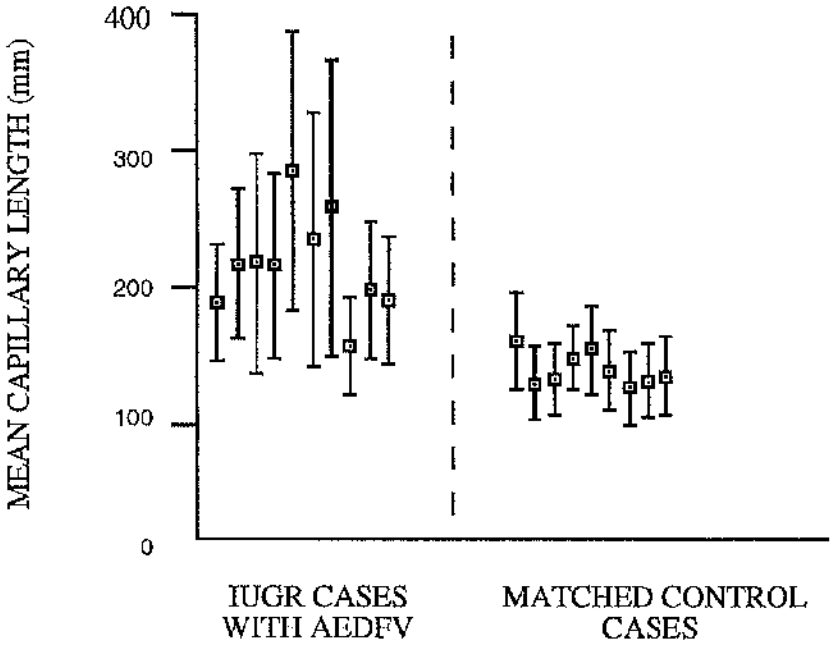

Figure 4.4 Mean capillary length

The mean length of capillaries in each of the 10 IUGR and 9 gestationally age-matched control placental vascular casts is marked by a box (|—| indicate one standard deviation). Capillaries in the IUGR placental casts were significantly longer than those seen in the control cases; $p<0.05$

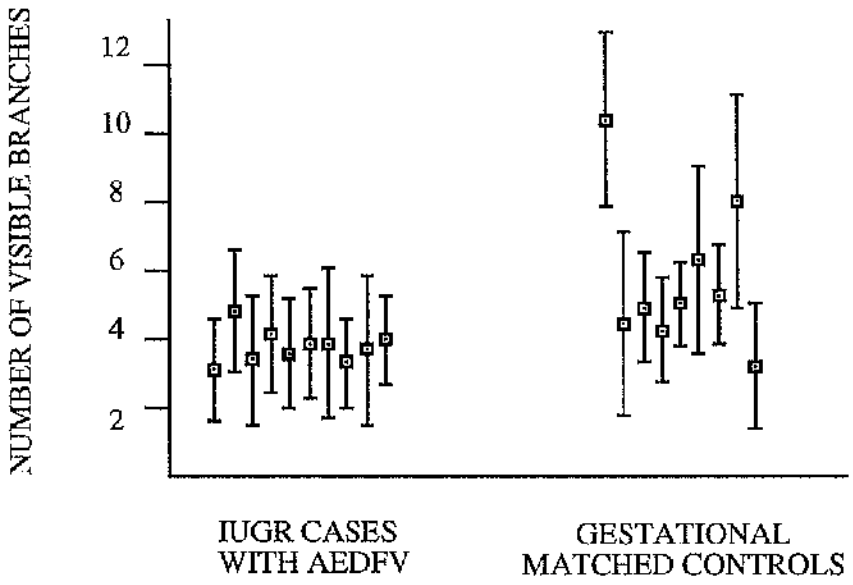

Figure 4.5 Mean number of capillary branches

Each box represents the mean number of branches present within the capillary loops of an individual case. |—| demonstrate one standard deviation. There were significantly fewer capillary branches present in the IUGR vascular casts than in the gestationally age-matched controls casts; $p<0.05$.

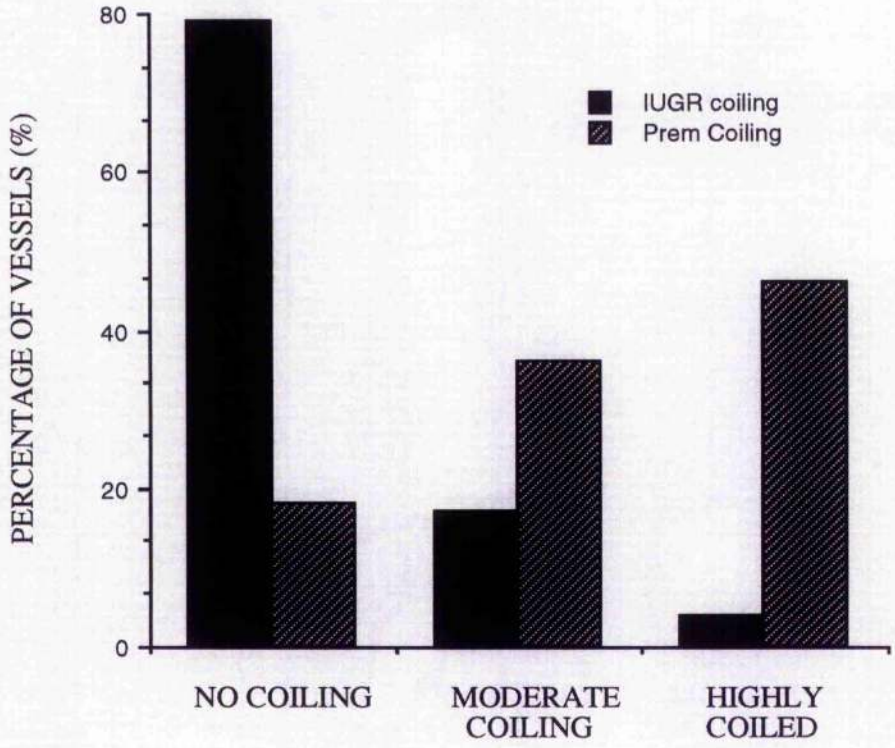

Figure 4.6 Capillary coiling

Degree of capillary coiling in the terminal capillary loops of IUGR and gestational age-matched control placental vascular casts. Significantly more IUGR capillaries were uncoiled when compared with the control capillary loops (79% vs. 18%; $p<0.05$). IUGR coiling- proportion of IUGR capillary loops which demonstrated coiling; Prem coiling - proportion of preterm control capillary loops which demonstrated coiling.

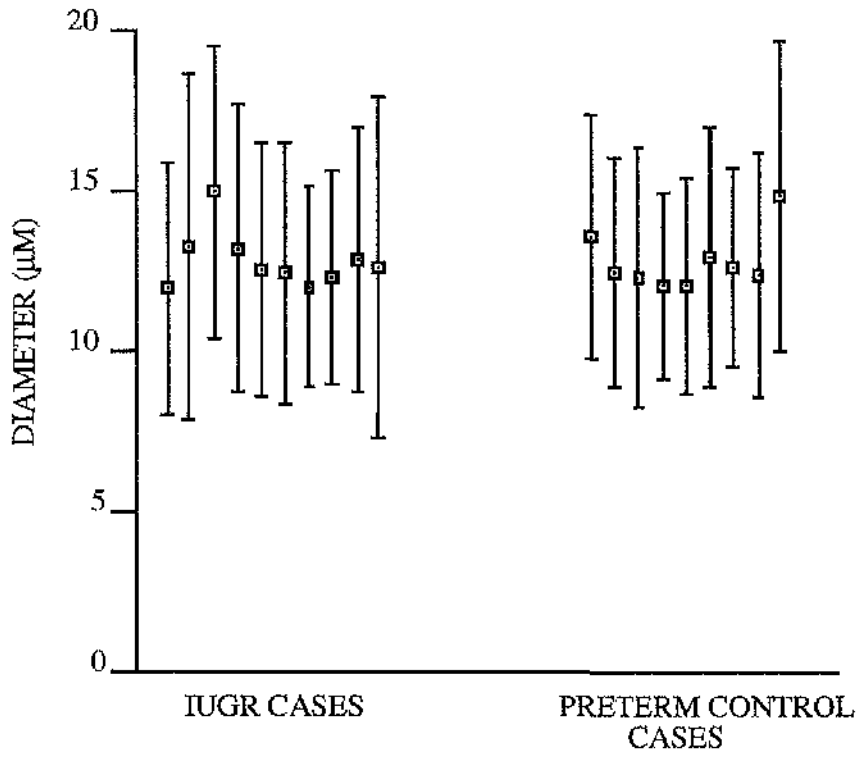

Figure 4.7 Mean (SD) diameter of terminal capillary vessels from 10 IUGR casts and 9 gestational age-matched preterm control vascular casts. ⊢⊣ indicate one standard deviation of each value. There was no significant difference between the mean capillary diameter of vessels in IUGR and control vascular casts.

CHAPTER 5

SCANNING ELECTRON MICROSCOPY OF TERMINAL VILLI

5.1 INTRODUCTION

The role of intermediate and terminal villi (peripheral villi) in the pathogenesis of intrauterine growth restriction has been largely ignored. However, the severely growth restricted fetus demonstrates biochemical and clinical evidence of intrauterine starvation (Nicolaides, Economides & Soothill 1989; Economides et al. 1989) and it is at the level of these peripheral villi that maximal feto-maternal exchange of oxygen and substrates occurs.

The obliterative theory of increased umbilical artery vascular resistance (Giles, Trudinger & Baird 1985, McGowan, Mullen & Ritchie 1987; Bracero et al. 1989) assumes that the biochemical changes, which occur in the IUGR fetus result from the functional loss of large areas of peripheral placental villi secondary to small stem arteriole occlusion. This is somewhat surprising as the normal placenta, which has a large functional reserve and can withstand inactivation of up to 40% of its villous tissue (Robinson et al 1979; Fox 1986), is capable of proliferation and adaptation throughout pregnancy. Placental size increases dramatically in pregnancies complicated by maternal anaemia for example, to compensate for the diminished content of oxygen available (Agboola 1975). In the event of stem vessel obliteration, compensatory growth in unaffected areas of the placental villous tree would therefore be expected.

Surprisingly therefore, stereological analysis of placentas from growth restricted pregnancies complicated by AEDFV have shown that the volume of peripheral villi in placentas from IUGR pregnancies is significantly diminished (Jackson et al. 1995), implying that rather than compensatory growth, peripheral villous growth may actually be impaired.

In chapter 4 we demonstrated that peripheral capillary growth within placentas from IUGR pregnancies was abnormal when compared to gestationally matched controls. Together with the results of other workers which have shown reduced intravascular capillary volumes in IUGR placentas (Hitschold et al. 1993), our findings would indicate that angiogenesis is impaired in placentas from growth restricted pregnancies and thus placental perfusion limited.

Fetal substrate provision requires not only adequate placental perfusion but also the effective transfer of these metabolites from the maternal uterine circulation across the syncytiotrophoblast membrane. The reduction of syncytial surface area suggested by stereology (Hitschold et al. 1993; Jackson et al. 1995) would therefore restrict the transfer of fetal substrates thus "starving" the fetus *in-utero*.

In order to determine three-dimensional villous structure in placentas from IUGR pregnancies, and thus establish if the reduction of villous tissue reported (Jackson et al. 1995; Hitschold et al. 1993) was simply due to depleted numbers of normal terminal/intermediate villi or as with the capillaries in chapter 4, the consequence of abnormal villous development, we examined tissue casts from IUGR and control placentas by scanning electron microscopy (SEM).

5.2 METHODS

5.2.1 Clinical details

Placental tissue from the ten IUGR and ten control patients used in Chapter 4 for the vascular cast study were also used on this occasion. The clinical details of the IUGR cases are shown in Table 2.1 [Case numbers 1-7, 9, 10 and 20] and details of the preterm control cases in Table 2.2 [Case numbers 21, 22, 24, 28 and 36-40]

5.2.2 Collection of material

5.2.2.1 Preparation of buffer

0.2M solution of disodium hydrogen orthophophate (DHO) was prepared by dissolving 2.83g DHO in 100 mls of distilled water [Sol A]. 2.81g of citric acid was then dissolved in 100 mls of distilled water to give a 0.1M solution of citric acid [Sol B]. Neutral phoshphate buffer was prepared by adding 86 mls of Sol A to 17.7 mls of Sol B. Prepared solutions were all stored in a refrigerator at 4^0C.

5.2.2.2 Preparation of 2.5% glutaraldehyde

2.5% buffered glutaraldehyde was prepared by adding 5 mls of 25% aqueous glutaraldehyde to 50 mls of phosphate buffer. Fresh buffered glutaraldehyde was prepared just prior to the specimen collection.

5.2.2.3 Specimen collection

Immediately following delivery of the placenta, a further two chorionic plate arteries supplying two randomly chosen peripheral cotyledons were cannulated with a 16 gauge venous cannula. These were secured with a black silk suture to prevent backflow or extrusion of the cannulae. Cotyledons affected by infarction were excluded. The chorionic veins draining each cotyledon were punctured at a point proximal to the cord root ensuring free venous drainage throughout the procedure and limiting artefacts which might arise from feto-maternal fluid shift (Burton, Ingram & Palmer 1987). The cotyledons were initially flushed with 5 mls of isotonic saline to irrigate the vascular bed then immediately perfusion-fixed at a constant pressure with 20 mls of 2.5% neutral buffered glutaraldehyde over a 5

minute period. The perfused area blanched with fixation and a minimum of 10 blocks of the fixed tissue (5 x 5 x 5 mm) were then excised from an area just above the basal plate. These were transferred into fresh 2.5% phosphate buffered glutaraldehyde for storage prior to processing. The total time from delivery of the placenta to fixation in glutaraldehyde was typically 2-3 minutes and never longer than 5 minutes.

5.2.2.3 **Critical point drying**

The expansion of intra and extra cellular fluid can cause structural artefacts in the tissue morphology. Tissue for SEM must therefore be processed in such a way that most of the intra and extra cellular fluid is removed simultaneously, a process known as critical point drying. Tissue for examination was prepared as follows; cubes of the fixed placenta were dehydrated through ascending concentrations of alcohol to replace tissue fluid with alcohol, then placed in the pre-cooled pressure chamber of a critical point dryer. Liquid carbon dioxide was then instilled through the pressure chamber over a period of 10 minutes until all the intermediate fluid - alcohol- was replaced. The temperature of the chamber was increased and the carbon dioxide converted into a gaseous form. By gradually reducing the pressure of the chamber, gaseous carbon dioxide was released ensuring the complete removal of extracellular and intracellular fluid with only minimal distortion of the villous structure.

During scanning electron microscopy, electrons may accumulate on the tissue surface, producing high electrostatic charges and impairing the quality of the image obtained. To limit this, the specimens were placed in a sputtering machine and coated with a thin layer of gold prior to SEM examination.

5.2.3. **Analysis of the tissue casts**

The specimens were all coded independently and viewed blind in a Zeiss 940 DSM scanning electron microscope. A minimum of three tissue blocks were examined from each case.

The tissue casts were examined first at low power (x 100) to assess the quality of tissue fixation, the degree of electrostatic charging and the amount of extravillous fibrin present. Areas comprised of terminal and intermediate villi were then identified at higher magnification (x 200). The distribution, shape and size of terminal villi were recorded. Thereafter the texture of the syncytiotrophoblast was evaluated and the microvillous surface examined at high magnification (x 5000) to assess the density of microvilli present.

5.3 RESULTS

5.3.1 Comparison of the study groups

The two groups were appropriately matched for gestational age. The mean gestational age of the IUGR group was 31.5 weeks [SD 2.83] and that of the control group 31.3 weeks [SD 2.87]. The mean birthweight of the IUGR group was significantly less than that of the controls (1150g [SD 379.5] vs. 1782g [SD 519.5]; $p<0.05$).

5.3.2 Terminal villous structure

Qualitatively, fewer villi were present in the IUGR placental casts than in the control casts. The peripheral villi, comprising intermediate and terminal villi, of the control placentas were characterised by a dense population of multiple, small bud-like villi, with areas, corresponding to sinusoidal dilatations present at the villous tips and randomly throughout most villous buds (Figure 5.1). In contrast the peripheral villi of the IUGR placental casts appeared as elongated drainpipes (Figure 5.2) with only tiny isolated areas showing any resemblance to the small villous buds seen in the control casts. There was little variation in the diameter of peripheral villi in the IUGR casts. The size, shape and pattern of peripheral villi matched the appearance of the capillary vessels in the vascular casts examined previously in chapter 4.3.2. Large plaques of fibrin, often extending between several villi, were present on the syncytial surface of 75% of the IUGR casts, particularly cases 1, 3 and 8, and was not specific for only those IUGR cases which were complicated in addition by maternal pre-eclampsia. In contrast, only one of the preterm control casts showed significant fibrin present. Furthermore, the syncytiotrophoblast surface over the IUGR terminal villi appeared to be piled up, forming characteristic wrinkles, bands and folds in the syncytiotrophoblast surface (Figure 5.3). These "wrinkles" were never seen in any of the control cases. No differences were detected in the density or appearance of microvilli on the syncytiotrophoblast surfaces of either IUGR or control cases.

5.4 DISCUSSION

Villous development follows a specific pattern in normal pregnancy. During the first and most of the second trimester, intermediate villi differentiate primarily into support stem villi while during the latter half of pregnancy exchange, terminal villi are formed in preference (Castellucci et al. 1989; Jackson, Mayhew & Boyd 1992). Since the majority of severely growth restricted pregnancies with abnormal umbilical Doppler are delivered prematurely, these placentas would naturally differ from normal term control cases. However, using appropriate gestational age-matched control pregnancies we have demonstrated that the placentas of growth restricted pregnancies demonstrate abnormal villous structure which is

not simply the consequence of preterm delivery. In contrast to the control placentas which were characterised by multiple globular terminal villous buds, the IUGR placentas were composed of elongated, non-budded villi (Figure 5.2); these bore a striking resemblance to the elongated, uncoiled capillary loops demonstrated in chapter 4 by vascular casting.

Terminal villi arise passively as outpockets from intermediate villi during the late second and the third trimesters of pregnancy, when longitudinal capillary growth exceeds that of the villi themselves (Bernischke & Kaufmann 1990). The development of these peripheral villi expands the villous surface area dramatically, providing a syncytial surface area of $8m^2$ by term (Leiser, Kosanke & Kaufmann 1991). The syncytiotrophoblast layer of the placenta is part of the interface between fetal and maternal blood and via several transport mechanisms is the means of transferring vital substrates from the maternal circulation to that of the fetus. The dramatic expansion of the syncytial surface area during the third trimester is therefore vital to cope with the increasing metabolic demands of the growing fetus. Our findings of elongated, non-budded villi, would suggest that villous growth and development is significantly deranged in placentas from IUGR pregnancies and the syncytial surface area thus diminished.

In chapter 4 we demonstrated, using vascular casts, that the structure of the capillary bed was abnormal in IUGR placentas; the appearance of the capillaries present suggested that capillary growth was defective. The three-dimensional structure of the placental villi and the external appearance of the syncytiotrophoblast (Figures 5.2 and 5.3) in the IUGR placental tissue casts which we examined would confirm this impression. The size and shape of the IUGR peripheral villi virtually mirrored that of the IUGR capillary casts. Furthermore, the syncytiotrophoblast over the IUGR placental villi was aggregated into wrinkles and folds which would suggest that the rate of villous growth, exceeded that of the capillaries to such an extent that "redundant" syncytiotrophoblast resulted, necessitating the formation of these syncytial folds. Since terminal villous development is intimately linked to longitudinal capillary growth (Kaufmann et al. 1985), the potential mechanisms regulating placental angiogenesis are valid in this setting also.

Capillary growth may not be the only determinant of terminal villous development. Since terminal villi arise from intermediate villi, alterations to the intermediate villous population would also influence terminal villous development. From the tissue cast images we obtained, it would appear that the total villous density of the IUGR placentas were significantly less in those of the controls. This is in keeping with the results of two-dimensional placental morphology studies which have indicated that villous volume is reduced in IUGR pregnancies (Boyd & Scott 1985; Hitschold et al. 1993; Jackson et al. 1995), though unfortunately, none of these studies have calculated specific villous class volumes. In

addition, gross studies in placental morphology would also suggest that villous proliferation is impaired in IUGR pregnancies as placentas from affected pregnancies have fewer cotyledons and are thinner than those from healthy control cases (Nordenvall et al. 1991).

The fetus depends on the transfer of maternal oxygen and nutrients across the trophoblast membrane and therefore a reduction of peripheral villous tissue will deplete the surface area available for nutrient transfer. Moreover, large portions of the syncytiotrophoblast in the IUGR placentas were covered with fibrin, often extending between several villi. Since it is the microvillous membrane of the syncytiotrophoblast which contains many of the receptors and carrier proteins necessary for substrate transfer, these plaques may additionally reduce the functional transport area available, limiting further, nutrient provision for the IUGR fetus.

It is not clear why excessive fibrin plaques might accumulate around the IUGR syncytiotrophoblast. In the normal placenta, fibrin may be deposited secondary to syncytiotrophoblast damage and in association with villi which are surrounded by slow intervillous blood flow. It is possible in the IUGR placentas that abnormal villous development may alter the circulation of blood around the placental bed and encourage fibrin deposition. Alternatively, in addition to the structural changes we have demonstrated, the functional capacity of the trophoblast layer may also be deranged. Under such circumstances, the anti-platelet and anticoagulant activity (Iloka et al. 1993; Uszynski 1991) of the normal syncytiotrophoblast may also be deficient resulting in fibrin deposition.

We have demonstrated that the reduction in peripheral villous tissue noted previously in IUGR pregnancies complicated by AEDFV, is the consequence of abnormal villous development and not simply due to reduced numbers of normal peripheral villi. The consequent reduction in the syncytiotrophoblast surface area may explain in part the fetal substrate deprivation characteristic of the IUGR fetus. It is likely that the lack of villous tissue reflects primary abnormalities in the expression of genetically controlled regulators of cytotrophoblast proliferation and highlights the vital need for the role and expression of these agents in placental maldevelopment to be established.

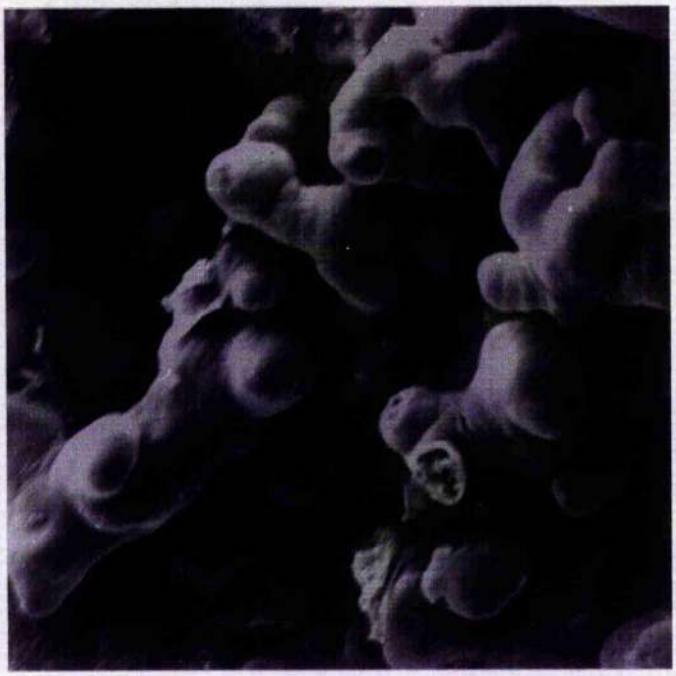

Figure 5.1 SEM of peripheral villi in an uncomplicated preterm placenta.

The peripheral villi in the control placentas were composed of multiple small, squat, wide-based buds, which correspond to the capillary loops identified in the vascular casts (magnification x 200).

Figure 5.2 SEM of peripheral villi within a placenta from a pregnancy complicated by IUGR.

The peripheral villi present in the IUGR placental casts showed little "bud" formation and appeared rather as elongated drainpipes. This appearance was in keeping with the elongated, uncoiled unbranched capillary loops previously identified in the IUGR vascular casts. To allow the entire length of the villi to be included the magnification is x 165.

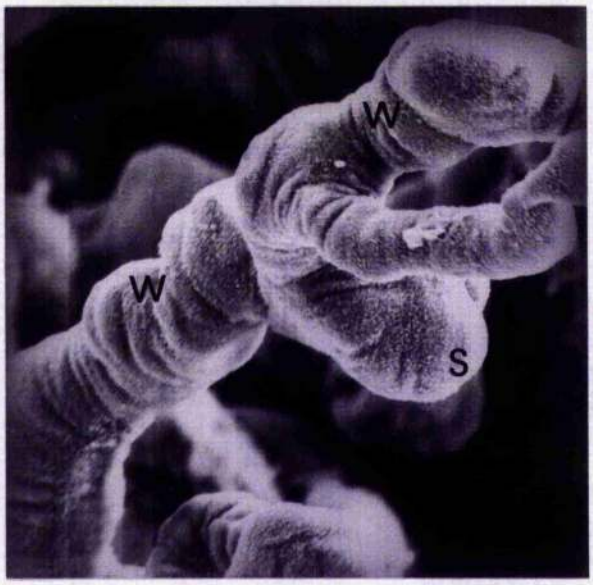

Figure 5.3 SEM of peripheral villi from an IUGR placental cast.

The syncytiotrophoblast (S) surface appeared to be compressed, forming characteristic folds and "wrinkles" (W) on the syncytiotrophoblast surface (x 500).

CHAPTER 6

THE ULTRASTRUCTURE OF TERMINAL VILLI

6.1 INTRODUCTION

Terminal villi are the site of maximal feto-maternal exchange and since umbilical venous blood is frequently observed to have a low oxygen content in AEDFV pregnancies (Nicolaides, Economides & Soothill 1989), it has been assumed that the placenta, at the level of these exchange villi, where transfer occurs, must also be hypoxic. Previous ultrastructural studies of the placenta in cases of fetal growth retardation have shown inconclusive results, primarily because these studies used varying definitions of growth retardation and in the main were confined to small-for-gestational age term pregnancies (Standstedt 1979; Aherne & Dunhill 1966). Since only a tiny proportion of these latter pregnancies are truly growth restricted *in-utero,* few of these placentas would have been pathological.

One recent study, in which the pathological and control group were both well defined and appropriately matched, demonstrated that the volume of terminal villi was significantly reduced in IUGR placentas with AEDFV (Jackson et al. 1995). This study however, addressed only the question of villous number and did not assess if the terminal villi present were normal in appearance. In the preceding chapters (Chapters 4 and 5), we presented evidence to show that both the external appearances of the terminal villi, and the fetoplacental capillaries within them, are grossly abnormal in AEDFV pregnancies and did not indicate hypoxia as a major determinant of placental development.

To evaluate if the internal appearance of terminal villi was also abnormal in IUGR pregnancies, and to assess in particular if these villi showed evidence of hypoxic change, we used placental tissue from our homogeneous group of IUGR pregnancies to conduct a detailed ultrastructural study of placental terminal villi.

6.2 METHODS

6.2.1 Clinical details

The clinical details of the 10 cases selected are shown in Table 2.1 (Case numbers 1-10). All the pregnancies were complicated by severe IUGR as defined in Chapter 2 (Section 2.2.1) and all were delivered by caesarean section. Five pregnancies were normotensive and five were complicated by pre-eclampsia as defined previously in 2.2.1. Birthweights after delivery, based on a local database (Smalls & Forbes 1983), were confirmed to be <10th centile - the majority being <3rd centile - in all but one case.

For comparison purposes a control series of eight preterm pregnancies were also selected. These pregnancies were uncomplicated and were matched for gestational age and smoking habit. Their clinical details are shown in Table 2.2 (Case numbers 20-27).

6.2.2 **Perfusion - fixation of specimens**

Immediately following delivery, the placenta was examined for evidence of gross pathology and weighed. Chorionic plate arteries supplying two separate peripheral cotyledons, chosen at random, were cannulated with a 16 gauge venous cannula (Venflon) and secured with a black silk suture to prevent backflow or extrusion of the cannulae. Cotyledons affected by infarction were excluded. To each cannula, two syringes, one filled with 0.9% isotonic saline and the other with 2% neutral phosphate buffered glutaraldehyde, were connected via a polyethylene three port connector. The chorionic veins draining each cotyledon were punctured at a point distal to the cannulae. This ensured free venous drainage throughout the procedure and limited artefacts which might arise from feto-maternal fluid shift (Burton, Ingram & Palmer 1988). The cotyledons were initially flushed with 5 mls of isotonic saline then immediately perfusion-fixed at a constant pressure with 20 mls of 2% neutral buffered glutaraldehyde over a 5 minute period. The perfused area blanched with fixation and a minimum of 10 blocks of the fixed tissue (5 x 5 x 5 mm) were then excised from an area just above the basal plate. These were transferred into fresh 2% phosphate buffered glutaraldehyde for storage prior to processing. The total time from delivery of the placenta to fixation in glutaraldehyde was typically 2-3 minutes and never longer than 5 minutes.

6.2.3 **Tissue Processing**

Tissue blocks were rinsed twice in phosphate buffer (5.2.2.1), then post-fixed for 45 minutes in 1% osmium tetroxide. Following a further 2 brief washing in phosphate buffer the tissue blocks were dehydrated in ascending concentrations of ethyl alcohol then embedded in araldite resin and polymerised at 60^0 C for 24 hours.

6.2.4 **Transmission Electron Microscopy**

The tissue specimens were examined blind to the clinical information. Semithin sections were prepared with an LKB ultramicrotome, stained with toludine blue and viewed with a Leica microscope, to identify areas free of perivillous fibrin in which terminal villi, as defined below, predominated. Three suitable blocks from each case were selected, the areas were isolated using a standard paring technique and ultrafine sections cut with the LKB ultramicrotome using a fresh glass knife. The cut sections were floated into a water filled truf and a minimum of three sections then retrieved onto a copper plated grid. These were subsequently stained for 10 minutes with uranyl acetate then submerged in lead citrate for a further 2 minutes. The grids were viewed in a Philips CM 10 transmission electron microscope. A minimum of 10 terminal villi (usually 15-20) seen in cross-section were

photographed individually at standard final magnifications (x 2025, x 4950, x 14850). Villi which were viewed in longitudinal or oblique sections, and those which showed evidence of branching patterns were excluded.

6.2.5 **Description of terminal villi**

Terminal villi were defined as terminal ramifications of villus trees, characterised by the absence of fetal arteries, arterioles, veins and venules. Since smooth muscle cells are not present in the vessel walls, the terminal villous vascular system is composed solely of capillaries with dilated segments known as sinusoids (Kaufmann et al. 1985). The effect of IUGR on the standard morphometric characteristics used to define normal terminal villi, such as the percentage of the villus occupied by capillaries, or the percentage of villus surface made up by vasculosyncytial membrane (Sen, Kaufmann & Schwiekhart 1979), is unknown. These features were therefore not included as mandatory in our identification of terminal villi

The diameter of each terminal villus was measured in two randomly chosen perpendicular planes and averaged; for each case studied, the mean value was calculated. Each terminal villus cross section was then described according to set criteria as follows;

6.2.5.1 TROPHOBLAST

The numbers and position of both syncytiotrophoblast and cytotrophoblast nuclei were counted. Evidence of syncytial nuclear senescence, such as syncytial nuclear clumping and increased electron density of the nuclear chromatin, was sought (Jones & Fox 1977). Vasculosyncytial membranes, defined as anuclear areas of syncytiotrophoblast where the fetal capillary endothelium is closely opposed, were identified and the shape and pattern of the syncytiotrophoblast microvillus surface, both in general and in particular over the vasculosyncytial membranes, was assessed in a minimum of 4 views (final magnification x 14850).

6.2.5.2 TROPHOBLAST BASAL LAMINA

Well-defined areas of trophoblast basal lamina (lamina densa) viewed in cross section were identified at a final magnification of x 14850 and the mimimum membrane thickness measured.

6.2.5.3 VILLUS STROMA

The density of the villus stroma was assessed qualitatively, looking in particular for the presence of collagen fibres and the density of background fibrillar material (Castellucci & Kaufmann 1982; Jones & Fox 1991). The type and number of cells within the stroma, and the presence of basal lamina-like material surrounding connective tissue cells [myofibroblasts (Gabbiani & Majno 1972)] was also noted for each case.

6.2.5.4 FETAL CAPILLARIES

Capillary endothelial cells were assessed for evidence of damage and the presence of luminal obstruction by platelet clumps, thrombus or red cell aggregates.

6.2.6 **Immersion fixation**

6.2.6.1 **Preparation of buffered formalin**

Phosphate buffer was prepared as follows; 3.12g of sodium dihydrogen orthophosphate was dissolved in 100 mls of distilled water forming a 0.2M solution of sodium dihydrogen orthophosphate [solution 1]. 0.2 M disodium hydrogen orthophosphate was then prepared by adding 2.83 g disodium hydrogen orthophosphate to 100 mls of distilled water [solution 2]. 28 mls of solution 1 was added to 72 mls of solution 2 forming 100 mls of phosphate buffer (pH 7.2). Neutral buffered formalin was then constituted by adding 80 mls of 10% formalin (containing 4% formaldehyde) to 120 mls of phosphate buffer. Prepared solutions were all stored in a refrigerator at 4^0C.

6.2.6.2 **Preparation of tissue**

From the remainder of the placenta, a minimum of four random, full-thickness blocks of tissue were excised and immersion fixed in 4% neutral buffered formalin for a minimum of 8 hours and not more than 24 hours. These were processed in a standard manner for wax-embedding

6.2.7 **Light microscopy studies**

Two semithin sections (3-5 μM) were cut from each paraffin block and mounted on glass slides. The mounted sections were dipped in haematoxylin, washed 3 times then immersed in water soluble eosin. Thereafter, they were dipped in grades of alcohol from 70% through to absolute alcohol, washed twice in xylene, then covered.

Congestion of fetal capillaries within terminal villi was evaluated in ten random fields (x100) from each case. Congestion was considered to be present in a field where the majority (>90%) of the fetal capillaries demonstrated densely packed erythrocytes.

6.2.8 **Statistical analysis**

All parameters were shown to approximate a normal distribution and thus quantitative data are shown as mean (standard deviation). Differences in mean values were assessed by an unpaired Student's t-test. P values <0.05 were considered significant.

6.3 RESULTS

6.3.1 **Comparison of the two groups**

The gestational age of the two groups was comparable; IUGR group (31.6 weeks [SD 2.6])); control group (30.5 weeks [SD 3.4]). Birthweight was significantly lower in the IUGR group (1068g [SD 333]) compared with the control group (1754g [SD 484]; p =0.001). Similarly there was a significant reduction in the placental weight of the IUGR group when compared to the controls (282g [SD 88] vs. 386g [SD 51]). There was no difference in the placental/fetal weight ratio between the two groups (0.25 [SD 0.07] vs. 0.27 [SD 0.09]; p=0.66). Four women in each group were smokers.

6.3.2 TROPHOBLAST

The mean diameter of terminal villi in the IUGR group was significantly less than in the control group (5.7 nm [SD 0.6] vs. 6.6 nm [SD 0.8]; p = 0.02). Terminal villi from the IUGR group had significantly increased numbers of syncytiotrophoblast nuclei (SN) as compared to the control group; 9.6 nuclei per villus cross section (SD 1.9) vs. 4.5 (SD 1.6); p < 0.001). Most of the SN in the IUGR group were aggregated into syncytial knots and demonstrated evidence of nuclear senescence (Figure 6.1). Within the IUGR cases, there was no difference in the number of SN when maternal pre-eclampsia was present (8.6 nuclei [SD 1.67]) or absent (7.8 [1.64]; p=0.47. This finding was confirmed by paraffin histology. By contrast, the numbers of cytotrophoblast nuclei (CN) were significantly less in the IUGR group when compared with the control group though again the presence or absence of maternal pre-eclampsia did not alter the findings; CN were found in only 40% of villus cross sections in the IUGR group and when present only 1 CN per villus cross section was normally seen. In the control group 80% of villus cross sections were found to have CN and in these cases 2 CN per villus cross section were normally seen (p < 0.01) (Figure 6.2). Sections of villus cytotrophoblast without nuclear profiles were excluded.

The trophoblast basal lamina (lamina densa) in the IUGR group was approximately twice as thick when compared with the control group (1.3 nm [SD 0.29] vs. 0.6 nm [SD 0.23]; p < 0.001) (Figure 6.3). No obvious differences in the microvillous surface of the syncytiotrophoblast were identified between the two groups.

6.3.3 VILLUS STROMA

An increased density of background fibrillar material was seen within the stroma of the IUGR group when compared with the gestational age-matched control group and this difference was irrespective of the presence or absence of maternal pre-eclampsia. In addition, there appeared to be an increase in the number of collagen fibres in the IUGR group, as identified by their characteristic banding pattern. The villus stroma in the IUGR

group contained a large number of fibroblast cells. These fibroblast-like cells which were arranged in parallel to the long axis of the villus, demonstrated ultrastructural features typical of myofibroblasts namely an incomplete basal lamina together with intracytoplasmic bundles of filaments and area densae (Gabbiani & Majno 1972) [Figure 6.4]. Winding bands of basal lamina-like material were noted to surround these cells. By contrast, the stroma in the control group contained only a minimal amount of background fibrillar material, and collagen fibres were only occasionally identified (Figure 6.5). No myofibroblast like cells were identified in any of the terminal villi from the control group.

6.3.4 FETAL CAPILLARIES

In general no differences in endothelial cell structure were observed between the two groups. However, congestion of fetal capillaries by erythrocytes was observed in three of the IUGR cases, each of which was in addition complicated by pre-eclampsia. The fetal erythrocytes in these three cases appeared to be moulded together, and varied in their electron density. In places these aggregates showed signs of lysis of the erythrocyte cell membrane (Figure 6.1). At the light microscopic level, congestion was also more evident in the IUGR group when compared with the control group (4.5 fields examined [SD 1.4] vs. 0.75 fields examined [SD 2.9]; $p<0.01$) Congestion was also more obvious in those IUGR cases which were in addition complicated by pre-eclampsia (5.8 fields [SD 2.4] vs. 3.5 fields [SD 2.9]) but this difference was not statistically significant.

Transmission electron microscopy demonstrated no evidence of platelet clumping within the capillaries in any of the IUGR cases, nor in any of the controls. A summary of the data, comparing terminal villi from IUGR and gestation age-matched control pregnancies is displayed in Table 6.1.

6.4 DISCUSSION

Until recently, the investigation of placental structure in pregnancies complicated by abnormal fetal growth has been hampered by the lack of a reliable method to distinguish the small but healthy normal fetus from one whose growth has been restricted *in-utero*. By identifying reduced umbilical artery perfusion, Doppler ultrasound distinguishes the severely growth-restricted fetus from the small but healthy normal one (Laurin et al. 1987; Burke et al. 1990; McParland, Steer & Pearce 1991). Therefore the study required, in addition to the identification of a small fetus with oligohydramnios, the presence of AEDFV as a diagnostic criterion for IUGR. Moreover, to ensure that any findings were not solely the effect of

preterm gestation, a group of normally-grown gestational age-matched fetuses with normal umbilical artery perfusion were selected to serve as a control group.

Both immersion and perfusion fixation provides satisfactory villous fixation. However, immersion fixation is associated with considerable post-partal vessel collapse (up to 33%) and consequently a significant reduction in the villous volume and surface area (Burton Ingram & Palmer 1987) while the morphological data obtained by perfusion fixation is very similar to that from *in-situ* biopsies of the placenta (Sen, Kaufmann & Schweikhart 1979). The cases used in this study were therefore prepared by perfusion fixation with glutaraldehyde. Since free venous drainage does not significantly affect the degree of post-partal vessel collapse and limits villous oedema, resulting from feto-maternal fluid shift, the venous outflow tracts were always punctured.

Perfusion fixation of large areas of the placenta is associated with incomplete removal of fetal blood and therefore suboptimal fixation. Moreover even short term ischaemia may significantly alter the ultrastructure of the placenta (Kaufmann 1985c). Therefore, only two cotyledons were perfused to limit the degree of artefact resulting from ischaemia while retaining an adequate sample size. Within these areas, multiple specimens were taken, excluding the basal and chorionic plate areas (Boyd, Brown & Stewart 1980).

Several major ultrastructural differences were identified in the terminal villi from pregnancies complicated by IUGR when compared with gestational age-matched controls. The mean cross-sectional diameter of the terminal villi in the IUGR group was significantly smaller than in the control group. These observations are in agreement with a recent light-microscopic study which suggested that both the mean cross-sectional area of terminal villi and the degree of vascularisation within them, was significantly reduced in IUGR pregnancies with reduced umbilical artery perfusion (Jackson et al. 1995). Other studies (Hitschold et al. 1993; Boyd et al. 1985) which evaluated villous diameter did not find any difference in the placentas of small babies, but these studies looked only at term SGA infants (Boyd & Scott 1985) or compared SGA pregnancies with pregnancies which were not matched for gestation (Hitschold et al. 1993). Since terminal villi are ideally adapted for diffusional exchange - because of their high vascularisation and low materno-fetal diffusion distance (Sen 1979) - these changes may have major functional implications for the fetus. Fetal hypoxia has been associated with placentas which demonstrate a significant reduction in the number of terminal villi (Kaufmann et al. 1985a).

Increased numbers of syncytiotrophoblast nuclei (SN), which were usually arranged in clusters known as syncytial knots, were noted in the IUGR group (Jones & Fox 1977). These nuclei also demonstrated typical features of senescence, such as chromatin clumping

and nuclear moulding, which have been described previously in the trophoblast of the normal term placenta (Martin & Spicer 1973; Jones & Fox 1977; Jones & Fox 1991). Syncytial knot formation, amongst other functions, is thought to be a mechanism by which aged nuclei are removed from the syncytiotrophoblast layer (Cantle et al. 1987; Bernischke & Kaufmann 1990) and is indicative of placental maturity (Fox 1965). By contrast, syncytial knots were rarely identified in the gestational age-matched control group. These data indicate that syncytiotrophoblast maturation is more advanced in the IUGR group.

The numbers of cytotrophoblast cells, identified by the presence of nuclei, were reduced in the IUGR group when compared with the gestational age-matched controls. These cells are the stem cells of the syncytiotrophoblast. During the first trimester they form a complete layer beneath the syncytium but with advancing gestation, they become more sparse and are seen only infrequently in the term placenta. Cytotrophoblast cells are incorporated into the syncytiotrophoblast by the creation of gap junctions followed by the formation of intercellular bridges and subsequent fusion of the cellular membranes (Contractor et al. 1977; Firth, Farr & Baumann 1980). It is well known that syncytial fusion of the cytotrophoblast is not only a mechanism effecting syncytial growth, but is also vital for continuous functional regeneration of the latter; failure to incorporate cytotrophoblast into the syncytiotrophoblast will result in degeneration within a few days (for review see Bernischke & Kaufmann 1990). Since the syncytium in the IUGR placentas showed degenerative changes in the presence of reduced numbers of cytotrophoblast cells, it is likely that this reflects reduced syncytial fusion resulting from a primary deficiency in cytotrophoblast cell proliferation. This hypotheses could be tested using markers of cell proliferation

Numerous receptors and transporter systems within the microvillus surface of the syncytiotrophoblast ensure adequate transfer of nutrients and waste across the feto-maternal barrier. The alterations in trophoblast structure identified in IUGR placentas may therefore have functional implications for placental transport. Microvillous membranes prepared from pregnancies complicated by IUGR at term have demonstrated a reduced activity of the system A amino acid transporter (Mahendran et al. 1993). Reduced circulating concentrations of the amino acids carried by this transporter have been found in umbilical venous blood from pregnancies complicated by IUGR (Cetin et al. 1990; Pardi et al. 1993). No ultrastructural differences were observed in the appearance of the microvillus surface of the syncytiotrophoblast layer in the IUGR cases. However, in view of the findings which suggest an accelerated ageing of the syncytiotrophoblast, studies employing electron immunohistochemistry, or physiological studies with membrane preparations, are warranted to search for other potential transport defects within the trophoblast in IUGR.

Other properties of the trophoblast may similarly be affected. Trophoblast is known to express the constitutive form of the enzyme nitric oxide synthase (Myatt et al. 1993). The physiologic production of nitric oxide by villous trophoblast may prevent platelet aggregation in the intervillous space or may act as a second messenger between cyto and syncytiotrophoblast. By inference, a defect in this mechanism, as a result of premature ageing of the trophoblast may explain why intervillous platelet aggregation, thrombosis and infarction occurs in IUGR.

The basal lamina densa beneath the trophoblast layer was approximately twice as thick in the IUGR group when compared with the gestational age-matched controls. Furthermore, there was a striking increase in the density of fibrillar material and fibroblasts within the stroma and an increase in the number of collagen fibres present. This may be of functional significance since the oxygen diffusive conductance of villi is proportional to the thickness of both the trophoblast and stromal compartments (Mayhew, Jackson & Haas 1990).

A group of fibroblast-like cells which demonstrated ultrastructural features similar to those of myofibroblasts were also noted in the stroma of the IUGR terminal villi. Myofibroblasts are characterised by contractile filaments, packed rough endoplasmic reticulum, nuclear indentations or folds and a well defined layer of basal lamina-like material separated from the cell membrane by a translucent area (Skalli & Gabbiani 1988). Myofibroblasts were first described in granulation tissue (Gabbiani & Majno 1972) where they are thought to play a role in wound retraction. Since then they have been identified in several "remodelling" situations such as lung fibrosis (Adler et al. 1981) and are involved in the stromal response to neoplasia (Harris & Ahmed 1977). Several groups have also suggested the existence of such cells in the human placenta (Fellar et al. 1985), but convincing proof has only been found in the larger stem villi (Demir et al. 1992; Graf, Frank & Oney. 1992; Kohnen et al. 1993). Their presence in the terminal villi of IUGR placentas is unexpected; it is tempting to speculate that these extravascular contractile cells are responsible for the increased deposition of basal lamina material such as collagen IV and laminin within the villous stroma thus reducing the compliance of these villi. Furthermore since myofibroblasts are contractile and responsive to vasoactive agents such as ANP, endothelin and angiotensin, they may as a consequence also alter impedance to blood flow within the fetoplacental circulation (Macara, Kingdom & Kaufmann 1993). The origin of myofibroblasts is probably variable; some arising from fibroblast cells and others from smooth muscle cells but the stimulus for their development is unclear. Several candidates such as fibroblast growth factor, platelet derived growth factor and transforming growth factor-ß have been postulated to be stimulators of myofibroblast differentiation. These factors are also thought to be involved in placental angiogenesis. It is interesting to speculate that a derangement of normal placental angiogenesis might induce vascular remodelling by sequentially promoting myofibroblast

differentiation. The circulating concentration of angiotensin II, a smooth muscle mitogen in-vitro (Lyall, Lever & Morton 1988), in umbilical venous blood is elevated in pregnancies complicated by IUGR (Kingdom et al. 1993a). This may be a further possible mechanism through which myofibroblasts may be recruited into the more distal parts of the fetoplacental circulation in IUGR.

Many of the ultrastructural changes which we have observed in these IUGR cases with abnormal umbilical artery perfusion have previously been reproduced in the rhesus monkey placenta (which is a haemochorial placenta similar to that of the human) by either fetectomy or by ligation of the umbilical cord (Panigel & Myers 1972). Syncytial knot formation, loss of cytotrophoblast, an increase in stromal collagen fibres, and a thickening of the basal lamina were all consistently observed in the periphery of the villous trees after a minimum of 14 days following surgery. Since there was no fetoplacental circulation to extract oxygen, these manoeuvres had resulted in an elevation of uterine venous pO_2 by up to 80 mmHg and though not quantified, must also have resulted in a relative increase in intervillous pO_2.

Similarly, examination of placentas from macerated stillborn fetuses demonstrates many of these features at the light-microscope level: in particular an increase in stromal cellularity and the presence of collagen fibres (Fox 1968). Fetal oxygen consumption has obviously ceased in such pregnancies, though uteroplacental perfusion continues. Therefore the changes observed in the human placenta following fetal death may be the result of this relative increase in oxygen tension within the non-perfused terminal villi; furthermore, a positive correlation is thought to exist between oxygen tension and collagen production by stromal cells (for review of oxygen effects see Kaufmann 1993). In view of these reports, the findings from our cases of IUGR with reduced umbilical artery perfusion would suggest that the oxygen tension within the terminal villi was relatively increased compared to the control cases. Moreover the time intervals involved suggest that this is a chronic rather than an acute process.

This hypothesis contradicts the generally held belief that the placenta (or to be more precise the terminal structures of the fetal villous trees) in addition to the fetus, is chronically-hypoxic in IUGR. Uteroplacental blood flow is reduced in pregnancies complicated by IUGR and blood returning to the IUGR fetus along the umbilical vein has a reduced oxygen content. However, reduced blood flow does not necessarily equate with uteroplacental hypoxia. One of the few reports evaluating uteroplacental oxygen content demonstrated that the oxygen content of intervillous blood was normal despite reduced oxygen concentrations in the umbilical vein (Nicolaides et al. 1986). These observations can be reconciled if there is either a failure of blood flow within the placenta or a failure of the fetal villous circulation to deliver oxygenated blood to the fetus. This conclusion is supported by data indicating a

negligible umbilical arterio-venous gradient for oxygen in IUGR pregnancies with grossly-abnormal umbilical artery Doppler waveforms. (Tyrell, Lilford & Obaid 1989; Nicolaides Economides & Soothill 1989). In addition, sampling of the uterine vein at the time of caesarean section for IUGR (Pardi et al 1992) indicates a 33% increase in oxygen content when compared with matched controls (from 4.46 to 5.92 mmol/L). It is therefore likely that the fetus is indeed centrally hypoxic because the placenta fails to extract oxygen adequately from the intervillous space.

Congestion of the fetal capillaries, while observed by light microscopy in a small proportion of control cases, was much more widespread in the IUGR cases - especially if the pregnancy was complicated by pre-eclampsia. Our electron microscopy studies identified this process in three of the pre-eclamptic cases studied. These erythrocytes varied in their electron density, and in places the cell membranes showed disintegration. It is therefore unlikely that these observations are perfusion artefacts, rather they suggest occlusion of the capillaries during the antenatal period. If there is prolonged antenatal capillary stasis blood flow and therefore oxygen transfer to the fetus will be impaired in growth retarded pregnancies - without the placenta itself being hypoxic at the level of the terminal villi.

The data also provides a pathologic basis for the longitudinal studies which have examined changes in Doppler waveforms from the umbilical artery in IUGR fetuses. The umbilical artery waveform can deteriorate to the point of AEDFV over a variable period of time, a process which generally proceeds faster if the pregnancy is also complicated by pre-eclampsia (Ribbert et al. 1993; Arduini, Rizzo & Romanini 1993). This deterioration may be due to progressive congestion of capillaries within terminal villi. Serial Doppler ultrasound studies of the umbilical artery are likely to detect this congestive process (Morrow et al. 1989), and may thus explain the observation from clinical studies that Doppler appears superior to cardiotocography for surveillance of the preterm IUGR fetus (Trudinger et al. 1987a; Almstrom et al 1992).

In summary, several important differences in the structure of terminal villi from pregnancies complicated by IUGR with reduced umbilical artery perfusion have been identified. No evidence of hypoxic change was identified in the IUGR cases, rather the differences are compatible with a relative increase in oxygen tension within the terminal villi. The fetal hypoxia which is observed in IUGR is likely to be due to impaired oxygen transfer from the terminal villi to the fetus, resulting from a combination of an abnormal terminal capillary structure and congestion of these vessels by fetal erythrocytes. In addition, the abnormalities seen in the trophoblast layer could result in impaired nutrient transport across the placenta.

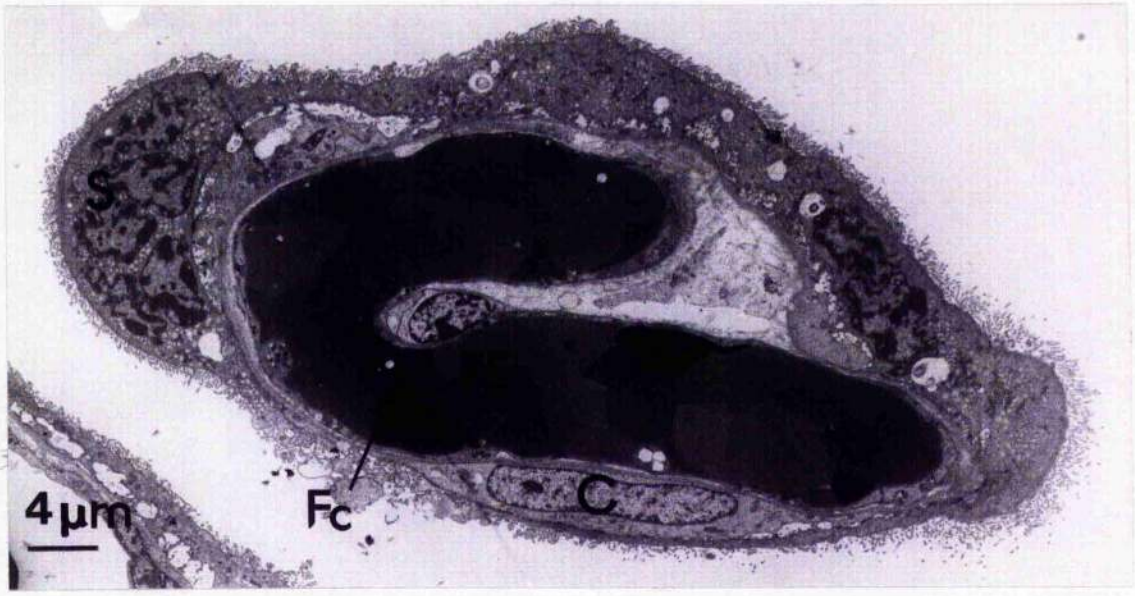

Figure 6.1. Transmission electron micrograph of a terminal villus from an IUGR pregnancy

The terminal villi from IUGR pregnancies were characterised by an increased number of syncytial nuclei which were aggregated together in clumps (SN); few cytotrophoblast cells were identified (CN) and within the fetal capillaries (Fc) there was evidence of antenatal congestion with moulding of the fetal erythrocytes (FE) and lysis of the erythrocyte cell membrane

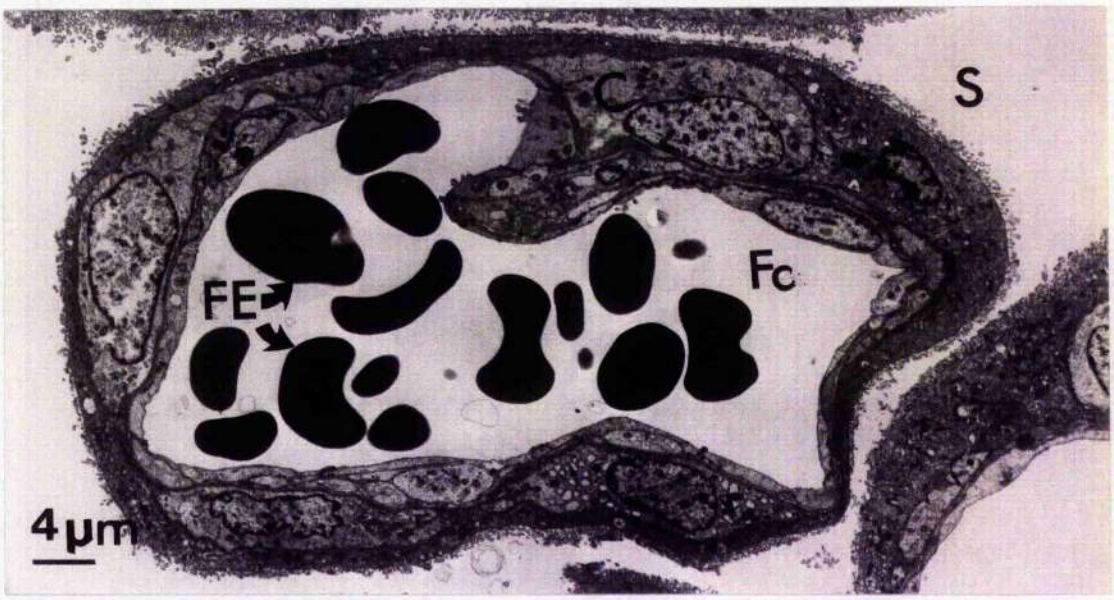

Figure 6.2 TEM of a terminal villus from a normal preterm placenta.

The terminal villi in the gestation age-matched control placentas had significantly more cytotrophoblast cells (CN), less syncytial nuclei (SN) and little evidence of capillary congestion (Fc) when compared with the placentas from IUGR pregnancies.

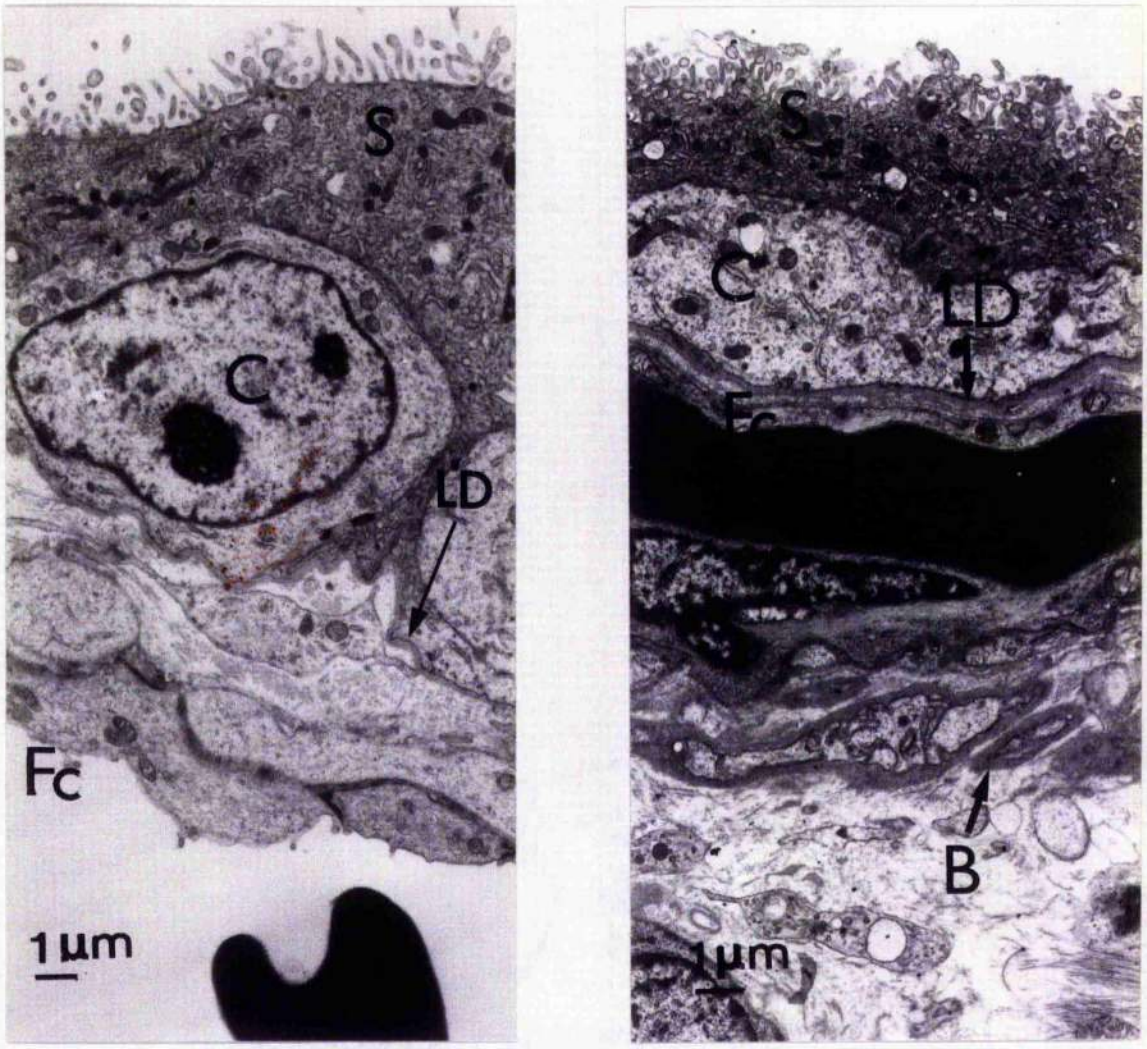

Figure 6.3 Basal lamina densa (LD) in terminal villi from an IUGR (1) and matched control placenta (2). The mean basal lamina densa (LD) was significantly thicker in the IUGR terminal villi than in the controls. In addition, basal lamina-like substance was also very prominent within the stroma of the IUGR placentas. (S)-syncytiotrophoblast; (C)-cytotrophoblast; (Fc) - fetal capillary

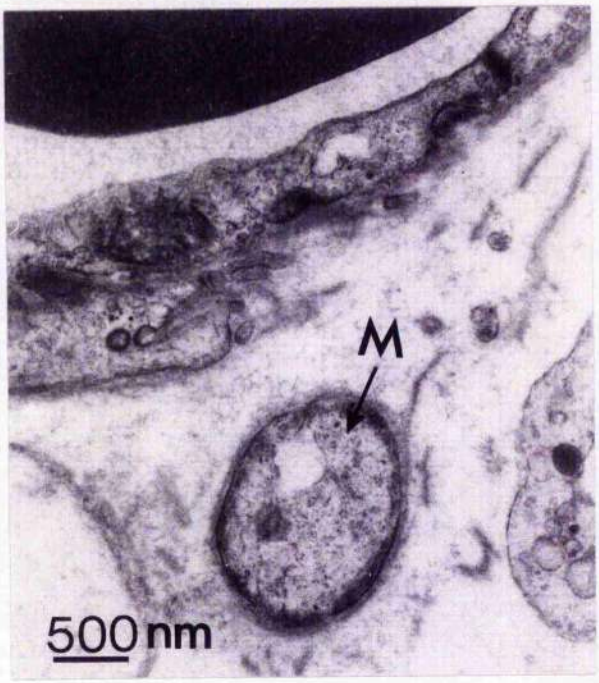

Figure 6.4 Transmission electron micrograph of terminal villous stroma from an IUGR placenta, demonstrating the myofibroblast cell (M) in cross-section

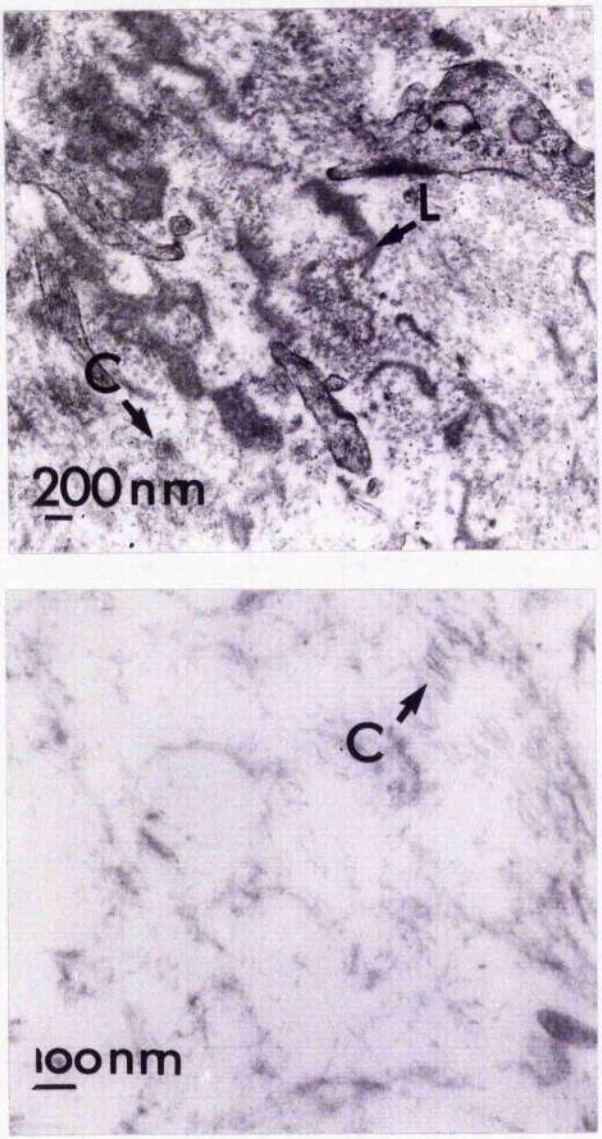

Figure 6.5 Villus stroma in terminal villi from an IUGR (1) and control (2) placenta. The villus stroma in the IUGR placentas had significantly more collagen fibres (C) and an increased density of background basal lamina-like material (L) when compared with villi from the control placentas. The control villus stroma is more highly magnified in order to illustrate that collagen fibres, though scanty, were indeed present.

PARAMETER	IUGR CASES	PRETERM CONTROL CASES	
MEAN VILLUS DIAMETER (μM)	57 (6.0)	66 (8.0)	*
SYNCYTIAL NUCLEI NUMBER	9.6 (1.9)	4.5 (1.6)	*
CYTOTROPHOBLAST NUCLEI NUMBER	1 nucleus/cross-section; seen in 40% of sections	2 nuclei/cross-section; seen in 80% of sections	*
LAMINA DENSA (μM)	1.3 (0.29)	0.6 (0.23)	*
VILLUS STROMA DENSITY	Fibroblast like cells +	Fibroblast like cells 0	
	Fibrillar material ++	Fibrillar material +	
	Collagen fibres +++	Collagen fibres +	

Table 6.1 Ultrastructural characteristics of terminal villi in placentas from pregnancies complicated by IUGR and normal gestation age-matched controls. Values are described as means (standard deviation). *indicates $p < 0.05$

CHAPTER 7

VILLOUS CELL PROLIFERATION

7.1. INTRODUCTION

Following implantation, the early placenta intrudes through the uterine endometrium to penetrate maternal spiral arteries within the underlying endometrial and myometrial layers (Schlafke & Enders 1975). This process of invasion occurs in two distinct phases (Pijnenborg et al. 1980); the first, which establishes an intervillous circulation of maternal blood from endometrial vessels, occurs near the end of the first trimester (Hustin & Schapps 1987); the second, in which myometrial spiral arteries are invaded, around week 18 of gestation. Though placental invasion to deeper layers of the uterus may cease at this stage, placental growth and maturation does not (Sands & Dobbing 1985); continuing fetal development demands increasing supplies of vital substrates and thus the placental and vascular surface area available for exchange must also expand. Of necessity therefore, the placenta must retain the capacity for cell proliferation and growth, until the fetus is delivered (Teasdale 1980).

It is now well established that cytotrophoblast cells alone have the capacity for replication and cell division, the syncytiotrophoblast being a post-mitotic tissue (Pierce & Midgeley 1963; Tao & Hertig 1965; Weinberg et al. 1970). Within the cytotrophoblast cell population, there are two distinct groups of cytotrophoblast cells, though both arise from the same stem cell; firstly cytotrophoblast cells responsible for placental invasion (Robertson & Warner 1974) and secondly those involved in placental growth and repair.

The mechanisms by which normal villous cytotrophoblast cells differentiate into invasive cytotrophoblast cells, known as extravillous or intermediate cytotrophoblast, is not known (Kurman, Main & Chen 1984) Their appearance coincides with the formation of decidual cells within the uterine stroma (Ran & Braustein 1991) and it is therefore likely that a local uterine or maternal endocrine signal may act as the trigger. It is these invasive cytotrophoblast cells which penetrate the maternal spiral arteries, converting them into flaccid in-elastic vessels, thus accommodating the increase in uterine blood flow. This invasive phase of cytotrophoblast cell behaviour appears to be strictly limited to the first 20 weeks of pregnancy. It is unclear how such behaviour is so tightly regulated, though it would appear to be the result of an intrinsic cytotrophoblast cell programme in which invasive cytotrophoblast cell behaviour ceases after 20 weeks gestation (Aplin 1991).

In contrast, growth and repair of the villous tree is modulated by villous cytotrophoblast cells and occurs throughout gestation. During the first trimester in particular, these cytotrophoblast cells are numerous, forming a complete layer beneath the syncytiotrophoblast (Jones & Fox 1991) and demonstrating great proliferation potential (Cotte et al. 1980; Yeger et al. 1989). With advancing gestation, and maturation of the placenta fewer cytotrophoblast

cells are present but they may still be seen in 20%-40% of the villous trophoblast (Bernischke & Kaufmann 1990).

Increased numbers of cytotrophoblast cells have been noted in pregnancies complicated by "hypoxia" *in-utero* (Fox 1968). *In-vitro* data obtained using villous explants would appear to confirm this since cytotrophoblast cell proliferation is induced in hypoxic conditions (Fox 1970; McLennan et al. 1972).

Since umbilical venous blood from IUGR pregnancies demonstrates evidence of a low oxygen tension (Nicolaides, Economides & Soothill 1989) it is assumed that the IUGR placenta like the IUGR fetus must also be chronically hypoxic and therefore cytotrophoblast cell proliferation stimulated. However, we have previously shown by morphological assessment using TEM (chapter 6) that there is no evidence of cytotrophoblast cell proliferation in IUGR placentas, rather the cytotrophoblast cell population was reduced.

In order to validate the TEM findings of Chapter 6, cytotrophoblast cell proliferation was evaluated in both IUGR and gestational-aged matched control placentas using the proliferation marker MIB-1. This monoclonal Ki-67 antibody reacts with a nuclear cell proliferation associated antigen that is expressed in all cells which are in the active phase of the cell proliferation cycle, and absent from quiescent cell in G_0 (Gerdes et al. 1983)

7.2. **METHODS**

7.2.1 **Clinical details**

Pregnancies complicated by IUGR, as defined in section 2.2.1 were identified. The clinical details of the 8 cases selected are shown in Table 2.1 (Case numbers 5, 10-16). All were delivered by caesarean section. Four pregnancies were normotensive and four were complicated by pre-eclampsia defined as previously (Section 2.2.1). Birthweight centiles, calculated from the local delivery database (Smalls & Forbes 1983) were <10th centile (except for case 11), the majority being <3rd centile. All the neonates, including case 11, showed clinical evidence of intrauterine starvation.

For comparison purposes a control series of eight preterm pregnancies were also selected; these were matched for gestational age and smoking habit. Their clinical details are shown in Table 2.2 (Case numbers 26, 29-35). Of the three cases which were admitted in spontaneous unexplained preterm labour, two were delivered by caesarean section.

7.2.2 **Collection of specimens**

Placental tissue was collected at the time of delivery (Section 2.6). In addition to the TEM blocks obtained by perfusion-fixation, a minimum of 4 full-section (from chorionic to basal plate) blocks of non-perfused tissue were excised from random areas of the placenta and immersion fixed in 4% neutral buffered formalin as described in Section 6.2.6

7.2.3 **Preparation of sections**

The glass slides used for the experiments were all pre-treated with ethyl alcohol, incubated with 2% APES for 20 seconds, then washed in acetone and aqua dest (1 minute each). This improved adhesion of the tissue sections to the slides.

Serial sections (3-5 μM) of tissue were cut from the paraffin blocks, mounted on the pre-treated slides and incubated at 37^{0}C overnight. The following day they were deparaffinised in xylene (2 x 10 minutes) and graded ethanol (100 and 96% ethyl alcohol for 5 minutes each). After blocking endogenous peroxidase by incubation, with 1% hydrogen peroxidase in methanol, the sections were washed in PBS [sol] (prepared by diluting 500 mls of PBS concentrate in 10 l of distilled water). The sections were then immersed in 10 mM citric acid and pre-treated 4 x 5 minutes in a microwave at 600 Watts, to enhance expression of the antigen.

7.2.4 **MIB-1 Immunohistochemistry**

A commercial kit (Histostain SP Kit), based on the streptavidin-biotin reaction was used. Primary antibodies were diluted in PBS [dil] (prepared by dissolving one PBS tablet in 100 mls of distilled water) with 1.5% BSA. The bound antibody was visualised using AEC.

MIB-1:

After a 20 minute incubation with 10% non-immune goat serum to eliminate background staining, the sections were incubated with the primary antibody, MIB-1 [dilution 1:20; (Key et al. 1993)] for 60 minutes. The slides were washed three times in PBS [sol] then incubated with the secondary biotinylated goat anti-mouse antibody for 30 minutes. After a further wash in PBS [sol] the sections were incubated for 5 minutes with the streptavidin-peroxidase conjugate then washed again in PBS [sol]. The bound antibody was visualised by incubating the sections with an AEC chromagen/hydrogen peroxide mixture for 15 minutes. Before counterstaining the sections with haematoxylin (for 3 minutes) they were washed in distilled water (2 x 5 minutes).

Controls were generated by replacing the primary antibody with PBS [sol]/1.5% BSA. The controls all remained negative. The antibody was being used concurrently in other studies into placental proliferation. These slides acted as positive controls

7.2.5 **MIB-1 Analysis**

A 22 x 22 mm grid box, containing 400 squares was generated and 100 fields marked by random allocation. These grid boxes were placed with an identical orientation over each sections. For each case the 100 selected fields were examined and the numbers of positively-staining red nuclei (representing the number of proliferating cells) counted separately for both the trophoblast and villus stromal compartments (Figure 7.1).

7.2.6 **Estimation of villous volume**

Since the proportion of villous tissue present within each section differed widely, the villus cross-sectional surface of each case was estimated using a standard 20 point counting method as described in Section 3.2.5.2

7.2.7 **Verification of methods**

To ensure that an adequate sample was evaluated, 25% of the placental fields were examined In addition, to verify that the results were reproducible, the counts were also repeated on two of the cases at separate time intervals. No significant differences were found.

7.2.8 **Statistical analysis**

The data obtained on the number of proliferating cells were not normally distributed; these data were therefore first log transformed [log(x)] before analysis and expressed as geometric mean (standard deviation). The data on villous volume were normally distributed and are shown as mean ± standard deviation. Differences between the mean values of the parameters measured were assessed by unpaired Student's t-test with p values <0.05 considered significant.

7.3 **RESULTS**

7.3.1 **Comparison of the groups**

Gestational age in the IUGR group (30.8 weeks [SD 2.4]) was similar to the control group (31.8 weeks [SD 2.4]). However, as expected, the birthweight of the IUGR was significantly lower than that of the preterm controls (1064 g [SD 334] vs. 1891g [SD 454]; $p<0.05$). The placental weight of the IUGR group was also significantly smaller than that of the controls (271g [SD 98] vs. 450g [SD 31]; $p<0.05$). There were no significant differences in the placental/fetal weight ratio between the two groups. Four women in each group were smokers.

7.3.2 **Villous Volume**

The proportion of villous tissue within each case is summarised in Table 7.1. There was significantly less villous tissue in the IUGR placentas when compared with gestational age matched controls (56.8% [SD 7.9] vs. 70.3% [SD 10.8]; $p<0.05$) but within the IUGR group, there was no difference between those with maternal pre-eclampsia and those without (56.5 [SD 9.6] vs 58.25 [SD 5.9]; p=0.766).

7.3.3 **MIB-1 morphometric analysis**

The morphometric analysis of the MIB staining is summarised in Table 7.1. Since the proportion of sectional area occupied by villi was significantly lower in the IUGR group the nuclear counts are expressed as numbers of positively staining nuclei per cm^2 of villi in each case. Even after correction, the numbers of proliferating trophoblast nuclei per cross sectional area was significantly lower in the IUGR group as compared to the gestational age-matched controls (2.5 [SD 0.33] vs. 2.92 [SD 0.24]; $p<0.05$) (Figure 7.2). Again, within the IUGR group, there was no difference between those with maternal pre-eclampsia and those without (2.553 [0.19] vs 2.451 [SD0.46]; p=0.7).

The MIB-1 immunohistochemical study demonstrated no significant differences in the number of positively staining stromal cells between the IUGR group and the gestational age-matched control group (2.19 [SD 0.29] vs. 2.31 [SD 0.27]; p=0.37)

7.4 **DISCUSSION**

As for the TEM study (chapter 6), severely growth retarded pregnancies were carefully identified. A group of normally-grown preterm pregnancies with normal umbilical artery perfusion were also selected to serve as controls. Since the two groups were appropriately matched for gestation placental data from the two groups were comparable.

The number of cytotrophoblast cells within placentas from IUGR pregnancies was significantly reduced, even after correction for the volume of villous tissue present. Since cytotrophoblast cells are the germinative zone of the placenta it is by proliferation of this layer that new villous sprouts and thence mesenchymal villi (the precursor of all villi) are formed. Thus the MIB counts confirm the impression of TEM examination (Chapter 6) that trophoblast proliferation is reduced or impaired in placentas from pregnancies complicated by IUGR and AEDFV (Table 7.1). Furthermore these changes were not altered by the presence or absence of maternal pre-eclampsia within the IUGR group

"Unexplained" intrauterine growth retardation is attributed to placental ischaemia, a consequence of poor trophoblast invasion and reduced utero-placental blood flow. Our confirmation of a reduction in cytotrophoblast cell number is therefore all the more surprising since based on both *in-vitro* and *in-vivo* data cytotrophoblast cell proliferation is stimulated by hypoxia. *In-vitro*, villous explants maintained in conditions of low oxygen tension (6%) show increased cytotrophoblast cell numbers when compared with villous explants maintained in identical culture conditions but with 26% oxygen (McLennan et al. 1972; Fox 1970). While *in-vivo*, cytotrophoblast cells are found in the vicinity of vasculosyncytial membranes, where fetal vessels come in close proximity to maternal blood. In these areas, the oxygen gradient from maternal to fetal blood is maximal and the oxygen tension therefore least (Bernischke & Kaufmann 1990). More-over, excessive numbers of cytotrophoblast cells are found in pregnancies complicated by pre-eclampsia, particularly those with early onset, severe disease, thought to be a response to placental ischaemia (Wigglesworth 1962; Fox 1965). Within our study there was no evidence of excessive cytotrophoblast cell proliferation in the IUGR pregnancies complicated by maternal pre-eclampsia, suggesting perhaps that the primary pathology in these cases was impaired fetal growth and the development of hypertension, a secondary maternal response to the fetal condition.

We found no evidence of increased cytotrophoblast cell proliferation by light or electron microscopy, rather our findings would suggest impaired cytotrophoblast proliferation. Had the number of cytotrophoblast cells been directly proportional to the volume of villous tissue, it could be concluded that cytotrophoblast proliferation was appropriate for the small placental size. However, even after correcting for the smaller villous volume, cytotrophoblast cell proliferation was significantly impaired. This would suggest that cytotrophoblast cell proliferation is specifically inhibited.

While hypoxia stimulates cytotrophoblast cell proliferation, higher oxygen tensions inhibit proliferation. We have previously noted evidence of impaired blood flow in terminal villi from IUGR pregnancies (Chapter 6). Such ante-natal vessel congestion would permit equilibration of fetal and maternal blood and reduce the oxygen gradient normally present between maternal and fetal blood, resulting in relatively high oxygen tensions being present within the placental villi. Since placental villi normally have a steep oxygen gradient and low oxygen tension, an increase in villous oxygen tension, though still within physiological limits, would produce relatively hyperoxic conditions and inhibit cytotrophoblast cell proliferation.

Reduced turnover and production of trophoblast may not only affect placental size but also placental function. Syncytiotrophoblast is the interface between maternal and fetal tissues and though gaseous transfer occurs by simple diffusion, many of the substrates necessary for

fetal growth must be actively transported across this membrane. Without regeneration of the trophoblast layer, the functional capacity of the trophoblast will be diminished. Further investigations are required to evaluate the distribution and function of transport pathways in IUGR placentas.

In contrast, there was no significant difference in the number of proliferating stromal cells between IUGR and control placentas, once these values were corrected for villous volume. Stromal cells are comprised of reticular cells, fibroblasts and Hofbauer cells. Both reticular cells and fibroblasts are involved in mechanical support within the villous, producing collagen fibrils and forming channels for the passage of Hofbauer cells (Jones & Fox 1991). These latter cells, of uncertain origin, have a variety of functions including immunological activity (Bulmer & Johnstone 1984; Mues et al. 1989) and remodelling of the villous stroma (Castellucci et al. 1980). Though placental fibroblast proliferation rates appear to be predetermined or "programmed" as a function of gestational age (Fant 1991), their functional activity may be modified by factors such as endothelins (Fant et al. 1992). The increase in stromal collagen noted previously by TEM may therefore not simply be related to stromal cell number but to the effect of vasoactive agents on these cells.

Immunohistochemical studies with the proliferation marker MIB-1 have confirmed that cytotrophoblast cell turnover is significantly reduced in IUGR placentas. Rather than indicating a role for placental ischaemia, these findings suggest that relatively high oxygen tensions develop within placental villi from IUGR placentas, inhibiting cytotrophoblast cell proliferation and thus impairing trophoblast formation.

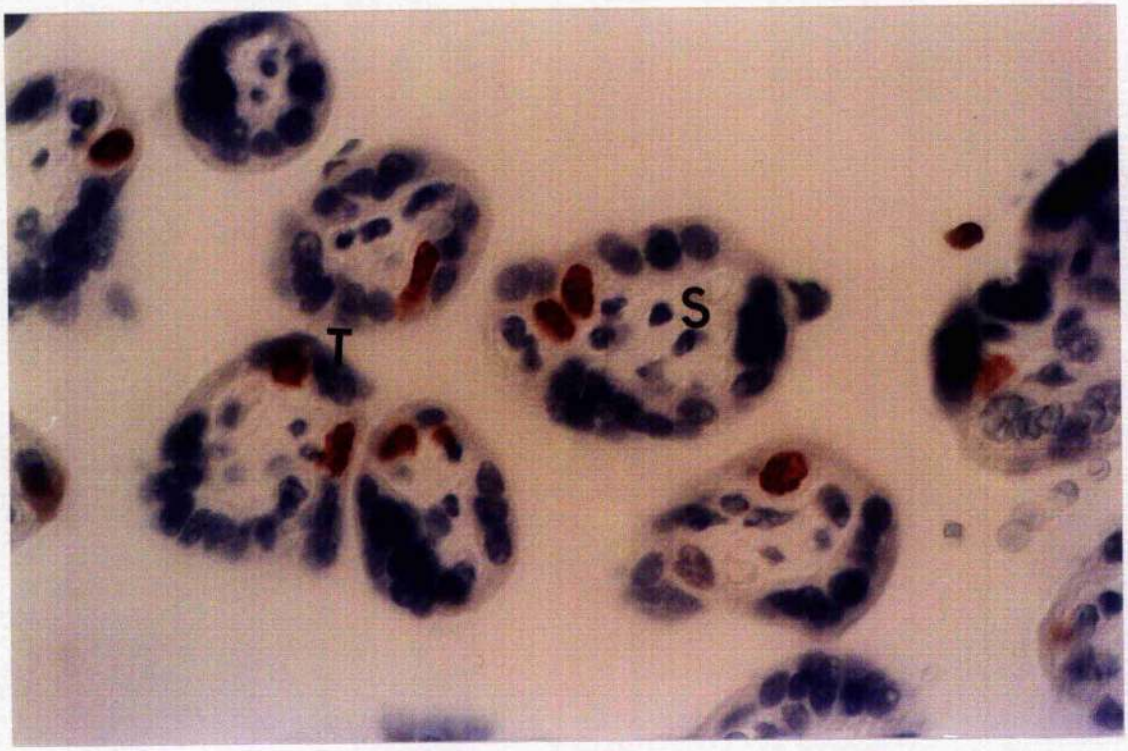

Figure 7.1 Cross-section of placental villi from a normally grown pregnancy stained with the proliferation marker MIB-1. Cells in the active phase of replication stain are positive for the antibody and are stained red. Several such cells may be seen within the trophoblast (t) and stroma (s) of the placental villi. (magnification x 400)

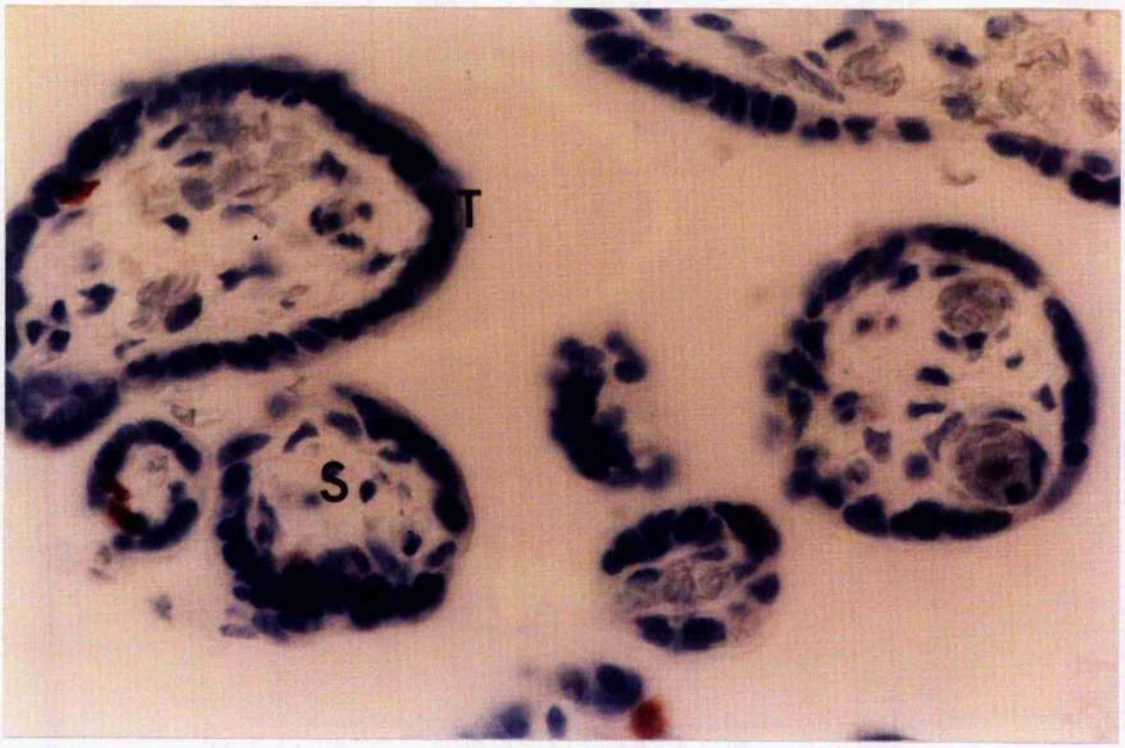

Figure 7.2 Cross-section of placental villi from a pregnancy complicated by IUGR and AEDFV stained with the proliferation marker MIB-1. Actively proliferating cells stain red, however, these villi were characterised by an absence of positive (red) cells within the trophoblast (t) or stroma (s). When corrected for villous volume, the number of positively staining trophoblast cells was significantly less than that seen in the villi from normal pregnancies. (magnification x 400)

IUGR CASES

CASE NUMBER	% VILLUS TISSUE	NUMBER OF MIB-1 POSITIVE NUCLEI IN TROPHOBLAST	NUMBER OF MIB-1 POSITIVE NUCLEI IN VILLOUS STROMA
5	59%	237	49
10	43%	326	183
11	57%	535	219
12	61%	199	116
13	60%	92	86
14	64%	1172	406
15	50%	250	138
16	65%	520	278

PRETERM CONTROL CASES

22	80%	655	135
25	83%	607	107
26	75%	676	190
27	67%	844	429
28	67%	2760	547
29	83%	995	284
30	78%	812	182
31	81%	417	100

Table 7.1 Villous volume and MIB-1 positive proliferating cell counts in trophoblast and stroma of IUGR and gestational-age matched preterm control placentas.

In each case the number of MIB-1 positive cells has been corrected for the % villus tissue present. There were significantly fewer proliferating cells in the IUGR trophoblast compared with the control trophoblast [2.5 vs. 2.9; $p<0.014$] but similar numbers of proliferating stomal cells in both IUGR and preterm control placentas [2.1 vs. 2.3 $p=0.37$].

CHAPTER 8

THE VILLOUS STROMA

8.1 INTRODUCTION

The TEM images obtained in Chapter 6, suggested an apparent increase in the number of collagen fibres and the concentration of fibrillar material within the villous stroma of terminal villi in IUGR pregnancies. To confirm our initial observations and also to evaluate the type of collagens involved, we submitted placental paraffin sections from IUGR and gestational matched pregnancies for stromal immunohistochemical studies. In addition, since larger areas of the placenta could be evaluated, we were able to assess if the changes were widespread throughout the placenta of IUGR pregnancies.

8.2 METHODS

8.2.1 Selection of patients

Eight women with pregnancies complicated by severe intrauterine growth retardation as defined in section 2.2.1 were identified antenatally. Three of the pregnancies were also complicated by pre-eclampsia as defined in 2.2.1; the remaining five pregnancies were normotensive. There were no other complicating maternal or fetal conditions. All the women were delivered by caesarean section. Following delivery the birthweight centiles of the neonates, based on a local database (Smalls & Forbes 1983), were all confirmed to be less than the 10th centile for gestational age. The clinical details of the cases used are given in Table 1.1 [Case numbers 1-7 and 20]

For the purposes of comparison, a group of 8 women with normally grown, healthy fetuses who were delivered prematurely were also selected. In total five of the pregnancies were delivered by caesarean section. The clinical details of these cases are given in Table 1.2 [Case numbers 22-24, 28, 34, 36, 37 and 40]. Four women in each group were smokers.

8.2.2 Collection of specimens

Tissue samples were collected, processed and embedded in paraffin as described in chapter 3 (section 3.2.2)

8.2.3 Preparation of sections

Glass slides were prepared in the following manner; the slides were washed in ethyl alcohol, immersed for 20 seconds in acetone with 2% APES then washed twice (for 60 seconds each time) in pure acetone. Serial sections (3-5 μM) were cut from the tissue blocks obtained from the cases listed above and mounted on the prepared glass slides.

The mounted sections were deparaffinised as follows; the sections were washed in distilled water, immersed in xylene for 10 minutes then transferred through a graded series of alcohol (100, 96 and 90%) for 5 minutes in each solution. After rinsing the sections with methanol, endogenous peroxidase was blocked by incubation with a methanol/hydrogen peroxide solution (60mls:1ml) for 30 minutes before a further wash with PBS [sol].

8.2.4 **Immunohistochemistry**

The following antibodies were employed:

Polyclonal Antibodies - Fibronectin; Collagen I; Collagen II; Collagen III; Collagen IV
Monoclonal Antibody - Laminin

The sections for each stromal antibody were all processed simultaneously during a single run.

8.2.5 **Staining with polyclonal antibodies**

The sections were covered with a serum blocker (20% normal swine serum in PBS) for 20 minutes to reduce non-specific background staining. After tipping the blocker from the slides, the sections were incubated with the primary antibody for 18 hours at 4^{0}C then washed three times in PBS [sol] (3 x 10 minutes). A secondary antibody (swine anti-rabbit 1:100 in 4% NSS/PBS) was then applied for 45 minutes. The sections were washed a further 3 times in PBS [sol] (3 x 10 minutes) then incubated with peroxidase conjugated streptavidin (1:800 in 4% NSS/PBS) for 45 minutes. After rinsing in PBS (3 x 10 minutes), AEC was applied to the sections for 15 minutes. The sections were washed twice in distilled water then counterstained with haematoxylin for 3 minutes before mounting with coverslips.

8.2.6 **Staining with monoclonal antibodies**

A similar technique was used for monoclonal antibody staining with the following modifications: 2% BSA in PBS was used as an initial blocking solution; the secondary antibody applied was peroxidase conjugated rabbit anti-mouse (1:50 in 2% BSA/PBS); for a third layer peroxidase conjugated avadin (1:100 in 4% NSS/PBS) was used. These sections were similarly visualised using AEC.

Negative controls were generated for each antibody by omitting the primary antiserum and the specificity of the procedure verified using pre-adsorbed specific antiserum. Hepatic and vascular tissue, previously used in our laboratory served as positive controls.

8.2.7 **Analysis of sections**

The sections were all coded independently and therefore examined blind to any clinical information. Each section was examined qualitatively in a systematic fashion. The pattern

and density of staining present for each antibody in terminal, intermediate and stem villi was noted. The scoring system for staining density was as follows; no staining 0, light staining 1, moderate staining 2 and dense staining 3.

8.3 RESULTS

8.3.1 Comparison of the study groups

There was no difference between the two groups in terms of the gestational age at delivery (31.25 weeks [SD 3.24] vs 31.25 weeks [SD 3.28]. The birthweight of the IUGR group was significantly less than that of the controls (1100g [SD 368] vs 1739g [SD 488]; $p<0.05$). Likewise the placentas from the IUGR pregnancies were significantly lighter than the placentas from the control cases (246g [SD 55.4] vs 448 [SD 69.4]; $p<0.05$)

8.3.2 Collagen Type 1

CONTROL GROUP: Collagen type I antibody staining (Grade 1) was well localised to the basement membranes of intermediate and terminal villi and formed a fine fibrillar network within the stroma of these villi (Figure 8.1). The distribution of staining was similar in stem villi but was more intense.

IUGR GROUP: Collagen type I antibody staining was generally more intense throughout all the villi of the IUGR placentas. Within intermediate and terminal villi, thick duplicated layers were present in the basement membrane. These merged with well defined bands which were also present in the villous stroma. In other areas a fairly dense homogeneous staining pattern was seen within the villous stroma (Figure 8.2). In stem villi the collagen I antibody staining formed dense sheets throughout the villous stroma .

8.3.3 Collagen Type II

CONTROL GROUP: Virtually no staining was present in any of the villi from the control placentas.

IUGR GROUP: Light (grade 1) staining was present around the basement membranes of large arteries within the stem villi. Patchy, grade 1 staining was also present within the stroma of stem, intermediate and terminal villi but this was variable in appearance and distribution.

8.3.4 Collagen Type III

CONTROL GROUP: Grade 2 staining was identified around the vessels, the basement membrane, and within the interstitium of the stem villi. Intermediate and terminal villi

showed almost no staining, but when present the staining was light and localised to the stroma (Figure 8.3).

IUGR GROUP: In stem villi, antibody staining was distributed in thick heavily stained bands around the fetal vessel walls; these bands also extended into the stromal portion of the villi. Virtually every intermediate and terminal villus was stained with collagen III antibody forming a meshwork in the stroma and discrete layers around the fetal vessel walls (Figure 8.4).

8.3.5 **Collagen Type IV**

CONTROL GROUP: Moderate staining for the antibody was present throughout the stroma of stem villi and around both the trophoblast and fetal vessel basement membranes. In intermediate and terminal villi, the staining formed a well defined line outlining the basement membranes of the fetal blood vessels and the trophoblast. There was minimal antibody staining in the stroma of these villi (Figure 8.5).

IUGR GROUP: Densely stained concentric rings of antibody staining were present in the stem villi around the fetal arterial vessels and extending into the villous stroma to form a web-like net. The same pattern of staining was also present in the intermediate and terminal villi such that the basement membranes of the trophoblast and fetal vessels could not be distinguished from the stromal antibody web of staining (Figure 8.6).

8.3.6 **Collagen V, VI and VII**

CONTROL GROUP: Patchy, grade 1 staining was present within the villous stroma of all villous classes.

IUGR GROUP: The pattern of staining was identical to that of the control group.

8.3.7 **Fibronectin Antibody**

CONTROL GROUP: For all villous classes, staining for this antibody was primarily seen in the basement membrane areas of the trophoblast and the fetal vasculature. A fine meshwork of fibrillar staining was also present in the villous stroma. Within stem villi, this fibrillar staining formed almost a continuous sheet.

IUGR GROUP: Complete sheets of positve staining were seen in the stroma of stem villi. The stromal fibrillar staining pattern was identical to the control group in intermediate and terminal villi but was slightly more intense.

8.3.7 **Laminin**

CONTROL GROUP: The laminin antibody formed a very thin pencil-thin outline around the basement membranes of both fetal vessels and villous trophoblast in all villous classes (Figure 8.7).

IUGR GROUP: In all villi, the basement membrane outlines around the trophoblast and vessel walls appeared irregular and thickened. Duplication of the laminin layer was identified, particularly in the trophoblast basement membrane of the terminal villi (Figure 8.8).

For each of the antibodies evaluated, it was impossible to distinguish any difference in the staining pattern of the IUGR cases which were "uncomplicated" and those which were additionally characterised by maternal pre-eclampsia.

8.4 **DISCUSSION**

Collagen is the generic name for a family of proteins which form the major fibrous constituent of skin, tendon, bone and the extracellular matrix. Tropocollagen, the basic molecule of all collagen proteins is composed of three polypeptide chains, arranged in a triple helix, which is cleaved from a larger precursor molecule secreted, in the placenta, from fibroblasts and Hoefbauer cells within the villous stroma. A complex series of steps is required for this process; the collagen gene is first switched on, the message then transcripted, translated, pre-processed, and the molecule then converted ultimately from pro-collagen to the stable collage molecule. It is recognised that in some tissues and pathological states the rate of collagen production can be significantly stimulated or altered at one, or many of these sites.

Within the placenta, collagenous and non-collagenous proteins are major constituents of the villous stromal matrix and basement membranes. The extracellular villous matrix was previously thought to be an inert tissue, but it is now clear, that by varying the proportions of matrix proteins present, structural and functional properties of the extracellular space may be altered (Hay 1981). Alterations therefore, to the composition of the villous stroma has major functional implications for the feto-placental unit. Having demonstrated by TEM (Chapter 6) that the basement membrane lamina densa was thickened in IUGR pregnancies and that the villous stroma appeared to contain increased amounts of collagen material, we wished to confirm these findings and establish the types of collagens/non-collagens involved. The staining patterns of all the collagens subtypes previously identified within placenta (Amenta

et al. 1986) and the two non-collagenous glycoproteins fibronectin and laminin were examined.

Collagen I is the main structural component of the villous stroma and is the major collagen present within the mature term placenta. Collagen I antigens are localised almost exclusively on the thick cross-banded collagen fibers which can be visualised by TEM (Boselli et al. 1981; Clark et al. 1982; Martinez-Hernandez 1984) and are distributed within the stroma, particularly around blood vessels. Increased staining for collagen I antibody was present within the villous stroma of terminal villi from the IUGR placentas, forming almost a homogeneous mass within the stroma. A relative increase in collagen I content, particularly within stem villi (Locci et al. 1993) may increase placental vascular impedence and so contribute to the development of abnormal Doppler waveforms.

Antibodies against collagen III are located on thin, non-cross-banded fibers (Amenta et al. 1986) within the interstitium and are often closely associated with collagen I fibers. Not surprisingly therefore, the pattern of collagen III antibody staining closely mirrored that of collagen I antibody. It is likely that the increase in background fibrillar material, which was noted in the villous stroma of terminal villi from IUGR pregnancies (Chapter 6), was due to an increase in collagen III. The functional relevance of this finding is unclear.

Collagen IV and laminin are both normally located within the basement membrane, laminin in the lamina rara and collagen IV preferentially in the lamina densa (Martinez-Hernandez & Amenta 1983). While both these components showed increased staining in the basement membrane, surprisingly, significant staining was also present within the villous stroma. Collagen IV and laminin are only occasionally noted in the villous stroma of normal placentas and are thought to be a consequence of villous remodelling; basement memebrane material being deposited into the villous stroma, prior to digestion and removal (Amenta et al. 1986).

Within other organs, vascular re-modelling is associated with significant increases in collagen production. Pulmonary arteries have been shown to actively stimulate collagen production in primary pulmonary hypertension, though the initial stimulus for this is not yet recognised (Botney et al. 1993). Furthermore in chronic hypoxia, increased collagen production is seen in both large and small arterial vessels and is additionally associated with neomuscularisation of non-muscularised vessels (Rabinovitch et al. 1986). Significantly, prior treatment with a collagen inhibitor dramatically reduces the degree of pulmonary hypertension which ensues (Kerr et al. 1987).

We have shown previously that the terminal villous and vascular network present in the placentas of IUGR pregnancies are structurally very abnormal (Chapter 5). A depleted capillary vascular network cannot cope with the increasing volume of placental blood necessary to meet the demands of the growing fetus. In response to the increased blood volume processed, it is possible that vascular remodelling is initiated in a similar manner to that seen in the lungs, thus resulting in an increased deposition of collagen IV and laminin around the vascular basement membranes and within the villous stroma.

In conjunction with this, myofibroblast cells, which we have previously demonstrated within terminal villi from IUGR placentas, are also involved in tissue remodelling and produce basal lamina material. The presence of these cells in terminal villi from IUGR placentas may indicate further that "remodelling" is occurring and provide an additional source of interstitial basal lamina substances.

Thickening and duplication of the basal lamina have been noted previously in pregnancies complicated by pre-eclampsia (Fox 1968b), diabetes mellitus (Okudaira et al. 1966) and maternal smoking (Van der Veen & Fox 1982; Van der Velde et al. 1985). Since basal laminal substances are produced by epithelial cells, its density in the placenta, is thought to correlate with the number of cytotrophoblast cells present (Jones & Fox 1980). Thus increased basal lamina thickening in pre-eclampsia was related to the increased numbers of cytotrophoblast cells seen in the placentas of affected pregnancies. However, we were unable to demonstrate any difference between the IUGR pregnancies with and without pre-eclampsia, though it is acknowledged that the numbers within each of our groups may be too small to detect these changes. Likewise we were unable to show any difference in the appearance of the basal lamina between smokers and non-smokers in our study. Although, Van der Velde (1985) previously demonstrated thickening of the basal lamina amongst his group of smokers, this was not associated with increased numbers of cytotrophoblast cells. It is clear therefore that multiple factors mediate the regulation of basal lamina production.

Since we and others have shown that the volume of terminal villi present in placentas from IUGR pregnancies is significantly less than that present in normal control cases, it is not possible from these studies to assess if the increased staining present is simply a feature of the reduced villous volume leading to an increased collagen concentration, the direct result of increased collagen production, or the effect of a failure in collagen degradation.

Collagen production is known to be stimulated by enviromental factors such as increased oxygen tension and vascular stasis (Fox 1968a; Paningel & Myers 1972). We have previously demonstrated evidence to indicate that both vascular stasis and relative hyperoxia occur within terminal villi from IUGR placentas (Chapters 6 and 7). Outside the placenta,

collagen production is known to be stimulated by growth factors, notably, transforming growth factor ß (TGF-ß) but others such as TGF-∂ and epidermal growth factor (EGF) are also involved. TGF-ß1 is released from platelets during platelet activation (Sporn & Roberts 1986) and has been noted not only to induce collagen synthesis, particulaly collagen type I synthesis, but also to inhibit cell proliferation (Fine et al. 1987; Creely et al. 1992). Though TGF-ß1 appears to be expressed primarily in the syncytiotrophoblast and not the placental villous core during normal pregnancy (Dungy et al. 1991) indicating a potential role in placental invasion, its distribution and function in pathological pregnancies has not yet been evaluated. In addition, since it is recognised that platelet turnover is increased in pregnancies complicated by IUGR (Van den Hof 1990), activation of this system may well occur in IUGR pregnancies. Increased expression of TGF-ß in early pregnancy, resulting perhaps, from a maternal cytokine or inflammatory stimulus may contribute both to the inhibition of villous proliferation and the promotion of collagen production.

Alternatively the relative increase in collagen may reflect a failure in collagen degradation. In view of its stability, collagen is almost exclusively degraded by the collagenase enzyme. Basic fibroblastic growth factor (b-FGF) promotes collagenase production and in addition, is a potent stimulator in neovascularisation (Rifkin & Moscatelli 1989). There is a clear failure of vascular growth in the placentas of IUGR pregnancies and the excess collagen present, which compounds problems for the feto-placental unit, may simply be an added feature, reflecting poor vascular developement due to a lack of growth factor stimulation.

Further studies which quantify collagen content per villous volume and evaluate collagen messenger RNA are clearly needed to answer these questions and distinguish the relative contribution of these potential pathways to the features we have demonstrated. Only as these issues are addressed can our findings be reliably interpreted.

Irrespective of the methods by which these changes are demonstrated, the potential for long term "remodelling" must not be ignored. Barker (1992) and others have shown clearly that the fetal enviroment may programme future patterns of disease. Increased collagen production and neomuscularisation are classical pathological features of extra-uterine hypertension. If the changes we have demonstrated in the placenta of pregnancies complicated by IUGR are mirrored in the fetal systemic circulation the precursor lesions of adult hypertension may well be established by the time of birth.

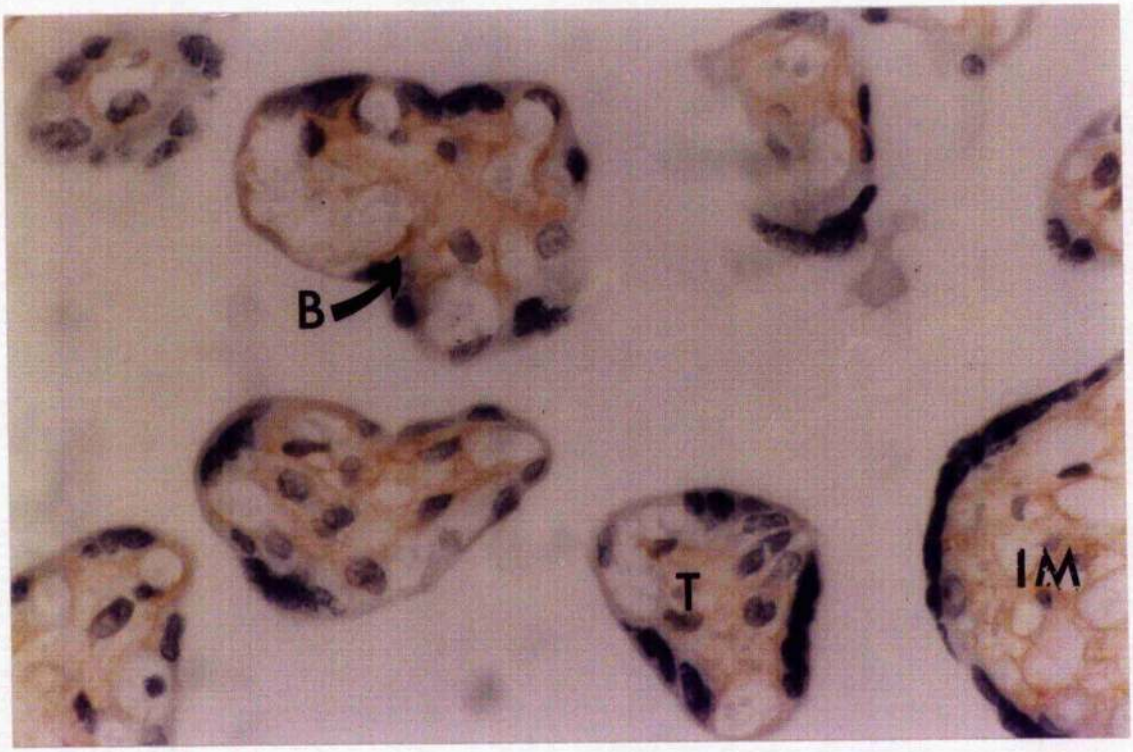

Figure 8.1 Collagen Type I antibody staining in a normal preterm placenta

Staining for collagen type I antibody was well defined to the villous and vascular basement memebranes (B) of the intermediate (IM) and terminal (T) villi. (magnification x 400)

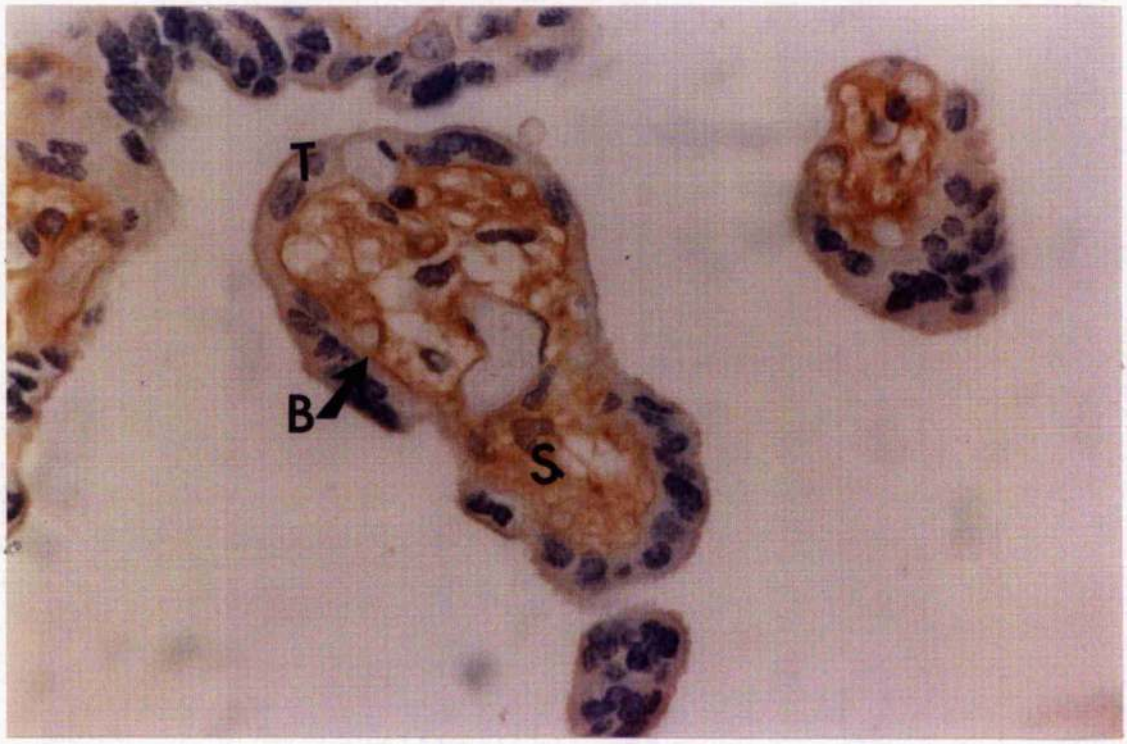

Figure 8.2 Collagen type I antibody staining in a placenta from a pregnancy complicated by IUGR and abnormal umbilical artery Doppler waveforms.

The antibody staining formed thick bands in both the basement membranes (B) and villous stroma (S) of (T) villi. (magnification x 400)

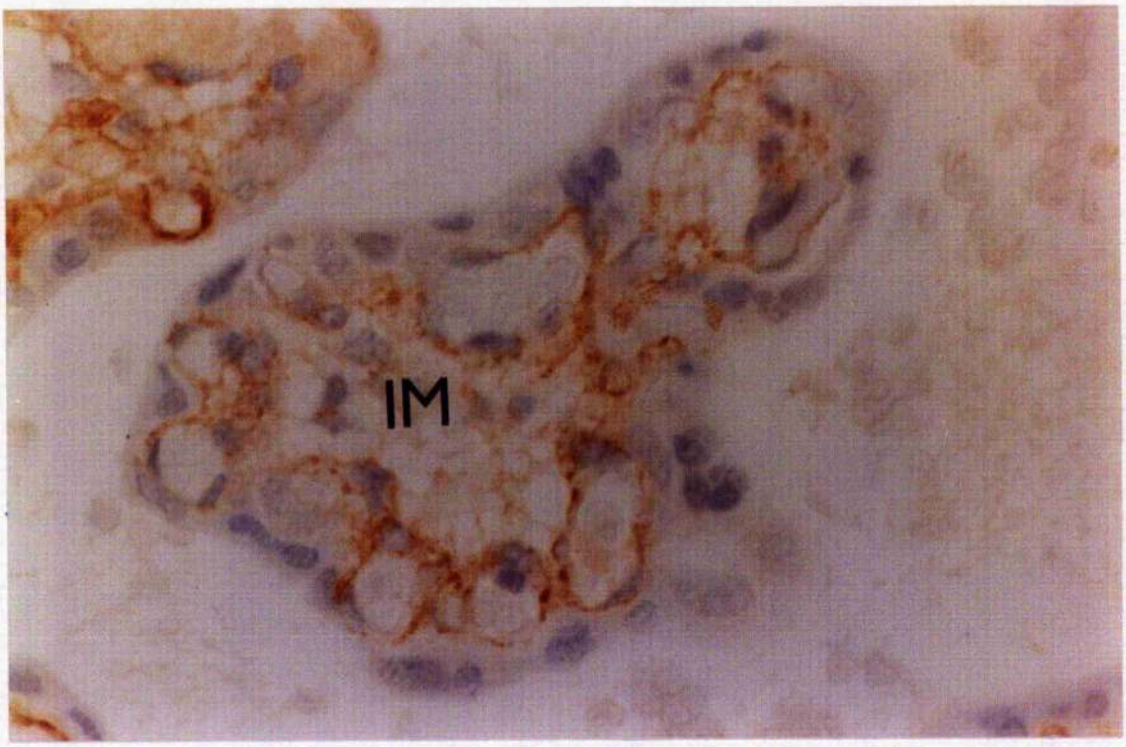

Figure 8.3 Collagen type III antibody staining in a placenta from a normal preterm control pregnancy.

Antibody staining was primarily associated with the villlous and vascular basement membranes in stem villi. In the intermediate (IM) villi antibody staining was minimal in the villous stroma and formed a pencil line associated with the basement membrane. (magnification x 400)

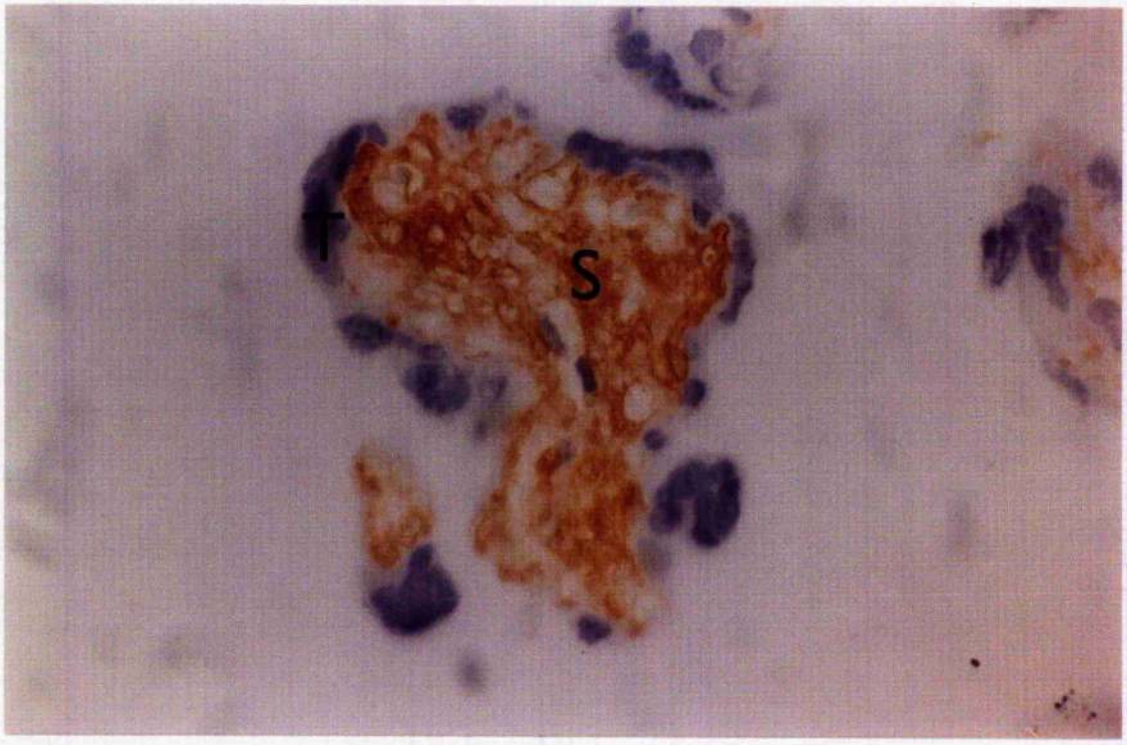

Figure 8.4 Collagen type IIII antibody staining in villi from an IUGR pregnancy

Collagen III antibody staining formed a dense meshwork in the villous stroma (S), even within terminal (T) villi. (magnification x 400)

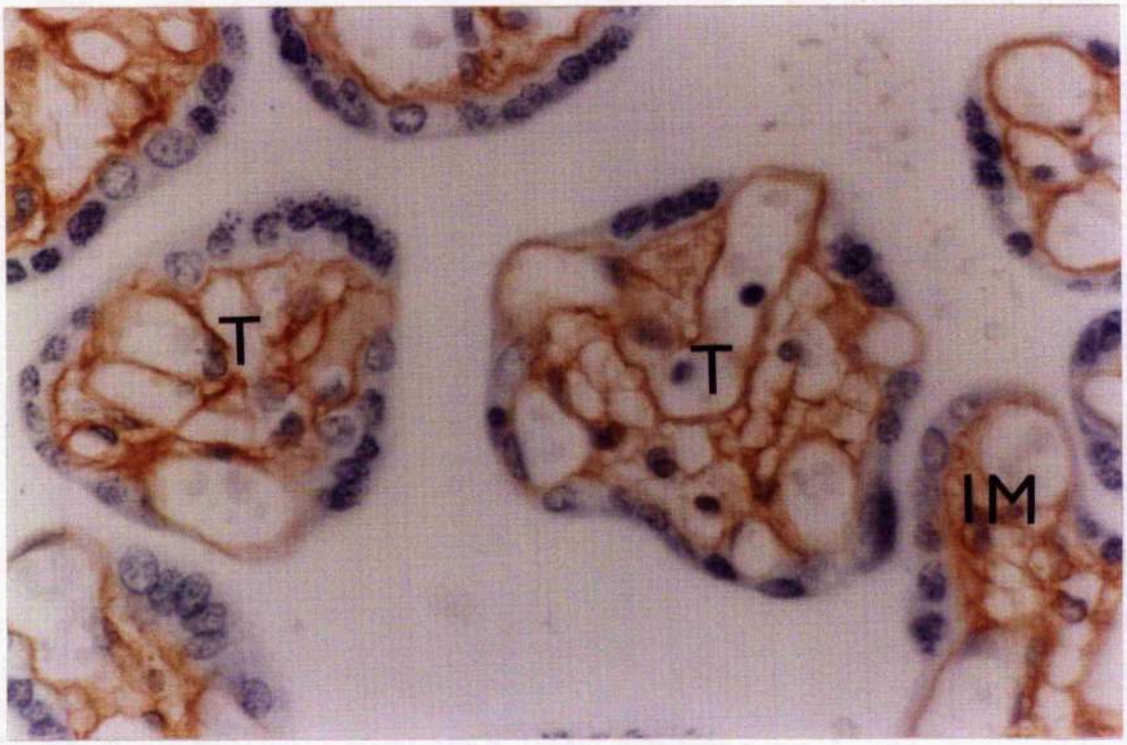

Figure 8.5 Collagen type IV antibody staining in villi from a normal preterm control pregnancy.

Grade 2 antibody staining was localised to the villous and vascular basement membranes within terminal (T) and intermediate (IM) villi. (magnification x 400)

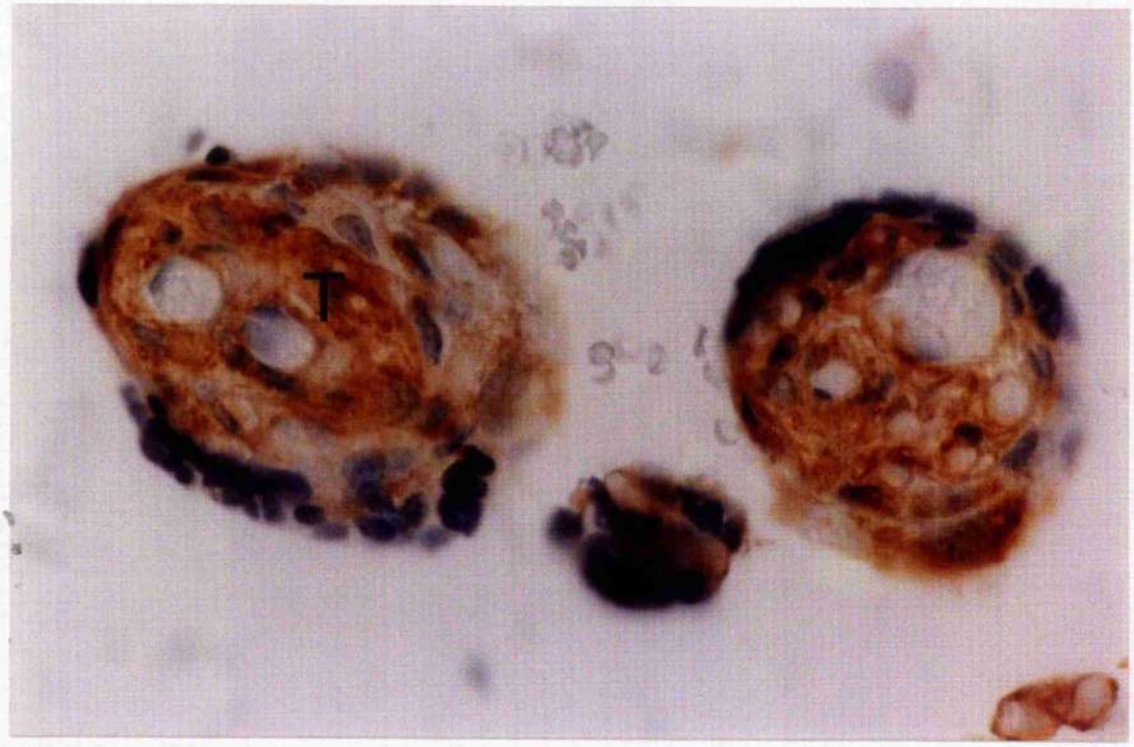

Figure 8.6 Collagen type IV antibody staining in villi from an IUGR pregnancy

Dense staining was present throughout the villous stroma of terminal (T) villi, forming a homogeneous sheet of staining in some villi. (magnification x 400)

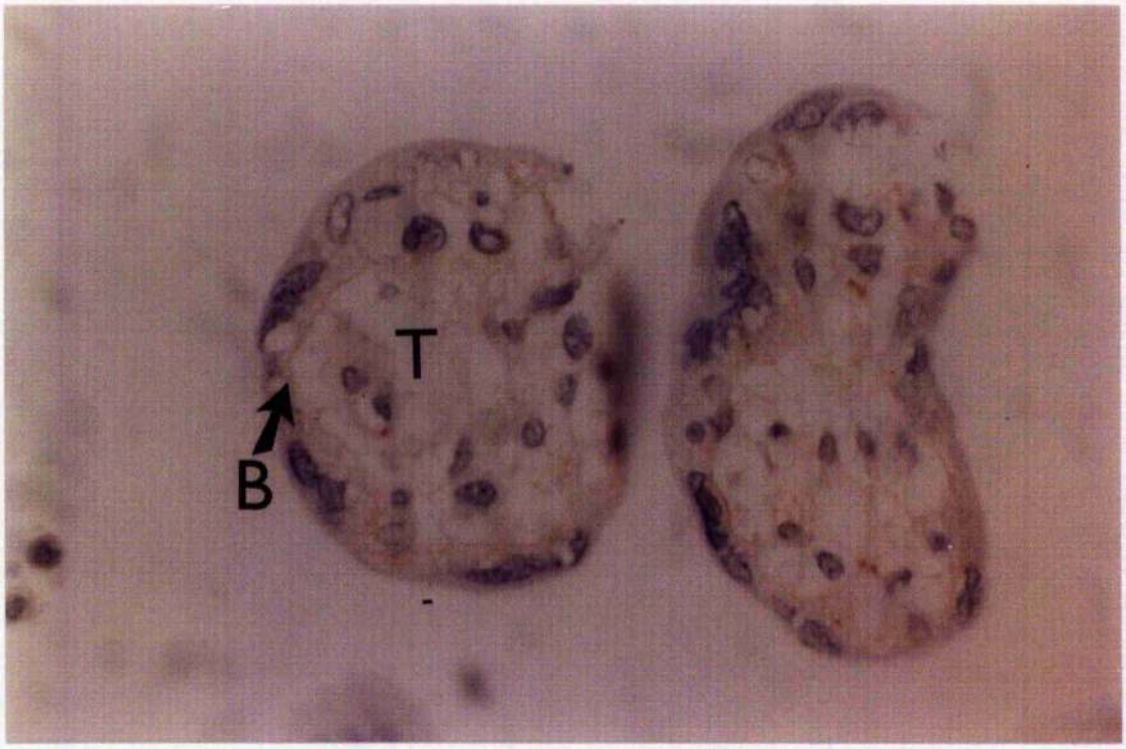

Figure 8.7 Laminin antibody staining in normal preterm placental villi

Grade 1 staining for the laminin antibody was present, forming a thin pencil line, around all basement memebranes (B) in all villous classes; (T) - terminal villi. (magnification x 400)

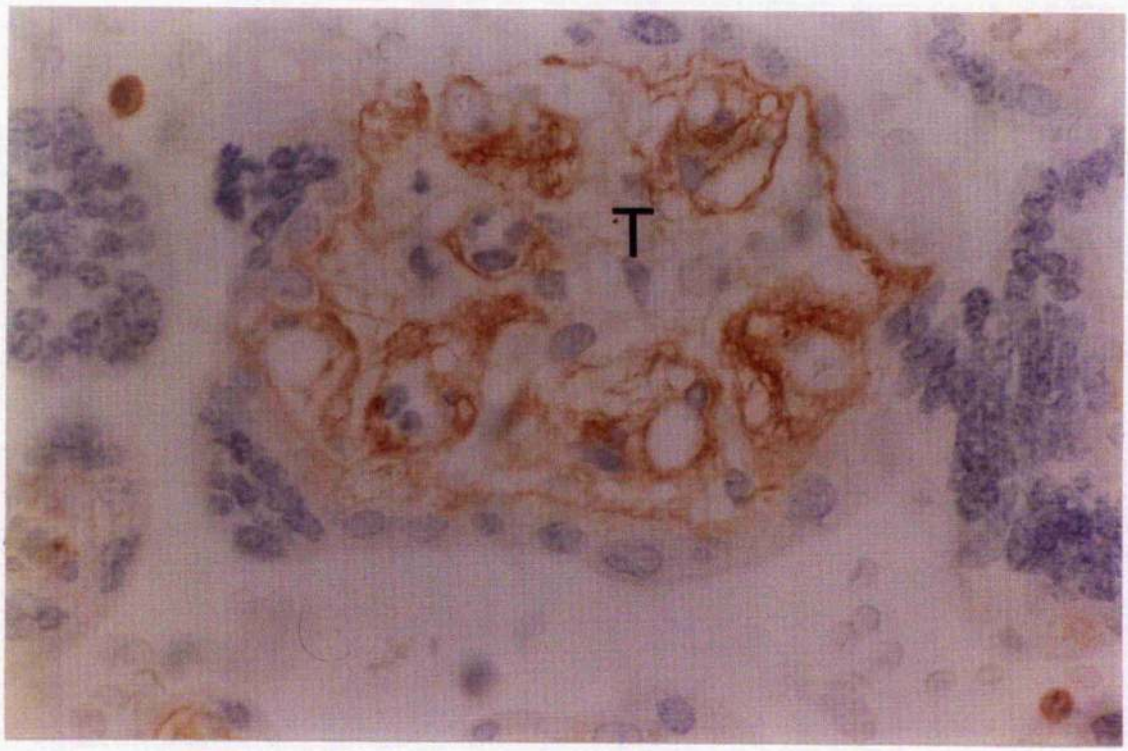

Figure 8.8 Laminin antibody staining in villi from an IUGR pregnancy

The antibody staining for laminin was more intense in the basement membranes of all villous classes of the IUGR villi: (T) - terminal villi. (magnification x 400)

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

9.1 REVIEW

The severely growth restricted fetus is characterised antenatally by abnormal umbilical artery Doppler waveforms and biochemical evidence of both hypoxia and intrauterine starvation (Nicolaides, Economides & Soothill 1989; Economides & Nicolaides 1989; Nicolini et al. 1990). Since intrauterine growth restriction is not only a leading cause of perinatal mortality but is also linked with the development of adult cardiovascular and endocrine disease patterns (Barker 1992; Hinchcliff et al. 1992; Rona, Gulliford & Chinn 1993), it is imperative that we understand the mechanisms which underlie abnormal fetoplacental blood flow; manifest as abnormal umbilical artery Doppler waveforms and ensuing intrauterine hypoxia/starvation. In so doing we may establish the pathophysiology of intrauterine growth restriction which would thus allow us to develop well directed programmes to prevent and/or treat this condition.

The current thinking on the origins of IUGR may be summarised as follows: Impaired trophoblast invasion of the maternal spiral arteries (Shepherd & Bonner 1976; Brosens, Dixon & Robertson 1977; Khong et al. 1986) is thought to result in poor maternal blood flow to the placental bed. As a consequence, the maternal supply of oxygen and nutrients to the fetus is presumed to be deficient and fetal growth thus restricted. Under such circumstances, the placenta like the fetus is exposed to these adverse conditions, but several questions remain unresolved. The "deficient substrate supply theory" does not explain the development of abnormal umbilical artery Doppler waveforms in severely growth restricted pregnancies. Furthermore, although the IUGR fetus is hypoxic and has reduced concentrations of amino acids, blood samples from the placental bed of normal and IUGR pregnancies shows no difference in the concentrations of oxygen present (Nicolaides et al. 1986). In addition, simultaneous measurements of maternal and fetal amino acids concentration ratios would suggest that the maternal concentrations of amino acids are identical for both IUGR and control pregnancies, and indicate that it must therefore be the transfer of these substrates to the fetus which is impaired in IUGR pregnancies and not the maternal substrate supply (Economides et al. 1989).

Since the feto-placental circulation is not innervated, abnormal umbilical artery Doppler waveforms can only be generated by vasomotor agents or alterations in placental vascular anatomy. Currently, there is no conclusive evidence to suggest a primary vasomotor basis for abnormal Doppler (Macara, Kingdom & Kaufmann 1993), though obviously these agents may compound the effects of other factors. Anatomical studies have focused on the number of small stem arterioles and demonstrated reduced numbers of these vessels thought to result from progressive vessel occlusion by platelet emboli or ischaemic sclerosis. Later animal studies have since confirmed that loss of the placental vascular bed can induce similar Doppler changes to those seen in IUGR pregnancies (Trudinger et al. 1987b; Morrow et al

1989b). However, since stem villi are not primarily involved in villous gas and nutrient transfer, the anatomical theories proposed for abnormal Doppler would not explain the presence of fetal hypoxia. Moreover, platelet emboli have never been seen in any portion of the vascular tree in human IUGR placentas and the number of small arterioles has never been related to the rest of the IUGR placental vascular tree to determine if these changes might reflect more widespread hypovascularisation and not simple isolated vessel occlusion.

The original observations from IUGR studies indicated that trophoblast invasion of the maternal spiral arteries was deficient but failed to address the question of why this might occur. Spiral artery invasion occurs secondary to placental growth and therefore the findings of impaired trophoblast invasion would suggest that primary placental growth and development is deranged in IUGR pregnancies. Under such circumstances, the villous surface area for substrate transfer would be significantly depleted and substrate deficiency ensue. Moreover, since villous and vascular development are intimately linked, hypovascularisation might also ensue, thus altering vascular impedance, and the umbilical artery Doppler waveform within the placental bed.

This thesis investigated various aspects of placental anatomical structure in pregnancies complicated by IUGR and AEDFV, in order to evaluate if such anatomical changes might occur and thus contribute to the biochemical status of the growth restricted fetus and the development of increased placental vascular resistance.

9.2 REDUCED STEM VILLOUS VESSEL NUMBERS - VESSEL OBLITERATION OR A FAILURE OF VASCULAR DEVELOPMENT ?

In chapter 3, using the antibody, anti α-smooth muscle actin, we were able to demonstrate that there was no difference in the relative proportions of small and large villous vessels between IUGR and gestational age-matched control placentas. Specifically, we were unable to identify any selective loss of vessels in the 20-90μM interval which might support the vessel obliteration theory of abnormal Doppler. The reduced numbers of tertiary stem villous arterioles reported previously must therefore simply reflect a generalised impairment of vascular development in the IUGR placenta and not the specific loss of small arterioles by occlusion and subsequent obliteration.

The reduction in arterial vessel number was not secondary to depleted numbers of stem villi since proportionately, the villous volume of stem villi was similar in both groups. Therefore vascular growth and development must be specifically impaired in IUGR placentas. However, we also demonstrated that the villous volume of peripheral villi was significantly

reduced in the IUGR cases and thus villous growth, at least at the level of intermediate and terminal villi, must also be modified in pregnancies complicated by IUGR.

9.3 THE LOSS OF PERIPHERAL VILLI - SIMPLE NUMERICAL REDUCTION OR PRIMARY PLACENTAL MALDEVELOPMENT?

In order to establish if the reduction in peripheral villous volume was due solely to depleted numbers of normal villi or secondary to abnormal patterns of peripheral villous development, we examined the three-dimensional structure of peripheral villi and the capillaries within them using scanning electron microscopy of tissue and microvascular casts. Placentas from normally grown pregnancies were composed of multiple small bud-like terminal villi containing a highly branched network of short, highly coiled capillary loops. Large dilated areas, sinusoidal dilatations, were present throughout the capillary loops; these reduce vascular impedance and thus improve materno-fetal exchange. In sharp contrast, the casts from pregnancies complicated by IUGR were characterised by relatively few, extremely elongated unbranched, uncoiled villi which contained capillary loops of similar dimensions. Sinusoidal dilatations were virtually absent from these loops. Since terminal villous buds and terminal capillary loops arise passively during the second and third trimesters of pregnancy as a result of excessive capillary growth, the vascular casts from these IUGR pregnancies which demonstrated a lack of capillary loops, further confirmed that vascular development was impaired in IUGR pregnancies. Our findings were all the more interesting since it is well established that hypoxia stimulates profuse capillary and villous growth (Bacon et al. 1984; Scheffen et al. 1991). The deficient capillary network of the IUGR vascular casts suggests that these IUGR placentas did not develop in an hypoxic environment and the fetal hypoxia which occurs in the growth restricted fetus must therefore arise at a higher level.

9.4 REGULATION OF PLACENTAL VILLOUS AND VASCULAR GROWTH

Recent work has highlighted the role of growth factors in placental angiogenesis. Platelet derived growth factor (PDGF), a dimeric protein, with two polypeptide subunits designated A and B which has been shown to act both as a mitogenic (Bar et al. 1989) and a chemotactic factor for vascular endothelial cells (Westermark et al. 1990). PDGF-B corresponds to the *c-sis* gene. High concentrations of both PDGF-B genes and PDGF-receptor genes have been localised within the endothelial cells of the placental microvascular circulation and within the fibroblasts/smooth muscle cells of larger vessels (Holmgren et al. 1991). Suppression of the

proliferation B-phenotype in placental vessels, perhaps through maternal or paternal genetic imprinting, could therefore modify or inhibit normal placental angiogenesis.

Vascular endothelial derived growth factor (VEGF), also known as vascular permeability factor (VPF), is another member of the PDGF family and though produced in cells close to the vascular network is targeted to vascular endothelial cells (Dvorak et al. 1991) where it promotes mitogenesis and fibroblast migration (Sengar et al. 1993). It is thus a potent angiogenic factor. Interestingly, hypoxia upregulates the production of VEGF *in-vitro* (Shweiki et al. 1992), which may in part explain the hypervascularisation seen in hypoxic conditions such as pre-eclampsia and high altitude. The regulation and expression of these and other agents, such as interleukin-6, angiogenin and transforming growth factor B (TGF-B), in normal placental angiogenesis obviously warrants further investigation. Thereafter, the possible contribution of these factors to the pattern of capillary growth seen in IUGR pregnancies may be established.

New placental villi arise by the proliferation of cytotrophoblast cells which form new villous columns or villous sprouts. These obtain a stromal core and following vascularisation are classified as mesenchymal villi, the precursor of all villous types. Since first trimester cytotrophoblast cells are known to demonstrate a greater proliferation potential than their third trimester counterparts (Yeger et al. 1989) this process would appear to be governed by gestational age, suggesting that cytotrophoblast cell proliferation is intrinsically programmed or regulated.

A wide variety of growth factors and genetic regulators are now recognised to influence normal cytotrophoblast cell proliferation. Amongst the proto-oncogenes investigated, *c-myc* gene expression directly correlates with cytotrophoblast cell proliferation (Kelly et al. 1983; Klein and Klein 1986). Moreover, *c-myc* gene expression is maximal during the early first trimester and declines dramatically as gestational age advances (Ohlsson & Pfeifer-Ohlsson 1986), in keeping with the concept of rapid cytotrophoblast proliferation during the first trimester. Though not entirely proven, since the *c-myc* gene demonstrates features similar to many transcriptional factors (Johnson & McKnight 1989), it is likely that *c-myc* mediates cell proliferation by augmenting post-receptor gene transcription. The *c-sis* proto-oncogene, identified as the B-chain of PDGF, acts via growth factor receptors and is a potent growth stimulator. Though both the *c-sis* and the PDGF-A chain gene are expressed in placenta throughout pregnancy, PDGF-A appears to be restricted to proliferative cytotrophoblast cells in the first trimester but remains actively expressed in the mesenchymal stroma until term. Holmgren et al. 1991; Jackson et al. 1994). PDGF-B or the *c-sis* gene is co-expressed with the *c-myc* gene in first trimester proliferative cytotrophoblasts (Goustain et al. 1985) and has been shown in vitro to effect proliferation of term cytotrophoblast cells also (Holmgren,

unpublished data). In addition, the invasive cytotrophoblast cells found in the maternal decidua likewise express this gene and its receptor (Franklin et al. 1993).

Other growth factors must also be involved in this complex network since the absence of a functional insulin like growth factor-2 (IGF-2) gene in mice may also impair placental growth (DeChiara et al. 1990). IGF-2 is highly expressed in human proliferative cytotrophoblast cells (Ohlsson et al. 1989a) and is thought to modulate cytotrophoblast proliferation via the developmentally regulated IGF-1 receptor (Ohlsson et al. 1989b). It is not yet clear however, whether one specific factor is predominant in stimulating cytotrophoblast proliferation or all interact together, in specific sequence to activate this pathway.

It is likely that inhibitory factors are also necessary for normal placental development, thus preventing excessive villous growth. Glucocorticoids inhibit cell proliferation through suppression of the *c-myc* proto-oncogene (Reed, Nowell & Hoover. 1985) and induce ageing of the trophoblast (Wellman & Volk 1972). Receptors for glucocorticoids are abundant in term cytotrophoblasts (Robinson et al. 1988) and their increasing expression with advancing gestation may be one mechanism by which cytotrophoblast cell proliferation is inhibited in later pregnancy.

Normal placental growth and development requires both maternal and paternal genetic input (Solter 1988; Surani et al. 1988). Excessive trophoblast tissue, hydatidiform mole, is formed in pregnancies arising from paternal chromosomes alone (Kajii & Ohama 1977). In contrast, pregnancies comprised from only maternal genomic material may implant but fail to survive beyond midgestation, presumably because despite early implantation, placental development does not progress sufficiently to support fetal life.

In addition to these trophoblast genes, expression of other genes may likewise be regulated by the specific suppression or induction of portions of the maternal and paternal genome; for example, mice heterozygous for paternally deleted IGF-2 gene produced very small placentas while those with the maternally deleted genome showed little effect (DeChiara, Robertson &Estratiadis 1990). It is clear therefore that cytotrophoblast proliferation, and therefore placental/vascular growth may be significantly altered by placental gene expression.

9.5 ULTRASTRUCTURE OF TERMINAL VILLI - IS THERE EVIDENCE OF PLACENTA HYPOXIA IN IUGR ?

Since these cast findings were unexpected, we examined the ultrastructure of terminal villi for evidence of changes typical of hypoxia and found none. In contrast these villi had changes compatible with relatively high intravillous oxygen tensions; reduced numbers of cytotrophoblast cells, increased stromal collagens and clumping of the syncytiotrophoblast nuclei. Thus these findings suggest that increased oxygen tension may be present in the fetal placenta in IUGR. The increase in intravillous oxygen tension would suggest relative stasis of the fetal blood in the terminal capillary network thus permitting equilibration of the two circulations. In addition, the failure of cytotrophoblast cell proliferation, and thus the lack of new syncytiotrophoblast may have functional implications for placental transport.

These ultrastructural changes were not limited to the small areas of the placenta examined by TEM. In chapters 7 and 8 immunocytochemistry was used to evaluate the stromal collagen and cytotrophoblast cell proliferation patterns, previously identified by TEM, in additional areas of these placentas and confirmed that these changes were widespread throughout the IUGR placentas.

9.6 SUMMARY OF PLACENTAL STRUCTURE IN IUGR

Our results would suggest that villous and vascular development is deranged in the placentas of pregnancies complicated by IUGR and the lack of maternal spiral artery trophoblast invasion noted many years ago, may simply reflect this primary problem. The deficiency in placental villous turnover was confirmed by the MIB-1 studies in chapter 7 and the lack of villous tissue dramatically visualised by the tissue casts SEM images of chapter 5.

In order to obtain sufficient nutrients to meet the metabolic demands of normal growth and development, the fetus must process increasing volumes of blood through the placenta. This is only possible by a compensatory fall in placental vascular impedance with the rapid increase of the placental vascular network and the extensive growth of the placental villous surface area. If the growth retarded fetus is to survive and maintain placental blood flow, despite the severely depleted villous and vascular network we have shown, fetal arterial blood pressure is likely to rise and systemic peripheral resistance increase. These early adaptations to increased fetal blood pressure *in-utero* may predispose the fetus to develop persistent systemic hypertension and cardiac disease in later adult life and explain some of the histological changes seen.

However, *in-utero*, though an increase in arterial pressure may maintain placental blood flow at reasonable levels, the arterio-arterial vessels within the placenta are liable to become patent. Since the poorly developed terminal capillary network we have demonstrated offers a relatively high resistance pathway, blood may be shunted in significant quantities through these low resistance arterial shunts, thus bypassing the sites of maximal materno-fetal exchange within the terminal capillary network. As in the lung, shunting of blood through relatively "unventilated" portions of the placental tree will result in mixing of well-oxygenated and poorly-oxygenated blood, thus lowering the total oxygen concentration of blood in the umbilical vein. Such events would explain how the IUGR fetus can be hypoxic though oxygen concentrations in the placenta and uterine bed remain within normal levels. In addition therefore, though maternal oxygen therapy may improve the umbilical vein pO_2 of the small fetus born at high altitude, it is less likely to be of any benefit to the IUGR fetus (Ribbert, Lingen &Visser 1991).

Since the villous surface area, particularly that of the peripheral villi, is significantly reduced and the syncytiotrophoblast shows evidence of "ageing" as shown in chapter 6, the IUGR fetus may be substrate deprived both as a result of malfunction of the transporter proteins within the syncytiotrophoblast and/or depletion in the number of transporter systems available. In addition, poor blood flow through the terminal capillary network, evidenced by ante-natal capillary congestion, and the possible shunting of blood through arterial anastomoses will compound these problems, limiting further the nutrient resources available to the fetus.

9.7 INTRAUTERINE GROWTH RETARDATION - FUTURE DIRECTIONS

The findings we have demonstrated would suggest that abnormal expression and/or function of candidate growth factors and oncogenes is responsible, at least in part, for the alterations in villous and vascular growth patterns in the IUGR placenta. Future work should therefore investigate the role of agents such as PDGF, VEGF, c-myc and c-jun which modulate normal placental angiogenesis and cytotrophoblast proliferation in pathological growth retarded pregnancies. In addition, since it is clear that the function of genes may be altered by the pattern of maternal and paternal inheritance (eg. IGF-2 gene in mice), the effects of maternal and paternal disomy on placental phenotype must also be established. Clearly the functional capacity of the abnormal terminal villi we have demonstrated, must also be determined since for example, the transporter systems within the syncytiotrophoblast may not only be depleted in number but may also be functionally abnormal. The availability of *in-situ* methods to localise not only the receptors for these proteins but to actually measure m-RNA expression

may facilitate quantification of transporters, while membrane preparations from affected pregnancies could permit actual function to be evaluated.

It is clear from our findings that severe IUGR is not a condition acquired by the fetus in late pregnancy but the reflection of fundamental alterations in basic placental implantation and development. In view of this, any attempts to address the underlying pathophysiology of severe IUGR must focus not on the mid-gestation fetus but rather on the factors regulating the development of the early placenta and conceptus.

REFERENCES

ABBAS A, Snidjers RJM, Nicolaides KH. Serum ferritin and cobalamin in growth retarded fetuses. Br J Obstet Gynaecol 1994; 101:215-219

ADAMSON SL, Morrow RJ, Langille BL, Bull SB, Ritchie JWK. Site-dependant effects of increases in placental vascular resistance onthe umbilical artery flow velocity waveform in fetal sheep. Ultrasound Med Biol 1990; 16:19-27

AGBOOLA A. Placental changes in patients with a low haematocrit. Br J Obstet Gynaecol 1975; 82:225-227

AHERNE W, Dunhill MS. Quantitative aspects of placental structure. J Path Bact 1966; 91:123-139.

ALDER KB, Craighead JE, Vallyathan NV, Evans JN. Actin-containing cells in human pulmonary fibrosis. Am J Pathol 1981; 102:427-437

AL-GHAZALI W, Chita SK, Chapman MG, Allan LD. Evidence of redistribution of cardiac output in symmetrical growth retardation Br J Obstet Gynaecol 1989; 96:697-704

AL-GHAZALI WH, Chapman MG, Rissik JM, Allan LD. The significance of absent end-diastolic flow in the umbilical artery combined with reduced fetal cardiac output estimation in pregnancies at high risk for placental insufficiency. J Obstet Gynecol 1990; 10:271-275

ALMSROM H, Axelsson O, Cnattingius S et al. Comparison of umbilical-artery velocimetry and cardiotocography for surveillance of small-for-gestational-age fetuses. Lancet 1992; i:936-940.

AMENTA PS, Gay S, Vaheri A, Martinez-Hernandez A. The extracellular matrix is an integrated unit: Localisation of collagen type I, III, IV, V, VI, fibronectin and laminin in human term placenta. Collagen Rel Res 1986; 6:125-152

APLIN JD. Implantation, trophoblast differentiaition and haemochorial placentation: mechanistic evidence in-vivo and in-vitro. J Cell Science 1991; 99:681-692

ARDUINI D, Rizzo G. Fetal renal artery velocity waveforms and amniotic fluid volume in growth retarded and post-term fetuses. Obstet Gynecol 1991; 77:370-373

ARDUINI D, Rizzo G, Romanini C. The development of abnormal heart rate patterns after absent end-diastolic velocity in umbilical artery-analysis of risk factors. Am J Obstet Gynecol 1993; 168:43-50.

BACON BJ, Gilbert RD, Kaufmann P, Smith AD, Trevino FT, Longo LD. Placental anatomy and diffusing capacity in guinea-pigs following long-term maternal hypoxia. Placenta 1984; 5:475-488.

BAR S, Boes M, Booth BA, Dake BL, Henley H, Hart MN. The effects of platelet-derived growth factor in cultured microvessel endothelial cells. Endocrinol 1989; 124:1841-1848.

BARKER DJP, Osmond C, Golding J, Kuh D, Wadsworth MEJ. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. Br Med J 1989; 298;564-567.

BARKER DJP, Bull AR, Osmond C, Silva PA. Fetal and placental size and risk of hypertension in adult life. Br Med J 1990; 301:259-262.

BARKER DJP. The fetal and infant origins of adult disease. British Medical Journal Books, London: 1992.

BARKER DJP, Martyn CN, Osmond C, Hales CN, Fall CHD. Growth in utero and serum cholesterol concentrations in adult life. Br Med J 1993; 307:1524-1527

BEATTIE RB, Dornan JC. Antenatal screening for intrauterine growth retardation with umbilical artery Doppler ultrasonography. Br Med J 1989; 298:631-635

BENEDETTO C, Barbero M, Rey L, et al. Production of prostacyclin, 6-keto PGF 1_a and thromboxane B2 by human umbilical vessels increases from the placenta towards the fetus. Br J Obstet Gynaecol 1987; 94: 1165-1169.

BENIGNI A, Gaspari F, Orisi S et al. Human placenta expresses endothelin gene and corresponding protein is excreted in urine in increasing amounts during normal pregnancy. Am J Obstet Gynecol 1991; 164:844-848

BERMAN W, Goodlin RC, Heymann MA, Rudolph AM. Relationships between pressure and flow in the umbilical and uterine circulations of the sheep. Circ Res 1976; 38: 262-268.

BERNARDINI I, Evans MI, Nicolaides KH, Economides DL, Gahl WA. The fetal concentrating index as a gestation age-independant measure of placental dysfunction in intrauterine growth retardation. Am J Obstet Gynecol 1991; 164:1481-1490

BERNISCHKE K, Kaufmann P. Pathology of the Human Placenta. Spinger. New York, 1990.

BERTRAND C, Duperron L, St.Louis J. Umbilical and placental vessels: modifications of their mechanical properties in pre-eclampsia. Am J Obstet Gynecol 1993; 168:1537-1546.

BEWLEY S, Cooper D, Campbell S. Doppler investigation of utero-placental blood flow resistance in the second trimester: a screening study for pre-eclampsia and intrauterine growth retardation. Br J Obstet Gynaecol 1991; 98:871-879.

BLAIR E, Stanley F. Intrauterine growth and spastic cerebral palsy II. The association with morphology at birth. Early Hum Dev 1992; 28:91-96.

BLOCK BSB, Liaos AJ, Creasy RK. Responses of the growth retarded fetus to acute hypoxia. Am J Obstet Gynecol 1984; 148:878-885

BOE F. Studies on placental circulation in rats. II. Vascular pattern illustrated by corrosion preparations. Acta Endocrinol 1950; 5:365-369.

BOE F. Studies on the vascularisation of the human placenta. Acta Obstet Gynecol 1953; 32:Suppl 5, 1-92

BOE F. Studies on the human placenta. III. Vascularisation of the young fetal placenta. A. Vascularisation of the young chorionic villus. Acta Obstet Gynecol Scand 1969; 48:159-166.

BOSELLI J, Macarak EJ, Clark CC, Browncll AG, Martinez-Hernandez A. Fibronectin: its relationship to basement membranes. Collagen Rel Res 1981; 1:391-404

BOTNEY MD, Liptay MJ, Kaiser LR, Cooper JD, Parks WC, Mecham RP. Active collagen synthesis by pulmonary arteries in human primary pulmonary hypertension. Am J Path 1993; 143:121-129

BOURA ALA, Walters AWA. Autocoids and the control of vascular tone in the human umbilical placental circulation. Placenta 1991; 12:453-477.

BOWER S, Bewley S, Campbell S. Improved prediction of preeclampsia by two stage screening of uterine arteries using the early diastolic notch and color Doppler imaging. Obstet Gynecol 1993; 82:78-83.

BOYD JD, Hamilton WJ. The Human Placenta. W Heffer, Cambridge (1970)

BOYD PA, Brown RA, Stewart WJ. Quantitative structural differences within the normal term human placenta: A pilot study. Placenta 1980; 1:337-344.

BOYD PA, Scott A. Quantitative structural studies on human placentas associated with pre-eclampsia, essential hypertension and intrauterine growth retardation. Br J Obstet Gynaecol 1985; 92:714-721.

BRACE RA. Amniotic fluid dynamics. In: Maternal-fetal medicine. Creasy RK, Resnik R, eds. WB Saunders Company, Philadelphia, 1989

BRACERO LA, Beneck D, Kirshenbaum N Pieffer M, Stalter P, Schulman. Doppler velocimetry and placental disease. Am J Obstet Gynecol 1989;161:388-393

BRAR HS, Platt LD. Reverse end-diastolic flow velocity on umbilical artery velocimetry in high risk pregnancies: an ominous finding with adverse pregnancy outcome. Am J Obstet Gynecol 1988; 159:559-561

BROSENS I, Robertson WB, Dixon HG. The physiological response of the vessels of the placental bed to normal pregnancy. J Path Bacteriol 1967; 93:569-579.

BROSENS I, Dixon HG, Robertson WB. Fetal growth retardation and the vessels of the placental bed. Br J Obstet Gynaecol 1977; 84: 656-663.

BROUGHTON-PIPKIN F, Symonds EM. Factors affecting angiotensin II concentrations in the human infant at birth. Clin Sci Mol Med 1977; 52:449-456

BULMER JN, Johnson PM. Macrophage populations in the human placenta and amniochorion. Clin Exper Immunol 1984; 57:393-403

BURCHILL RC. Arterial blood flow in the human intervillous space. Am J Obstet Gynecol 1967; 98:303-311.

BURKE G, Stuart B, Crowley P, Ni Scanaill S, Drumm J. Is intrauterine growth retardation with normal umbilical artery blood flow a benign condition? Br Med J 1990;300:1044-1045.

BURTON GJ, Ingram SC, Palmer ME. The influence of mode of fixation on morphometrical data derived from terminal villi in the human placenta at term: a comparison of immersion and perfusion fixation. Placenta 1987; 8:37-51

BURTON GJ, Palmer ME. Short communication: Eradicating fetomaternal fluid shift during perfusion fixation of the human placenta. Placenta 1988; 9; 327-352

CAI WQ, Bodin P, Sexton A, Loesch A, Burnstock G. Localisation of neuropeptide Y and ANP in the endothelial cells of human umbilical blood vessels. Cell Tiss Res 1993; 272:175-181

CAMPBELL S, Wilkin D. Ultrasonic measurement of fetal abdomen circumference in the estimation of fetal weight. Br J Obstet Gynaecol 1975; 82:689-697.

CAMPBELL S, Pearce JMF, Hackett G, Cohen-Overbeek T, Hernandez C. Qualitative assessment of utero-placental blood flow: Early screening test for high risk pregnancies. Obstet Gynecol 1986; 68:649-653

CANTLE SJ, Kaufmann P, Luckhardt M, Schweikhart G. Interpretation of syncytial sprouts and bridges in the human placenta. Placenta 1987; 8:221-234.

CASTELLUCCI M, Kaufmann P. A three dimensional study of the normal human placental villous core: II Stromal architecture. Placenta 1982; 3:269-286

CASTELLUCCI M, Scheper M, Scheffen I, Celona A, Kaufmann P. The development of the human placental vascular tree. Anat Embryol 1989; 181:117-128

CETIN I, Corbetta C, Sereni LP et al. Umbilical amino-acid concentrations in normal and growth retarded fetuses sampled in utero by cordocentesis. Am J Obstet Gynecol 1990; 162:253-261.

CHABES A, Peroda J, Perez N et al. Morphometry of human placenta at high altitude. Am J Obstet Gynecol 1967; 14a-15a

CHAIET L, Wolf FS. The properties of streptavidin, a biotin-binding protein produced by streptomyces . Arch Biochem Biophys 1964; 106:1-5

CHAMBERLAIN PF, Manning FA, Morrison I, Harman CR, Lange IR. Ultrasound evaluation of amniotic fluid volume. Am J Obstet Gynecol 1984; 150:245-249.

CHATFIELD WR, Rodgers TGH, Brownlee BEW, Rippon PE. Placental scanning with computer-linked gamma camera to detect impaired blood flow and intrauterine growth retardation. Br Med J 1975; 2:120-122.

CHAUDHURI G, Buga GM, Gold ME, Wood KS, Ignarro LJ. Characterisation and actions of human umbilical endothelium derived relaxing factor. Br J Pharm 1991; 102:331-336.

CHITTY LS, Altman DG, Henderson A, Campbell S. Charts of fetal size: 3. Abdominal measurements. Br J Obstet Gynaecol 1994; 101:125-131.

CLAPP JF, Szeto HH, Larrow R, Hewitt J, Mann LI. Umbilical blood flow response to embolization of the uterine circulation. Am J Obstet Gynecol 1980; 60: 60-67

CLARK CC, Crossland J, Kaplan G, Martinez-Hernandez A. Localization and identification of the collagen found in the 14.5 day rat embryo visceral yolk sac. J Cell Biol 1983; 93:251-260

COHEN OVERBECK T, Pearce JMF, Campbell S. The antenatal assessment of uteroplacental and feto-placental blood flow using Doppler ultrasound. Ultrasound Med Biol 1985; 11:329-339

CONTRACTOR SF, Banks RW, Jones CP. Fox H. A possible role for placental lysosomes in the formation of the villous syncytiotrophoblast. Cell Tiss Res 1977; 178:411-419

COOKE SF, Craven DJ, Symonds EM. A study of angiotensin II binding sites in human placenta, chorion and amnion. Am J Obstet Gynecol 1981; 140: 689-692

COONS AH, Creech HJ, Jones RN, Berliner E. The demonstration of pneumoccal antigen in tissues by the use of a fluorescent antibody. J Immunol 1942; 45:159-170

COPEL JA, Schlafer D, Wentworth R, Belanger K, Kreitzer L, Hobbins JC, Nathanielsz PW. Does the umbilical artery systolic/diastolic ratio reflect flow or acidosis? Am J Obstet Gynecol 1991; 163:751-756

COTTE C, Easty G, Neville AM, Monaghan P. Preparation of highly purified cytotrophoblast from human placenta with subsequent modulation to form syncytiotrophoblast in monolayer cultures. In-Vitro 1980; 16:639-646

CREELY JJ, Dimari SJ, Howe AM, Haralson MA. Effects of transforming growth factor-ß on collagen synthesis by normal rat epithelial cells. Am J Path 1992; 140:45-55

DAFFOS F, Cappela-Pavlovsky M, Forestier F. Fetal blood sampling during pregnancy with use of a a needle guided by ultrasound: a study of 606 consectutive cases. Am J Obstet Gynecol 1985; 158:132-136

DAVEY DA, MacGillvray I. The classification and definition of the hypertensive disorders of pregnancy. Am J Obstet Gynecol 1988; 158:892-898

DECHIARA TM, Efstratiadis A, Robertson EJ. A growth deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. Nature 1990; 345:78-80

DECHIARA TM, Robertson E, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor 2 gene. Cell 1990; 64:849-859.

DEMIR R, Kaufmann P, Castellucci C, Erbengi T, Kotowski A. Fetal vasculogenesis and angiogenesis in human placental villi. Acta Anatomica 1989; 136:190-203.

DEMIR R, Demir N, Kohnen G et al. Ultrastructure and distribution of myofibroblast-like cells in human placental stem villi. Elect Micros 1992; 3:509-510.

DEMPSTER J, Mires GJ, Patel N, Taylor DJ. Umbilical artery velocity waveforms : poor association with small-for-gestational age babies. Br J Obstet Gynaecol 1989; 96:692-696

DESOYE G, Hartmann M, Blaschitz A, Dohr G, Hahn T, Kohnen G, Kaufmann P. Insulin receptors in syncytiotrophoblast and fetal endothelium of human placenta. Immunohistochemical evidence for developmental changes in distribution pattern. Histochem 1994; 101:277-285

DIVON MY, Guidetti DA, Bravermann JJ, Oberlander E, Langer O, Merkatz IR. Intrauterine growth retardation - a prospective study of the diagnostic value of real time sonography combined with umbilical artery flow velocimetry. Obstet Gynecol 1988; 72:611-614

DIVON MY, Girz BA, Leiblich R, Langer O. Clinical management of the fetus with markedly diminished umbilical artery end-diastolic flow. Am J Obstet Gynecol 1989; 161:1523-1527.

DOUGLAS SA, James S, Hiley CR. Endothelial modulation and changes in endothelin presser activity during hypoxia in the rat isolated perfused superior mesenteric arterial bed. Br J Obstet Gynaecol 1991; 103:1441-1448

DUNGY LJ, Siddiqi TA, Khan S. Transforming growth factor-ß expression during placental development. Am J Obstet Gynecol 1991; 165:853-857

DVORAK HF, Sioussat TM, Brown LF et al. Distribution of vascular permeability factor (vascular endothelial growth factor) in tumours; concentration in tumour blood vessels. J Exp Med 1991; 174:1275-1278

EBRAHIM GJ. Care of the newborn. Br Med J 1984; 289:899

ECONOMIDES DL Nicolides KH. Blood glucose and oxygen levels in small-for-gestational age fetuses. Am J Obstet Gynecol 1989; 160:385-389

ECONOMIDES DL, Nicolaides KH, Gahl WA, Bernardini I, Evans MI. Plasma amino acids in appropriate and small for gestational age fetuses. Am J Obstet Gynecol 1989; 161:1219-1227.

ECONOMIDES DL, Crook D, Nicolaides KH. Hypertriglygeridaemia and hyoxemia in small for gestational age fetuses. Am J Obstet Gynecol 1990; 162:382-386

EIK-NES S, Brubakk AO, Ulstein M. Measurement of human fetal blood flow. B Med J 1980; i:283-284

EIK-NES SH, Marsal K, Kristofferson K. Methodology and basic problems related to blood flow studies in the human fetus. Ultrasound Med Biol 1984; 10:329-337

ERSKINE RLA, Ritchie JWK. Umbilical artery blood flow characteristics in normal and growth retarded fetuses. Br J Obstet Gynaecol 1985; 92:605-610

ESCOBAR GJ. Prognosis of surviving very low birthweight infants: still in the dark. Br J Obstet Gynaecol 1992; 99:1-4.

FAIRLIE FM, Moretti M, Walker JJ, Sibai BM. Determinants of perinatal outcome in pregnancy induced hypertensionwith absence of end-diastolic frequencies. Am J Obstet Gynecol 1991; 164:1084-1089

FAIRLIE FM, Moretti M, Walker JJ, Sibai BM. Umbilical artery and uteroplacental velocity in pregnancies complicated by idiopathic low birthweight centile. Am J Perinatol 1992; 9:250-253

FANT ME. In-vitro growth rate of placental fibroblasts is developmentally regulated. J Clin Invest 1991; 88:1697-1702.

FANT ME, Nanu L, Word RA. A potential role for endothelin-1 in human placental growth: Interactions with the insulin-like growth factor family of peptides. J Clin Endocrinol Metab 1992; 74:1158-1163

FELLER AC, Schneider H, Schmidt D, Parwaresch MR. Myofibroblasts as a major cellular constituent of villous stroma in human placenta. Placenta 1985; 6:405-415.

FINE A, Goldstein RH. The effect of TGF-ß on cell proliferation and collagen formation by lung fibroblasts. J Biol Chem 1987; 262:3897-3902

FIRTH JA, Farr A, Baumann K. The role of gap junctions in trophoblastic cell fusion in the guinea-pig placenta. Cell Tissue Res 1980; 205: 311-318.

FISHER SJ, Leitch MS, Kantor MS, Basbaum CB, Kramer RH. Degradation of extracellular matrix by trophoblastic cells of first trimester human placentas. J Cell Biochem 1985; 27:31-41

FITZGERALD DE, Drumm JE. Non-invasive measurement of human fetal circulation using ultrasound: a new method. Br J Obstet Gynaecol 1977; 2:1450-1451
FLEISCHER A, Schulmann H, Farmakides G et al. Umbilical artery velocity waveforms and intrauterine growth retardation. Am J Obstet Gynecol 1985; 151:502-505

FOK RY, Pavlova Z, Bernischke K, Paul R, Platt LD. The correlation of arterial lesions with umbilical artery Doppler velocimetry in the placentas of small-for-dates pregnancies. Obstet Gynecol 1990; 75:578-583.

FOX H, The pattern of villous variability in the normal placenta. J Obstet Gynaecol Brit Cwlth. 1964; 71:479-458

FOX H. The significance of villous syncitial knots in the human placenta J Obstet Gynecol Br Cwlth 1965; 72:347-355.

FOX H. Morphological changes in the human placenta following fetal death. J Obstet Gynaecol Br Cwlth 1968a; 75: 839-843.

Fox H. Pathology of the Placenta Saunders, London, 1968b.

FOX H. Effect of hypoxia on trophoblast in organ culture: a morphologic and autoradiographic study. Am J Obstet Gynecol 1970; 107:1058-1064

FOX H. Pathology of the placenta. Clinics Obstet Gynaecol 1986; 13;501-519

FOX SB, Khong TY. Lack of innervation of human umbilical cord. An immunological and histochemical study. Placenta 1990; 11:59-62.

FRANKLIN GC, Holmgren L, Donovan M et al. Expression and control of PDGF stimulatory loops in the developing placenta. Troph Res 1993; 7:287-303

FREIDMAN DM, Rotkowski M, Snyder JR, et al. Doppler blood velocity waveforms in the umbilical artery as an indicator of fetal well-being. J Clin Ultrasound 1985; 13:161-165

GABBE SG. Intrauterine growth retardation. In: Obstetrics: normal and problem pregnancies. Gabbe SG, Neibyl JR, Simpson JL eds. New York: Churchill Livingstone, 1991:923 - 944.

GABBIANI G, Majno G. Dupuytrens contracture: fibroblast contracture. Am J Path 1972; 66:131-138.

GENNSER G, Rymark P, Isberg PE. Low birthweight and risk of high blood pressure in adulthood. Br Med J 1988; 298:1498-1499.

GERDES J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 1983; 31:13-20

GILES WB, Trudinger BJ, Baird P. Fetal umbilical artery flow velocity waveforms and placental resistance: pathological correlation. Br J Obstet Gynaecol 1985; 92:31-38

GILES WB, Trudinger BJ. Umbilical cord whole blood viscosity and the umbilical artery flow velocity time waveforms: a correlation. Br J Obstet Gynecol 1986; 93:466-470

GILES WB, Lingman G, Marsal K, Trudinger BJ. Fetal volume blood flow and umbilical artery flow velocity waveform analysis: a comparison. Br J Obstet Gynaecol 1986; 93:461-465.

GILES W, Trudinger B, Cook C, Connelly A. Placental microvascular changes in twin pregnancies with abnormal umbilical artery waveforms. Obstet Gynecol 1993; 81:556-559

GLANCE DG, Elder MG, Bloxham DL, Myatt L. The effects of the components of the renin-angiotensin system on the isolated perfused human placental cotyledon. Am J Obstet Gynecol 1984; 149: 450-454.

GOSLING RG, King DH. Ultrasound angiology. In Arteries and Veins. Marcus AW, Adamson L, eds. Churchill Livingstone, Edinburgh. p61-98.

GOUSTAIN AS, Betsholtz C, Pfeifer-Ohlsson S, Persson H, Rydnert J, Bywater M et al. Co-expression of the sis and myc proto-oncogenes in developing human placenta suggests autocrine control of trophoblast growth. Cell 1985; 41:301-312

GRAF R, Frank HG, Oney T. Histochemical and immunohistochemical investigations of the fetal extravascular and vascular contractile system in the normal placenta and during preeclampsia. In: Risk assessment of prenatally-induced adverse health effects. Neubert D Ed. Springer: Heidelberg, 1992; 537-550.

GRAHAM PC, Kingdom JCP, Raweily EA, Gibson AAM, Lindop GBM. Distribution of renin containing cell in the developing human kidney: an immunohistochemical study. Br J Obstet Gynaecol 1992; 99: 765-769.

GRIFFIN D, Cohen-Overbeck T, Campbell S. Fetal and utero-placental blood flow. Clin Obstet Gynecol 1983; 10:565-602

GRIFFIN D, Bilardo K, Masini L, Diaz-Recasens J, Pearce JM, Wilson K, Campbell S. Doppler blood flow waveforms in the descending thoracic aorta of the human fetus. Br J Obstet Gynaecol 1984; 91:997-1006.

GUPTA I, Hillier VF, Edwards JM. Multiple vascular profiles in the umbilical cord; an indication of maternal smoking habits and intrauterine distress. Placenta 1993; 14:17-123

HABASHI S, Burton GJ, Steven DH. Morphological study of the fetal vasculature of the human term placenta: scanning electron microscopy of corrosion casts. Placenta 1983; 4:41-56

HACKETT GA, Campbell S, Gamsu H, Cohen-Overbeek T, Pearce JMF. Doppler studies in the growth retarded fetus and prediction of neonatal necrotising enterocolitis, heamorrhage and neonatal morbidity. Br Med J. 1987; 294:13-16

HAHN P. Effect of litter size on plasma cholesterol and insulin and some liver and adipose tissue enzymes in adult rodents. J Nut 1984; 114:1231

HARRIS AK, Ahmed A. The ultrastructure of tubular carcinoma of the breast. J Pathol 1977; 123:79-83.

HARTIKAINEN-SORRI A-L, Vuolteenaho O, Leppaluoto J, Ruskoaho H. Endothelin in umbilical artery vasospasm. Lancet 1991; 337:619.

HAY ED. Cell biology of the extracellular matrix. ed. Hay ED., Plenum Press, New York 1981

HEIDA HS, Gomes-Sanchez CE. Hypoxia increases endothelin release in bovine endothelial cells in culture, but epinephrine, noepinephrine, serotonin and angiotensin II do not. Life Sci 1990; 47:247-251

HEMSEN A, Gillis C, Larson O, Haegerstrand A, Lundberg JM. Characterisation, localisation and actions of endothelin in umbilical vessels and placenta of man. Acta Physiol Scand 1991; 43:395-404

HENDRICKS SK, Sorensen TK, Wang KY, Bushnell JM, Seguin EM, Zingheim RW. Doppler umbilical artery waveform indices - Normal values from fourteen to forty-two weeks. Am J Obstet Gynecol 1989; 161:761-765.

HERTIG AT, RockJ. Two human ova of the pre-villous stage having a developmental age of about seven and nine days respectively. Contrib Embryol 1945; 29:127-156.

HILL DJ, Clemmons DR, Riley SC, Bassett N, Challis JRG. Immunohistochemical localization of insulin-like growth factors (IGFs) and IGF binding proteins -1, -2 and -3 in human placenta and fetal membranes. Placenta 1993; 14:1-12.

HINCHLIFFE SA, Lynch MRJ, Sargent PH, Howard CV, Van Velzen D. The effect of intrauterine growth retardation on the development of renal nephrons. Br J Obstet Gynaecol 1992; 99:296-301

HITSCHOLD T, Weiss E, Beck T, Hunterfering H, Berle P. Low target birthweight or growth retardation? Umbilical Doppler velocity waveforms and histometric analysis of fetoplacental vascular tree. Am J Obstet Gynecol 1993; 168:1260-1264.

HODDE KC, Nowell AJ. Scanning electron microscopy of micro-corrosion casts. Scann Elect Microsc 1980; II:89-106

HOLMGREN G, Heldin CH, Westwermark B, Ohlsson R. Co-expression of the sis and myc proto-oncogenes in developing human placenta suggests autocrine control of trophoblast growth . Cell 1985; 41:301-312.

HOLMGREN L, Glaser A, Pfeifer-Ohlsson S, Ohlsson R. Angiogenesis during human extraembryonic development involves the spatiotemporal control of PDGF ligand and receptor gene expression. Development 1991; 113: 749-754.

HOLMGREN L, Claesson-Welsh L, Heldin CH, Ohlsson R. The expression of PDGF a and b receptors in subpopulations of PDGF producing cells implicates autocrine stimulatory loops in control of proliferation of cytotrophoblasts which have invaded the maternal endometrium. Growth Factors 1992; 6:219-231.

HUMBERT JR, Abelson H, Hathaway WE, Battaglia FC. Polycythemia in small for gestational age infants. J Pediatr 1969; 75: 812-814.

HUSTIN J, Schaaps JP. Echocardiographic and anatomic studies of the maternotrophoblastic border during the first trimester of pregnancy. Am J Obstet Gynecol 1987; 157:162-168

HUSTIN J, Schaaps JP, Lambotte R. Anatomical studies of the uteroplacental vascularisation in the first trimester of pregnancy. Troph Res 1988; 3:49-60.

HUSTIN J, Jauniaux E, Schaaps JP. Histological study of the materno-embryonic interface in spontaneous abortion. Placenta 1990; 11:477-486

IHARA Y, Taii S, Mori T. Expression of renin and angiotensinogen genes in the human placenta tissues. Endocrinologia Japonica 1987; 34: 887-896

ILOKA H Akada S, ShimamotoT, Yamada Y, Sakamoto Y, Yoshida T, Ichijo M. Studies on placental inhibition of platelet aggregation: A comparison of human syncytiotrophoblast brush border and basal plasma membranes. Placenta 1993; 14:519-528

INAYATULLA A, Chemtob S, Nuwayhid B, Varma DR. Repsonses of placental arteries from normotensive and preeclamptic women to endogenous vasoactive agents. Am J Obstet Gynecol 1993; 168:869-874

INDIK JH, Reed KL. Variation and correlation in human fetal umbilical doppler velocities and fetal breathing: evidence of the cardiac-placental connection. Am J Obstet Gynecol 1990; 163:1792-1796

INGLIS GC, Kingdom JCP, Nelson DM et al. Atrial Natriuretic hormone: A paracrine or endocrine role within the human placenta? JCEM 1993; 76:1014-1018

IRION GL, Clark KE. Relationship between the ovine fetal umbilical artery blood flow waveform and umbilical vascular resistance. Am J Obstet Gynecol 1990; 163:222-229

IRION GL, Mack CE, Clark KE. Fetal haemodynamic and fetoplacental vascular response to exogenous arginine vasopressin . Am J Obset Gynecol 1990; 162:1115-1120

JACKSON M, Joy CF, Mayhew TM, Haas JD. Stereological studies on the true thickness of the villous membrane in human term placenta: a study of placentae from high altitude pregnancies. Placenta 1985; 6:249-258

JACKSON M, Mayhew T, Haas JD. On the factors which contribute to thinning of the villous membrane in human placentae at high altitude. II An increase in the degree of peripheralization of fetal capillaries. Placenta 1988; 9:9-18.

JACKSON MR, Walsh AJ, Morrow RJ, Mullen BM, Lye SJ, Ritchie JWK. Reduced placental villous tree elaboration in small-for-gestational age pregnancies: relationship with umbilical artery Doppler waveforms. Am J Obstet Gynecol 1995 (in press)

JACKSON MR, Mayhew TM, Boyd PA. Quantitative description of the elaboration and maturation of villi from 10 weeks of gestation to term. Placenta 1992; 13:357-370.

JACKSON MR, Carney EW, Lye SJ, Ritchie JWK. Localisation of two angiogenic growth factors (PDECGF and VEGF) in human placentae throughout gestation. Placenta 1994; 15:341-353.

JACOBSON RL, Brewer A, Eis A, Siddiqi TA, Myatt L. Transfer of aspirin across the perfused human placental cotyledon. Am J Obstet Gynecol 1991; 165:939-944.

JAUNIAUX E, Burton GJ, Moscoso GJ, Hustin J. Development of the early human placenta: a morphometric study. Placenta 1991; 12:269-276.

JAUNIAUX E, Jurkovic D, Campbell S, Hustin J. Doppler ultrasonographic features of the developing placental circulation: correlation with anatomic findings. Am J Obstet Gynecol 1992; 166:585-587

JAUNIAUX E, Burton GJ. Correlation of umbilical Doppler features and placental morphometry: the need for uniform methodology. Ultrasound Obstet Gynecol 1993; 3:233-235

JOHNSON PF, McKnight SL. Eukaryotic transcriptional regulatory proteins. Ann Rev Biochem 1989; 58:799-839

JONES CP, Fox H. Syncytial knots and intervillous bridges in the human placenta: an ultrastructural study. J Anat 1977; 124: 275-286.

JONES CP, Fox H (1991) Ultrastructure of the normal human placenta. Electron Microsc Rev 4; 129-178

JONES DC, Sanchez-Romez L. Effect of angiotensin II infusion during normal pregnancy on flow velocity waveforms in the uteroplacental and umbilical circulations. Obstet Gynecol 1990; 76: 1093-1096.

JOUPPILA P, Kirkinen P. Umbilical vein blood flow as an indicator of fetal hypoxia. Br J Obstet Gynaecol 1984;91:107-110

KAAR K, Jouppila P, Kuikka J, Luotola H, Toivanen J, Rekonen A. Intervillous blood flow in normal and complicated late pregnancy measured by means of intravenous ^{133}Xe method. Acta Obstet Gynecol Scand 1980; 59:7-10

KAFFE S, Godmilow L, Walker BA, Hirschhorn K. Prenatal diagnosis of bilateral renal agenesis. Obstet Gynecol 1977; 49:478-485

KAJII T, Ohama K. Androgenetic origin of hydatidiform mole. Nature 1977; 268;633-634.

KARIMU AL, Burton GJ. The effects of maternal vascular pressure on the dimensions of the placental capillaries. Br J Obstet Gynaecol 1994; 101:57-63

KAUFMANN P, Stark J. Enzymhistochemische untersuchungen an reifen menschlichen placentazoten. I. reifungs-und alterungsvorgange am trophoblasten. Histochemistry 1972; 29:65-82.

KAUFMANN P. Development and differentiation of the human placental villous tree. Bibl Anat 1982; 22:29-39

KAUFMANN P, Bruns U, Leiser R, Luckhart M, Winterhager E. The fetal vascularisation of term human placental villi. II. Intermediate and terminal villi. Anat Embryol (Berl) 1985a; 173:203-214

KAUFMANN P. Basic morphology of the fetal and maternal circuits in the human placenta. Cont Gynec Obstet 1985b; 13:5-17.

KAUFMANN P. Influence of ischemia and artificial perfusion on placental structure and morphometry. Cont Gynec Obstet 1985c; 13:18-26.

KAUFMANN P, Luckhardt M, Leiser R. Three-dimensional representation of the fetal vessel system in the human placenta. Troph Res 1988; 3:113-137

KAUFMANN P, Kohnen G, Kosanke G. Wechselwirkungen zwischen Plazentamorphologie und fetaler Sauerstoffversorgung. Gynaekologe 1993; 26:16-23.

KELLY K, Cochrane B, Stiles CD, Leder P. Cell specific regulation of the c-myc gene by lymphocyte mitogens and platelet derived growth factor. Cell 1983; 35:603-610.

KERR JS, Ruppert CL, Tozzi CA, Neubauer JA, Frenkel HM, Yu SY, Riley DJ. Reduction of chronic hypoxic pulmonary hypertension in the rat by an inhibitor of collagen production. Am Rev Resp Dis 1987; 135:300-306

KEYURRANGUAL V, Samani NJ, Craven DJ, Symonds EM. Expression of components of the renin-angiotensin system in human placenta and foetal membranes. Abstracts from Medical Research Society October 1991.

KEY G, Becker MHG, Baron B et al. New Ki-67-equivalent murine monoclonal antibodies (MIB 1-3) generated against bactrially expressed parts of the Ki-67 cDNA containing three 62 base pair repetitive elements encoding for the Ki-67 epitope. Lab Invest 1993; 68:629-636.

KHONG TY, De Wolf F, Robertson WB, Brosens I. Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small for gestational infants. Br J Obstet Gynaecol 1986; 93: 1049-1059.

KINGDOM JCP, Ryan G, Whittle MJ, et al. Atrial natriuretic peptide: a vasodilator of the fetoplacental circulation? Am J Obstet Gynaecol 1991; 165:791-800

KINGDOM JCP, McQueen J, Ryan G, Connell JMC, Whittle MJ. Characterisation of placental vascular receptors for atrial natriuretic peptide from normal, pre-eclamptic and growth retarded pregnancies. Placenta 1991; 12:408

KINGDOM JCP, McQueen J, Connell JMC, Whittle MJ. Maternal and fetal atrial natriuretic peptide levels at delivery from normal and growth retarded pregnancies. Br J Obstet Gynaecol 1992; 99:845-49

KINGDOM JCP, McQueen J, Connell JCM, Whittle MJ. Fetal angiotensin II levels and vascular (type I) angiotensin receptors in pregnancies complicated by intrauterine growth retardation. Br J Obstet Gynaecol 1993a; 100:476-483

KINGDOM JCP. Neth-Jesse L, Czekierdowski A, Miller RK. Placental release of endothelin-1 under hypoxic conditions in vitro (abstract). Placenta 1993b; A37

KINGDOM JCP, McQueen J, Ryan G, Connell JMC, Whittle MJ. Fetal vascular atrial natriuretic peptide receptors in human placenta: Alteration in intrauterine growth retardation and preeclampsia. Am J Obstet Gynecol 1994; 170:142-147

KLEIN G, Klein E. Conditioned tumorigenicity of activated oncogenes. Cancer Res 1986; 46:3211-3224

KOHNEN G, Kosanke G, Korr H, Kaufmann P. Comparison of various proliferation markers applied to human placental tissue. Placenta 1993; 14:A38.

KOHNEN G, Castellucci M, Graf R, Kaufmann P. Contractile filaments of extravascular stromal cells in human placental villi. Placenta 1993; 14:A39.

KURJAK A, Zalud I, Salihagic A, Crvenkovic G, Matijevic R. Transvaginal color Doppler in the assessment of abnormal pregnancy. J Perinat Med 1990; 19:155-165

KURMAN RJ, Main CS, Chen HC. Intermediate trophoblast: a distinctive form of trophoblast with specific morphological, biochemical and functional features. Placenta 1984; 5:349-370

LAMETSCHWANDTNER A, Lametschwandtner U Weiger T. Scanning electron microscopy of vascular corrosion cast - technique and applications: Updated review. Scann Microsc 1990; 4:889-941.

LAURIN J, Lingman G, Marsal K, Persson P-H. Fetal blood flow in pregnancies complicated by intrauterine growth retardation. Obstet Gynecol 1987; 69: 895-902.

LAW CM, de Swiet M, Osmond C, et al. Initiation of hypertension *in utero* and its amplification throughout life. Br Med J 1993;306:24-27.

LEE MML, Yeh M. Fetal microcirculation of abnormal human placenta. Am J Obstet Gynecol 1986; 154:1133-1139

LEVER AF. Slow pressor mechanisms in hypertension: a role for hypertrophy of resistance vessels. J Hypertens 1986; 4:515-524

LEISER R, Kohler T. The blood vessels of the cat girdle placenta. Observations on corrosion casts, scanning electron microscopical and histological studies. I. Maternal vasculature. Anat Embryol 1983; 167:85-93

LEISER R. Fetal vasculature of the human placenta: scanning electron microscopy of microvascular casts. Contrib Gynecol Obstet 1985; 13:27-31.

LEISER R, Kosanke G, Kaufmann P. Human placental vascularisation: structural and quantitative aspects. In: Placenta-Basic science for clinical application. Soma H, ed. Tokyo: Karger publications, 1991

LIM KH, Bass K, Kosten K, Damsky CD, Fisher S. Developmental delay of cytotrophoblast differentiation along the invasive pathway. In : Proceedings of the fortieth annual meeting of the Society for Gynecologic Investigation, Toronto, Canada, March 17-20, 1993. 40th Society for Gynecological Investigation Meeting 1993 (abstract S166), Toronto, Canada.

LOCCI M, Nazzaro G, de Placido A et al. Correlation of Doppler and placental immunohistochemical features in normal and intrauterine growth-retarded fetuses. Ultrasound Obstet Gynecol 1993; 240-245.

LOKE YW, Gardner L, Burland K, King A. Laminin in human trophoblast - decidua interaction. Human Reproduct 1989; 4:457-463.

LOW JA. The current status of maternal and fetal blood flow velocimetry. Am J Obstet Gynecol 1991; 164:1049-1063

LUCAS A. Programming by early nutrition in man. In: The childhood enviroment and adult disease. Brock GR, Whelan J, eds. Chichester: Wiley, 1991:38-50

LUCKETT WP, Beier HM. Origin and differentiation of the yolk sac and extraembryonic mesoderm in presomite human and rhesus monkey embryos. Am J Anat 1978;152:59-97

LUNELL NO, Sarby B, Lewander R, Nylund L. Comparison of uteroplacental blood flow in normal and in intrauterine growth-retarded pregnancy. Gynecol Obstet Invest 1979; 10:106-118

LYALL F, Lever AF, Morton JJ. Vascular hypertrophy and hypertension: A role for growth factors? Acta Physiol Scand 1988; 133:189-196.

MACARA LM, Kingdom JCP, Kaufmann P. Control of the fetoplacental circulation. Fetal Maternal Med Rev 1993; 5:167-179

MAHENDRAN D, Donnai P, Glazier JD, D'Souza SW, Boyd RDH, Sibley CP. Amino acid (system A) transporter activity in microvillous membrane vesicles from the placentas of appropriate and small for gestational age babies. Pediatr Res 1993; 34:661-665

MAK KKW, Gude NM, Walters WAW, Boura ALA. Effects of vasoactive autocoids on the human umbilical-fetal placental vasculature. Br J Obstet Gyanecol 1984; 91:99-106

MANNING FA, Baskett TF, Morrison I, Lange I. Fetal biophysical scoring: A prospective study in 1,184 high-risk patients. Am J Obstet Gynecol 1981; 140:289-294

MANDSAGER NT, Brewer A, Myatt L. Vasodilator effects of parathyroid hormone parathyroid hormine related peptide and calcitonin gene related peptide in the human fetal placental circulation. In : Proceedings of the fortieth annual meeting of the Society for Gynecologic Investigation, Toronto, Canada, March 17-20, 1993. 40th Society for Gynecological Investigation 1993; abstract P265

MARI G, Russel LD. Arterial blood flow velocity waveforms in normal and small for gestational age fetuses. Am J Obstet Gynecol 1992; 166:1262-1270

MARLOW N, Hunt LP, Chiswick ML. Clinical factors associated with adverse outcomes for babies weighing 2000g or less at birth. Arch Dis Child 1988; 63:1131-1136

MARTINEZ-HERNANDEZ A, Amenta PS. Biology of disease: the basement membrane in pathology. Lab Invest 1983; 48:656-677

MARTINEZ-HERNANDEZ A. The hepatic extracellular matrix 1: Electron immunohistochemical studies in normal rat liver. Lab Invest 1984; 51:57-74

MARTIN BJ, Spicer SS. Ultrastructural features of cellular maturation and ageing in human trophoblast. J Ultrastr Res 1973; 43:133-

MAYHEW T, Jackson MR, Hass JD. Microscopical morphology of the human placenta and the effects on oxygen diffusion: a morphometric model. Placenta 1986; 7:121-131

MAYHEW T, Burton G. Methodological problems in placental morphometry: Apologia for the use of stereology based on sound sampling practice. Placenta 1988; 9:565-581

MAYHEW TM, Jackson MR, Haas JD. Oxygen diffusive conductances of human placentae from term placentae at low and high altitudes. Placenta 1990; 11:493-503.

MAYHEW T, Jackson MR, Boyd PA. Changes in oxygen diffusive conductances of human placentae during gestaion (10-41 weeks) are commensurate with the gain in fetal weight. Placenta 1993; 14:51-61

McCARTHY A, Woolfson RG, Shant KR, Poston L. Abnormal endothelial cell function of resistance arteries from women with pre-eclampsia. Am J Obstet Gynecol 1993; 164:507-513

McGOWAN LM, Mullen BM, Ritchie JWK. Umbilical artery flow velocity waveforms and the placental vascular bed: pathological correlation. Am J Obstet Gynecol 1987; 157:900-902.

McLAREN M, Greer IA, Walker JJ, Forbes CD. Reduced prostacyclin production by umbilical arteries from pregnancies complicated by severe pregnancy induced hypertension. Clin Exp Hypertens 1987; [B] 2:365-374

MacLENNAN AH, Sharp F, Shaw-Dunn J. The ultrastructure of human trophoblast in spontaneous and induced hypoxia using a system of organ culture: a comparison with ultrastructural changes in pre-eclampsia and placental insufficiency. J Obstet Gynaecol Brit Commonw 1972; 79:113-121

McPARLAND P, Steer SA, Pearce JMF. The clinical implications of absent or reversed end-diastolic frequencies in the umbilical artery flow velocity waveforms. Eur J Obstet Gynaecol Reprod Biol 1991; 37:15-23.

McQUEEN J, Jardine AG, Kingdom JCP, Templeton A. Interaction of angiotensin II and atrial natriuretic peptide in the human feto-placental unit. Am J Hypertens 1991; 3: 641-644.

McQUEEN J, Kingdom JCP, Whittle MJ, Connell JMC. Characterization of atrial natriuretic peptide receptors in the human fetoplacental vasculature. Am J Physiol 1993a; 264:H798-804

McQUEEN J, Kingdom JCP, Connell JMC, Whittle MJ. Fetal endothelin levels and placental vascular endothelin receptors in intrauterine growth retardation. Obstet Gynecol 1993b; 82:992-998

MICHEL MZ, Khong TY, Clark DA, Beard RW. A morphological and immunohistochemical study of human placental bed biopsies in miscarriage. Br J Obstet Gynaecol 1990; 97:984-988.

MILNER P, Loesch A, Burnstock G. Rapid release of endothelin and ANP from isolated aortic endothelial cells expressed to increasing flow. Biochem Biophys Res Commonw 1990; 170:649-656

MIRONOV VA, Katcher OV, Lebedeva IM. Three-dimensional morphology of placental villous tree and anthropometric estimations of newborns from Kirgizian women living at high altitude. Placenta 1991; 12: 421.

MIRES G, Dempster J, Patel NB, Crawford JW. The effect of fetal heart rate on umbilical artery flow velocity waveforms. Br J Obstet Gynaecol 1987; 94:665-669.

MOORE TR, Brace RA. Amniotic fluid index (AFI) in the term ovine pregnancy: a predictable relationship between AFI and amniotic fluid volume. In: Proceedings of the thirty-fifth annual meeting of the Society for Gynecologic Investigation, Baltimore, Maryland, March 17-20, 1988. Baltimore: Society for Gynecologic Investigation, 1988.

MOORE TR. Superiority of the four-quadrant sum over the single-deepest-pocket technique in ultrasonographic identification of abnormal amniotic fluid volumes. Am J Obstet Gynecol 1990; 163:762-767

MORROW RJ, Ritchie JWK, Bull SW. Fetal and maternal hemodynamic responses to exercise in pregnancy assessed by Doppler ultrasonography. Am J Obstet Gynecol 1989; 160:138-140

MORROW RJ, Adamson SL, Bull SB, Ritchie JWK. Effect of placental embolisation on the umbilical artery waveform in fetal sheep. Am J Obstet Gynecol 1989b; 161:1055-60

MORROW RJ, Adamson SL, Bull SB, Ritchie JWK. Hypoxic acidemia, hyperviscosity, and maternal hypertension do not affect the umbilical arterial velocity waveform in fetal sheep. Am J Obstet Gynecol 1990; 163:1313-1320

MORROW RJ, Adamson SL, Bull SB, Ritchie JWK. Acute hypoxemia does not affect the umbilical artery flow velocity waveform in fetal sheep. Obstet Gynecol 1990; 75:590-593.

MORROW RJ, Adamson L, Ritchie JWK, Pearce M. Pathophysiological basis of abnormal flow velocity waveforms - check. In: Doppler Ultrasound in Perinatal Medicine. Pearce M ed. London: Churchill Livingstone, 1992. p196-218

MORTON JJ, Beattie EC, McPherson F. Angiotensin II receptor antagonist Losatan has persistant effects on blood pressure in the young spontaneously hypertensive rat: lack of relation to vascular structure. J Vasc Res 1992; 29:264-269

MUES B, Langer D, Zwaldo G, Sorg C. Phenotypic characterisation of macrophages in human term placenta. Immunology 1989; 67:303-307

MYATT L, Brewer A, Brockman DE. The action of nitric oxide in the perfused human fetal-placental circulation. Am J Obstet Gynecol 1991; 164:687-692.

MYATT L, Langdon G, Brewer AS, Brockman DE. Endothelin-1 induced vasoconstriction is not mediated by thromboxane release and action in the human fetal-placental circulation. Am J Obstet Gynecol 1991; 165:1717-1722.

MYATT L. Control of vascular resistance in the human placenta. Placenta 1992; 13:329-341.

MYATT L, Brockmann D, Eis ALW, Pollock JS. Immunohistochemical localisation of nitric oxide synthase in the human. Placenta 1993; 14: 487-495

NEILSON JP, Grant A. Ultrasound in pregnancy. In: Effective care in pregnancy and childbirth. Chalmers I, Enkin M, Kierse MJNC eds. Oxford University Press 1989

NEILSON JP. The measurement of fetal growth. In: Chamberlain G, eds. Modern Antenatal care of the fetus. Blackwell Scientific Publications, London. 1990; 71-88

NELSON DM, Walsh SW. Aspirin differentially affects thromboxane and prostacycline production by trophoblast and villous core compartments of human placental villi. Am J Obstet Gynecol 1989; 161:1593-1598.

NICOLAIDES KH, Soothill PW, Rodeck CH, Campbell S. Ultrasound guided sampling of umbilical cord and placental blood to assess fetal well-being. Lancet 1986; i:1065-1067

NICOLAIDES KH, Bilardo CM, Soothill PW, Campbell S. Absence of end-diastolic frequencies in the umbilical artery: a sign of fetal hypoxia and acidosis. Br Med J 1988; 297:1026-1027

NICOLAIDES KH. Economides DL, Soothill PW. Blood gases, PH, and lactate in appropriate and small for gestational age fetuses. Am J Obstet Gynecol 1989; 161:996-1001

NICOLINI U, Hubinont C, Santolaya J, Fisk NM, Coe A, Rodeck CH. Maternal-fetal glucose gradient in normal pregnancies and pregnancies complicated by alloimmunisation and fetal growth retardation. Am J Obstet Gynecol 1989; 161:924-927

NICOLINI U, Nicolaidis P, Fisk NM, et al. Limited role of fetal blood sampling in prediction of outcome in intrauterine growth retardation. Lancet 1990; 336:768-772.

NORDENVALL M, Ullberg I, Laurin J, Lingman J, Standstedt B, Ulmsten U. Placental morphology in relation to umbilical artery blood flow velocity waveforms. Eur J Obstet Gynaecol Reprod Biol 1991; 40:179-90.

OHLSSON R, Holmgren L, Glaser A, Szpecht A, Pfeifer-Ohlsson S. Insulin like growth factor 2 and short range stimulatory loops in control of human placental growth. EMBO J 1989a; 8:1993-1999

OHLSSON R, Larsson E, Nilsson O, Wahlstrom T, Sundstrom P. Blastocyst implantation preceeds induction of insulin-like growth factor II gene expression in human trophoblasts. Dev 1989b; 106:555-559

OKUDAIRA Y, Hirota K, Cohen S, Strauss L. Ultrastructure of the human placenta in diabetes mellitus. Lab Invest 1966; 15:910-926

OSMOND C, Barker DJP, Winter PD, Fall CHD, Simmonds SJ. Early growth and death from cardiovascular disease in women. Br Med J 1993; 307:1519-1524

PALMER RMJ, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987; 327: 524-526.

PANIGEL M, Myers RE. Histological and ultrastructural changes in rhesus monkey placenta following interruption of fetal placental circulation by fetecomy or interplacental umbilical vessel ligation. Acta Anat 1972; 81:481-506.

PARDI G, Cetin I, Marconi AM et al. Venous drainage of the human uterus: respiratory gas studies in normal and fetal growth-retarded pregnancies. Am J Obstet Gynecol 1992; 166:699-706

PARDI G, Cetin I, Marconi AM et al. Diagnostic value of blood sampling in fetuses with growth retardation. N Eng J Med 1993; 328:692-696.9
PARER JT. The effects of acute maternal hypoxia in fetal oxygenation and the umbilical circulation in sheep. Eur J Obstet Gynecol Reprod Biol 1980; 10:125-136

PEARCE JM, Campbell S, Cohen-Overbeek T, Hackett G, Hernandez J, Royston JP. Reference ranges and sources of variation for indices of pulsed Doppler flow velocity waveforms from the uteroplacental and fetal circulation. Br J Obstet Gynaecol 1988; 95:248-256

PEETERS LLH, Sheldon RE, Jones MD, Makowski EL, Meschia G. Blood flow to fetal organs as a function of arterial oxygen content. Am J Obstet Gynecol 1979; 135:637-646

PERKINS RP. Perspectives on perinatal brain damage. Obstet Gynecol 1987; 69:807-816

PFEIFER-OHLSSON S, Rydnert J, Goustain AS, Larsson E. Betsholtz C, Ohlsson R. Cell-type-specific pattern of myc proto-oncogene expression in developing human embryos. Proc Natl Acad Sci USA 1985; 82:5050-5054

PHILIPSON EH, Sokol RJ, Williams T. Oligohydramnios: Clinical associations and predictive value for intrauterine growth retardation. Am J Obstet Gynecol 1983; 146:271-278.

PIERCE GB, Midgley AR. The origin and function of human syncytiotrophoblast giant cells. Am J Pathol. 1963; 43:153-173

PINJEBORG R, Dixon G, Robertson WB, Brosens I. Trophoblastic invasion of human decidua from 8-18 weeks of pregnancy. Placenta 1980; 1:3-19.

POURCELOT L. Applications cliniques de l'examen Doppler transcutane. In: Velocimetric ultrasonor Doppler. Peronneau P, ed. INSERM 7-11 Octobre 1974; 34:213-240

PUISTOLA U, Ronnberg L, Martikainen H, Turpeenniemi-Hujanen T. The human embryo produces basement membrane collagen (type IV collagen) degrading protease activity. Hum Reprod 1989; 4:309-311

RABINOVITCH M, Gamble W, Nadas AS, Meittinen OS, Reid L. Rat pulmonary circulation after chronic hypoxia: hemodynamic and structural features. Am J Physiol 1979; 236:H818-H827

RABINOVITCH M, Bothwell T, Hayakawa et al. Pulmonary artery endothelial abnormalities in patients with congenital heart defects and pulmonary hypertension. Lab Invest 1986; 55:632-653

RAN SG, Braustein G. Decidua produces a protein that inhibits choriogonadotrophin release from human trophoblasts. J Clin Invest 1991; 87:326-330

REED JC, Nowell PC, Hoover RG. Regulation of *c-myc* mRNA levels in normal human lymphocytes by modulators of cell proliferation. Proc Natl Acad Sci USA 1985; 82:4221-4224

REILLY FD, Russell PT. Neurohistochemical evidence supporting an absence of adrenergic and cholinergic innervation in the human placenta and umbilical cord. Anat Rec 1977; 188:277-286

RIBBERT LSM, van Lingen RA, Visser GHA. Continuous maternal Hyperoxygenation in the treatment of early fetal growth retardation. Ultrasound Obstet Gynaecol 1991; 1:331-335

RIBBERT LSM, Visser GHA, Mulder EJH, Zonneveld MF, Morssink LP. Changes with time in fetal heart rate variation, movement indices and haemodynamics in intrauterine growth retarded fetuses - a longitudinal approach to the assessment of fetal well being. Early Hum Dev 1993; 31:195-208.

RIFKIND B, Moscatelli D. Recent developments in the cell biology of *b*-fibroblastic growth factor. J Cell Biol 1989; 109:1-6

ROBINSON BG, Emanuel RL, Frim DM, Majzoub JA. Glucocoricoid stimulates expression of cortico-trophin-releasing hormone gene in human placenta. Proc Natl Acad Sci USA 1988; 85:5244-5248

ROBERTSON WB, Warner B. The ultrastructure of the human placental bed. J Path 1974; 112:203-211

ROBILLARD JE, Weitzman RE, Burmeister L, Smith FG. Developmental aspects of the renal response to hypoxemia in the lamb fetus. Circ Res 1981; 48:128-138.

ROBINSON JS, Kingstone EJ, Jones CT, Thorburn GD. Studies of experimental growth retardation in sheep: the effect of removal of endometrial caruncles on fetal size and metabolism. J Devel Physiol 1979; 1:379-398

ROCHELSON B, Schulman H, Farmakides G, et al. The significance of absent end-diastolic velocity in umbilical artery velocity waveforms. Am J Obstet Gynecol 1987; 156:1213-1218

RODESCH F, Simon P, Donner C, Jauniaux E. Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. Obstet Gynecol 1992; 80:283-285

RONA RJ, Gulliford MC, Chinn S. Effects of prematurity and intrauterine growth on respiratory health and lung function in childhood. B Med J 1993; 306:817-820

ROUBAT C, Mondon F, Bandet J, Ferre F, Cavero I. Regional distribution and pharmacological characterization of endothelin - 1 binding sites in human fetal placental vessels. Placenta 1991; 12:55-67.

RUDOLPH AM, Heymann MA. Circulatory changes during growth in the fetal lamb. Circ Res 1970; 26:289-299.

RUTHERFORD S, Phelan J, Smith C, Jacobs N. The four-quadrant assessment of amniotic fluid volume: an adjunct to antepartum fetal heart rate testing. Obstet Gynecol 1987; 70:353-360.

RYDNERT J, Pfeifer-Ohlsson S, Goustain AS, Ohlsson R. Temporal and spatial pattern of cellular *myc* oncogene expression during human placental development. Placenta 1987; 8:339-345.

SANDS J, Dobbing J. Continuing growth and development of the third trimester human placenta. Placenta 1985; 6:13-22

SALAS SP, Power RF, Singleton A, Wharton J, Polak JM, Brown J. Heterogenous binding sites for a-atrial natriuretic peptide in human umbilical cord and placenta. Am J Physiol 1991; 261:R633-R638

SCHAAPS JP, Hustin J. In-vivo aspect of the maternal-trophoblastic border during the first trimester of gestation. Troph Res 1988; 3:39-48

SCHEFFEN, Kaufmann P, Philippens L, Leiser R, Geisen C, Mottaghy K. Alterations of the fetal capilary bed in the guinea pig placenta folowing long-term hypoxia. In Oxygen transport to tissue, Vol XII. Piiper, Goldstick, Meyer, Eds. Plenum Press, New York 1991.

SCHLAFKE S, Enders AC. Cellular basis of interaction between trophoblast and uterus at implantation. Biol Reprod 1975; 12:41-65

SCHMIDT KG, di Tommaso M, Silverman NH, Rudolph AM. Evaluation of changes in umbilical blood flow in the fetal lamb by Doppler waveform analysis. Am J Obstet Gynecol 1991; 164:1118-1126

SCHUHMANN RA, Wynn RM. Regional ultrastructutal differences in placental villi in cotyledons of a mature human placenta. Placenta 1980; 1:345-353

Scottish Low Birthweight Study Group. The Scottish low birthweight study: I Survival, growth, neuromotor and sensory impairment. Arch Dis Child 1992; 67:675-681.

Scottish Low Birthweight Study Group. The Scottish low birthweight study: II. Language attainment, cognitive status and behavioural problems. Arch Dis Child 1992; 67:682-686

SEEDS JW. Impaired fetal growth: Definition and clinical diagnosis. Am J Obstet Gynecol 1984; 64:303-310

SEMEDLER AC, Faxelius G, Bremme K, Lagerstrom M. Psychological development in children born with very low birthweight after severe intrauterine growth retardation: a 10-year follow-up study. Acta Paediatr 1992; 81:197-203

SEN DK, Kaufmann P, Schweikhart G. Classification of human placental villi. II. Morphometry. Cell Tiss Res 1979; 200:425-434.

SENGAR DR, Dvorak HF. In: Human astrocytomas, Black PMcC, Lampson L eds. Blackwell Scientific Publications, Oxford 1993

SHEPPARD BL, Bonnar J. The ultrastructure of the arterial supply of the human placenta in pregnancy complicated by fetal growth retardation. Br J Obstet Gynaecol 1976; 83:948-959

SHEPPARD BL, Bonnar J. An ultrastructural study of utero-placental spiral arteries in hypertensive and normotensive pregnancy and fetal growth retardation. Br J Obstet Gynaecol 1981; 88:695-705

SHWEIKI D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia initiated angiogenesis. Nature 1992; 359:843-845.

SIJMONS EA, Reuwer PJHM, van Beek E, Bruinse HW. The validity of screening for small-for-gestational age and low weight for length infants by Doppler ultrasound. Br J Obstet Gynaecol 1989; 96:557-561

SKALLI O, Ropraz P, Trzeciak A, Benzonana G, Gillessen D, Gabbiani G. A monoclonal antibody against a-smooth muscle actin: a new probe for smooth muscle differentiation. J Cell Biol 1986; 103:2787-2796.

SKALLI O, Gabbianai G. The biology of the myofibroblast. Relationship to wound contraction and fibrocontractive diseases. In: The molecular and cellular biology of wound repair. Clark RMF, Henson PM Eds. Plenum Publishing Corporation 1988; 373-402.

SKIDMORE R, Woodcock JP. Physiological interpretation of Doppler-shift waveform - 1. Theoretical considerations. Ultrasound Med Biol 1980; 6:7-10

SMALLS M, Forbes JF. Centile values of birthweight for gestational age in Scottish infants. Glasgow; Social, Paediatric and Obstetric Research Unit, University of Glasgow 1983.

SNIJDERS RJM, Sherrod C, Gosden CM, Nicolaides KH. Fetal growth retardation: associated malformations and chromosomal abnormalities. Am J Obstet Gynecol 1993; 168:547-555.

SNIJDERS RJM, Abbas A, Melby O, Ireland RM, Nicolaides KH. Fetal plasma erythropoietin concentration in severe growth retardation. Am J Obstet Gynecol 1993; 168:615-619

SOLTER D. Differential imprinting and expression of maternal and paternal genomes. Annu Rev Genet 1988; 22:127-146

SOOTHILL P, Nicolaides KH, Rodeck CH, Campbell S. Effect of gestational age on fetal and intervillous blood gas and acid-base values in human pregnancy. Fetal Therapy 1986; 1:168-175.

SOOTHILL PW, Nicolaides KH, Campbell S. Prenatal asphyxia, hyperlacticaemia, hypoglycaemia and erythroblastosis in growth retarded fetuses. Br Med J 1987; 294:1051-1053

SPORN MB, Roberts AB. Peptide growth factors and inflammation, tissue repair and cancer. J Clin Invest 1986; 78:329-332

STANDSTED B. The placenta and low birthweight. Curr Top Pathol 1979; 66:1-55.

STEEL SA, Pearce JMF, Nash G, Christopher B, Dormandy J, Bland JM. Correlation between Doppler flow velocity waveforms and cord blood viscosity. Br J Obstet Gynaecol 1989; 96:1168-1172.

STUART MJ, Clark DA, Sunderji SG, Allen JB, Yambo T, Elrad H, Slott JH. Decreased prostacyclin production: a characteristic of chronic placental insufficiency syndromes. Lancet 1993; i:1126-1128

SURANI MAH, Barton SC, Howlett SK, Norris ML. Influence of chromosomal determinants on development of androgenetic and parthenogenetic cells. Development 1988; 103:171-178.

TANNIRANDORN Y, Fisk NM, Shah V et al. Plasma renin activity in fetal disease. J Perinat Med 1990; 18:229-231

TAO TW, Hertig AT. Viability and differentiation of human trophoblast in organ culture. Am J Anat 1965; 116:315-327

TAYLOR DJ, Howie PW. Fetal growth achievement and neurodevelopmental disability. Br J Obstet Gynaecol 1989; 96:789-794

TEASDALE F. Gestational changes in the functional structure of the human placenta in relation to growth: a morphometric study. Am J Obstet Gynecol 1980; 137:773-781

TEMPLETON AGB, Whittle MJ, McGrath JC. The role of endogenous thromboxane in contractions to U46619, oxygen, 5-HT and 5-CT in the human isolated umbilical artery. Br J Pharmacol 1991; 103:1079-84.

TEMPLETON A, Kingdom JCP, Whittle M, McGrath J. Contractile responses of the human umbilical artery from pregnancies complicated by hypertension and intrauterine growth retardation. Placenta 1991; 12:439

TENCE M, Petit A. Characterisation of angiotensin II binding sites in the human term placenta. Molecular Cell Endocrinol 1989; 63:111-119.

THALER I, Manor D, Itskovitz J et al. Changes in uterine blood flow during human pregnancy. Am J Obstet Gynecol 1990; 162:121-125

THOMPSON RS, Trudinger BJ, Cook CM, Giles WB. Umbilical artery velocity waveforms: normal reference values for A/B ratio and Pourcelot ratio. Br J Obstet Gynaecol 1988; 95:589-591

THORP JA, Walsh SW, Brath PC. Comparison of the vasoactive effects of leucotrienes with thromboxane mimic in the perfused human placenta. Am J Obstet Gynecol 1988; 159:1376-1380

TODD AL, Trudinger BJ, Cole MJ, Cooney GH. Antenatal tests of fetal welfare and development at age 2 years. Am J Obstet Gynecol 1992; 167:66-71

TODROS T, Guiot C, Pianta PG. Modelling the fetoplacental circulation : 2. A continuous approach to explain normal and abnormal flow velocity waveforms in the umbilical arteries. Ultrasound Med Biol 1992; 18:545-551

TOMPSETT DH. Anatomical techniques 2nd edition. Churchill Livingstone, Edinburgh 1970; p283

TRUDINGER BJ, Cook CM, Giles WB, Connelly A, Thompson RS. Umbilical artery flow velocity waveforms in high risk pregnancy: randomised controlled trial. Lancet 1987; i:188-190

TRUDINGER BJ, Stevens D, Connelly A et al. Flow velocity waveforms and the placental resistance: the effects of embolization of the umbilical circulation. Am J Obstet Gynecol 1987b; 157:1443-1449.

TRUDINGER BJ, Cook CM, Thompson RS, Giles WB, Connelly A. Low dose aspirin therapy improves fetal weight in umbilical placental sufficiency. Am J Obstet Gynecol 1988; 159:681-685.

TRUDINGER BJ, Connelly AJ, Giles WB, Hales JR, Wilcox GR. The effects of prostacyclin and tromboxane analogue (U46619) on the fetal circulation and umbilical flow velocity waveforms. J Dev Physiol 1989; 11:179-184

TYRRELL SM, Lilford RJ, Obaid AH. Relationship between umbilical artery Doppler waveforms and cord gases. Obstet Gynecol 1989; 88:871-879.

USZYNSKI M. Tissue anticoagulant in human placenta: preliminary study with heparin-like anticoagulant and review of the literature. Gynecol Obstet Invest 1991; 32:129-133.

UZAN S, Beaufils M, Breart G, Bazin B, Capitant C, Paris J. Prevention of fetal growth retardation with low dose aspirin: findings of the EPREDA trial. Lancet 1991; 337:1427-1431

VANE JR, Anggard EE, Botting RM. Regulatory mechanisms of the vascular endothelium. N Eng J Med 1990; 323:27-36.

VAN den HOF MC, Nicolaides KH. Platelet count in normal, small and anaemic fetuses. Am J Obstet Gynecol 1990; 162:753-59

VAN der VEEN F, Fox H. The effects of cigarette smoking on the human placenta: a light and electron microscopic study. Placenta 1982; 3:243-256

VAN der VELDE, Peereboon Stegeman JHJC, Treefers PE, James J. Basal lamina thickening in the placentae of smoking mothers. Placenta 1985; 6:329-340

VAN DEN WIJNGAARD JA, van Eyck, Wladimiroff JW. The relationship between fetal heart rate and Doppler blood flow velocity waveforms. Ultrasound Med Biol 1988; 14:593-597

VAN DER VOORDE J, Vanderstichele H, Leusen I. Release of endothelium-derived relaxing factor from human umbilical vessels. Circ Res 1987; 60:517-522

VYAS S, Nicolaides KH, Bower S, Cambell S. Middle cerebral artery flow velocity waveforms in fetal hypoxaemia. Br J Obstet Gynaecol 1990; 97:797-803

WALLENBERG HC, Rotmans N. Prevention of recurrent idiopathic fetal growth retardation by low dose aspirin and dipyridamole. Am J Obstet Gynecol 1987; 157:1230-1250.

WEINBERG PC, Cameron IL, Parmley T, Jeter JR, Pauerstein CJ. Gestational age and placental cellular replication. Obstet Gynecol 1970; 36:692-696

WEINER CP, Robillard JE. Atrial natriuretic factor, digoxin-like immunoreactive substance, norepinephrine, and plasma renin activity in human fetuses and their alteration by fetal disease. Am J Obstet Gynecol 1988; 159:1353-1360

WEINER CP, Williamson RA. Evaluation of severe growth retardation using cordocentesis: hematologic and metabolic alterations by etiology. Obstet Gynecol 1989; 73:225-229.

WEINER Z, Farmakides G, Schulman H, Penny B. Central and peripheral heamodynamic changes in fetuses with absent end-diastolic flow velocity in the umbilical artery: correlations with computerised fetal heart rate patterns. Am J Obstet Gynecol 1994; 170:509-515.

WELLMAN KF, Volk BW. Fine structural changes in the rabbit placenta induced by cortisone. Arch Path 1972; 94:147-157

WENTWORTH P. The placenta in cases of haemolytic disease of the newborn. Am J Obstet Gynecol 1967; 98:283-289.

WESTERMARK B, Siegbahn A, Heldin CH, Claesson WL. B-type receptor for platelet derived growth factor mediates a chemotactic response by means of a ligand-induced activation of the receptor protein-tyrosine kinase. Proc Natl Acad Sci USA 1990; 87:128-132

WHARTON J, Anderson RH, Springall D et al. Localisation of atrial natriuretic peptide immunoreactivity in the ventricular myocardium and conduction system of the fetal and adult heart. Br Heart J 1988; 60: 267-274.

WIGGLESWORTH JS. The Langhans layer in late pregnancy: a histological study of normal and abnormal cases. J Obstet Gynaecol Brit Commonw 1962; 69:355-365

WILCOX GR, Trudinger BJ. Fetal platelet consumption: a feature of placental insufficiency. Obstet Gynecol 1991; 77: 616-21

WILKES BM, Mento PF, Hollander AM, Maita ME, Sung S, Girardi EP. Endothelin receptors in human placenta: relationship to vascular resistance and thromboxane release. Am J Physiol 1990; 258: 864-870.

WILKES BM, Krim E, Mento PF. Evidence for a functional renin-angiotensin system in full term feto-placental unit. Am J Physiol 1984; 249:E864-E870.

WILLIAMS S, St. George IM, Silva PA. Intrauterine growth retardation and blood pressure at age seven and eighteen. J Clin Epidemiol 1992; 45:1257 - 1263.

WLADIMIROFF JW, Tonge HM, Stewart PA. Doppler ultrasound assessment of cerebral blood flow in the human fetus. Br J Obstet Gynaecol 1986; 93:471-475

WLADIMIROFF JW, Huisman TWA, Stewart PA. Fetal cardiac flow velocities in the late first trimester of pregnancy; a transvaginal Doppler study. J Am Coll Cardiol 1991; 17:1357-1359

WOO JSK, Liang ST, Lo RLS, Chan FY. Middle cerebral artery Doppler flow velocity waveforms. Obstet Gynecol 1987; 70:613-616

WOO JSK, Laing ST, Lo RLS. Significance of an absent or reversed end diastolic flow in Doppler umbilical artery waveforms. J Ultrasound Med 1987; 6:291-297

de WOLF F, Robertson WB, Brosens I. Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small for gestational age infants. Br J Obstet Gynaecol 1986; 93:1049-1059

YANAGISAWA M, Kurihara H, Kimura S et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 1988; 332: 411-415.

YEGER H, Lines LD, Wong P-Y, Silver MS. Enzymatic isolation of human trophoblast and culture on various substrates: comparison of first trimester with term trophoblast. Placenta 1989; 10:137-151.

ZANZANI ED, Ascendo JL. Erythropoietin. Transfusion 1989; 29:46-57