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Histological and immunohistochemical events during placentation in pigs

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The early morphological events in pig placental development are summarized and related to the known data on differences in placental vascular efficiency between Meishan and US breeds. The activation and localization of a number of factors, the ligands and their receptors, such as insulin-like growth factor (IGF), transforming growth factor β (TGFβ), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), as well as retinoids and calcium, is described. The comparison between these factors gives a strong impression of their complex interactions and hormonal relationships during placentation and vascular development in pigs. This review also emphasizes that retinoids are of great importance for placental function and that the transport of vitamin A appears to take place in the areolar gland complex only, whereas based on histochemistry and electron energy dispersive analysis, the calcium transport may be confined to the interareolar route across the interhaemal barrier.

Introduction

The placenta develops as a temporary organ to provide a highly regulated transfer of nutrients and waste products from mother to fetus and vice versa, including facilitation of the exchange of oxygen and carbon dioxide. Placenta is composed of a very close apposition between the uterine mucous membrane and the genetically different fetal allogenetic membrane, the allantochorion, which, in some species, may invade the endometrium in a highly regulated manner as seen during placentation in cows, horses, mustellids, mice and humans (Wooding and Flint, 1994).

However, pig placenta is non-invasive and, in addition, it is classified morphologically as diffuse, folded, epitheliochorial and indeciduate, with subunits composed by the interareolar part for haemothrophic exchange and the areolar gland complexes for transfer of large molecules called histiotroph (Wooding and Flint, 1994; Dantzer, 1999). Neovascularization of this 'new developing organ' very early in gestation is essential for cell growth and as an efficient transport route for gaseous and nutrient exchange between mother and embryo.

Morphological events during initial placentation

The morphological events preceding and during initial placentation in pigs have been described in detail (Dantzer, 1985; Keys and King, 1990; Stroband and Van der Lende, 1990).

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In brief, at day 13 after coitus, conceptus elongation has almost ceased; conceptus migration within the uterine horns has taken place and the elongated conceptus (1.0–1.5 m) is now apposed closely to the elaborated circular folds of the endometrium. Anchoring of the conceptus to the uterine luminal surface occurs through newly developed epithelial proliferations in the endometrium that are initiated at the mesometrial side. These events are followed closely by adherence, close apposition and formation of interdigitating microvilli between maternal epithelium and developing trophoblast at days 15–16 after coitus.

At the same time, from day 15 after coitus, the vasculature of the subepithelial capillary network at the mesometrial side becomes distended, denser in its network and positioned very close to the base of the uterine epithelium (Keys and King, 1990; Dantzer *et al.*, 1991; Dantzer and Leiser, 1994; Leiser and Dantzer, 1994). With continued development, the maternal subepithelial capillary network appears to create the basis for the architecture of complex microscopic folding leading to the formation of maternal–fetal complementary ridges and furrows (Dantzer and Leiser, 1994). At day 32 after coitus, the vasculature develops a counter- to crosscurrent maternal–fetal interrelationship that maintains this basic architecture, although it is elaborated continually throughout gestation (Leiser and Dantzer, 1988).

Neovascularization in the fetal membranes is delayed by about 2 days compared with that of the maternal endometrium. Both are initiated close to the embryonic disc, progressing from the mesometrial side to the anti-mesometrial side as the blastocyst expands along the length of the uterine horn and continues along the elongated blastocyst towards its two tips (Dantzer *et al.*, 1991).

The uterine epithelium undergoes further changes in the composition of cell organelles and secretory activity as the materno–fetal contact becomes more and more close and indented. The endometrium undergoes mutual microscopic folding and under this process there must be an intimate interaction between the uterine epithelial cells, the stroma of the lamina propria, the vascular endothelial cells and developmental processes in the complementary allantochorion.

Signalling substances in the pig placental interhaemal barrier

Studies of Chinese Meishan pigs, which farrow 3–5 more viable piglets than do US or European breeds, have indicated that Meishan pigs remain relatively small compared with commercial breeds. Embryonic survival after day 30 after coitus is higher in Meishan pigs. Although Meishan fetuses in the later stages of gestation have smaller placentae compared with US breeds, placental efficiency is increased by a markedly increased growth of placental blood vessels at the fetal–maternal interface. Further experiments have indicated that uterine type modulates conceptus size and that the genotype of the conceptus controls placental vascular efficiency (Ford, 1997; Biensen *et al.*, 1999). In gilts evaluated for the different genotypes of oestrogen receptor, the B allele was associated with larger litter size and significantly longer placentae compared with AA genotype gilts. Hearts of AA×BA fetuses were significantly heavier compared with BB×AB and BB×BB fetuses, and the fetuses of AA×AA genotype were the lightest. Differences in heart weight would suggest that there is a relationship with placental vascularity; however, further investigations are required (Van Rens and Van der Lende, 2000; Van Rens *et al.*, 2000).

Insulin-like growth factor

Insulin-like growth factors (IGFs) are important mitogenic peptides that stimulate cell division and differentiation during fetal and placental growth (DeChiara *et al.*, 1990; Liu *et al.*,

1993). In a recent paper on expression of IGF and IGF binding proteins (IGFBPs) in guinea-pig placenta, Han *et al.* (1999) emphasized the importance of comparative studies between species, as there are significant differences in the spatial distributions of IGF-II and IGFBPs between the placentae of various species such as rhesus monkeys, sheep and rats. In a summary of the pig IGF system, Simmen *et al.* (1998) indicated that there is a relationship between nutrition and systemic/local IGF and that IGF is involved in the regulation of placental cellular responses to protein energy restriction to retain sufficient placental efficiency during gestation.

During the initial stages of placentation up to day 30 of gestation, IGF-I is immunolocalized in the uterine luminal and glandular epithelium, and in the endothelium and vascular smooth muscle cells, and there is weak activity in the trophoblast and fetal mesenchymal cells of the placenta (Persson and Rodriguez-Martinez, 1997; Persson *et al.*, 1997). In pregnant endometrium, expression of the oestrogen receptor gene is correlated with IGF-I gene expression, which is indicative of a common regulator, probably conceptus-derived oestrogens. Expression of IGF-I gene in the endometrium is consistent with variations in IGF-I immunoreactivity in the uterine glands and their secretory activity. The consistent strong immunostaining in the vascular smooth muscle cells indicates that IGF-I may be active in endometrial vascular development and function together with a number of other factors.

IGF-II gene expression in the interhaemal placental barrier was studied from day 8 to day 40 after coitus. IGF-II appears in the pig conceptus during early pregnancy, as it is activated in the extra-embryonic blood vessels between day 16 and day 24 after coitus, and at day 30 after coitus in the trophoblast, whereas IGF-II transcripts were not detected in pig endometrium. IGFBP-2 gene expression was present in both trophoblast and uterine epithelium at day 30 after coitus, and was very strong in the latter, whereas IGFBP-2 was not expressed in blood vessels (Fig. 1) (Persson, 1996). These results indicate an activation of IGF-II in the epitheliochorial placenta, in which IGF-II is first expressed in the trophoblast, thereby indicating that IGF-II interacts in the developmental pathways of pig placentation.

On the basis of *in vitro* experiments it was shown that an IGF-II analogue with selected affinity for IGF-II (type II) receptor increased thymidine uptake in pig uterine glandular cells two-fold compared with untreated cells. In addition, it was shown that a combination of IGF-I and IGF-II or IGF-II alone stimulated thymidine incorporation to a greater extent than did IGF-I alone. Therefore, it was suggested that IGFBP modulation of uterine gland cell growth may involve both IGF-dependent and -independent pathways in a complex interplay of IGF system components in regulation of uterine endometrial growth in pigs (Badinga *et al.*, 1999).

Transforming growth factor b (TGFb)

Gupta *et al.* (1998a,b) demonstrated that, in the peri-implantation period (day 10 to day 14 after coitus), uterine expression of TGFβ-1, -2 and -3 genes, as well as immunocytochemical localization of TGFβ receptors, is pregnancy-specific and that bioactive TGFβs are present at the conceptus–maternal interface. TGFβs may be involved in autocrine–paracrine interactions between these two genetically different tissues in a period during which marked uterine remodelling and conceptus differentiation take place by affecting cellular communication, proliferation, differentiation, extracellular matrix protein and integrin modification, tissue repair, angiogenesis and immunosuppression (Lawrence, 1996). However, the presence of TGFβs and, thus, suggested functional interaction with other factors during placentation, still needs to be investigated. Changes in glycan composition during gestation in pigs might be an important base for further studies (Jones *et al.*, 1995).

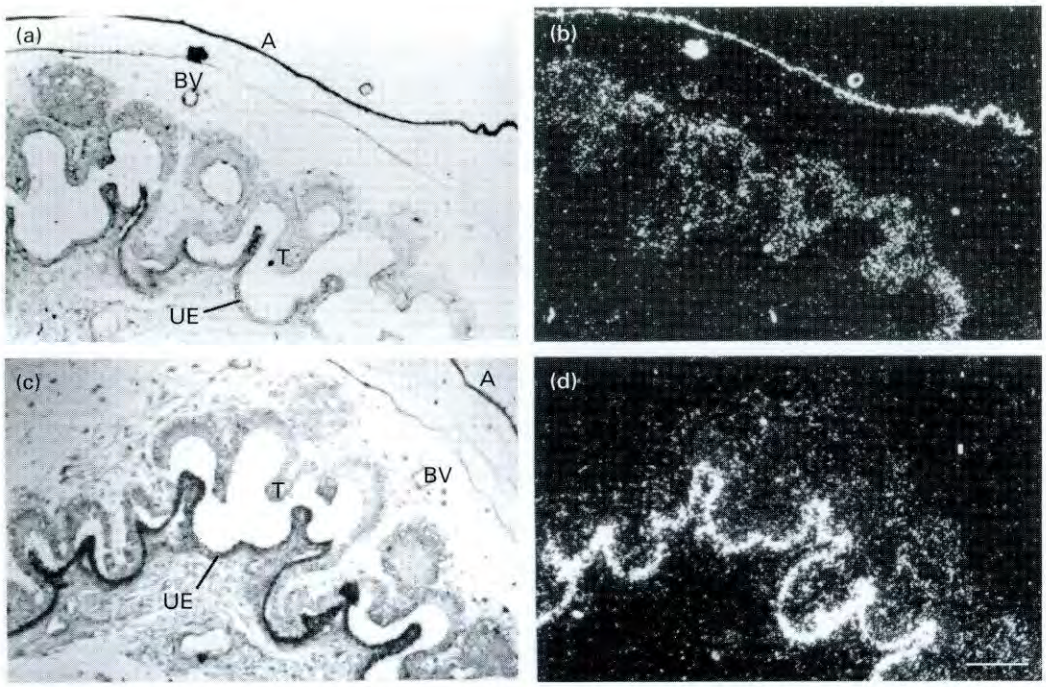

Fig. 1. The insulin-like growth factor binding protein 2 (IGFBP-2) gene was expressed in both the maternal and embryonic parts of the placenta and compared with the expression of the IGF-II gene. (a,c) Bright-field and (b,d) dark-field images of *in situ* hybridization of IGF-II and IGFBP-2 gene expression in the endometrium and placenta. (a,b) IGF-II transcripts are visible in the allantoic endoderm (A), trophoblast (T) and blood vessels (BV), but not in the uterine epithelium (UE). (c,d) IGFBP-2 transcripts are abundant in the surface epithelium of the endometrium (UE) and can also be detected in the trophoblast (T), but not in allantoic epithelium (A) or blood vessels (BV). Scale bar represents 100 μm. Reproduced from Persson (1996), with permission.

Retinoids in pig placentation

Retinoids, including vitamin A (retinol) and its active metabolite, retinoic acid, are unstable hydrophobic compounds that are indispensable for cellular differentiation and growth in general (Blomhoff, 1994), and for placental and embryonic development in particular (Soprano *et al.*, 1986; Baavik *et al.*, 1996). Transport mechanisms and metabolism are regulated tightly by the retinoid-binding proteins consisting of the 21 kDa plasma retinol-binding protein (RBP), the cellular RBPs (CRBP I and II) and cellular retinoic acid binding protein (CRABP I and II) of approximately 16 kDa. The RBPs are involved in cellular transport of retinol, whereas CRBP I participates in cellular transport of retinol and its metabolism into retinoic acid. In addition, the CRABPs are involved in the retinoic acid signalling pathways, regulation of the availability of retinoic acid to the nuclear receptors and modulation of retinoic acid metabolism (Chambon, 1996; Gustafson *et al.*, 1996; Li and Norris, 1996; Napoli, 1996). The relative amounts of RBP transcripts and the immunohistochemical location of RBP have been studied in pig placenta and uterus (Harney *et al.*, 1990, 1994a,b; Trout *et al.*, 1992; Schweigert *et al.*, 1999). However, Johansson *et al.* (2001) investigated the localization of RBP, CRBP-I and CRABP-I within the interareolar region and the areolar gland complex by immunohistochemistry and revealed the transepithelial route across the pig interhaemal barrier throughout gestation (Fig. 2). The results reveal that staining intensity of the RBPs in the pig placenta far exceeds the reactivity observed

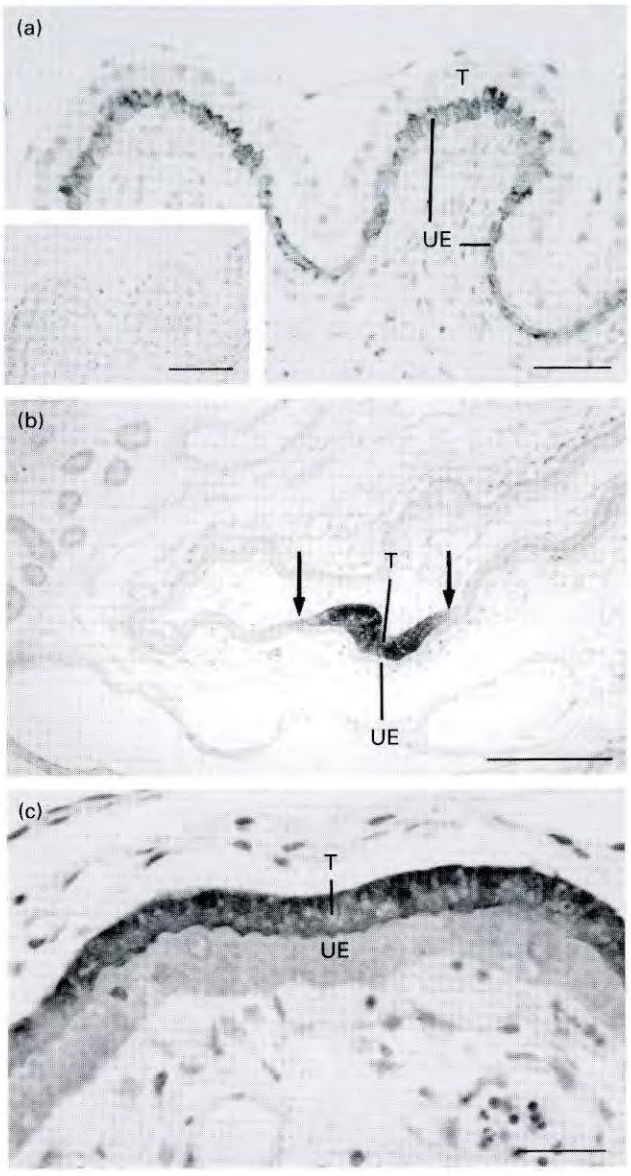

Fig. 2. Localization of retinol-binding protein (RBP) and cellular retinoid-binding protein (CRBP) in pig uterus and placenta during the first third of gestation. (a) In the interareolar region on day 20 after coitus, immunoreactivity of RBP is restricted to the uterine epithelium (UE) and uterine glands (not shown), whereas the trophoblast (T) is non-reactive. The control is shown in the inset. (b) At day 25 after coitus, the immunoreactivity for CRBP shows very strong reactivity in the trophoblast of the areola (T) and none in the areolar uterine epithelium (UE). The areola is marked by arrows. The low reactivity in the interareolar region and uterine gland (upper left side) is just visible at this low magnification with both regions included. (c) Detail from the interareolar region at day 25 after coitus showing the cellular retinoid acid binding protein (CRABP), which is located exclusively to the trophoblast (T), whereas the uterine epithelium (UE) is non-reactive. Scale bars represent (a) 100 μm, ((a) inset and (b)) 250 μm and (c) 50 μm. From Johansson *et al.* (2001), with permission.

in mouse and human placentae (Johansson *et al.*, 1997, 1999; Sapin, 1998). The RBP immunoactivity was located to the uterine and glandular epithelium but not to the maternal epithelium lining the areolar cavity, whereas at the fetal side only the trophoblast of the areolae showed immunostaining. The CRBP immunostaining was co-localized with RBP in maternal endometrium, but appears as fine granularities within the cytoplasm. At the fetal side, strong staining was located in the trophoblast of areolae and there was less and finer granular staining in the trophoblast of the interareolar region. In contrast, CRABP immunostaining was located exclusively to the trophoblast of the interareolar regions of the placentae. These results were consistent with previous investigations in human and mouse placentae (Johansson *et al.*, 1997, 1999), but revealed that the areolar gland complex is the transport route for vitamin A to the conceptus, and that RBPs are needed in the development and growth of pig placenta.

Vascular endothelial growth factors (VEGFs)

Recent reviews on angiogenesis in placentation and during implantation summarized different factors of importance for vascular growth, and inhibition during early placental growth and embryonic development (Reynolds and Redmer, 2001; Sherer and Abulafia, 2001). One of the most important growth factors involved with angiogenesis is vascular endothelial growth factor (VEGF). VEGF is a potent mitogen, morphogen and chemoattractant for endothelial cells, and is stimulated by hypoxia, cytokines and various hormones (Neufeld *et al.*, 1994). *In vitro* experiments with endometrial carcinoma cell lines indicate that oestradiol and progesterone increase the expression of VEGF mRNA (Charnock-Jones *et al.*, 1993). In pigs, embryonic production of oestrogens modulates the secretion of uterine proteins (Simmen and Simmen, 1990), secures the maternal recognition of pregnancy (Geisert *et al.*, 1990; Bazer *et al.*, 1998) and affects myometrial contractility (Pope *et al.*, 1982; Scheerboom *et al.*, 1987). The increase in angiogenesis observed at the mesometrial side of the uterus, where the first contact is established close to the embryonic disk (Keys and King, 1988; Dantzer and Leiser, 1994), may be stimulated by a paracrine effect of oestrogen secretion by the blastocyst, which may stimulate VEGF release (Charnock-Jones *et al.*, 1993).

Neovascularization of pig placenta has been investigated recently with some surprising results (Winther *et al.*, 1999). Immunohistochemical studies of VEGF and two of its receptors, Flt 1 (VEGFR-1) and KDR (VEGFR-2), revealed a high correlation in spatiotemporal distribution between the ligand and its receptors. Immunoreactivity of VEGF and VEGF receptors increased markedly in the capillary endothelium from day 13 after coitus through the first half of early pregnancy and remained almost constant until term compared with non-pregnant endometrial capillaries. In late stages of gestation there was a slight decrease in VEGF and KDR, whereas Flt-1 remained high. Vascular smooth muscle cells also showed a positive immunoreactivity for VEGF and its receptors. In addition, VEGF and its receptors were related to several non-endothelial cells such as uterine luminal and glandular epithelium, and trophoblast. The luminal epithelium shows a decrease in activity in the first half of early placentation (days 13–21), whereas in the trophoblast a decrease in immunostaining was observed in the second half of early gestation (days 21–31). Thereafter, the immunostaining increased and remained high to term with a slight decrease for KDR in late gestation (days 71–105 after coitus; Fig. 3a,b). Uterine glands also showed a marked increase in KDR staining from the late luteal phase of the oestrous cycle to days 13–21 after coitus and remained high in the late stage of gestation. These observations are in accordance with studies of human placenta concerning VEGF activity in uterine smooth muscle cells (Brown *et al.*, 1997) and trophoblast (Clark *et al.*, 1996). Furthermore, KDR is needed during the initial increase in angiogenesis, vasculogenesis and blood island formation (Shalaby *et al.*,

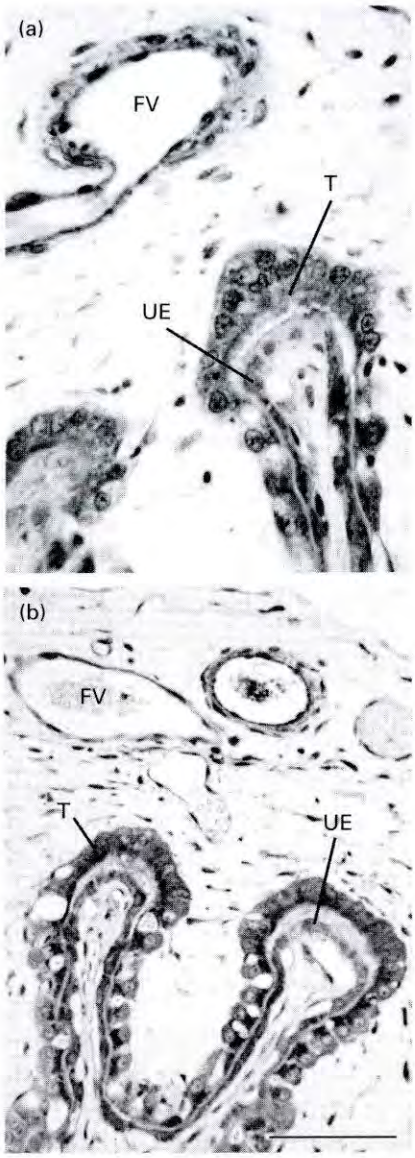

Fig. 3. Immunohistochemistry of vascular endothelial growth factor (VEGF) from the last third of gestation in pigs. (a) VEGF shows a strong reactivity in uterine epithelium (UE), trophoblast (T) and in endothelium and smooth muscle cells of fetal vessels (FV). (b) Localization of KDR (VEGF receptor 2) in the same cells as the ligand, namely uterine epithelium, trophoblast and in endothelial cells and smooth muscle cells of fetal vasculature. Scale bar represents 50 μm. From Winther *et al.* (1999), with permission.

1995), whereas Flt-1 plays a role in mediation of calcium-dependent nitric oxide release and regulation of human trophoblast activity (Ahmed *et al.*, 1997). These results imply that, during placentation in pigs, the VEGF ligand–receptor system may not only participate in the regulation of angiogenesis but also influences cellular differentiation and transport capabilities in the maternal–fetal interface, including the uterine glandular epithelium.

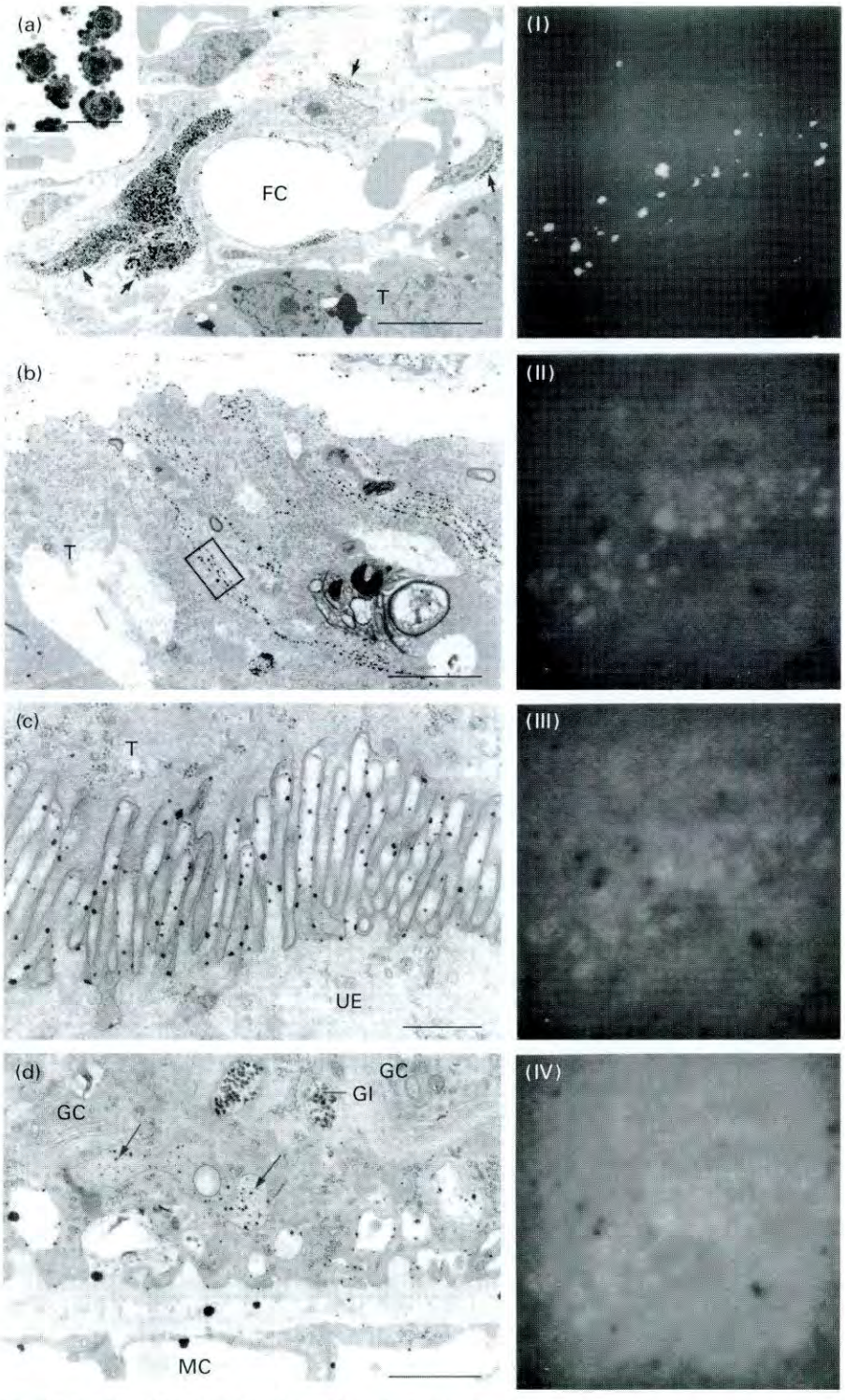

Fig. 4 (a)–(d) and (I)–(V). For legend see facing page.

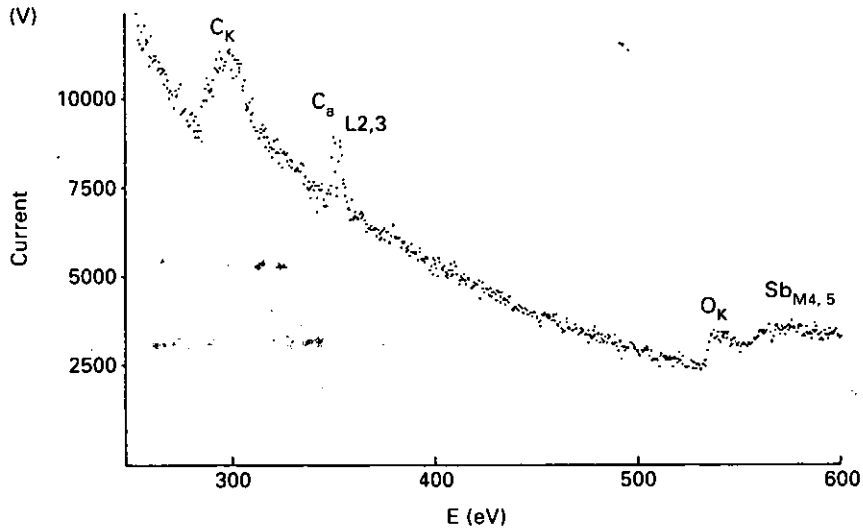

Fig. 4. Localization of calcium, seen as electron-dense antimonate precipitates, in the interhaemal barrier and mesenchyme of pig placenta from day 48 of gestation. Electron spectroscopic imaging (ESI) of the marked area in (b) is shown in the right-hand column (I, II, III, IV) and electron energy loss spectroscopy (EELS) is shown in (V). (a) Basal part of the trophoblast (T) and underlaying mesenchyme with fetal capillaries (FC) and prominent antimonate precipitates in the spherite-like inclusions (arrows). Notice also the precipitates at the luminal side of the fetal endothelium. Inset: a higher magnification view of the precipitate in the mesenchyme showing the concentric substructure. (b) Low magnification, of a 40 nm unstained Gauss image model, ESI at 250 eV, used for ESI (see panels I–IV) and EELS analysis (see panel V) in the marked area, showing the base of the trophoblast and the antimonate precipitates related closely to the lateral plasma membranes and in cytoplasmic vesicles. (c) Detail of the interdigitated microvilli between the uterine epithelium (UE) and the trophoblast, with antimonate precipitates related to the interdigitating microvilli. (d) The basal part of the uterine epithelium close to a maternal capillary (MC). Antimonate precipitates are visible in relation to the folded basal plasma membrane, in vesicles (arrows) as well as in a few stacks of the Golgi complex (GC). Glycogen: Gl. Scale bars represent (a) 10 μm, (inset to (a) and (c)) 500 nm, (b) 2 μm and (d) 1 μm. (I) Calcium distribution, pixel-wise, calculated from the other three images, II–IV, and here freed from background and reinforced to be clearly visible. (II) Electron spectroscopic image at 355 eV, containing a calcium-absorptive edge, the primary picture. (III) First background image for calcium at 355 eV. (IV) Second background image for calcium at 325 eV. (V) EELS analysis of the marked area in (b), showing the element distribution and demonstrating the content of calcium (Ca) and antimonate (Sb). Background for calcium at 320 eV and calcium edge, background for Sb+0 at 500 eV and Sb-O edge at 600 eV.

Platelet-derived growth factor (PDGF)

PDGF is a mitogen that exerts pleiotrophic effects on growth and motility of mesenchymal-derived cells. The PDGF-A ligand and receptors were immunolocalized in vascular smooth muscle cells. The PDGF receptors are expressed strongly in the endothelial and perivascular areas of the subepithelial layer, whereas PDGF is present in epithelial cell layers of the placenta (Persson and Rodriguez-Martinez, 1997). These findings indicate that PDGF interacts in an autocrine as well as a paracrine manner in the modulation of angiogenesis within the pig placenta.

Calcium

Calcium is important during fertilization (Lane and Bavister 1998) and during embryonic and fetal development, as intracellular calcium concentrations regulate many important cellular

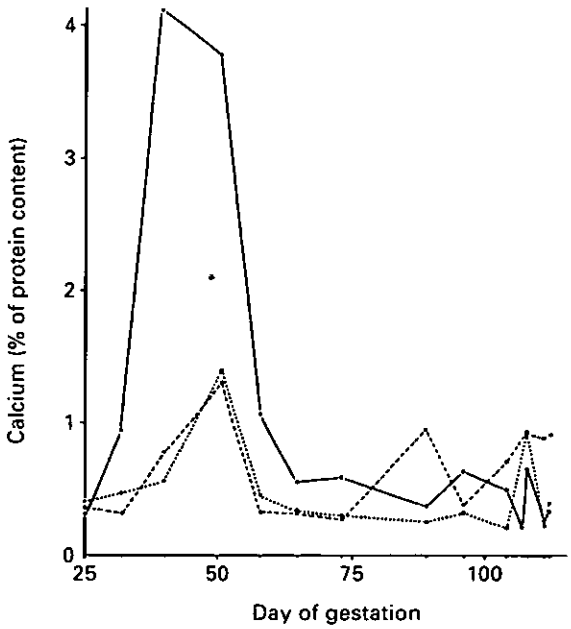

Fig. 5. Diagram showing the content of calcium in fetal pig membranes: allantochorion (——), amniochorion (----) and in the blind, non-vascularized paraplacental ends (.....). It can be seen that calcium, measured as percentage of the protein content, accumulates mainly in the allantochorion, peaking between day 36 and day 55 of gestation.

functions such as cell division, membrane fusion, exocytosis, cell–cell communication and metabolism, as well as playing a role in many second messenger systems (Campbell, 1983). However, during gestation the increases in ossification and growth of the skeletal system will increase the demand for transfer of calcium from the dam to the fetus. During the 114 days of gestation in pigs, primary ossification centres appear at about day 34 after coitus, with a second period of ossification at day 100 (Patten, 1948; Hodges, 1953). There is a marked increase in fetal growth rate from day 60 of gestation to term (Marrable, 1971). After day 80 of gestation there is a three-fold increase in the deposition of fetal calcium: in ten fetuses evaluated on days 80 and 100 after coitus, 1.2 g and 4.0 g of calcium, respectively, were deposited daily (Moustgaard, 1971). In classical studies (Brambel, 1933; Wislocky and Dempsey, 1946) and studies with electron microscopy (Dantzer *et al.*, 1989), large masses of lime infiltrations have been described in the mesenchyme between the chorionic and allantoic epithelium, which are seen as a temporal storage related to the interareolar regions.

Calcium deposits in placenta from 17 pregnant sows collected between day 25 and day 112 after coitus were investigated by measurement of calcium content and by using different methods for light and electron microscopy. Minor infiltrations, observed as spherite-like bodies, were localized in the mesenchyme just beneath the base of the interareolar trophoblast and related closely to vessels (Fig. 4a), particularly from day 33 to day 60 after coitus. However, calcium could not be measured by X-ray energy dispersive microanalysis after conventional processing for electronmicroscopy at pH 7.4, but could be measured in fresh cryofixed and ultrathin cryosectioned tissues (performed by Dr M. H. Nielsen and Dr L. Bastholm, University of

Copenhagen, Denmark) (data not shown). These results indicate that at this pH calcium may be bound loosely.

A histochemical method for calcium localization described by Borgers *et al.* (1983) was modified using 1% (w/v) potassium pyroantimonate in 7% (w/v) sucrose, 0.1 mol potassium phosphate buffer l^{-1} (> pH 8.5) in all preparatory steps from perfusion fixation in glutaraldehyde to embedding for electron microscopy to investigate the presence of calcium in the spherite-like inclusions in the mesenchyme and to locate and follow calcium intracellularly in the interhaemal barrier. For a negative control, the tissue was pretreated with EGTA, a calcium binder, before being processed by the calcium-binding potassium pyrantimonate solution. The presence of calcium in the antimonate precipitates was confirmed subsequently by electron spectroscopic imaging (ESI) (Fig. 4b; I–IV) taken at the specific element (calcium) edge and for estimation of the net calcium distribution by electron energy loss spectroscopy (EELS) (Fig. 4b; V) (analysis provided by Dr W. Probst, Zeiss, Oberkochen, Germany).

The antimonate precipitates indicate the presence of Ca^{2+}, Na^{+} and Mg^{2+}, but in this study the precipitate was predominantly calcium as shown by the three different types of analysis. The precipitates, here described from the maternal to the fetal side, were scattered at the maternal endothelial plasma membrane and in the maternal uterine epithelium along the basal plasma membrane. Intracellularly, the precipitates were observed in large vesicles located basally and in the large membranous lysosomes typical of the maternal epithelium in pigs (Dantzer, 1984) and as fine precipitates in some of the inner stacks of the Golgi complexes (Fig. 4d). At the interdigitating microvilli between maternal and fetal epithelium, precipitates are mainly observed at the maternal side and at the inner side of vesicles opening to the narrow intercellular space between these two compartments (Fig. 4c). At the fetal side, calcium precipitates are visible apically in the trophoblast, in endocytic tubules and in large apical vesicles, as well as at the lateral plasma membranes and to some extent at the basal cell border (Fig. 4b,c). In both epithelia of the materno–fetal interface, fine precipitates were also detected in mitochondria. Precipitates are also clearly visible in the large spherite-like inclusions in the mesenchyme and at the luminal side of the fetal endothelium (Fig. 4a). The confirmation of calcium in these precipitates was done by electron spectroscopy imaging ESI (Fig. 4b; I–IV) and by electron energy loss spectroscopy EELS (Fig. 4b; V), using antimonate precipitates at the folded lateral plasma membranes of the trophoblast for demonstration (Fig. 4b). The localization of calcium to the basolateral plasma membranes of the trophoblast indicates that these membranes are linked to membrane bound Ca^{2+}-ATPase activity, which is one of the largest of the calcium transporters assisting in calcium extrusion (Carafoli *et al.*, 1990; Bronner, 1991).

The content of calcium was determined in fetal membranes collected between day 25 and day 112 after coitus. The fetal membranes were separated from the maternal endometrium and dissected into allantochorion, amniochorion and the blind nonvascularized ends of the conceptus (calcium analysis was done by Dr S. Boisen, Danish Agricultural Reseach Centre, Foulum, Denmark). The results (Fig. 5) indicate a rapid increase in calcium content from day 25 to day 36 after coitus and a rapid decrease from day 50 to day 65 after coitus in the allantochorionic membrane, with only a slight increase in the two other compartments of the fetal membranes. These findings, together with the observation that there were no apparent calcium antimonate precipitations in the areolar gland subunit, give a clear indication of transplacental transport of calcium across the interareolar compartment of pig placenta. This finding is in contrast to the supposed route for transepithelial placental transport in other species with an epitheliochorial placenta, such as sheep, cows and horses (Wooding *et al.*, 1996; Morgan *et al.*, 1997; Nikitenko *et al.*, 1998; Wooding *et al.*, 2000), determined by

9 kDa calcium binding protein, an apparent valid marker for epithelial-mediated active transcellular transporter protein (Kumar, 1995). These studies found that placental calcium transport in cow, sheep and mare placenta takes place over the areolar gland subunit only. Although the morphological methods differ, it appears that another pathway, the interareolar region, is used in pigs, which is also in accordance with the temporary storage of loosely bound calcium at the fetal side in the mesenchyme close to the fetal vessels of the interareolar regions described above.

Furthermore, these results also give a clear indication of the allantochorion as a reservoir for loosely bound calcium, preceding the rapid increase in growth and ossification of the skeleton. In a comparative study of carbonic anhydrase in six different types of placenta (Ridderstråle *et al.*, 1997), the highest activity in maternal capillaries, uterine epithelium and trophoblast was observed in pigs, in contrast to low activities in cows and horses. Therefore, it is possible that the high carbonic anhydrase activity in pig placenta is needed in placental transport and metabolism of calcium, as suggested for human placenta by Aliakbar *et al.* (1990).

Conclusion

This description of the activation and localization of a variety of important ligands and their receptors, including IGFs, TGFβ, VEGF and PDGF, during pig placentation gives a strong impression of the complex interactions between these factors and hormones during pig placentation and vascular development. However, it should also be emphasized that more studies combining analysis of genotypes, molecular biology, gene activation and a variety of histological techniques are required to achieve a better understanding of regulatory mechanisms during pig placentation. In addition, it is recognized that retinoids are of great importance for placental functions and that the transport of vitamin A from the maternal to the fetal side appears to take place in the areolar gland complex only, whereas calcium transport in pigs may be confined to the interareolar route across the interhaemal barrier.

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