Morphology of pig uterine subepithelial capillaries after topical and systemic oestrogen treatment

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The ultrastructure of subepithelial capillaries in pig endometrium was studied after intrauterine and systemic oestradiol treatment. Gilts were killed on day 13 or 19 ($n = 4$ per day per treatment) after surgical introduction of Silastic beads containing either oestradiol or cholesterol into the uterine lumina on day 10 after oestrus. An additional group of gilts was injected i.m. with 5 mg oestradiol valerate on days 11–15 of the oestrous cycle and killed on day 13 or day 19 ($n = 4$ per day). Light and electron microscope studies of endometrial samples revealed that cholesterol beads did not appear to affect subepithelial capillary structure. Both intrauterine and systemic treatment of gilts with oestradiol were associated with regional modification of the capillary wall involving attenuation and fenestration of the face directly underlying the uterine epithelium, accumulation of caveolae in the opposite face of the capillary, and the occurrence of discontinuous, multilayered capillary basal laminae. The similarity of these structural changes to those observed in capillaries at the time of embryonic attachment suggests that oestrogens of blastocyst origin may function to modify capillary morphology and possibly facilitate increased transcapillary traffic during the establishment of pregnancy.

Introduction

The uteri of a variety of mammals respond to the presence of preimplantation blastocysts by exhibiting increased uterine vascular permeability. This phenomenon, which appears to be crucial to placental and embryonic development, is thought to be induced by vasoactive mediators (Psychoyos, 1973). The structural basis and possible mediators for the profound capillary permeability accompanying the invasive implantation process of rodents (see Psychoyos, 1973) and rabbits (Hoffman *et al.*, 1990) have been well documented. However, the nature and extent of capillary modification during embryonic attachment in species exhibiting noninvasive epitheliochorial placentation remains to be fully elucidated. Previous study of subepithelial capillaries in the uteri of cyclic and pregnant gilts indicated that structural indices of permeability were associated with the development of a close association between the blastocyst and uterine mucosa (Keys *et al.*, 1986; Keys and King, 1988). Apposition between the conceptus and luminal surface of the endometrium before attachment in pigs may facilitate paracrine interactions between the trophoblast and uterus that result in modification of the uterine microvasculature.

Pig blastocysts, which are capable of oestrogen synthesis as early as day 10 after conception, are the main source of these compounds *in utero* during early pregnancy (Perry *et al.*, 1973;

King and Ackerley, 1985). The extensive growth of pig conceptuses before implantation is accompanied by the secretion of large amounts of trophoblast-derived oestrogen into the uterine lumen, beginning on about day 11.5 (Perry *et al.*, 1973, 1976; Geisert *et al.*, 1982a). Interaction between the conceptus and maternal epithelium in pigs is coincident with localized modification in endometrial structure (Keys and King, 1990), many features of which can be simulated by systemic or intrauterine application of oestradiol (Keys and King, 1992). Given the temporal relationship between the onset of oestrogen production by pig conceptuses and the structural evidence of increased uterine vascular permeability (Keys and King, 1988), the objective of this study was to investigate whether the ultrastructure of pig uterine subepithelial capillaries is influenced by application of exogenous oestradiol.

Materials and Methods

Animals

Twenty-four nulliparous crossbred gilts (Yorkshire $\times$ Landrace) of similar ages (about 225 days old) and 90–110 kg body mass were housed in groups of 6–8 per pen (3×3.7 m) and fed about 2.5 kg per day per animal of a corn–soybean diet. Water was available *ad libitum* from drinking nipples. Gilts were observed daily for oestrus in the presence of a vasectomized boar, and the first day of standing oestrus was designated as day 0. After exhibiting two oestrous cycles of

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normal duration (18–22 days), each animal was assigned randomly to a treatment group and slaughter date. One group of eight gilts received an i.m. injection of 5 mg oestradiol valerate (Squibb Pharmaceutical, Montreal) in 0.5 ml sesame oil once a day at 16:00 h on days 11–15 of the oestrous cycle. Sixteen additional gilts were laparotomized on day 10 after oestrus. Anaesthesia was induced with Brietal Sodium (Eli Lilly, Toronto) at a dose of 1 mg kg⁻¹ i.v. and maintained with halothane (Somnothane, Hoechst, Montreal). Eight animals received oestradiol-impregnated Silastic beads and the rest received cholesterol-containing beads within their uterine lumina. Four beads were introduced into the lumen of each horn at each of the uterotubal junctions and mid-point through small puncture incisions. Each puncture was closed with a single suture to prevent herniation of the endometrium and escape of the beads. Details of the preparation and oestradiol release rates of the oestradiol- and cholesterol-containing beads were published by Keys and King (1992). Animals subjected to laparotomy were housed individually in gestation crates from the time of recovery from anaesthetic until slaughter. Four animals from each of the three treatment groups were killed by exsanguination on days 13 and 19.

Tissue preparation for electron microscopy

Immediately after slaughter, the right uterine horn was placed on ice, trimmed, and opened along its antimesometrial aspect; two 20 cm strips of tissue were pinned endometrial side up in a tray containing 0.2 mol cacodylate buffer l⁻¹ (pH 7.4). Tissue with beads *in situ* could not be properly infiltrated and sectioned. The endometrial folds surrounding oestradiol-impregnated beads were gently separated and the beads removed. Cholesterol beads, which did not become engulfed by endometrial folds, were simply lifted off the endometrium. Flagpins were inserted lateral to the mesometrial region to mark bead sites; the tray was drained and flooded with 0.2 mol cacodylate buffer l⁻¹ containing 3% glutaraldehyde (w/v; pH 7.4), and the tissue was left to harden. Multiple tissue samples directly associated with a bead, immediately adjacent to a bead, and distal to bead locations were processed from each animal. Six areas were selected randomly at the mesometrial region of two endometrial strips from each gilt injected with oestradiol valerate.

Endometrial samples were processed for light and electron microscopy using methods described by Keys and King (1989) and embedded in methacrylate and epon, respectively. Thick sections (1 µm) were cut from epon blocks at each of six sites per animal, treated with Paragon multiple stain (King *et al.*, 1979) and screened for cross-sectional profiles of uterine epithelium. Ultrastructural observations were limited to capillaries and the terminal ends of arterioles and venules that were positioned immediately subjacent to the luminal epithelium. These vessels, which lack a muscular coat, are usually involved in phenomena of altered permeability (Cotran and Majno, 1964; Zweifach, 1973). Regions of the cross-sectional capillary wall were designated as proximal, distal and lateral relative to the uterine epithelium for comparison between sampling sites and treatment groups (Fig. 1).

Results

The cholesterol beads had migrated from the incision sites and were located randomly on the endometrial surface at day 13. Most of the cholesterol beads were contained in the cervix or had passed out of the reproductive tract at day 19, and the ovaries contained avascular regressing corpora lutea and developing follicles. The thick firm mesometrium and loose oedematous endometrium of each uterus indicated pro-oestrous changes. Presumably luteolysis had occurred and animals were in the follicular phase of the subsequent cycle.

Beads containing oestradiol had also migrated from incision sites on day 13 and day 19 but were engulfed by fusion of vascular endometrial folds along the entire mesometrial aspect of the uterus. Vascularized corpora lutea and very small follicles characterized the ovaries of all animals in both oestradiol treatment groups at day 19.

Plasma progesterone concentrations have been reported for these animals (Keys and King, 1992). Significant increases in plasma progesterone concentrations on day 19 in oestradiol-injected (13.28 ± 2.93 ng ml⁻¹) and oestradiol bead-treated (9.39 ± 1.72 ng ml⁻¹) groups ($P < 0.05$), relative to cholesterol bead-treated (0.79 ± 0.26 ng ml⁻¹) gilts, indicated that both routes of application of oestradiol had extended luteal life span beyond the normal time of luteolysis.

Cholesterol bead treatment

Consistent differences in the morphology of capillaries with respect to sampling site and day were not found in gilts in which cholesterol beads had been inserted. Nuclear position within the capillary wall was variable relative to the uterine epithelium and all aspects of the endothelial wall were usually of uniform thickness (Fig. 1). In less than 15% of capillaries, endothelial thickness was highly variable proximal to the uterine epithelium and attenuated segments were occasionally observed on days 13 and 19. A few fenestrations were observed in one animal on day 13, while a single fenestration was observed at one site in one gilt on day 19. When present, attenuated regions and fenestrations were restricted to the proximal endothelium, regardless of treatment group. Small vesicles were prominent at the luminal and external faces, and were scattered throughout the endothelium. Filopodia were pleomorphic and ranged in number from few to innumerable. Numerous dense inclusions and vacuoles of variable dimension were scattered throughout the cytoplasm. Endoplasmic reticulum and mitochondria were abundant near the nucleus at day 13 but were less prominent at day 19. Junctional complexes were long, tortuous and terminated in zonula occludente on the luminal side of the capillary. Intercellular gaps were not observed. The basal lamina was fuzzy, laminated and appeared to be discontinuous in many cases at day 13. This layer was intact at day 19, and in only one case did the basal lamina appear to comprise more than one layer.

Oestradiol bead treatment

Differences in capillary structure were not observed between sites at, adjacent to, or away from beads. In contrast to

Fig. 1. Pig endometrium at day 13 of cholesterol bead treatment. The uterine epithelium lies beyond the left edge of the micrograph. The closest portion of the capillary wall to the uterine epithelium is designated as the proximal face (P); the distal face (D) is the furthest away; the lateral faces (L) connect these to complete the capillary wall. The capillary wall lacks morphological evidence of regional modification. Caveolae (c) and vesicles are abundant at the luminal aspect. A few filopodia (f) are evident. Polyribosomes and endoplasmic reticulum are scattered throughout the endothelial wall. The basal lamina is diffuse (arrowhead), and a zonula occludens occurs at the luminal extremity of an interendothelial junction (J). Scale bar represents 1 μ m.

cholesterol bead-treated animals, marked regional modification of the capillary wall was observed at day 13 in oestradiol bead-treated animals (Fig. 2). Nuclei were predominantly located lateral or distal to the uterine epithelium and were associated with thick areas of cytoplasm containing numerous mitochondria, polyribosomes, plentiful endoplasmic reticulum and large vacuoles. Many small vesicles were primarily disposed at the luminal and external aspects of the endothelium. Dense inclusions were scattered throughout. Filopodia, which occurred on the lateral and distal faces and sometimes formed a complex network, varied in number and form, as observed in the cholesterol group.

The endothelium underlying the uterine epithelium varied in thickness but generally formed long sinuous regions, several of which were studded with fenestrations (Fig. 3). Although a few dense inclusions were sometimes observed along the proximal endothelial face, this area was otherwise featureless internally. Vesicles and filopodia were very rare. The appearance of the basal lamina was variable, appearing as an intact single or multilayered form, discontinuous and multilayered, or was absent from other capillaries. Endothelial junctions were not altered.

In contrast to the extensive regional modification of the capillary wall at day 13, animals containing oestradiol beads

Fig. 2. Pig endometrium at day 13 of oestradiol bead treatment. Marked regional modification characterizes the capillary wall. The proximal face exhibits attenuation with several fenestrated areas (small arrowheads), and the long axis of the capillary is parallel to the uterine epithelium (UE). The distal face is thicker and contains an abundance of organelles, vesicles and complex filopodia (f). The basal lamina below the uterine epithelium appears distinct and intact (large arrowhead), while that of the capillary is discontinuous and consists of several thin layers (*). Scale bar represents 1 μ m.

in utero at day 19 had subepithelial capillaries that lacked evidence of regionalization and were characterized by a paucity of organelles indicative of metabolic activity (Fig. 4). Nuclear position varied, as did endothelial thickness. Thinning proximal to the luminal epithelium was restricted to one short attenuated segment that lacked fenestrations. The inner surface was irregular with a few small filopodia. The basal lamina was discontinuous, sometimes thick and diffuse in appearance, and often composed of several layers.

Oestradiol injection

Modifications of capillary structure which were similar to those at day 13 of oestradiol bead treatment were also observed in gilts at day 13 of oestradiol injection. The endothelium of the proximal face, which was consistently very close to and followed the convolutions of the uterine epithelium and associated basal lamina (Fig. 5), usually contained fenestrated segments. The wall was consistently thicker lateral and distal to these regions and contained a large number of dense inclusions, small vesicles and plentiful endoplasmic reticulum and mitochondria (Fig. 6). Filopodia were either

Fig. 3. Pig endometrium at day 13 of oestradiol bead treatment. The uterine luminal epithelium is evident in the upper right corner. The attenuated proximal face contains three discrete fenestrations closed by diaphragms (arrows). The capillary basal lamina (cbl) is diffuse and discontinuous. Scale bar represents 1 μ m. Inset: Higher magnification of left fenestration.

Fig. 4. Pig endometrium at day 19 of oestradiol bead treatment. The proximal face lacks attenuation. A paucity of organelles is evident except for small vesicles, which are particularly abundant at the luminal and external faces of the endothelium. The basal lamina (bl) remains multi-layered. UE: uterine epithelium. Scale bar represents 1 μ m.

Fig. 5. Pig endometrium at day 13 of oestradiol injection. Structural features of the extensively attenuated area of proximal endothelium are limited to multiple fenestrations (arrows), a few vesicles and dense inclusions (i). The capillary basal laminae (cbl) are diffuse and discontinuous, while the basal lamina associated with the uterine epithelium (ebl) is thick and separated from the basal epithelial plasmalemma in some areas. Scale bar represents 1 μ m.

absent or present as a few blunt forms. Junctional complexes were extensive. The appearance of the basal lamina was highly variable, as described for intraluminal oestradiol treatment.

Attenuated endothelium, usually containing fenestrated regions, occurred commonly subjacent to the epithelium at day

19 in the oestradiol injection group. The capillaries were generally oriented with their long axis parallel to the uterine epithelium, presenting a broad face of endothelium adjacent to this tissue (Fig. 7). Irregular wall thickness or uniform thickness with organelles dispersed throughout characterized other non-fenestrated capillaries. Abundant small vesicles were primarily disposed near the lumen. Numerous dense inclusions, mitochondria and endoplasmic reticulum were found on the distal face. The occurrence of filopodia on the distal face ranged from a few to an extensive network, but these extensions were rare along the proximal face. The basal lamina remained discontinuous.

Discussion

Incubation of beads *in vitro* (Keys and King, 1992) indicated that the pattern of steroid release differed from that of developing conceptuses (Robertson *et al.*, 1978; Bazer *et al.*, 1982; Stone and Seamark, 1985). Although the large surge of oestradiol released from the beads on days 11 and 12 simulated production of this steroid by pig conceptuses, the subsequent release of much smaller daily amounts indicated that the beads failed to mimic the second period of increased release from conceptuses starting on day 15 of pregnancy. In addition to an initial oestrogen stimulation on day 11, a secondary prolonged exposure to increased oestrogen beginning on day 14 is required to completely induce pseudopregnancy (Geisert *et al.*,

Fig. 6. Pig endometrium at day 13 of oestradiol injection. The epithelial–stromal interface lies to the left of the image. This capillary lacks fenestrated regions and contains polyribosomes, endoplasmic reticulum, mitochondria (m), intracellular vesicles (v), and caveolae (c) which are dispersed throughout the wall. Junctions (J) remain tortuous and are sealed by a dense appearing zonula occludens. The basal lamina is diffuse, incomplete and folded in some areas (arrows). Scale bar represents 1 μ m.

Fig. 7. Pig endometrium at day 19 of oestradiol injection. Uterine epithelium (UE) occupies the upper portion of the micrograph. This overview indicates parallel orientation of the long axis of the capillary to the uterine epithelium, with a long attenuated proximal endothelial face in extensive close proximity to the uterine epithelium. Scale bar represents 1 μ m.

lacking at day 19 of oestradiol bead treatment, although the basal lamina remained discontinuous, thick, and multilayered. The amount of steroid released from the beads into the uterine lumen at this stage (Keys and King, 1992) was probably inadequate to maintain the alterations. In contrast, capillary modifications similar to those observed in pregnant gilts and in oestradiol bead treated gilts on day 13 persisted until day 19 in the oestradiol-injected group. Differences in the time course of the vascular changes between intraluminal and systemic application of oestrogen may be explained by a more stable amount of oestrogen entering the uterus during the latter treatment. A positive correlation between concentrations of this steroid and the number of uterine intra-endothelial vesicles, vesicle fusions and fenestrations has been observed in other species (Wolff and Merker, 1966; Friederici, 1967; Martin *et al.*, 1973). Oestradiol may act directly in structural modification of the endothelium through interaction with the endothelial oestradiol receptor (Colburn and Buonassisi, 1978), or by inducing synthesis of vasoactive substances such as histamine and prostaglandin.

Morphological signs of general metabolic activation of the endothelium at day 13 of oestradiol bead treatment and days 13 and 19 of oestradiol injection included extensive granular endoplasmic reticulum, numerous mitochondria, and an abundance of vesicles and caveolae. Metabolically activated endothelium has been described at implantation sites in many species (Enders and Schlafke, 1979) and the ultrastructural similarity to capillaries exposed to angiogenic stimuli (Christofferson and Nilsson, 1988) indicates that these features reflect angiogenesis.

Regional modifications in capillary morphology after oestradiol treatment suggest an increase in transcapillary traffic by transport of material across the endothelium via fenestrae at the attenuated proximal face and exchange by vesicular transport at the thicker distal face. The incidence of caveolae and intra-endothelial vesicles, which provide a vehicle for transport of large molecules such as ferritin (Bruns and Palade, 1968), horseradish peroxidase (Graham and Karnovsky, 1966) and dextrans (Simionescu *et al.*, 1972), was markedly increased. Laforest and King (1992) demonstrated that ferritin injected directly into a uterine artery of one gilt on day 15 of pregnancy is transported across the capillary wall by endothelial vesicles and transendothelial channels. Fusion of vesicles with the endothelial surface is involved in the formation of fenestrations (Elfvin, 1965; Wolff and Merker, 1966; Palade and Bruns, 1968; Casley-Smith, 1970). Small numbers of vesicles in flattened

1987). Therefore, although the beads released sufficient oestrogen to stimulate endometrial changes and prolong luteal function until day 19, concomitant modifications in capillary structure must be interpreted with caution since the beads produced what may be considered pharmacological rather than physiological doses during the initial stages, and yet the amounts released after the initial peak may not have been sufficient to extend luteal function beyond 28 days (King and Rajamahendran, 1988).

Animals that received cholesterol beads had subepithelial capillaries that were not structurally different from the corresponding days of the cycle (Keys and King, 1988). In contrast, the subepithelial capillaries of animals that had received oestradiol beads exhibited morphology similar to that at days 13 and 19 of pregnancy (Keys and King, 1988), indicating that modifications were due to the steroid the beads contained, rather than their physical presence. These alterations included marked regional modification of the capillary wall, with attenuated, fenestrated endothelium forming the proximal face, plentiful vesicles and filopodia on the thicker distal face, as well as a discontinuous and sometimes multilayered basal lamina. In contrast to animals at day 19 of pregnancy (Keys and King, 1988), structural indications of increased permeability were

areas near fenestrations or in non-fenestrated capillaries have been observed in many tissues; this suggests that vesicles are used during the process of elongation of vessels and enlargement of endothelial surfaces (Wolff, 1966; 1977).

Fenestrations are restricted in distribution to endothelia across which large amounts of solute or fluid must traverse (Yamada, 1955; Casley-Smith, 1971; Renkin, 1978; Buonassisi and Colburn, 1980) and are densely packed in fields of extensively flattened endothelium (Wolff, 1977), as was the case along the proximal face of subepithelial capillaries in the oestrogen-treated uteri examined, as well as in the uteri of pregnant pigs (Keys and King, 1988). Although these pores are generally closed by diaphragms (Maul, 1971), molecules as large as ferritin pass through (Casley-Smith, 1970). Increased numbers of fenestrated capillaries at implantation sites in rats suggest that fenestrae also contribute to the increased vascular permeability in this species (Abrahamsohn *et al.*, 1983). Fenestrations proliferate under the influence of steroids (Wolff and Merker, 1966) and in response to unidentified factors seemingly produced by epithelial cells (Campbell and Uehara, 1972), which would explain their consistent presence along the capillary wall directly underlying the uterine epithelium. Functionally, extensive profiles of capillary wall parallel, and in close proximity, to the uterine epithelium would increase the area of the inner vessel surface for exchange, and attenuation of the capillary wall would decrease the interhaemal distance, both of which favour maternal-fetal exchange (Battaglia, 1981).

Differences in the tortuous nature of cell junctions or the presence of tight junctions were not observed at any of the stages or in any of the treatments examined. Fusion of the external leaflets of the plasmalemmae of endothelial cells at intercellular clefts (Simionescu *et al.*, 1975) are a physical determinant of permeability. In species exhibiting more invasive forms of placentation, implantation is associated with marked increases in the number of interendothelial gaps and frank capillary leakage (Abrahamsohn *et al.*, 1983; Hoffman and Hoos, 1984; Hoffman *et al.*, 1990); both of these microcirculatory effects can be reproduced in immature rats by systemic injection of oestradiol (Ham *et al.*, 1970). Differences between our observations in pigs and those reported for rodents indicate clear species differences in microvascular response to oestradiol as a participant in placental development.

The basal lamina, produced and organized by the endothelium and adhering cells (Pierce and Nakane, 1967; Kefalides and Denduchis, 1969; Bar and Wolff, 1972) disintegrates in response to vasoactive agents or degeneration (Cotran and Majno, 1964). If regeneration occurs within a reasonable time, a new basal lamina forms inside the old scaffold, resulting in a multi-layered structure. Multi-lamellar and thickened basal laminae characterize endothelial damage (Pierce and Nakane, 1969; Vracko and Benditt, 1972), as well as angiogenesis (Findlay, 1987). Degradation of the basal lamina of a microvessel is thought to reflect the action of collagenase and plasminogen activator secreted by the endothelial cells in response to an angiogenic stimulus (Findlay, 1987). The occurrence of discontinuous, diffuse, laminated basal laminae in oestrogen-treated and pregnant pigs (Keys and King, 1988) reflects a subepithelial vascular network which is being restructured and rapidly expanded. Given the complex nature of angiogenesis, involving cell division, chemotaxis and

elaboration of basal lamina (Folkman, 1985), many factors including ovarian and conceptus steroids are probably involved. The basal lamina regulates the exit of material from uterine capillaries following oestradiol treatment, as illustrated using mercuric sulphide (Friederici, 1967) and carbon (Ham *et al.*, 1970).

The increased incidence of fenestrations and endothelial vesicles following oestrogen administration is probably related to increased transcapillary transport rather than to a marked increase in capillary permeability. Ultrastructural features of the subepithelial capillaries in oestrogen-treated gilts were also consistent with angiogenic activity. These changes mimic those observed in the microvasculature of pregnant uteri, in temporal association with apposition between the elongated blastocyst and endometrium, and elaboration of oestrogen by the conceptus. They also coincide with two previously documented uterine responses to intraluminal or systemic oestrogens: increased blood flow to the gravid uterine horns and histotroph production (Ford and Christenson, 1979; Ford, 1985; Geisert *et al.*, 1982a, b). On the basis of the amount of aromatase activity (Flint *et al.*, 1979), tissue concentrations of oestrogens (Gadsby and Heap, 1978) and increased oestrogen concentrations in the uterine flushings of pregnant pigs (Zavy *et al.*, 1980; Ford *et al.*, 1982), it is evident that the blastocysts are the main source of oestrogen *in utero* during this period. These facts indicate that the conceptus can orchestrate the expansion and modification of the maternal vasculature necessary to ensure placentation. Concomitant increases in transcapillary traffic, uterine blood flow and histotroph production, as well as attenuation of the subepithelial capillary wall, undoubtedly facilitate the passage of nutrients toward the rapidly developing conceptus and the transfer of blastocyst-induced products into maternal circulation. Disturbances in the exchange of materials across the uterine subepithelial capillaries during this period of rapid embryonic growth may be a factor contributing to the high early embryonic mortality associated with this phase.

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