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Endothelial Cell Hyperproliferation and Stratification in Uteroplacental Blood Vessels of the Black Mastiff Bat, *Molossus rufus*

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Abstract

Placentation was studied histologically and immunocytochemically in black mastiff bats obtained at frequent intervals throughout pregnancy. These were bred in a captive colony or collected from a reproductively-synchronized wild population. During late pregnancy, the single fetus was largely sustained by a discoidal, hemochorial placenta located at the cranial end of the right uterine horn. This invariant positioning was determined by a vascular tuft that developed there both during early pregnancy and non-pregnant cycles. This provided a scaffold for early placental morphogenesis. As development proceeded, small arterioles and venules serving the tuft were converted to large uteroplacental vessels. Within the base of the placenta, these became lined by an unusual vascular epithelium composed of intermingled patches of multilayered endothelial cells and cytotrophoblast. Initially, the endothelium became multilayered by hypertrophy, proliferation, and infolding of its basal lamina. These created endothelial bilayers usually insinuated between basal laminae. The development of temporary gaps in the laminae then permitted further enlargement of the vessels and proliferation of the endothelial cells as monolayer sheets or chains. The latter were interconnected, forming a complex, stratified, cellular network associated with a prominent meshwork of basal laminae. Throughout much of pregnancy, these endothelial cells were cuboidal to columnar and possessed an abundance of basal glycoprotein granules presumably containing basal lamina precursors. The cells also expressed vimentin and frequently von Willebrand factor, but not cytokeratins or desmin. Pronounced thickening of the endothelia and amplification of their basal laminae likely evolved to greatly strengthen the walls of the uteroplacental vessels.

Keywords

Angiogenesis; Comparative placentation; Cytotrophoblast; Endothelial cell; Uteroplacental vessel

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Introduction

Bats are noteworthy amongst small mammals for having small litter sizes, relatively long gestation periods, and delivering infants that are large relative to the size of their mother [1, 2]. Most species bear a single infant, although some members of the family Vespertilionidae have from two to four young.

Two ways were previously suggested in which a relatively large neonatal body mass might be advantageous. As bats do not provide insulating nests for their young, the thermal inertia of relatively large neonates might ameliorate drops in their body temperature during the mother's absence. Furthermore, large body size may be associated with greater neuromuscular maturity. This probably enables newborn bats to cling tightly to their mothers or their roosts, which in turn would be protective against dangerous falls and some predators [2]. More precocious development at birth would also presumably be correlated with enhanced or earlier behavioral maturity that could be protective for similar reasons.

While the picture is still fragmentary, it is clear that the production of large infants is associated with a variety of maternal reproductive and placental adaptations. Many bats have highly localized, predetermined implantation sites that are probably situated to provide for optimal vascularization of the placenta [3, 4]. Furthermore, in several bat species maternal blood vessels, involved in the morphogenesis and vascularization of the placenta, exhibit unusual adaptations. As described below, this includes the hyperproliferation and stratification of maternal endothelial cells in uteroplacental vessels of the black mastiff bat, *Molossus rufus*. This species has also been widely referred to as *Molossus ater* [5].

To fully understand these observations, a brief review of the female reproductive biology of *M. rufus* is required. This bat possesses two ovaries and a bicornuate uterus; however, ovulation only occurs from the right ovary, and the single conceptus is only carried in the right uterine horn. During pregnancy, three different vascular placentae are formed in that horn – a choriovitelline placenta that functions briefly, a diffuse labyrinthine endotheliodichorial placenta that is prominent and extensive during mid-gestation, and a discoidal hemochorial placenta that functions as the major site of feto-maternal exchange late in pregnancy [6-10].

Several aspects of development and structure of the discoidal placenta in *M. rufus* are unusual: this always forms in the same location at the cranial end of the right uterine horn; during a substantial portion of its early development, no maternal blood circulates through this placenta; and at approximately mid-gestation, maternal blood flow through the discoidal placenta commences and the diffuse placenta involutes [7, 8].

The invariant positioning of the discoidal placenta is related to the presence of an unusual vascular tuft in the endometrium immediately surrounding the cranial end of the lumen in the right horn. Such tufts develop after ovulation in both uterine horns, but only that in the right horn participates in formation of a placenta. Each tuft consists of a concentration of maternal capillaries and possibly endothelial tubules. These are connected to distinctive arterioles and venules that run parallel to the intramural portion of the uterotubal junction. During early pregnancy, the smallest tuft vessels provide a scaffold upon which cytotrophoblast proliferates preferentially to initiate formation of the discoidal placenta [8, 9]. The arterioles and venules are then converted into much larger, uteroplacental vessels that are the focus of this paper.

In the absence of an early, ongoing pregnancy, luteal regression occurs. This is associated with extensive endometrial sloughing and associated bleeding (i.e., true menstruation), including complete loss of the vascular tufts. The tufts are then reformed after the next

ovulation [9]. This represents the most pronounced example of normal (non-pathogenic) angiogenesis thus far observed in adult mammals. The tufts are extensively modified during pregnancy [8] and must therefore also be reformed after parturition.

2. Materials and methods

All of the bats used in this study were captured with mist nets on Trinidad, West Indies [5]. Most were maintained in captivity and many were bred with husbandry procedures described previously [6–9, 11]. These provided 47 gravid and 40 non-gravid reproductive tracts. Most major stages of implantation and placental development appear to have been well represented. Thirteen additional uteri were collected from the reproductively-synchronized wild population for immunocytochemical studies.

For histological studies, the bats were killed by the administration of an intraperitoneal injection of sodium pentobarbital at a dosage of about 90 mg/kg body weight. Their reproductive tracts were then removed, fixed in Zenker's fluid for 10–12 hr, and processed to paraffin wax. When conceptuses could readily be dissected out of the uterus, these were fixed, weighed and their C-R lengths recorded. The entire reproductive tracts or portions of particular interest were then serially-sectioned, in a frontal plane, at 6 μ m. [5–9].

The histological sections were stained with hematoxylin and eosin, Masson's trichrome procedure, or by the periodic acid-Schiff (PAS) technique and counterstained with hematoxylin. Many sections of each tract were incubated for 1 hr at 37°C in 0.1% α -amylase (1,4- α -D-glucan-glucanohydrolase; Sigma Chemical Company, St. Louis, MO) dissolved in a 0.02M phosphate buffer (pH 6) to remove any glycogen before being processed according to the PAS procedure.

Immunocytochemical methods for intermediate filament proteins (cytokeratins, vimentin and desmin) and von Willebrand factor (factor VIII-related antigen) were used to confirm some of the cell identifications in this study [12]. For this purpose, 13 advanced pregnant tracts were collected from animals in the reproductively-synchronized wild population on Trinidad on April 29, 1994 and April 29, 1995. These carried fetuses with C-R lengths of 24.0–33.5 mm. Some tracts were removed from the females and immersion-fixed in cold Zamboni's fluid for 48 hrs. Other females were anesthetized and then perfused via the left ventricle with approximately 15 ml of lactated Ringer's solution containing 5% dextrose (Baxter Healthcare Corp., Deerfield, IL 60015). This was followed immediately by cold, 4% methanol-free formaldehyde in 0.1 M phosphate buffer (pH 7.4). The discoidal placenta, adjacent portion of the uterine wall, right ovary and oviduct were then dissected free from the remainder of the tract and immersion-fixed for 24 hrs in the same solution. Following fixation, the specimens were washed and transported in cold 0.1 M phosphate-buffered saline (pH 7.4). Upon arrival in New York, the specimens were processed to paraffin wax and sectioned at a thickness of 6 μ m [12].

Antibodies used included the following: CAM 5.2 (Becton Dickinson, San Jose, CA, USA), a mouse monoclonal antibody directed against human cytokeratins 8 and 18; AE1/AE3 (Zymed Laboratories, Inc., South San Francisco, CA, USA), a mouse monoclonal cocktail directed against cytokeratins 1–8, 10, 14–16 and 19; a mouse monoclonal directed against human leiomyoma desmin (Zymed Laboratories, Inc.); a mouse monoclonal directed against porcine lens vimentin (Zymed Laboratories, Inc.); and a rabbit polyclonal directed against von Willebrand factor (human factor VIII-related antigen; Zymed Laboratories, Inc.). After this and each subsequent step the sections were rinsed with PBS. The sections were then incubated for 30 min with a biotinylated anti-mouse or anti-rabbit secondary antibody (Vector Laboratories), followed by 30 min with the enzyme conjugate in the

Vectastain Elite ABC Kit (Vector Laboratories), and finally for 10 min in a solution of diaminobenzidine and hydrogen peroxide to develop a colored reaction product.

Negative controls were performed by replacing the primary antibody with an isotype-matched irrelevant antibody at the same concentration for the monoclonal antibodies, or purified normal rabbit immunoglobulin at a similar concentration for the polyclonal antibodies directed against von Willebrand factor. Some sections were counterstained with Gill's hematoxylin.

3. Results

During pregnancy, major maternal vessels within the base of the discoidal, hemochorial placenta of *M. rufus* develop a unique epithelial lining which, in routine histological sections, has a stratified appearance. This paper focuses upon the development, cellular nature and probable function of that lining.

3.1. Development and early changes in the endometrial vascular tufts

During the non-pregnant reproductive cycle and early pregnancy, distinctive vascular tufts developed in the endometrium around the cranial end of each uterine horn. Irrespective of whether the female became pregnant or not, the endometrium in both horns decidualized as the luteal phase progressed. This was associated with hypertrophy of the tuft endothelial cells, which was greatest in the venules, less so in the arterioles and least in the capillaries and possible endothelial tubules. All of these vessels also developed more prominent basal laminae. In the case of the venules, many of the endothelial cells accumulated round glycoprotein granules in their basal cytoplasm and rested on infolded basal laminae. As early pregnancy progressed, this infolding became more pronounced in venules of the right tuft. Less pronounced infolding and smaller accumulations of granules appeared then in arterioles as well. These vascular differences persisted until the embryo reached a C-R length of about 9.7 mm [8, 9].

3.2. Conversion of tuft arterioles and venules to uteroplacental vessels

In tracts carrying slightly larger embryos (11.4 – 12.3 mm), further changes had become evident in the tuft arterioles and venules. Within the base of the developing discoidal placenta, mitotic figures were more common in their endothelial cells and the total number of endothelial cells had greatly increased (Figure 1). This gave rise to a multilayered endothelium. This also modified the arterioles and venules to such a degree that they could no longer be readily distinguished within the placenta. Vessel types could then be determined only by tracing them out into the myometrium, where distinctive characteristics remained. It was common in regions of the greatest endothelial cell proliferation to observe the retention of a central, vascular lumen. Prior to the commencement of significant maternal blood flow through the discoidal placenta, however, these were generally narrow and could have been significantly occluded *in vivo*.

Although some of these endothelial cells still had prominent accumulations of basal glycoprotein granules, most now contained only modest to low numbers of granules (Figure 1). Particularly in the case of the venules, this represented a diminution in granule concentration from previous stages [8]. This may have been attributable to enhanced mitotic activity and/or an exocytosis of granules to expand the endothelial cell basal laminae. Whether all endothelial cells remained associated with intact basal laminae, which were now frequently difficult to trace, is uncertain.

At this stage, endothelial cells were identified on the basis of their position inside of the original, much-thickened basal laminae and the presence (in some) of the granules. Most

cells outside this basal lamina were identified as cytotrophoblast. The cytotrophoblast cells generally had larger, more euchromatic nuclei, more prominent nucleoli, more basophilic cytoplasm, and a significant content of glycogen. Some cytotrophoblast had also spontaneously canalized to form vascular channels (Figure 1) which eventually became linked to the placental labyrinth. Gaps had also begun to develop in the original (most peripheral) vascular basal laminae of the tuft arterioles and venules. These gaps permitted some endothelial cells to grow outward, bringing them into direct contact with the cytotrophoblast (Figure 1).

In uteri carrying conceptuses with a C-R length of 16.7-19.0 mm, the original basal laminae of the tuft arterioles and venules were more frequently absent or discontinuous (Figure 2). This was associated with further outward proliferation of endothelial cells and inward growth of cytotrophoblast. This provided a mechanism for the vessels to expand greatly in diameter. Because of the frequent ramification of endothelial layers noted in these vessels in subsequent stages, discontinuities also presumably developed in infolded segments of their basal laminae. By this stage, the cytological differences (noted above) between endothelial cells and cytotrophoblast were even more pronounced (Figure 2C).

In uteroplacental vessels associated with a more advanced embryo (C-R length of 24.3 mm), basal glycoprotein granules were more abundant in many of the endothelial cells, and the endothelial cell basal laminae had frequently become more obvious again (Figure 3). This made it easy to recognize that many of the intervening endothelial cells were now arranged as interconnected monolayers, rather than as bilayers or clusters. Mitotic activity remained high and was often evident deep within the endothelium.

At this stage, large portions of the individual endothelial cells appeared to be associated with basal laminae, and glycoprotein granules were typically abundant in the adjacent cytoplasm. This may be attributable to their change in cell shape (from squamous to rotund) and/or more of the cell surface assuming a basal character. Where the endothelial cells were still arranged as bilayers or clusters, small clefts or lumina were sometimes seen between their apposing apical surfaces. Their presence indicates a lack of apical, cell-to-cell binding.

Cytotrophoblast intermingled in the walls of the developing uteroplacental vessels varied from scattered cells to large patches. In addition to being relatively rich in glycogen (Figure 2C), this cytotrophoblast was also generally surrounded by a thin, PAS-positive layer. The latter presumably represents a basal lamina and possibly secreted extracellular matrix material. This never became as prominent as the basal laminae associated with the endothelial cells (Figure 4).

The luminal surface of the uteroplacental vessels was covered by a layer of cells that could not always be identified with certainty in ordinary histological sections. Immunocytochemical staining (see below) established that these could be either endothelial or cytotrophoblast cells.

By late in pregnancy (fetal C-R lengths of 24.3 – 34.8 mm), the diffuse endotheliochorial placenta had involuted, and maternal blood flow had largely shifted instead to the discoidal placenta [7, 8]. Uteroplacental vessels at the base of the discoidal placenta were then generally engorged with blood and lined by a continuous, thick, multilayered epithelium that did not appear to differ between arteries and veins (Figure 4). This consisted of large areas of endothelial cells with interspersed cytotrophoblast cells, sometimes also in sizeable masses. Mitotic figures continued to be common in both cell types and were frequently evident deep within the endothelium (Figure 5).

Late in pregnancy, there was considerable variability in the abundance of glycoprotein granules in the endothelial cells, and in the arrangement of endothelial cells within the vascular epithelium. In general, there seemed to be an inverse relationship between their content of granules and prominence of the adjacent basal lamina, suggesting that exocytosis of the granules augmented the basal lamina. Some endothelial cells were still organized as clusters or bilayers, particularly in the basal-most layers of the epithelium and in channels where the vascular epithelium was relatively thin. Most appeared, however, as individual cells or tortuous monolayers associated with very prominent basal laminae (Figure 5).

The organization of the epithelium in the uteroplacental vessels late in pregnancy suggests that the much of the endothelium had now become stratified, rather than pseudostratified as in earlier pregnancy (see Discussion). Furthermore, the interconnected endothelial basal laminae in those vessels must have then constituted a highly complex, three dimensional meshwork. Despite their large size, these vessels contained no smooth muscle in their walls (at least within the placenta), and their collagenous adventitia (provided by allantoic mesenchyme) was relatively thin (Figure 5).

During late pregnancy, it was also common to see areas of dead tissue in and around the uteroplacental vessels (Figure 4A). These could include endothelial and cytotrophoblastic cells, as well as surrounding connective tissue and decidua.

3.3. Immunocytochemical observations

Immunocytochemical studies confirmed cell identifications for the uteroplacental vessels. The multilayered endothelial cells reacted positively with antibodies directed against vimentin (Figure 6A). These were never reactive with the antibodies directed against cytokeratins (CAM 5.2 and monoclonal cocktail AE1/AE3) (Figure 6B, C) or desmin. Many of the multilayered endothelial cells in these vessels also reacted positively with antibodies directed against von Willebrand factor (Figures 6D, 7A). Expression of this factor was granular (presumably in Weibel-Palade bodies), and had a less polarized distribution than glycoprotein granules in the endothelial cells. Endothelial cells in more ordinary blood vessels in the same sections were strongly reactive with these antibodies (Figure 7B).

Cytotrophoblast in and around the uteroplacental vessels was always reactive with the antibodies directed against cytokeratins (Figure 6B, C). It was non-reactive with the antibodies directed against desmin, vimentin (Figure 6A) and von Willebrand factor. Some exceptional cytotrophoblast became highly invasive and penetrated as far as the broad ligament, oviduct and ovary on the right (gravid) side of the reproductive tract via intramural (not endovascular) migration along maternal blood vessels [12]. This strongly co-expressed cytokeratins and vimentin in the myometrium and outside of the uterus. It generally exhibited a weaker staining reaction for vimentin when present in the walls of uteroplacental arteries passing through the decidua or when intermingled with endothelial cells in uteroplacental vessels immediately adjacent to the decidua. Cytotrophoblast intermingled with endothelial cells elsewhere in these vessels did not exhibit obvious vimentin expression.

With immunocytochemical staining, it was confirmed that cytokeratin-positive and vimentin-negative cytotrophoblast sometimes grew as a monolayer over multilayered endothelial cells (Figure 6B).

4. Discussion

Studies of gravid uteri from *M. rufus* established that maternal vessels within the base of this species' discoidal placenta were derived from arterioles and venules serving an unusual

vascular tuft that developed during early pregnancy in the endometrium of the right horn. These vessels became lined by a multilayered, luminal epithelium as pregnancy progressed. This was largely composed of maternal endothelial cells, but also frequently contained interspersed cytotrophoblast. The endothelial cells differed from those in most other mammalian blood vessels in being highly proliferative and hypertrophied. At most stages, they also possessed an abundance of basal glycoprotein granules and/or had prominent basal laminae.

Although an initially similar vascular tuft developed in the left horn, pregnancies were never carried there. Endothelial cells of the arterioles and venules belonging to that tuft also hypertrophied, accumulated glycoprotein granules, developed thickened basal laminae, and exhibited basal lamina infolding (albeit limited); however, they never proliferated to the same extent or became stratified as in the gravid horn [8]. This suggests that endothelial proliferation may be stimulated in a paracrine manner by growth factors emanating from closely-associated cytotrophoblast and/or allantoic mesenchyme present only in the gravid horn. This proliferation may also be driven by relatively hypoxic conditions in and around the uteroplacental vessels during much of their development [13], when they are not functional. The nearest functional capillary beds during this period would be in the surrounding allantoic mesenchyme [8].

Normally, in most blood vessels of adult mammals, endothelial cells are disposed as squamous monolayers and have low proliferative indices. In the uteroplacental vessels serving the discoidal placenta of *M. rufus*, maternal endothelial cells clearly break these rules. Initially at least, a multilayered state was attained by endothelial hypertrophy and hyperplasia on progressively expanded and infolded basal laminae. This gave rise to a unique vascular epithelium in which most endothelial cells were arranged as bilayer sheets insinuated between basal laminae. This presumably reflected a continued effort by the endothelial cells to grow as monolayers (Figure 8).

When the embryo reached about 11.4 mm, however, several important changes became evident: 1) the number of endothelial cells within the original vascular basal laminae markedly increased; 2) gaps frequently developed in the endothelial basal laminae; and 3) glycoprotein granules temporarily became less common in the endothelial cells. Depending upon the location of the gaps, these had probably been created by proteinases secreted by either the endothelial cells or cytotrophoblast. Both cells types have this capability in other species [14-17]. That said, it should be noted that a very frequent branching of endothelial chains was observed within the vascular epithelium, while trophoblastic infiltration was minimal or absent in many areas. This suggests a central role for endothelial proteinases in much of the basal lamina breakdown.

As placental development proceeded from this stage, the concentration of glycoprotein granules again increased in the endothelial cells, and their basal laminae became progressively more prominent. With reappearance of the latter, it also became evident that many of the endothelial cells were now arranged as interlaced, monolayer chains or sheets of rotund cells separated by basal laminae (Figure 8).

This change from a bilayer to a monolayer arrangement may occur in three possible ways: 1) partial basal lamina dissolution appears to free monolayer patches of endothelium; 2) ramification of the endothelial chains or sheets appears to set the stage for their further proliferation as monolayers; and 3) there may be a widespread merging of paired endothelial sheets into monolayers. Evidence of basal lamina breakdown and endothelial ramification during the appropriate time period was actually observed. The last mechanism initially seems less likely, as it would necessitate the merger of adjacent monolayer sheets with

opposite polarities and basal lamina attachments. This is not inconceivable, however. During normal branching morphogenesis, the epithelium of elongating mammary ducts transiently reorganizes into a highly proliferative, incompletely polarized, multilayered state. It then returns to a quiescent, highly polarized, bilayered, epithelial architecture [18].

Classification of the epithelium in the large uteroplacental vessels of *M. rufus* presents a challenge. Early in pregnancy, all of their endothelial cells rested on a basal lamina, albeit a progressively infolded one. This would then constitute a pseudostratified epithelium. As pregnancy progressed, however, all of the largest vessels became lined by a much-thickened epithelium consisting predominantly of endothelial cells with some intermingled cytotrophoblast. Many of the cytotrophoblast cells were clustered as stratified masses. Although the endothelial cells continued to proliferate as monolayers, this was associated with the development of many breaks in their basal laminae and frequent branching of the chains or sheets. Ultimately, this created a stratified endothelium, but in a clearly unique manner.

Considerable effort has been made to define the complex cellular and molecular mechanisms controlling blood vessel formation, the transformation of small into large vessels, and the regulation of luminal size. All of these processes involve endothelial cells and are of high pathophysiological relevance [17, 19-22]. Further work with the discoidal placenta of *M. rufus* should provide new insights into these processes, because development of its large uteroplacental blood vessels deviates so markedly from what is seen in more commonly-studied vascular model systems. This includes: 1) the rapid proliferation of many endothelial cells with no direct exposure to blood or a patent vascular lumen; 2) the folding and branching of monolayer endothelial sheets, but no tubule formation or tubular outgrowth into the surrounding mesenchyme; 3) the formation of a multilayered endothelium, although continued growth of the endothelial cells as monolayers seems to be retained; 4) the development of endothelial cells with a cuboidal or columnar form and a greatly enhanced capability to form basal laminae; 5) a possible expansion of the basolateral membrane domain of the endothelial cells; 6) no exposure of the endothelial cells to regular blood flow, high shear stresses, or pressure during a significant portion of the vessels' growth; and 7) the rapid transformation of small tuft arterioles and venules to much larger uteroplacental vessels with no recruitment of vascular smooth muscle, at least around their intraplacental segments.

Several lines of evidence suggest why this unique epithelium may have evolved. *M. rufus* develops two successive chorioallantoic placentae – an extensive diffuse endotheliochorial placenta (functional during mid-gestation) and a discoidal hemochorial placenta (functional late in pregnancy). Development of the discoidal placenta may have permitted the mothers to give birth to larger, more precocious infants than could have been supported only by the diffuse placenta. This may be protective against injurious falls and predation (e.g., by owls and snakes) in the bats' natural roosts. Weights for newborn *M. rufus* are not available; however, the two largest fetuses obtained in the present study constituted 16.6 – 17.6 % of the mothers' mass with the fetuses still *in utero* or 19.9 – 21.5 % with the fetuses removed. The neonatal mass of *Molossus molossus* was reported to be about 23.2 % of maternal mass [23]. Uteroplacental blood vessels serving the discoidal placenta clearly become much larger than those in the diffuse [7], but relative blood flows and efficiency of physiological exchange for the two placentae are unknown.

As uteroplacental vessels serving the discoidal placenta develop from very small arterioles and venules [7-9], significant strengthening of their walls must become necessary. These vessels not only help to attach the placenta to the uterine wall, but would also presumably be subject to considerable mechanical stress when heavily pregnant females return from

foraging. *M. rufus* commonly roost under roofs or in tree hollows [10]. This means that their abdomens must absorb substantial impact upon landing at small openings along the eaves of buildings or the entrances to tree hollows. These are fast-flying bats that do not hover, although they probably decelerate to some extent immediately before landing. The impact problem may also be aggravated as pregnancy progresses by the foraging pattern of *M. rufus*. Rather than feeding periodically throughout the night, foraging seems to be highly concentrated around dusk and dawn (JJ Rasweiler IV, unpublished observations).

Strengthening of the tuft arterioles and venules during this conversion appears to involve the proliferation of both endothelial cells and trophoblast. The larger, endothelial component is presumably reinforced by amplification of its basal laminae, in the form of a complex three-dimensional meshwork, as well as the tethering of the vimentin-rich cytoskeleton of its cells to adhering junctions and to extracellular matrix via focal adhesions [24]. Most of the cytotrophoblast cells, on the other hand, contain a cytokeratin-rich cytoskeleton no doubt linked to adhering junctions. Where cytotrophoblast completely replaces the stratified endothelium, the cytotrophoblast must be capable of supplanting the presumed mechanical function of the latter. This has important implications with respect to some of what abundant trophoblast may be doing in the uterine vessels of other bat species (see below).

Cytotrophoblast was intimately associated with both endothelial cells and endothelial cell basal laminae within the vascular epithelium of the uteroplacental vessels. Apparent binding of these cell types suggests that they probably express some of the same adhesion molecules. This has previously been noted for the cytotrophoblast cells that invade the spiral arterioles serving human and macaque placentae [25-29]. At least during early development of the discoidal placenta in *M. rufus*, cytotrophoblast also clearly has an affinity for the endothelial basal laminae of the vascular tuft and uses them as a morphogenetic scaffold [8].

Modifications that probably strengthen the uteroplacental vessels and maternal vascular channels within the placenta have also been observed in several other bat species. In the white-winged vampire bat, *Diaemus youngi*, large amounts of trophoblast invade maternal vessels serving its discoidal placenta via intramural routes and progress at least as far as the broad ligament. This trophoblast co-expresses cytokeratins and vimentin and is often associated with extracellular matrix material of trophoblastic origin [12]. Mechanical reinforcement provided by these components could be particularly important in the case of arterial vessels, because smooth muscle fibers in their tunicae mediae dedifferentiate as pregnancy progresses to mesenchymal-like cells. This presumably facilitates physiologically-important vessel dilatation. It seems most unlikely that endothelial cells significantly strengthen these vessels, as they remain as squamous monolayers throughout gestation. One must suspect that the highly invasive trophoblast may also play a central role in inducing dedifferentiation of the vascular smooth muscle [12].

It is likely that the uteroplacental vessels of heavily gravid *D. youngi* are subject to significant mechanical stress upon landing while foraging or at roosts. This is both a cave and tree hollow dwelling species [30]. The birth weight of *D. youngi* is unknown; however, the largest fetus collected was furred and weighed 27.3% of the mother with the fetus still *in utero* [12]. Furthermore, such stress would presumably be even greater, when heavily-gravid females are also carrying recently-ingested blood meals. The weight of blood consumed by the closely-related, common vampire bat (*Desmodus rotundus*) is known to create significant flight problems for the animals immediately after feeding [31]. Highly invasive trophoblast has been observed in the walls of blood vessels supplying the uterus of *D. rotundus* [Rasweiler, unpublished observations].

In *D. youngi*, *D. rotundus* and many other bats, the interhemal barrier in the placenta also contains an unusual extracellular layer, the intrasyncytial lamina. This is thought to be derived at least initially from the basal lamina of maternal endothelial cells [12, 32-36]. One important function of the intrasyncytial lamina appears to be to strengthen maternal vascular channels [33, 37]. Clearly, this is a recurring problem for these animals.

Maternal blood vessels serving the placenta of another bat, *Nycteris thebaica* undergo major modifications involving their endothelial cells, possibly for similar structural reasons. As the main artery enters the junctional zone beneath the placenta, it becomes dilated and acquires a thick matrix. This is filamentous and contains embedded cells apparently derived from the luminal endothelium. The walls of major veins in the area also have a prominent matrix with numerous embedded cells, some of which are confluent with maternal endothelial cells lining the vessel lumina. How these modifications develop is unknown [38].

In platyrrhine primates, some intraplacental maternal vessels persist until term. These are characterized by cuboidal to columnar endothelial cells, surrounded by thickened subendothelial basal laminae and an outer, fibril-containing layer [39, 40]. Ultrastructural observations indicate that the endothelial cells probably synthesize the basal laminae. It has been suggested that this subendothelial sheath may develop to provide mechanical support for the vessel walls and/or to resist trophoblastic invasion [40].

Pronounced endothelial hypertrophy has also been noted in endometrial vessels associated with the implantation site and maternal veins immediately adjacent to the trophoblastic shell of macaque placentae [16, 41]. In the latter situation, this appears at low magnification to be associated with a possible clustering or multilayering of the endothelial cells close to the shell. This begs further morphological characterization, and its functional significance remains obscure.

In view of the pronounced endothelial proliferation that occurs in the uteroplacental vessels serving the discoidal placenta of *M. rufus*, the question arises as to what role endothelial progenitor cells [42, 43] might play. Several lines of evidence suggest that this may be minimal or nil. For a significant portion of the period when such proliferation is pronounced, the discoidal placenta is not yet functional as an organ of physiological exchange. Blood flow through the developing uteroplacental vessels only occurs after small tubules of the placental labyrinth become patent [7, 8]. Thus, it would be impossible for bone marrow-derived progenitor cells to even gain access to much of the highly proliferative endothelium, except possibly in those limited regions where blood flow has been blocked. Finally, both before and after flow recommences through these vessels, mitotic figures are commonly observed deep within their multilayered endothelium. It seems unlikely that these would involve newly-seeded progenitor cells.

Although the pronounced proliferation and stratification of endothelial cells in some uteroplacental vessels of *M. rufus* are highly unusual, one can envision how this novel method of strengthening the vessel walls may have evolved. In the event of pregnancy, small vessels of the vascular tuft in the right horn provide a scaffold upon which cytotrophoblast proliferates to initiate development of the discoidal placenta. This suggests that the tuft endothelial cells may be secreting factors that influence trophoblastic proliferation and differentiation, and that these are at least partially incorporated into their unusually prominent endothelial basal laminae [8]. In the evolution of *M. rufus*, natural selection probably worked to strengthen the uteroplacental vessels by further enhancing the proliferative capabilities and basal laminae of their endothelial cells. The development of an appropriately-timed process for creating frequent gaps in their basal laminae was also no

doubt essential. This permits a marked increase in vascular diameter and wall thickness, as well as the interlocking of layers within the stratified endothelium.

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Fig. 1.

(A) Section through a uteroplacental vessel within the base of the developing discoidal placenta associated with an 11.4 mm embryo. Endothelial cells (e) had proliferated greatly within, and to some extent outside of, the original basal lamina (b) of the vessel. The vessel was surrounded by a cuff of more basophilic cytotrophoblast (cyt) that had become extensively canalized to form channels. These will convey maternal blood into and out of the labyrinth. Lumen of uteroplacental vessel (l). Amylase-PAS-hematoxylin. Scale bar: 50 μ m. (B) Higher power view of a parallel section of the same vessel shown in (A). Numerous mitotic figures (arrowheads) are evident within the now multilayered endothelium (e). Note that gaps (*) are present in the original basal lamina (b) of the vessel, and endothelial cells had proliferated outside it. The adjacent cytotrophoblast cells (cyt) were generally more basophilic than the endothelial cells. Clusters of glycoprotein granules (gp) were prominent in some, but not all, of the endothelial cells. Lumen of vessel (l). Amylase-PAS-hematoxylin. Scale bar: 25 μ m.

Fig. 2.

(A) Section of uteroplacental vessel associated with a 19.0 mm embryo. Glycoprotein granules (gp) and segments of basal laminae are now more prominent within the endothelium (e) than in Fig. 1. A sizeable gap (*) had developed in the original basal lamina (b) of the vessel, and many endothelial cells had proliferated outside it (e.g., at X). Vascular channels (vc) for maternal blood had formed in the cytotrophoblastic cuff (cyt) surrounding the uteroplacental vessel. Lumen of vessel (l). Amylase-PAS-hematoxylin. Scale bar: 50 μ m. (B) Section of developing uteroplacental vessel associated with an 18.5 mm embryo. A continuous, thickened basal lamina is no longer evident between the endothelium (e) and the cytotrophoblastic cuff (cyt) surrounding the vessel. The magenta pink staining of the cytotrophoblast cytoplasm is attributable to its greater content of glycogen. Glycoprotein granules (gp) are more prominent within the endothelium (e.g., around e on the right) than in Fig. 1. Vascular channel (vc) that had formed within the cytotrophoblastic cuff. Lumen of vessel (l). PAS-hematoxylin. Scale bar: 50 μ m. (C) Higher power view of the same section shown in (B). Glycoprotein granules are evident in many of the endothelial cells (e), while the cytotrophoblast (cyt) has a magenta pink coloration due to staining of its glycogen. Note

also the larger, more euchromatic nuclei and more prominent nucleoli in the cytotrophoblast cells. Lumen of vessel (l). Tubule of the placental labyrinth (pl). Vascular channel (vc) for maternal blood that had developed within the cytotrophoblastic cuff. PAS-hematoxylin. Scale bar: 25 μ m. (D) Section of developing uteroplacental vessel associated with a 16.5 mm embryo. A long discontinuity is evident (between the arrowheads) in the original basal lamina (b) of the vessel. Some of the basal lamina approaching the arrowheads appears thinner and may have been newly-synthesized or stretched. PAS-positive glycoprotein granules and segments of basal lamina are more prominent within the endothelium (e.g., around e) than in previous figures. Some endothelium with these characteristics is now also clearly evident outside of the original basal lamina (e.g., at X). Cytotrophoblast (cyt) lines most of the large vascular channels (vc) that connect the uteroplacental vessels to the placental labyrinth (pl). Allantoic mesenchyme (am). Lumen of uteroplacental vessel (l). Amylase-PAS-hematoxylin. Scale bar: 50 μ m.

Fig. 3.

Sections of the walls of two uteroplacental vessels associated with a 24.3 mm embryo. (A) Segments of basal laminae with associated glycoprotein granules (arrowheads) are much more prevalent than in previous figures. In some areas, the endothelial cells (e) appear to be organized as bilayers (bi) or acinar-like clusters (a) due to endothelial folding. Note that most of the endothelial cells constitute part of an extensive, interconnected network. Some cells with larger, more euchromatic nuclei and prominent nucleoli are probably cytotrophoblast (cyt). Allantoic mesenchyme (am). Lumen of vessel (l). Amylase-PAS-hematoxylin. Scale bar: 25 μ m. (B) In this section, most endothelial cells (e) appear to be organized as monolayers. Glycoprotein granules are abundant (e.g., at arrowhead). In many areas (e.g., around *) two basal laminae with an intervening gap separate adjacent layers of endothelial cells, again as a result of folding. Two mitotic figures (arrows) are evident deep within the endothelium. Some cytotrophoblast cells (cyt) with larger, more euchromatic nuclei are also present. Allantoic mesenchyme (am). Lumen of the vessel (l). Amylase-PAS-hematoxylin. Scale bar: 25 μ m.

Fig. 4.

(A) Section containing portions of two adjacent uteroplacental vessels associated with a 24.3 mm embryo. Sizeable patches of endothelial cells (e) and cytotrophoblast (cyt) are present in their walls. In the vessel on the left, cytotrophoblast (*) has overgrown the luminal side of the endothelium. In the vessel on the right, cytotrophoblast had grown through the vessel wall nearly to the lumen (l); however, a covering layer of endothelium persists. A few cytotrophoblast cells are also intermingled with the endothelium (e.g., at arrowheads). Patch of necrotic tissue (nt). Amylase-PAS-hematoxylin. Scale bar: 50 μ m. (B) Higher power view of the section depicted in (A). The thin, PAS+ layer surrounding many of the cytotrophoblast cells (e.g., at the arrowhead) presumably represents their basal lamina and/or secreted extracellular matrix material. Note that in many areas (e.g. around *) two basal laminae separate adjacent chains of endothelial cells. Lumen of vessel (l). Amylase-PAS-hematoxylin. Scale bar: 25 μ m. (C) Section of uteroplacental vessel associated with a 27.1 mm embryo. In this region, much of this vessel wall is composed of stratified cytotrophoblast (cyt), although a small patch of endothelium (e) is evident as well. Cytotrophoblast-lined tubules of the placental labyrinth (pl). Lumen of the uteroplacental vessel (l). Amylase-PAS-hematoxylin. Scale bar: 50 μ m.

Fig. 5.

(A) Section of a uteroplacental vessel associated with a 24.3 mm embryo. Four mitotic figures (arrowheads) are evident well within the endothelium (e). Note that many of the endothelial cells constitute part of one continuous, interconnected network. Some of the cells on the surface of the epithelium (e.g., at *) may be cytotrophoblast. PAS-hematoxylin. Scale bar: 25 μ m. (B) Section of a uteroplacental vessel associated with a 33.3 mm embryo. This is lined predominantly with a multilayered endothelium (e), although a few probable cytotrophoblast cells (arrowheads) are also intermingled. A prominent meshwork of basal laminae is evident around the endothelial cells. At the arrow, there is a sharp transition from stratified endothelium to a monolayer of cytotrophoblast (*) typical of the placental labyrinth (pl). Cytotrophoblast-lined vascular channel (vc) leading to the placental labyrinth. Allantoic mesenchyme (am). Lumen of vessel (l). Amylase-PAS-hematoxylin. Scale bar: 50 μ m. (C) Higher power view of the epithelial lining of the vessel shown in (B). This consists predominantly of endothelial cells (e) apparently linked together in the form of a complex monolayer network and associated with a prominent meshwork of basal laminae. Glycoprotein granules are generally much less evident within the endothelial cells than in earlier stages. A cytotrophoblast-lined vascular channel (vc) leading to the placental

labyrinth is evident at the top of the figure. Allantoic mesenchyme (am). Lumen of vessel (l). Amylase-PAS-hematoxylin. (A), 25 μ m.

Fig. 6.

(A) Section of a uteroplacental vessel and surrounding placental parenchyma, associated with a 25.5 mm embryo, stained for the expression of vimentin. The vascular epithelium consists predominantly of vimentin-positive, maternal endothelial cells (e). A few vimentin-negative cytotrophoblast cells (cyt) are intermingled and also line a continuation of this vessel (at the bottom) as a monolayer. Fetal endothelial cells and mesenchymal cells surrounding tubules of the placental labyrinth (pl) are also vimentin-positive. Lumen of uteroplacental vessel (l). (B) Section of a uteroplacental vessel and surrounding placental parenchyma, associated with a 30.5 mm embryo, stained for the expression of cytokeratins with antibody mixture AE1/AE3. The lumen (l) of this vessel is lined either by maternal endothelial (e) or cytokeratin-positive cytotrophoblast (cyt) cells. Cytotrophoblast also underlines the endothelium at *. Small tubules of the placental labyrinth (pl) are entirely lined by cytotrophoblast. Fetal blood vessel (f) in the allantoic mesenchyme. (C) Section of a uteroplacental vessel, associated with a 30.5 mm embryo, stained for the expression of cytokeratins with antibody mixture AE1/AE3. The maternal endothelium (e) lining this vessel contains scattered, cytokeratin-positive cytotrophoblast cells (e.g., at arrowheads). A

small mass of cytotrophoblast also underlies the endothelium at *. Surrounding connective tissue (ct). Lumen of vessel (l). (D) Section of a uteroplacental vessel, associated with a 30.3 mm embryo, stained for the expression of von Willebrand factor. This was granular in distribution (arrowheads) and strongest in endothelial cells (e) close to the vessel lumen (l). Expression was also present, but weaker, in many of the deeper endothelial cells. The arrows denote the border between the vascular epithelium and surrounding connective tissue (ct). Cytotrophoblast-lined vascular channel (vc). Scale bars: 50 μ m (A-C), 25 μ m (D).

Fig. 7.

(A) Higher power view of a section through the stratified endothelium in the uteroplacental vessel depicted in Figure 6D, stained immunocytochemically for von Willebrand factor. Reactive granules are abundant in many of the endothelial cells (e.g., at arrowheads). Scale bar: 10 μ m. (B) Portion of the myometrium in the same section depicted in (A). Von Willebrand factor is also strongly expressed by the endothelial cells (e.g., at arrowhead) lining a more typical venule. Scale bar: 10 μ m.

Fig. 8.

Diagram depicting probable steps in the formation of the stratified endothelium in uteroplacental vessels serving the discoidal placenta of *M. rufus*. To facilitate understanding, the endothelial chains or sheets have been dispersed in this artistic rendition. No effort has been made to depict the final complexity of the stratified endothelium which was difficult to trace *in vivo*. (A) Vessel supplying the endometrial vascular tuft lined by a squamous endothelium with its associated basal lamina. (B) Vessel lined by a hypertrophied endothelium on an early, infolded basal lamina. (C) Vessel lined by a hypertrophied endothelium on a greatly infolded basal lamina and surrounded by a cuff of cytotrophoblast. 1. Acinar-like structure created by the sectioning of an endothelial fold or recess. (D) Vessel

lined with a stratified epithelium in which many endothelial cells are now in the form of monolayer chains or sheets. 1. Sites of breaks or gaps in the endothelial sheets and their basal laminae. These permit the outward proliferation of endothelial cells and the inward proliferation of cytotrophoblast. 2. Region of monolayer endothelial chains or sheets. Some endothelial cells are depicted without basal laminae, but the actual extent of basal lamina breakdown *in vivo* is uncertain. The appearance of the stratified endothelium in histological sections indicates that branch points and interconnections must also develop at frequent intervals in and between the chains or sheets. 3. Region with a persisting bilayer pattern of endothelial organization. 4. Inward proliferation of cytotrophoblast from the cuff surrounding the vessel. 5. Maternal blood spaces in the cytotrophoblastic cuff. These will provide links to the placental labyrinth.