New Cells and Old Vessels: The Remodeling of the Endometrial Vasculature during Establishment of Endometrial Cups


Department of Cell Biology & Human Anatomy, School of Medicine
Department of Reproduction, School of Veterinary Medicine
University of California, Davis, California 95616

ABSTRACT

Endometrial cups are very poorly vascularized, in striking contrast to most endocrine glands. The nature and establishment of the vascular bed supplying the cups were examined, particularly the role of blood vessels and lymphatic vessels in relation to the maintenance and function of this unique structure. Endometrial cups were examined by light and electron microscopy between Day 36 and Day 83 of pregnancy. At the time of invasion of trophoblastic girdle cells into the endometrium, an extensive vascular bed and subepithelial capillary plexus are present as well as dilated superficial lymphatic vessels. Enlarging cup cells enter and apparently fill these lymphatic vessels and then the deeper lymphatics. Cup cells soon occupy most of the endometrial stroma. The vasculature of the mature cup is sparse, as there is no evidence of angiogenesis or hypervascularity accompanying the ingression of trophoblast cells. Moreover, the extracellular matrix surrounding the vessels thickens and sometimes contains leukocytes. The elimination of lymphatic vessels and restricted permeability of blood vessels contribute to isolation of cup cells; this may initially facilitate immunological isolation but also may eventually lead to necrosis and consequently elicit rejection of the cups.

INTRODUCTION

As Steven [1982] reported in his review of the history of placentation in the mare, endometrial cups were seen and described [Schauder, 1912] long before Cole and his co-investigators established that these structures were the source of eCG [Cole & Hart, 1930; Clegg et al., 1954], and even longer before Allen and his associates established that the cup cells were derived from trophoblastic girdle cells [Allen & Moor, 1972; Allen et al., 1973; Hamilton et al., 1973]. Fully formed endometrial cups are readily visible, when the uterus is opened, as pale elongated raised regions on the otherwise pink endometrial surface. The epithelial nature of the cup and its poor vascularity produce this contrast, which is especially surprising in that most endocrine glands have a rich sinusoidal capillary network that is organized from a simpler capillary bed as the gland develops. In the present study, to verify that the endometrial cups are poorly vascularized, the vascularity of the mature cups (those in which the cup cells had completed their hypertrophy) was first examined. The second issue examined was the hypothesis that the basis of the poor vascularity is a lack of angiogenesis during formation of the cups from the time of initial girdle cell invasion through hypertrophy. Any changes in the nature of the blood vessels or the lymphatics were analyzed to consider the role of vascularization in allowing foreign epithelial cells to function within the endometrium.

MATERIALS AND METHODS

A series of stages of endometrial cups was collected from 11 mares on Day 36 (2), Day 37 (2), Day 38 (1), Day 42 (1), Day 45 (1), Day 51 (1), Day 67 (1), Day 68 (1), and Day 83 (1) of pregnancy. Mares were mated after detection of a preovulatory follicle and were examined by ultrasonography to confirm the presence of a conceptus. On the designated day of pregnancy expressed as days from ovulation, mares were killed with i.v. sodium pentobarbital, and the reproductive tracts were removed. Uteri were fixed initially by perfusion of the uterine artery with aldehyde fixative, and regions of endometrium containing cups were removed. After subsequent fixation for 2–4 h (2% formaldehyde-2% glutaraldehyde in 0.1 M phosphate buffer, or 4% formaldehyde in 0.1 M phosphate or 0.1 M cacodylate buffer), cups were further dissected in PBS, and slices from various regions of representative cups were selected. Tissues to be examined by transmission electron microscopy were osmicated and embedded.

1These studies were supported by grant HD10342 from the National Institute of Child Health and Human Development, and by the Equine Research Laboratory of the University of California.

2Correspondence. FAX: (916) 752-8520.
in Araldite epoxy resin. Representative slices were prepared for routine light microscopy, periodic acid-Schiff (PAS) staining, and immunocytochemical staining methods by embedding in paraffin.

Immunostain protocols were as follows. Sections of paraffin-embedded tissues were deparaffinized and rehydrated through a graded ethanol series; they were then treated with 0.1% trypsin in PBS for 25 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide, and nonspecific antibody binding was blocked with normal rabbit serum. The primary antibody used was a rabbit polyclonal antibody prepared against laminin (Sigma Chemical Co., St. Louis, MO). Sections were incubated in primary antibody for 1 h at room temperature and then in biotinylated secondary antibody (goat anti-rabbit IgG). After treatment with enzyme conjugate (streptavidin-peroxidase), the presence of peroxidase binding was visualized by a diaminobenzidine reaction product. In the control for this procedure, normal rabbit serum was substituted for the primary antibody; this in all cases eliminated specific staining.

To quantify the changes in endometrial gland and blood vessel distribution, 1-μm-thick sections of plastic-embedded tissues were chosen in which three to five contiguous fields could be counted at 60 × magnification, just beneath the surface epithelium in cup and noncup endometrium. The mean values and standard errors per field were converted to numbers of gland and vessel profiles intersected by the section per square millimeter (Table 1). For further verification of changes, the area occupied by glands and by vessels was calculated by transferring images of the subepithelial regions to a Macintosh Quadra 950 computer; the glands and vessels were outlined, and their areas were calculated as a percentage of the field occupied through use of the NIH-Image program (Table 2).

Sections from cups of five animals from Days 37 to 45 constituted the developing cup group, and the four animals from Days 51 to 83 were the source of the mature cup group. Endometrium from two animals in each of these groups and from a Day 36 animal were used for the endometrial samples. There was some variation as seen by the range, but the variation ap-

---

TABLE 1. Number of vessels at different stages.

<table>
<thead>
<tr>
<th>Region</th>
<th>Vessels/mm²</th>
<th>Range</th>
<th># Fields counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrium</td>
<td>20.7 ± 7.2*</td>
<td>18–33</td>
<td>15</td>
</tr>
<tr>
<td>Cup, Days 37–45</td>
<td>10.2 ± 2.0*</td>
<td>7–16</td>
<td>15</td>
</tr>
<tr>
<td>Cup, Days 51–83</td>
<td>6.1 ± 2.5*</td>
<td>4–13</td>
<td>24</td>
</tr>
</tbody>
</table>

* Mean ± SD.

---

TABLE 2. Percent of endometrial area occupied by glands and vessels.

<table>
<thead>
<tr>
<th>Region</th>
<th>Gland area*</th>
<th>Vessel area*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrium</td>
<td>32.97 ± 7.3%</td>
<td>6.7 ± 2.9%</td>
</tr>
<tr>
<td>Mature cups</td>
<td>6.70 ± 2.1%</td>
<td>1.00 ± 0.35%</td>
</tr>
</tbody>
</table>

* Mean ± SD.
RESULTS

The results are divided into findings that describe the mature endometrial cup (that having fully hypertrophied cup cells and including specimens collected on Days 51–83) and findings that describe stages in cup formation, Days 36–45. A number of representative cups from each animal were selected for examination by the various procedures.

Vasculature of the Mature Endometrial Cup

In the uterus near endometrial cups, radial vessels enter the endometrium and branch laterally to provide rich vascular beds to the glandular endometrium as well as an extensive capillary plexus beneath the surface epithelium. The vascular bed to the cup was sparse; there was larger spacing between capillaries and in deeper regions between arterioles and returning venules in the cups than in the endometrium lateral to the cups or in adjacent non-cup endometrial folds (Fig. 1). The vessels proceeded between cup cells to the area beneath the residual basal lamina of the luminal epithelium before retracing their pathway to the region of the endometrium beneath the cup. Consequently those girdle cells remaining above the luminal basal lamina were unvascularized. Thick sections of cups showed the vessels to the cups spreading from a central region and more sparsely distributed in the area that is rich in cup cells than at the periphery, which is rich in glands (Fig. 1). Although the vessels just beneath the cup and in the more loosely arranged basal regions of the cup often showed the rounded outline, thin walls, and expanded lumina associated with dilation, vessels within the cup (in contrast to the vessels of nearby non-cup endometrium) normally showed angular walls and narrow lumina.

Counts of the vessels in cup and non-cup endometrium graphically demonstrated the sparsity of vascularity within the mature cup and an intermediate decline in number of vessels per square millimeter in those cups in which invasion and hypertrophy of cup cells had not been completed. Cups with fully hypertrophied cup cells averaged only 6.1 vessels/mm², and immature cups 10.2 vessels/mm², whereas non-cup endometrium had approximately 3 times (20.7) the number of vessels per square
millimeter (Table 1). When the percentage of the area occupied by glands and vessels was calculated for mature cups, as opposed to endometrium, the decline in area occupied by both of these structures was obvious (Table 2).

In fully developed endometrial cups, in which hypertrophy of the cup cells had been completed (e.g., Days 51–68), the vessels were farther apart than in other regions of the endometrium. In the subluminal position, both vessels and glands were particularly sparse (Fig. 2). In addition, most of the vessels had a thick layer of extracellular matrix material surrounding them (Fig. 3). This material was PAS-positive, indicating that it contained glycosaminoglycans, and stained with an antibody to laminin (Fig. 4). It was therefore composed of materials similar to that of basement membrane and should probably be considered a thickened basement membrane, which is often present as more than a single layer.

The smallest vessels within the cup, the capillaries, usually lacked pore regions and were not as thin as the sinusoidal vessels of most endocrine glands. When an occasional area of endothelium with pores was found, it was short and had only a few pores. The endothelial cells of some of the venules had pericytes and perivascular fibroblasts within redundant layers of basal lamina material.

The large cup cells, many of which were binucleate, had extensive regions of ruffled borders whether they were in contact with one another or were adjacent to a capillary or the matrix associated with the vessels. Small granules were PAS-positive and were most numerous in Golgi regions of the cells, and accumu-
FIG. 4. A) In this micrograph of a Day 51 cup, the matrix surrounding the vessel has been stained by PAS, as has the secretion product in the apices of some of the cells in a tangentially sectioned gland (gl). B) Antibodies to laminin indicate that the matrix (arrows) surrounding the two vessels in the center is least partially composed of this basal lamina constituent; Day 64. ×320.

lations of the granules could be seen at the periphery of the cells. These remained small, and, although they were more numerous in some cells than in others, they never accumulated in the amount seen in the gonadotropin-producing cells of the pituitary.

No functional lymphatic vessels were found within the cup, and the deeper endometrial lymphatics of mature cups did not have any intruding cup cells.

The small areas of connective tissue adjacent to some of the vessels had a few fibroblasts, many of which were vacuolated and some of which showed cytoplasmic blebbing. In other areas, only extracellular materials intervened between the endothelium of the vessels and cup cells. The impression was that a number of fibroblasts must have disappeared from the original endometrium, but no counts of fibroblasts were made. Occasionally vessels were in contact with the basal lamina of the endometrial glands. However, since trophoblastic girdle cells invaded initially between the basal lamina of the glands and the gland cells, as well as directly into the stroma, cup cells separated the subepithelial vessels from most of the glandular epithelial cells, especially in the more superficial areas of the cup.

In cups from Day 68 and Day 83, the matrix surrounding the few vessels was thicker than in cups earlier in pregnancy. In addition, leukocytes were often seen within the matrix (Fig. 5). In cups from the Day 45 and 51 specimens, only a few of the leukocytes proceeded beyond the perivascular matrix; but in older stages, regions showing both leukocyte invasion and large numbers of lymphocytes and plasma cells were seen.

When there was appreciable necrosis either of the glands or, in later stages, of cup cells other than those above the residual uterine luminal basement membrane, there tended to be an increase in numbers of leukocytes entering areas of cup cells. Necrosis of the supraluminal cup cells did not appear to elicit a leukocyte response.

Origin of the Vasculature of the Forming Cup (Days 36–45)

Just before and at the time of invasion of girdle cells into the endometrium (Day 36), a rich endometrial vasculature, including a subepithelial network of capillaries (Fig. 6), was observed. When the girdle cells initially invaded into the endometrial stroma, they neither disturbed nor stimulated the vascular bed. Blood vessels were not damaged, and little if any extravasation of erythrocytes into the stroma occurred. At the same time, and in marked contrast to observations in non-equid species, trophoblast invasion apparently did not produce endothelial mitoses, as mitotic figures were not seen in endothelial cells at this stage. The preexisting vascular network therefore appeared to be neither disrupted nor augmented; the capillaries and venules were not dilated, nor were the endothelial cells of these vessels hypertrophied.

As the binucleate trophoblastic girdle cells invaded further into the endometrium, they encountered the superficial lymphatic vessels situated at the level where the gland necks en-
ter the coiled portion of the gland (Fig. 7). These lymphatics were entered by the invading girdle cells by Day 37, a day after initial invasion of the endometrium. At subsequent stages, masses of cup cells appeared in clusters with a defined border, suggesting the filling of lymphatic vessels with the enlarging cup cells (Fig. 8). At any rate, superficial lymphatics could no longer be identified within the cups.

Cups from Days 37, 38, and 42 showed progression of the girdle cells into the endometrium and the beginning of cup cell hypertrophy. In this process, they did not seem to disturb the blood vessels, but to separate them...
from the neighboring gland cells. The deeper lymphatic vessels among the basal regions of the glands were encountered at this stage, and again developing cup cells were found within the more superficial aspects of the lymphatics from one of the Day 37 specimens and from the single Day 38 and Day 42 specimens studied. It was in this location that such invasion was first noted [Enders & Liu, 1991]. As with the original invasion of the lymphatics, the invading cup cells were adherent to cup cells outside of the vessels, and no evidence of dehiscence of these cells into the vessels was seen.

**Hypertrophy into Cup Cells**

Since the binucleate cells that form the surface of the girdle are columnar, both change in shape and hypertrophy occur following invasion of the endometrium. The cup cells appeared more rounded and larger in Day 42 cups than in the earlier cups. In cups from the Day 45 specimen, the cup cells had largely hypertrophied, reaching an average diameter of nearly 40 μm and a cell volume more than 5 times that of the invading girdle cells. The cup cells occupied all but traces of the connective tissue space originally present. In addition, the glands were separated by a greater distance than before invasion; the spread of vessels also suggested that the area of initial migration had been expanded by the hypertrophy of the forming cup cells, as did the reduced areas for these structures seen in mature cups and reported above.

**Changes in Intra-Cup Endometrial Glands**

Interestingly, although a few areas remained in which cup cells were present between the basal lamina of glandular epithelium and its epithelial cells, for the most part the glands were both well organized and patent. However, the opening onto the cup surface appeared to be restricted, and in cups from the Day 51 specimen and later, the secretion of at least the more superficial regions of the glands appeared viscous. The deeper portions of the glands beneath cup cells were slightly dilated, but the content appeared more aqueous. The material accumulating in the forming cup was particularly well seen in cups from the Day 51 specimen, where individual masses of secretory material, perhaps corresponding to the secretions from single glands, could be visualized within the masses at the surface of the cups. Not only

---

**FIG. 6.** A) Endometrium beneath a region of unattached girdle on Day 37. Note the abundant subepithelial capillaries (arrows). B) Area of the same endometrium in a region where girdle cells have invaded the endometrial stroma. Note that both the subepithelial vessels (arrows) and deeper vessels are undisturbed. ×240.
did glands within the cup produce this viscous material; those on the periphery were usually dilated and also contributed this secretory material. Unlike the situation in blood vessels, mitotic figures were seen in the epithelium of the glands during cup formation. However, this hyperplasia appeared to result in longer glands rather than in greater numbers of glands per endometrial fold.

In cups from the Day 68 and 83 specimens, areas of disrupted glands and necrotic cup cells were found; and especially in the later specimen, areas of granulocyte and lymphocyte invasion were common. The pattern of the glands was disrupted within the cup and the adjacent glands were greatly dilated by accumulated secretory material near the gland necks. Nevertheless, the vessels within the cups were intact and had a thick circumferential layer of matrix around even the most superficial capillaries.

**DISCUSSION**

It appears that girdle cells in invading the endometrium expand a region of the endometrium without stimulating the development of more vessels. Since the endometrial glands survive separation from their capillary bed,
VASCULATURE OF ENDOMETRIAL CUPS

FIG. 8. Superficial region of a Day 45 cup. A) The cup cells have completed their hypertrophy and occupy most of the space between the glands. Vessels already have a surrounding dark-staining matrix, and no lymphatic vessels are found within the cup. However, clusters of cup cells (*) are in configurations suggesting the filling of a superficial lymphatic vessel. B) Higher magnification of the lower left of (A). Note the margin around the cluster of cup cells, suggesting that these cells may have filled an existing lymphatic vessel. A) \( \times 80 \); (B) \( \times 200 \).

The development of redundant basal lamina-like material around the vessels further separates these structures from the cup cells, and again decreases the efficiency of exchange. It appears likely that initially the invading girdle cells expand to the capacity of the existing vascular system but, perhaps due to the absence of angiogenic factors, do not promote new vessel formation. As the area around the vessels increases in thickness, cytolysis may well be promoted. Failure of vascularization was suggested by Allen [1982] as a possible cause of cytolysis.

Since it has been shown that girdle cells bear paternal histocompatibility factors [Antczak et al., 1987; Allen et al., 1993], the finding that leukocytes accumulate in the layers around the vessels suggests that these investments may have a role in protecting the cup cells from the consequences of their foreign histocompatibility. From this point of view, the vasculature can be considered to be a compromise between isolation of the cup cells and maintenance of a viable area of the endometrium. The early elimination of the lymphatic vessels, the manner in which the cup develops as a solid mass of cells, and the restricted permeability of the vessels would all contribute to partial isolation of the majority of the cup cells. The eventual necrosis of both cup and gland cells would not only release more antigen but in addition lead to loss of isolation and consequently allow the sloughing induced by the rejection as described by Allen [1982].
REFERENCES


Clegg MT, Boda JM, Cole HH, 1954. The endometrial cups and allantochorionic pouches, with emphasis upon the source of equine gonadotropin. Endocrinology 54:448–463.


