Conceptus Influences the Distribution of Uterine Leukocytes During Early Porcine Pregnancy

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ABSTRACT

Pregnancy in humans and rodents is associated with dramatic changes in leukocyte populations within the uterus. In these species, recruitment of leukocytes, mostly natural killer (NK) lymphocytes, accompanies decidualization of endometrial stroma even in the absence of pregnancy. In the pig, a nondecidualizing species, the predominant lymphocytes in the pregnant uterus are T and/or NK cells, but their distribution relative to embryonic attachment sites has not been reported. The objective of this study was to compare the abundance of leukocytes in porcine endometrium in contact with trophoblast with that between attachment sites during the early postattachment period. Uteri were recovered on Days 15–17 (n = 4), 18 and 19 (n = 4), 21 and 22 (n = 5), and 25–27 (n = 2) of gestation and from cycling pigs during the luteal phase (Day 15; n = 3). Leukocytes were identified in uterus obtained at versus between attachment sites using an antibody reactive with all leukocytes (CD44). In all pregnant animals, leukocytes were diffusely scattered throughout the endometrial stroma but were rare or absent in the luminal epithelium. Leukocyte density was ~3-fold greater in endometrium in contact with conceptuses than in endometrium between attachment sites throughout the early postattachment period. Leukocyte density during the luteal phase was similar to that between attachment sites, suggesting that leukocyte recruitment was a localized response to the embryo. The ability of an individual porcine conceptus to recruit maternal leukocytes to the adjacent stroma may be a vital step in early placental development and embryo survival.

INTRODUCTION

Pregnancy is associated with dramatic changes in immune cell populations within the uterus. Changes in the distribution of maternal leukocytes with respect to implantation sites have been most intensively studied in mice, in which the predominant leukocyte type is an unusual lymphocyte of the natural killer (NK) lineage. These cells are randomly dispersed throughout the endometrium and myometrium in the nonpregnant murine uterus [1]. Beginning at approximately midgestation, uterine NK cells aggregate in the mesometrium at each implantation site, forming a structure known as the metrial gland (reviewed in [2]). In these regions, but not between implantation sites, these cells undergo further differentiation, eventually becoming larger and more granulated than NK cells in the circulation [3, 4]. In humans, up to 35% of stromal cells in first trimester decidua are leukocytes [5]. Uterine leukocytes are prominent in the decidua basalis in close proximity to invading trophoblast and scattered through the decidual stroma as either clusters or individual cells [6–8]. The predominant leukocytes of the secretory (luteal) phase endometrium and first trimester decidua are large, granular NK cells that are phenotypically distinct from their counterparts in blood [5, 9].

The epitheliochorial placenta of the pig contrasts with that of humans and rodents in that the placenta forms through attachment of the chorion to the maternal epithelium and six tissue layers separate maternal and fetal blood supplies [10]. There is no invasion into the stroma, and no decidualization. Beginning on Day 12, the porcine conceptus undergoes rapid elongation, forming a thread up to 1 m in length by Day 16. As a result of complex folding and twisting, each conceptus occupies 10–20 cm of luminal surface on the mesometrial aspect of the uterus. By Day 13, the attachment process begins, starting at the embryonic disk and spreading outward [11]. Even at this early stage, proximity to the conceptus is associated with localized changes in the maternal epithelium [11] and morphological changes in subepithelial capillaries [12].

Little is known about effects of the semiallogeneic conceptus on uterine leukocyte distribution in the pig. The major leukocyte types in the porcine uterus are lymphocytes, macrophages, neutrophils, and dendritic-like cells [13–15]. As early as Day 10 of gestation, when embryos are still freely moving within and between uterine horns, the number of lymphocytes within the luminal epithelium on the mesometrial side is reduced relative to that on Day 10 of the cycle [16]. The number of intraepithelial lymphocytes decreases further through the first 3 wk of gestation [16] and remains low until at least Day 80 [15]. In the endometrial stroma, the number of CD2+ lymphocytes was increased on the antimesometrial side of the uterus at Days 18–21 of gestation relative to Days 10–14 [15]. On the mesometrial side of the uterus, Whyte and Binns [13] noted an infiltration of stromal leukocytes in endometrium directly associated with conceptuses beginning on Day 18, when maternal-fetal contact is well established. NK-like lytic activity is elevated in porcine endometrial cells obtained during early pregnancy but not during the luteal phase [17, 18]. The fact that this response is not mimicked by hormone-induced pseudopregnancy or intrauterine infusions of nonviable semen suggests that conceptus-derived factors may be involved [18].

Because leukocyte density in endometrium in contact with conceptuses has not been compared with that in interattachment endometrium in the same animal, it has not been possible to distinguish local influences of the porcine conceptus from systemic effects of pregnancy. As a litter-bearing species, the pig is an ideal species in which to

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address this question in the context of noninvasive placentation. To get an unbiased assessment of leukocyte density, tissues were probed with CD44, an antibody reactive with all leukocyte classes in peripheral blood [19], rather than subset-specific antibodies. CD44 is a transmembrane glycoprotein involved in a broad range of leukocyte activities, including migration, adhesion, and cytotoxicity [20]. Our objective was to compare the distribution of leukocytes in endometrium in contact with trophoblast with that between attachment sites during the early postattachment period.

MATERIALS AND METHODS

Collection of Tissue

Yorkshire sows 12–18 mo of age were bred naturally on the first day of standing estrus (Day 0) and again 24 h later. Reproductive tracts were recovered at slaughter on Days 15–17 (n = 4), 18 and 19 (n = 4), 21 and 22 (n = 5), or 25–27 (n = 2) of gestation and at Day 15 of the estrous cycle (n = 3). Breeding and slaughter procedures were approved by the University of Guelph Animal Care Committee and complied with the guidelines of the Canadian Council on Animal Care. Uteri were trimmed free of mesentery and opened along the antimesometrial border to avoid disrupting conceptus attachment sites. Attachment sites were identified visually, in some cases using ultraviolet light to detect the conceptus-associated autofluorescence characteristic of early porcine pregnancy [21]. Three or four strips of tissue approximately 2–4 mm wide were collected from each animal, both from attachment sites (including both uterine and conceptus tissue) and from regions between attachment sites. These tissue samples are referred to as “at” and “between” samples, respectively. Care was taken to minimize disruption of maternal-fetal contact. In nonpregnant pigs, tissue was collected from random sites along the mesometrial aspect of the uterus.

Fixation of Tissue

Although preservation of morphology is usually superior in tissue embedded in paraffin versus frozen tissue, leukocyte surface markers are difficult to detect paraffin-embedded tissues, particularly after fixation in solutions containing formaldehyde. Beckstead [22] assessed the effect of various fixatives on staining of human lymphoid tissues with five different leukocyte surface markers. He reported little or no staining in tissues fixed with routine formalin, zinc-formalin, paraformaldehyde, ethanol, or a variety of commercial fixatives (not containing formalin) recommended for improved antigen survival. Only tissues fixed in solutions containing zinc as the primary fixative exhibited staining comparable to that obtained with frozen tissues. In the present study, morphology and binding to a leukocyte surface marker were assessed in paraffin-embedded porcine uterine tissues fixed in three formalin-free fixatives: zinc chloride (0.5% in 0.1 M Tris base buffer plus 0.05% calcium acetate, pH 6.8–7), Zenker solution (5% mercuric chloride, 2.5% potassium dichromate, 1% sodium sulfate in water plus 5% acetic acid added just before use), and ethanol (100%). Frozen sections of porcine spleen served as the positive control for both fixation effects on antigenicity and the immunostaining procedure itself. Tissues fixed in 4% paraformaldehyde in PBS (pH 7.4, fixed overnight and then transferred to 70% ethanol before further processing) served as the positive control for preservation of morphology. Tissues fixed in zinc chloride and Zenker solution were fixed for 6 h and then transferred to 70% ethanol before further processing. Samples were processed routinely for paraffin embedding (1 h each in ethanol [70% × 2, 95% × 2, 100% × 3], Sli-debrite × 3 [Syntex attachment devices, Jones Scientific Products, Kitchener, ON, Canada], paraffin × 3), except that tissues fixed in ethanol were added to the tissue processor at the first 100% ethanol stage. Blocks were sectioned at 5 μm and placed on slides coated with 3-aminopropyltriethoxy silane (ICN Biomedicals Canada, St-Laurent, PQ, Canada). Immunostaining was carried out within 1 wk of sectioning.

Immunohistochemistry

Indirect immunohistochemistry was carried out using the Histostain-SP Kit (Zymed Laboratories, South San Francisco, CA). This kit is based on a labeled-streptavidin-biotin method in which the biotinylated secondary antibody (goat anti-mouse IgG) is applied to sections after the primary antibody, followed by horseradish peroxidase coupled to streptavidin, then the substrate/chromogen mixture (hydrogen peroxide and aminoethyl carbazol, respectively). The kit was used as recommended by the manufacturer with the addition of a heating step before the primary antibody incubation to reduce background. Heat-treating of sections using either a microwave oven or a pressure cooker is often recommended to obtain positive staining (heat-induced epitope retrieval). However, we found it an effective way to reduce nonspecific binding to connective tissue (endometrial stroma). Initially, slides were immersed in 0.01 M sodium citrate, pH 6.0, and heated on high power in a 700-watt microwave oven for four periods of 5 min each and then incubated in the hot buffer for a further 20 min. However, this heating period reduced specific as well as nonspecific staining in some tissues. Ultimately, the citrate buffer was preheated to boiling (2 min on maximum), and slides were then added and incubated in the hot buffer for 20 min.

Leukocytes were identified using a primary antibody recognizing the CD44 surface antigen (clone BAT31A, IgG1; VMRD, Pullman, WA), which has been shown to bind to 100% of porcine peripheral blood leukocytes [19]. Some types of nonhematopoietic cells also express certain CD44 isoforms [23], including uterine epithelial cells [24]. However, this antibody bound to leukocytes but not epithelial cells in the same sections, indicating that it was reactive with a hematopoietic isoform of CD44. The suitability of this antibody as a panleukocyte marker in porcine uterine tissues was confirmed by comparing numbers of positive cells in adjacent sections stained with an antibody reactive with CD45 (clone 74-9; VMRD), the porcine homologue of the leukocyte common antigen. Numbers of cells staining with CD44 versus those stained with CD45 differed by <4% (data not shown). Uterine leukocytes were further characterized by identifying T cells, based on CD3 expression. CD3, a component of the T-cell receptor complex, is expressed by all T-cell subsets. The recently available anti-pig CD3 antibody (clone 8E5, IgG1; VMRD) recognizes the CD3 ε-chain [25]. Negative controls consisted of adjacent sections receiving an isotype-matched control (mouse IgG1; Sigma, Mississauga, ON, Canada) in place of primary antibody.

Quantification of Leukocytes in Uterine Tissue

Numbers of CD44-positive cells (indicated by red cytoplasmic staining) were determined in both epithelial and stromal compartments with the aid of image analysis software (Sigma Scan, Version 4; Jandel Scientific, San Rafael, CA). Ten sites per section were scanned microscopically using the 40× objective and saved to disk. For each site, the number of positive cells was determined per length of luminal epithelium (expressed as cells/mm²) and in the associated area of endometrial stroma to approximately 150 μm from the luminal surface (expressed as cells/mm², referred to as density). Only cells in tissue, as opposed to blood vessels, were counted. Data for sites within a section were summed to generate a single number for epithelial and stromal regions. Within pregnant animals, effects of stage and location (at versus between attachment sites) were compared using a 4 × 2 variable ANOVA. Because more than one data point was collected from each sow, animal was included in the model as a random effect. Means were compared using the mixed linear models procedure of SAS (PROC MIXED; SAS, Cary, NC), and data are presented as least squares means. Statistical significance was inferred at \( P < 0.05 \).

RESULTS

Tissue morphology with zinc chloride fixation was not as well preserved as with paraformaldehyde fixation. In sections of zinc-fixed tissues, the compressed appearance of the deep uterine glands relative to those in paraformaldehyde-fixed sections suggested that the zinc fixative had not penetrated as well as had the paraformaldehyde. Blebbing on the apical surfaces of some luminal epithelial cells resembled the apical domes described by Parr and Parr in the rabbit uterus, which were artifacts of fixation (perfusion versus immersion) [26]. Despite the slight compromise in morphology, zinc fixation was clearly superior for immunostaining; no specific staining was detectable in tissue fixed with paraformaldehyde, ethanol, or Zenker solution, but strong positive staining was obtained in zinc-fixed sections. In contrast to paraformaldehyde, zinc chloride fixation was stable indefinitely at room temperature, and tissue could be left in it up to 8 days before processing with no loss in antigenicity.

In pregnant animals, uterine morphology was similar at
attachment sites (352 attachment sites (Fig. 1). Stromal leukocyte density at attachment sites was closely associated with conceptuses than in areas between attachment sites (126 cells/mm²) or luteal phase animals (62 cells/mm²). Leukocyte density in stroma associated with conceptuses was significantly higher relative to either interattachment sites (126 ± 15 cells/mm²) or luteal phase animals (62 ± 11 cells/mm²). Leukocyte density between attachment sites was similar to that in nonpregnant uterus (P > 0.05). Stromal leukocyte densities with respect to stage of gestation are shown in Figure 2B. In the ANOVA, the main effect of stage was not significant (P > 0.05), indicating that the increased leukocyte density in stroma associated with conceptuses was consistent throughout the early postattachment period.

The morphology of most CD44+ cells (round nuclei, low cytoplasm:nucleus ratio) suggested that they were lymphocytes. Previous histological studies had indicated that most of the lymphocytes in the porcine uterus were either T cells or NK cells. We attempted to probe tissues with an antibody reactive with CD16, which is expressed by porcine NK cells, most granulocytes, and neutrophils [27]. Using procedures established for CD44 and CD45, we were unable to demonstrate CD16 immunostaining, despite the fact that we have shown in a flow cytometric study that this antibody binds to porcine endometrial cells [28]. The epitope recognized by the CD16 antibody may be even more sensitive to fixation than are the other leukocyte surface markers. Although we were unable to localize NK cells, T cells were identified by assessing CD3 reactivity. The density of CD3+ stromal cells was significantly higher in pregnant than in nonpregnant animals (80 ± 12 cells/mm² versus 36 ± 9 cells/mm², respectively; P = 0.01). However, T-cell densities at versus between attachment sites were not significantly different at any stage (P > 0.05) or at any stage during the early postattachment period (Fig. 2C). When expressed as a percentage of CD44+ cells in adjacent sections, T cells made up a significantly smaller proportion of leukocytes in uterus at attachment sites than between attachment sites (29% ± 7% versus 67% ± 15%, respectively; P < 0.05). As with total leukocytes, the proportion of T cells in endometrium not associated with

FIG. 1. Porcine endometrium obtained at (A) and between (B) embryonic attachment sites at Day 22 of gestation. Leukocytes were localized by indirect immunohistochemistry using a panleukocyte marker (CD44) as the primary antibody. lu, Lumen. Paraffin, hematoxylin counterstain. Bar = 50 μm. A) CD44+ cells with lymphocyte morphology (encircled, arrows) at an attachment site are in a cluster between two blood vessels (bv). The trophoblast (tr) has been pulled away from the luminal epithelium during processing. B) In endometrium between attachment sites, CD44+ cells (arrows) are far fewer in number.

FIG. 2. Quantification of leukocytes (CD44+ cells) in luminal epithelium (A) and associated endometrial stroma (B), and stromal T lymphocytes (CD3+ cells, C) at and between conceptus attachment sites during early porcine pregnancy (least squares means ± SEM). A) Numbers of intraepithelial leukocytes declined with stage of gestation (P = 0.0002) but were not influenced by proximity to conceptus attachment sites (P = 0.55). B) Stromal leukocyte densities were not affected by stage of gestation (P = 0.02) but were significantly higher in endometrium obtained at versus between attachment sites at all stages of gestation (P = 0.0001). C) Densities of stromal T cells at versus between attachment sites were not significantly different at any stage (P > 0.05).
ceptuses was similar to that in luteal phase animals (63% ± 20%; $P > 0.05$).

**DISCUSSION**

Throughout the early postattachment period of gestation, leukocytes were approximately 3-fold more numerous in endometrial stroma in contact with conceptuses than in tissue obtained between attachment sites. In agreement with previous reports in the pig [13–15], leukocytes were diffusely scattered throughout the endometrial stroma. At attachment sites, stromal leukocytes were present as individual cells or in clusters in the loose connective tissue and in the vicinity of glands and blood vessels. Between attachment sites, leukocytes were sparsely scattered in the stromal connective tissue. Stromal leukocyte density during the luteal phase was similar to that between attachment sites in pregnant animals, suggesting that the infiltration observed at attachment sites was a localized response to the conceptus. This interpretation is consistent with results of functional studies in which endometrial NK lytic activity was elevated at Days 10 and 20 of gestation but low to undetectable in luteal phase and pseudopregnant pigs [18]. In this respect, the pig, a nondecidualizing species, contrasts with humans and rodents, in which decidualization of endometrial stroma without pregnancy triggers redistribution of uterine leukocytes comparable to that in normal pregnancy [29, 30]. In human ectopic pregnancies, NK cells are present in normal numbers in the decidua of the empty uterus but absent from tubal implantation sites [31–33].

Within the luminal epithelium, leukocytes were scarce by the earliest stage of gestation studied both at and between attachment sites. These observations are consistent with those of a previous study in the pig in which a progressive decline in intraepithelial lymphocytes was observed from Day 10 to Day 19 of gestation [16] and similar to findings in cattle and sheep [34, 35]. The fact that the reduction in intraepithelial lymphocytes is detectable prior to attachment [16] and is evident both at and between attachment sites (present study) suggests that contact with the conceptus is not involved in this response. However, later in gestation, lymphocytes have been reported to be absent from the luminal epithelium yet present in glandular epithelium and at areolar regions where trophoblast and maternal epithelium are separated [15]. Loss of lymphocytes from epithelial surfaces in more intimate contact with trophoblast has also been observed in sheep and cattle. Ruminants have a modified epitheliochorial placenta in which caruncles, specialized a glandular regions of the endometrium, interact with adjacent areas on the chorioallantoic membrane to form placentomes. As attachment progresses, chorionic cells migrate across the interface and fuse with uterine epithelial cells at placentomes, eventually forming syncytial regions that replace portions of the maternal epithelium. Lymphocytes are uniformly distributed in caruncular and intercaruncular uterus in nonpregnant sheep [36] but are greatly reduced at placentomes in both epithelium and stroma once chorionic cells have invaded the maternal epithelium [37–39]. Increases in stromal leukocytes similar to those in the present study have not been reported in ruminants. In intercaruncular regions, which are most comparable to the porcine placenta, the distribution of lymphocytes has been reported as similar to that in nonpregnant ewes [36, 39]. Unusual γδ-T cells in the intercaruncular epithelium of the ovine uterus greatly increase in size, granularity, and number beginning at midgestation [34, 40]. However, this response is due to systemic factors rather than local effects of the conceptus [41].

The leukocytes recruited to endometrial stroma at attachment sites had lymphocyte morphology, suggesting that they were T, B, and/or NK cells. Several lines of evidence support the notion that the conceptus-associated leukocytes were NK cells. Because they do not uniquely express any surface markers, these cells are often identified by ruling out the other lymphocyte types. B cells are scarce in the pregnant uterus of the pig [13, 42]. Largely based on expression of CD2, CD8, and CD4, previous researchers conducting histological studies have concluded that T cells are the major lymphocyte population [13, 15]. However, using CD3, a pan-T-cell marker, we found that although T cells make up a substantial proportion of stromal leukocytes, there were no significant differences related to proximity to conceptuses at any stage studied. T cells actually accounted for a smaller proportion of stromal leukocytes at attachment sites than either between attachment sites or in nonpregnant uterus. CD2+ lymphocytes that did not express CD4 or CD8 have been reported to be a major population during the implantation period [13, 14].

In flow cytometric studies, porcine NK cells have been shown to be CD2+ and either positive or dimly positive for CD8, depending on the specificity of the antibody used [43]. Given that low intensity CD8 expression might have been undetectable by immunohistochemistry, the CD2+ CD4+ CD8+ lymphocytes described in previous studies may have been NK cells. Elevated NK activity in endometrial stroma during early pregnancy [17, 18] and a substantial population of lymphocytes reactive with CD16, an NK cell marker, over the same period [28] further support the notion that the conceptus-associated leukocytes are NK cells.

The mechanism by which the porcine conceptus recruits maternal leukocytes is open to speculation. Epithelium-stromal interactions have been described in the nonpregnant murine uterus, based on recombination of uterine tissue types from steroid receptor knockout and wild-type mice. Estrogen regulation of secretory protein synthesis, progesterone receptor expression, and mitogenesis in epithelial cells requires estrogen receptors in the stroma [44–46]. We propose that recruitment of uterine leukocytes by the porcine conceptus involves interactions among trophoblast, epithelium, and stroma. The existence of such a communication loop in the pig is suggested by evidence that modifications in maternal epithelium and stromal capillaries associated with embryonic attachment [11] are mimicked by estrogen, a product of the trophoblast during this period [47]. After elongation on Day 12, each porcine conceptus covers a large area of luminal surface. It was thus somewhat surprising to observe such a marked and consistent increase in leukocyte density, evident as early as attachment sites could be identified, through to Day 27, when the placenta was well established. One possible explanation is that local concentrations of trophoblast-derived signaling molecules may be highest near the embryonic disk, as has been reported for estrogen [48]. Variations in the intimacy of maternal-fetal contact may also be a factor. Attachment commences in the region of the embryonic disk, and all stages of the process from simple apposition to microvillous interdigitation are likely to be present along the conceptus during the early attachment period [11]. The porcine trophoblast produces two types of interferons (IFNs) during this period, IFNγ and IFNδ, a novel type I IFN [49, 50]. Available evidence indicates that receptors for IFNγ are...
primarily in the epithelium, and those for IFNα are primarily in the stroma [51, 52]. Their role in porcine pregnancy is unclear, but IFNγ is both a well-known activator [53] and a major product of NK cells [54]. Mature NK cells in the mouse uterus are the main source of IFNγ in the metrial triangle [55, 56]. Recruitment of stromal lymphocytes in the porcine uterus may involve trophoblast-derived IFNα as an initial signal, which is then amplified within the stroma. In humans, the close physical association of uterine NK cells with stromal cells [5] is similar to that of the conceptus-associated leukocytes in the pig. Proliferation of human uterine NK cells and their responsiveness to interleukin (IL)-15, a cytokine crucial for NK cell differentiation [57], is enhanced by coculture with decidualized stromal cells [58, 59]. Expression of IL-15 is regulated by the transcriptional activator IFNγ regulatory factor 1 [60], which can be induced by a variety of stimuli, including IFNγ [61].

Although specialized leukocytes are a feature of the pregnant uterus in many species, their role in pregnancy is still poorly understood. In the mouse, there is mounting evidence to suggest that uterine NK cells, via their production of IFNγ, are important for modification of the uterine vasculature for pregnancy. In mice genetically deficient in NK cells, IFNγ, or IFNγ signaling pathways, abnormalities in the major decidual arteries become evident by midgestation [56, 62, 63]. Unlike humans and rodents, porcine conceptuses do not erode maternal tissues to gain better access to maternal blood. Success of this noninvasive placental strategy requires well-developed vascular beds on both maternal and fetal surfaces [64]. Variations in placental vascularity in late gestation have been associated with differences in embryo survival [65, 66]. Early embryonic mortality has been estimated at 25–40% in the pig, most of which occurs before Day 30 [67]. The ability of each conceptus to recruit maternal leukocytes to the stroma during this vulnerable period may be crucial to its ability to develop an effective placenta. Definition of these cells and determination of how they are recruited will be addressed in future investigations.

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