Glycosylation dependent cell adhesion molecule 1-like protein and L-selectin expression in sheep interplacentomal and placentomal endometrium

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Abstract

Glycosylation dependent cell adhesion molecule 1 (GlyCAM-1), a mucin component of sheep histotroph produced by glandular epithelium (GE) during early pregnancy, is hypothesized to function in implantation. However, GlyCAM-1 is present in uterine tissues subsequent to implantation suggesting additional functions of this L-selectin-binding ligand. This study focused on uterine GlyCAM-1 expression during placentome development in sheep. Western blot analysis of day 50 pregnant sheep identified 45, 40, and 25 kDa bands in interplacentomal endometrium, 40 and 25 kDa bands in placentomes, and 80 and 40 kDa bands in chorioallantois. The GlyCAM-1 proteins in interplacentomal regions were comparable to those detected in day 15–19 pregnant sheep, however, the 80 kDa form was unique to chorioallantois, and the absence of the 45 kDa GlyCAM-1 in placentomes indicated differences between interplacentomal and placentomal endometrium. Immunofluorescence identified GlyCAM-1 in luminal epithelium (LE), stromal fibroblasts, and vascular smooth muscle cells. To better define its cellular distribution, GlyCAM-1 was co-localized with either epithelium-specific cytokeratin, smooth muscle-specific alpha-smooth muscle actin (αSMA), or stromal-specific vimentin. In interplacentomal endometrium, GlyCAM-1 co-localized with cytokeratin in LE but not in GE. GlyCAM-1 did not co-localize with αSMA, and was localized in the extracellular matrix of vimentin-positive stroma. In placentomes, GlyCAM-1 did not co-localize with cytokeratin, but did co-localize with αSMA and vimentin. Thus, in contrast to interplacentomal regions, GlyCAM-1 in placentomes was predominantly localized in vasculature rather than epithelial cells. Further, leukocytes expressing L-selectin were localized to the endothelial surface of GlyCAM-1-expressing vessels within placentomes. These data suggest that GlyCAM-1 assumes distinct functions in compartment-specific regions of the sheep uterus.

Introduction

Glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) is a sulfated mucin-like glycoprotein that is secreted into endothelial venules of peripheral and mesenteric lymph nodes (Laskey et al. 1992). In lymph nodes, GlyCAM-1 functions as a ligand for the leukocyte cell surface adhesion molecule, L-selectin. Binding of GlyCAM-1 to L-selectin results in the activation of β1 and β2 integrins to promote firm adhesion to fibronectin for extravasation of blood-borne lymphocytes into secondary lymph nodes through a classic adhesion cascade (Rosen 1993, Hwang et al. 1996, Giblin et al. 1997, Dwir et al. 2001). GlyCAM-1 is also found on epithelial cells of the lung, cochlea, mammary gland, and uterus where it is hypothesized to fulfill a variety of functions including lubrication, epithelial protection, cellular transport and secretion of milk, and conceptus (embryo/fetus and associated placental membranes) adhesion for implantation (Dowbenko et al. 1993, Kanoh et al. 1999, Spencer et al. 1999, Genbacev et al. 2003). Clearly, GlyCAM-1 has different roles depending on whether it is expressed by endothelium or epithelium.

In the vasculature, leukocyte adhesion is mediated, in part, by L-selectin recognition of GlyCAM-1 secreted from the endothelium. These initial interactions allow leukocyte capture from flowing blood through rolling adhesion to the endothelium surface, and are rapidly followed by integrin activation and adhesion that tethers the leukocyte on the endothelium to allow for migration through the vessel wall (Alon & Feigelson 2002, McEver 2002). In a similar manner, the initial events of pregnancy involve a precisely orchestrated adhesion cascade resulting in attachment of the conceptus to the uterine lumenal...
expression in placentomal and interplacentomal endome-

study was to provide insight into the role(s) of GlyCAM-1

roles depending on whether it is expressed by endo-

conceptus development; and (3) GlyCAM-1 has different

endometrium provides epithelial histotrophic support for

vides hematotrophic support while interplacentomal

implantation period. In addition, the amount of immunoreactive

GlyCAM-1 in uterine flushings increases dramatically

during implantation (Genbacev et al. 2003). Indeed, trophoblast L-selectin

binds uterine LE to mediate adhesive interactions with

the uterine wall that may be critical for initial events of

implantation (Genbacev et al. 2003).

In pregnant sheep, immunoreactive GlyCAM-1 is

present in uterine LE, glandular epithelium (GE), and con-

ceptus trophoderm on days 13 to 19 of the peri-implan-

tation period. In addition, the amount of immunoreactive

GlyCAM-1 is a secretory product of the endometrial epi-

thelium (histotroph) that may be involved in cell–cell

interactions between conceptus and maternal

tissues during implantation (Spencer et al. 1999, Gray

et al. 2002).

The adult sheep uterine endometrium has distinct

aglandular caruncular and glandular intercaruncular

regions (Mossman 1937). The caruncular areas are the

sites of implantation and placentation. Synepitheliochorial

placentation in sheep involves conceptus trophoderm cell

migration and fusion with uterine LE to form syncytiotrophoblasts (Guillomot et al. 1981). Eventually fusion of

placentomes, which serve a primary role in hematotrophic

support of the fetus by the placenta during the latter

two-thirds of gestation (Boshier 1969). In contrast, inter-
caruncular areas eventually become interplacentomal

regions of the endometrium and contain GE that

synthesize and secrete histotroph, a variety of enzymes,

growth factors, cytokines, lymphokines, hormones, trans-

port proteins and other substances that support conceptus

growth and development (Martel et al. 1997, Gray et al.

2001, Hempstock et al. 2004). Placentomal and inter-

placentomal endometrium are evident after day 40 of

pregnancy in sheep. However, little is known about the
differences in gene expression within these compartment-
specific regions of the uterus that could provide insight

into their morphogenesis and function.

We hypothesized that GlyCAM-1 assumes distinct func-
tions in compartment-specific regions of the post-implan-
tation pregnant sheep uterus because: (1) GlyCAM-1 is

prominently expressed in endometrium during the peri-

implantation period; (2) placentomal endometrium pro-

vides hematotrophic support while interplacentomal

endometrium provides epithelial histotrophic support for

conceptus development; and (3) GlyCAM-1 has different

roles depending on whether it is expressed by endo-

thelium or epithelium. Therefore, the objective of this

study was to provide insight into the role(s) of GlyCAM-1

in sheep endometrium by examining GlyCAM-1 protein

expression in placentomal and interplacentomal endome-

trium from day 50 of pregnancy.

Materials and Methods

Animals

All experimental and surgical procedures complied with

the Guide for Care and Use of Agriculture Animals and

were approved by the Institutional Agricultural Animal

Care and Use Committee of Texas A&M University.

Mature ewes of primarily Rambouillet breeding were

observed daily for estrous behavior using vasectomized

rams, with all ewes exhibiting at least two estrous cycle of

normal duration (~16–18 days).

Sexually mature ewes were bred to intact rams at estrus

(day 0) and 12 h and 24 h postestrus. Ewes were randomly

assigned to be hysterectomized (n = 4 ewes/day) on days

15, 30, or 50 of pregnancy. Pregnancy was confirmed by

the presence of an apparently normal conceptus in uterine

flushes (day 15), or the presence of conceptus tissue on

days 30 and 50. At hysterectomy, several sections

(~1.5 cm) from the middle of each uterine horn, including

both placentomes and interplacentomal tissues from day

50 of pregnancy, were embedded in Tissue-Tek Optimal

Cutting Temperature (OCT) compound (Miles, Oneonta,

NY, USA), frozen in liquid nitrogen vapor, and stored at

−80 °C for subsequent immunofluorescence analysis.

Placentomes, interplacentomal endometrium, and chor-

ioallantois from day 50 of pregnancy were also physically

dissected from the myometrium, snap frozen in liquid

nitrogen, and stored at −80 °C prior to Western blot ana-

lysis. Endometrial tissues from days 15 and 30 of preg-
nancy were prepared for Western blot analysis as

described above without regard to whether they were car-

uncular or intercaruncular regions of the endometrium.

A unilaterally pregnant sheep model was used to further

evaluate GlyCAM-1 protein expression in uterine

milk/histotroph. In this model, only one uterine horn is

manipulated to produce a non-gravid horn that accumu-
lates glandular secretions to allow assessment of protein

components of histotroph that are not accessible during

normal placentation. The other uterine horn remains

unmodified and supplies a pregnancy with normal placentation.

Indeed, often twin lambs are generated in this

model. Sexually mature ewes (n = 3) were

checked daily for estrous behavior as described above.

Following a second estrous cycle of normal duration, the

ovary ipsilateral to the right uterine horn was removed. A

double ligature was placed on the base of the right uterine

horn at the uterine bifurcation. At the following estrus

(day 0), ewes were mated to intact rams to generate a

gravid and a non-gravid uterine horn in each sheep (Bazer

et al. 1979, Moffatt et al. 1987). All ewes were hysterecto-

mized on day 90 of gestation. A large volume (approxi-

mately 150 ml) of uterine fluids (uterine milk/histotroph)

was collected from the right uterine horn at the uterine

bifurcation. In addition, several sections (~1.5 cm) from

the middle of each gravid uterine horn were embedded in
Protein isolation and western blot analyses

Placentomes, endometrium, and chorioallantois were thawed and immediately homogenized in lysis buffer (1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.1 mM EGTA, 0.2 mM Na3VO4, 0.2 mM PMSF, 50 mM NaF, 30 mM Na4P2O7, 1 μg/ml leupeptin, 1 μg/ml pepstatin) at a ratio of 5 ml buffer per 1 g tissue. Cellular debris was cleared by centrifugation (12000 g, 15 min, 4°C). Uterine, allantoic and amniotic fluids were thawed and cellular debris removed by centrifugation as described above. The protein concentrations of the cell lysate supernatants and uterine tract fluids were determined using a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) against vimentin (Hybridoma 8.13), cytokeratin (Hybridoma V9), and smooth muscle α-actin (αSMA; Hybridoma 1A4). The secondary antibodies included fluorescein-conjugated goat anti-rabbit IgG (Chemicon), fluorescein-conjugated goat anti-mouse IgG (Zymed, San Francisco, CA, USA), as well as Texas Red-conjugated goat anti-rabbit and goat anti-mouse IgGs (Molecular Probes, Eugene, OR, USA).

Immunofluorescence analyses

For immunofluorescence staining of frozen sections, primary antibodies included affinity-purified rabbit anti-rat GlyCAM-1 IgG (CAM02; Singer & Rosen 1996), monoclonal mouse anti-L-selectin (Hybridoma DU1-29; VMRD Inc., Pullman, WA, USA), polyclonal rabbit anti-human Von Willibrand factor (Chemicon International, Temecula, CA, USA), and monoclonal IgGs (Sigma Chemical Company, St Louis, MO, USA) against vimentin (Hybridoma V9), cytoketatin (Hybridoma 8.13), and smooth muscle α-actin (αSMA; Hybridoma 1A4). The secondary antibodies included fluorescein-conjugated goat anti-rabbit IgG (Chemicon), fluorescein-conjugated goat anti-mouse IgG (Zymed, San Francisco, CA, USA), as well as Texas Red-conjugated goat anti-rabbit and goat anti-mouse IgGs (Molecular Probes, Eugene, OR, USA).

Localization of GlyCAM-1 alone (without co-localization with other proteins) was performed as previously described (Johnson et al. 1999). Briefly, frozen sections (~10 μm) of interplacentomal endometrium were cut with a cryostat (Hacker-BrightOTF, Hacker Instruments, Inc., Winnisboro, SC, USA) and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). Sections were fixed in −20°C methanol for 10 min, permeabilized at room temperature with 0.3% Tween 20 in 0.02 M PBS (rinse solution), and blocked in antibody dilution buffer (2 parts 0.02 M PBS, 1.0% BSA, 0.3% Tween 20 [pH 8.0] and one part glycerol) containing 10% normal goat serum for 1 h at room temperature. Sections were then dipped in rinse solution at room temperature and incubated overnight at 4°C with 4 μg/ml GlyCAM-1 antibody, and detected with fluorescein-conjugated secondary antibody. Slides were then overlaid with a cover-glass and Prolong antifade mounting reagent (Molecular Probes).

For co-localization of proteins, frozen sections of placentomes or interplacentomal endometrium were cut, mounted, fixed, permeabilized, and blocked as described above. Sections were then dipped in rinse solution at room temperature and incubated overnight at 4°C with 2 μg/ml of initial primary antibody (either anti-L-selectin, anti-Von Willibrand factor, anti-vimentin, anti-cytokeratin or anti-αSMA IgG). Following three washes in 4°C rinsing solution for 10 min each, sections were incubated with 2 μg/ml of initial secondary antibody (either FITC- or Texas Red-conjugated anti-mouse IgG) for 4 h at room temperature, and washed in 4°C rinsing solution 6 times for 10 min each. Sections were then incubated overnight at 4°C with 4 μg/ml of the second primary antibody (anti-GlyCAM-1 IgG). Following 6 washes in 4°C rinsing solution for 10 min each, sections were incubated with 2 μg/ml of the second secondary antibody (either FITC- or Texas Red-conjugated anti-rabbit IgG) for 2 h at 4°C, washed 6 times in 4°C rinsing solution for 10 min each, and dipped in distilled-deionized H2O. Slides were overlaid with antifade mounting reagent as described above.

Photomicrography

Photomicrographs of representative fields of immunofluorescence staining were evaluated with a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY, USA) fitted with an AxioCam HR digital camera. Digital images of FITC- and Texas Red-labeled antigens in sections were captured using AxioVision 3.0 or 4.3 software (Carl Zeiss). Digital camera settings were evaluated to confirm that no ‘spectral bleed through’ FITC signal was detectable in the Texas Red filter set and vice versa. In these studies, once the distribution of individual antigens was established, the co-distribution of two antigens was investigated simultaneously in individual sections using compatible primary and FITC- or Texas Red-secondary antibody combinations with appropriate filter sets. In co-localization studies, appropriate fluorophore-conjugated secondary antibodies were also switched to confirm co-distribution patterns (Fig. 5).
Individual fluorophore images were recorded sequentially with AxioVision 3.0 or 4.3 software and evaluated in multiple fluorophore overlay images. These images in ZVI file format were subsequently converted to TIF format and figures assembled in Adobe Photoshop 7.0.1.

**Results**

**Western blot analyses**

Immunoreactive 45-, 40-, and 25-kDa GlyCAM-1 proteins were detected in total endometrium from days 15, 30, and 50 of sheep pregnancy (Fig. 1A). These results agree with a previous report of GlyCAM-1 protein during the sheep peri-implantation period (Spencer et al. 1999), and indicate that the overall Western blot banding profile does not change for GlyCAM-1 through day 50 of gestation. However, immunoblotting of day 50 interplacentomal endometrium, placentomes, and chorioallantois revealed differences in protein banding profiles between tissues. Although the expected 45-, 40-, and 25-kDa proteins were present in interplacentomal endometrium, only the 40- and 25-kDa proteins were detected in placentomes, suggesting that GlyCAM-1 protein expression differs depending on whether the tissue is interplacentomal or placentalom within the same uterus (Fig. 1B). The 40-kDa as well as an 80-kDa immunoreactive GlyCAM-1 protein, that may represent an SDS-stable oligomer, were detected in day 50 chorioallantois (Fig. 1B).

A Western blot of uterine fluids from day 90 unilaterally pregnant sheep is shown in Fig. 2. High levels of the 45-kDa immunoreactive GlyCAM-1 were present in uterine milk from the non-gravid uterine horn. Therefore, GlyCAM-1 is a secretory product of the endometrial LE and GE through day 90 of pregnancy.

**Immunofluorescence analyses**

High levels of immunoreactive GlyCAM-1 were localized to the LE and stroma of day 50 interplacentomal endometrium (Fig. 3). GlyCAM-1 expression in placental epithelium was close to background levels of staining. When GlyCAM-1 (FITC conjugate) and cytokeratin (Texas Red conjugate) images were merged, the yellow fluorescence signal revealed excellent co-localization of GlyCAM-1 with the epithelium-specific intermediate microfilament cytokeratin in endometrial LE (Fig. 3; left column). However, although both GlyCAM-1 and the stromal-specific intermediate microfilament vimentin were present in the stromal compartment of the endometrium, a lack of true co-localization (yellow fluorescence) indicated that GlyCAM-1 is not intracellular, but is located in the extracellular matrix (Fig. 3; third column). GlyCAM-1 protein was nearly undetectable in GE, except in vesicle-like structures within the cytoplasm, suggesting that the protein is actively secreted into the GE lumen and does not accumulate in the GE appreciably (Fig. 3; second column, see Figures 1 and 2 for representative Western blots.).
inset). GlyCAM-1 was not expressed in the vasculature of interplacentomal endometrium (Fig. 3; fourth column).

In sharp contrast to interplacentomal endometrium, GlyCAM-1 was not detected at the conceptus-maternal epithelial interface of day 50 placentomes (Fig. 4; column 1). However, GlyCAM-1 expression in the endometrial stroma of placentomes was similar to that observed in interplacentomal endometrium (Fig. 4; column 2). Significantly, GlyCAM-1 and the vascular smooth muscle-specific intermediate microfilament αSMA were fully co-localized within the tunica media of endometrial vasculature of placentomes (Fig. 4; column 3).

GlyCAM-1 immunostaining in day 90 interplacentomal uterus was similar to that observed on day 50 of pregnancy, suggesting rapid secretion and a lack of accumulation of GlyCAM-1 protein in GE (Fig. 5). Interestingly, GlyCAM-1 was highly expressed in the myometrium on day 90 of pregnancy (Fig. 5).

Co-localization of GlyCAM-1 with its receptor L-selectin in day 50 endometrium showed that no L-selectin was detectable in interplacentomal endometrium or chorionic epithelium (data not shown). However, L-selectin immunoreactivity was present on unidentified immune cells within the endometrial mesenchyme of day 50 placentomes (Fig. 6). Immuno-co-localization of L-selectin with Von Willibrand factor, a marker for endothelium, confirmed that these immune cells were located at the surface of the vascular endothelium (Fig. 7). Therefore, immune cells may home to endometrial vessels via interactions between L-selectin on immune cells and GlyCAM-1 secreted from the smooth muscle cells of the tunica media of endometrial vasculature within the placentome.

Discussion

A previous study implicated GlyCAM-1 as a regulator of conceptus-maternal interactions during the peri-implantation period of pregnancy in sheep (Spencer et al. 1999). This study extends our understanding of GlyCAM-1 in the sheep uterus to the period of pregnancy that encompasses placentome development. These results illustrate that GlyCAM-1 continues to have a complex and compartment-specific pattern of expression within the sheep endometrium during placentation. GlyCAM-1 protein is temporally and spatially positioned to influence communication at the interface between maternal and conceptus tissues where it may influence histotrophic
and hematotrophic placentomes) support of conceptus development. Western blot analyses of endometrial extracts detected three immunoreactive GlyCAM-1 proteins of 45-kDa, 40-kDa, and 25-kDa. These results are consistent with a previous report in the sheep uterus (Spencer et al. 1999), and are also similar to protein profiles for GlyCAM-1 in mouse lymph nodes, suggesting that the immunoglobulin used in this study detects all known isoforms of GlyCAM-1. In lymph nodes, immunoreactive unglycosylated GlyCAM-1 migrates at 28-kDa, GlyCAM-1 modified with N-acetylgalactosamine migrates at 28–33-kDa, GlyCAM-1 modified with sialic acid, fructose, and sulfate migrates at 40–50-kDa, and mature endothelial GlyCAM-1 migrates at 50–60-kDa (Hemmerich et al. 1994, Crommie & Rosen 1995). The contribution of specific carbohydrate- and sulfate-based modifications to L-selectin binding is controversial, with disparate reports in competitive binding experiments (Hemmerich et al. 1994, Hemmerich & Rosen 1994, Crommie & Rosen 1995, Rosen 1999). It remains to be determined how the GlyCAM-1 present within the uterine environment of sheep is post-translationally modified. Indeed, it is not known how the 45-kDa and 40-kDa proteins evident in sheep Western blots correspond to reported post-translational modifications in lymph nodes. It is significant however that the accepted GlyCAM-1 ligand for L-selectin in lymph node vasculature migrates in a range from 50–60-kDa, and potentially corresponds to the 45-kDa form in sheep endometrium. Results of the present study indicate that GlyCAM-1 protein profiles observed during the peri-implantation period are maintained through day 50 of pregnancy in sheep. However, significant differences between interplacentomal endometrium and placentomes were evident. Interplacentomal endometrium expresses the 45-kDa immunoreactive GlyCAM-1 protein that was shown to be secreted into the uterine lumen of sheep (Spencer et al. 1999). A 50-kDa GlyCAM-1 protein is secreted in mouse lymph node organ cultures that is also present in mouse blood, and is a ligand for L-selectin (Brustein et al. 1992). Placentomes lack the 45-kDa GlyCAM-1 protein. It is intriguing that this form of GlyCAM-1 is present in interplacentomal endometrium which actively secretes histotroph from the epithelium and is present in uterine milk (histotroph) and allantoic fluid, but is absent in placentomes that lack this secretory ability. Notably, the chorioallantois contains an 80-kDa GlyCAM-1 protein that is not present in endometrium. An 80–90-kDa species of GlyCAM-1 was previously detected in mouse lymph nodes (Singer & Rosen 1996) which the authors hypothesized represented SDS-stable multimers of
Interestingly, high levels of another pregnancy-associated uterine secretory protein, osteopontin, are present as SDS-stable multimers in endometrium and allantoic fluid (Johnson et al. 2003a, F J White, R C Burghardt, G A Johnson, unpublished observations). Soluble multimeric adhesion proteins in uterine/placental tissues may have physiological relevance as adhesion molecules and extracellular matrix (ECM) contribute to the stabilization of the maternal–fetal interface, although further study is needed to test this hypothesis.

The most striking results of this study are the divergence in epithelial and vascular smooth muscle expression of GlyCAM-1 between interplacentomal endometrium and placentomes. Regarding these two tissue compartments, GlyCAM-1 is present in interplacentomal epithelium, whereas placentomes express GlyCAM-1 in the endometrial vasculature, but not epithelium. It was previously reported that GlyCAM-1 in the LE and GE increased between days 13 and 15, and remained high through day 19 of pregnancy (Spencer et al. 1999). The present results indicate that GlyCAM-1 continues to be expressed at high levels by interplacentomal LE through day 50. In contrast, GlyCAM-1 protein in day 50 and 90 GE, which is producing maximal levels of histotroph (Stewart et al. 2000), was low to undetectable except within distinct vesicle-like structures within the cytoplasm. It is reasonable to hypothesize that this spatial pattern of protein expression within GE cells is the result of processing of GlyCAM-1 into secretory vesicles leading to rapid secretion into the glandular lumen resulting in little intracellular accumulation of the protein. A similar localization for GlyCAM-1 has been observed in the high endothelial venules of peripheral lymph nodes. In these cells, immunogold labeling localized GlyCAM-1 in cytoplasmic vesicles, but failed to label the cell surface or cytoplasm even though the protein was present as a soluble factor in serum and lymph node organ culture medium (Kikuta & Rosen 1994). Additionally, large amounts of GlyCAM-1 are present in uterine milk (Fig. 2) suggesting secretion from LE and GE into the uterine lumen as a significant component of histotroph. Epithelial expression of GlyCAM-1 in reproductive tissues is not novel to the uterus. Nearly all epithelium in the mammary gland of lactating mice express GlyCAM-1 (Nishimura & Kohmoto 2001), and human mammary epithelium expresses multiple splice variants for GlyCAM-1 mRNA (Rasmussen et al. 2002). This expression is stimulated by prolactin (Hou et al. 2000), and results in

![Figure 5 Immunofluorescence analysis of GlyCAM-1 protein in frozen sections of sheep endometrium from the gravid uterine horn of a day 90 of unilaterally pregnant sheep. Immunoreactive GlyCAM-1 protein was detected using affinity-purified rabbit anti-rat GlyCAM-1 IgG (CAM02; Singer & Rosen 1996). Similar to day 50, high levels of GlyCAM-1 protein were present in LE and stroma (ST), but were nearly undetectable in GE on day 90 of pregnancy. Interestingly, GlyCAM-1 protein was also localized to myometrium (MYO). Tr, Trophoectoderm. Width of field is 350 μm.](https://www.reproduction-online.org)
Figure 6 Immunofluorescence co-localization of GlyCAM-1 with L-selectin in day 50 sheep placentome frozen cross-sections. GlyCAM-1 immunoreactivity was detected using Texas Red-conjugated anti-rabbit IgG (first column), or GlyCAM-1 immunoreactivity was detected using fluorescein-conjugated anti-rabbit IgG and L-selectin immunoreactivity was detected using Texas Red-conjugated anti-mouse IgG (second column). GlyCAM-1 is expressed in endometrial mesenchyme, whereas L-selectin is expressed by unidentified immune cells within sheep placentomes. Compare the antibody staining with staining using mouse and rabbit IgG (third column). Fetal, Chorionic villi; Maternal, Endometrial villi. Width of fields are 350 µm.

Figure 7 Immunofluorescence co-localization of L-selectin with the endothelium-specific Von Willibrand factor in day 50 sheep placentome frozen cross-sections. L-selectin immunoreactivity was detected using fluorescein-conjugated anti-mouse IgG (left column, top panel; green color), whereas the Von Willibrand factor was detected using Texas Red-conjugated anti-rabbit IgG (left column, middle panel; red fluorescence). L-selectin is expressed on immune cells associated with the endothelium of endometrial vessels (left column, bottom panel). Compare the antibody staining with staining using mouse and rabbit IgG (right column). Width of fields are 230 µm.
incorporation of GlyCAM-1 into the secreted milk fat globule membrane (Kester & Brunner 1982). While the role of GlyCAM-1 at the conceptus-interplacentomal endometrium interface is currently unknown, it has been hypothesized that this mucin is involved in conceptus-maternal interactions (Spencer et al. 1999) that are mediated through its receptor L-selectin (Nicholson et al. 1998, Genbacev et al. 2003). In the present study, L-selectin was not detected at the conceptus-maternal interface of interplacentomal endometrium (data not shown); however the presence of other GlyCAM-1 receptors such as P- and E-selectins cannot be ruled out (Singer & Rosen 1996).

This is the first report of GlyCAM-1 or L-selectin expression in the placentomes of ruminants. Placentomes are the point of interface between the fetal cotyledon and the uterine caruncles. Fetal chorionic villi protrude into crypts in the uterine caruncular tissue to provide maximal contact between endometrial and placental microcirculations for hematotrophic support of conceptus development (Boshier 1969). The localization of this ligand for selectins known to be involved in cellular transport, secretion, adhesion and leukocyte attachment to endothelium during extravasation (Dowbenko et al. 1993, Rosen 1999, Genbacev et al. 2003), to endometrial vessels in placentomes, highlights the functional specialization of caruncular tissue within the pregnant uterus. Indeed, the compartment-specific presence of GlyCAM-1 protein in placentomal vasculature strongly suggests roles in the transfer of molecules from the maternal uterine vasculature to the fetal membranes as a component of hematotrophic support for conceptus development during the latter two thirds of pregnancy in sheep. The specific molecular functions of this molecule in placentomes remain to be determined, but results of this study clearly indicate a temporal and spatial pattern of GlyCAM-1 expression that warrants further investigation. It is intriguing that the uterus is the only organ in which GlyCAM-1 has been reported to be expressed by smooth muscle, in both the tunica media of endometrial blood vessels and the myometrium (Spencer et al. 1999). In this study, strong myometrial immunostaining for GlyCAM-1 reinforced the previous report by Spencer and colleagues (1999). At present, speculation for a role for GlyCAM-1 as a component of the ECM of myometrium would be premature.

Although speculative, the presence of L-selectin bearing cells at the endothelial surface of these vessels suggests a role for GlyCAM-1 in the homing and tethering of circulating leukocytes to the pregnant sheep uterus. Indeed, leukocyte homing to the anti-mesometrial decidua of pregnant mice is, at least in part, mediated by L-selectin (Xie et al. 2005). In the previous report, it was concluded that GlyCAM-1 probably does not serve a role in leukocyte trafficking because it is not present in endothelium of endometrial vasculature. However, the present results showing vascular smooth muscle expression of GlyCAM-1, as well as L-selectin-positive leukocytes, at the endothelial surface in placentomes, but not interplacentomal endometrium strongly suggests physiological relevance. Because the placenta is designed to maximize exchange of gases, metabolic products, vasoactive factors, cytokines, and cells between maternal and fetal microcirculations, many arterioles/capillaries within this structure have gaps in the elastic laminae that separates the smooth muscle cells from the often discontinuous endothelium. These gaps cause vessels to be ‘leaky’, and facilitate the bidirectional transfer of products in and out of the vasculature. Therefore, similar to angiogenic vessels, molecules secreted from vascular smooth muscle can potentially arrive within the vessel lumen to contact circulating immune cells (Abramovitch et al. 1998). The physiological relevance of GlyCAM-1-mediated immune cell accumulation at vessels within the maternal component of the placentome remains to be investigated. L-selectin has widespread distribution on all classes of leukocytes suggesting a general function in a broad array of leukocyte-endothelial interactions (Picker & Butcher 1992, Rosen 1993). Certainly immune cells produce a wide array of cytokines and growth factors that influence tissue remodeling and angiogenesis essential for placentation (Zhang et al. 2003). Further, these immune cells have the potential to regulate immune tolerance at this most intimate point of contact between maternal and fetal tissues, the vasculature of the placentome (Hunt et al. 2000).

A surprising result of this study was the presence of immunoreactive GlyCAM-1 protein in the endometrial stroma of both interplacentomal and placentomal sheep endometrium. This expression is apparently induced following the initial attachment phase of implantation, because stroma does not express GlyCAM-1 through day 19 of pregnancy (Spencer et al. 1999). Interestingly, this pattern of expression is similar to that observed for osteopontin, another adhesion protein that is typically expressed by the epithelial linings of secretory organs (Johnson et al. 2003b). In addition, classic markers of endometrial stromal differentiation of decidualizing species, including desmin and αSMA, also increase by day 35 of pregnancy. Concurrent with changes in gene expression, stromal cells increase in size and become more polyhedral in shape in this non-decidualizing species (Johnson et al. 2003b). Therefore, GlyCAM-1 appears to be part of a stromal cell differentiation program that occurs after conceptus attachment to the uterus. It has been hypothesized that this stromal differentiation may affect endometrial receptivity to syncythial placentation in sheep. The large mucin-like GlyCAM-1, with its extensive glycosylation and extended structure, appears to be well positioned within the ECM of the stroma to limit conceptus trophoblast invasion into the uterine wall. Both GlyCAM-1 and the mesenchymal-specific intermediate microfilament vimentin were co-distributed within the stromal compartment of the endometrium. However the true co-localization (overlaying of green and red fluorescence to produce yellow) was not evident. Because vimentin is an intracellular protein, it is reasonable to...
conclude that GlyCAM-1 is not present in the cytoplasm of stromal cells, but is either secreted and incorporated into the ECM, or expressed at the surface of stromal cells.

In summary, these data suggest that GlyCAM-1 assumes distinct functions in compartment-specific regions of the sheep uterus during placentation. In interplacentomal endometrium, GlyCAM-1 is present in the LE where it may be involved in cell and tissue interactions at the conceptus–maternal interface. During placentome development, GlyCAM-1 is present in the vasculature of endometrial villi where it may participate in nutrient-based transfer for hematotrophic support of conceptus development. In endometrial stroma, GlyCAM-1 is present in the ECM where it may affect uterine receptivity for implantation. Clearly, the extensive spatial pattern of GlyCAM-1 described here implies physiological relevance and highlights the complexity of placentation in mammals.

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