

# Actions of Progesterone on Uterine Immunosuppression and Endometrial Gland Development in the Uterine Gland Knockout (UGKO) Ewe

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**ABSTRACT** In ewes, the uterine gland knockout (UGKO) phenotype is caused by neonatal exposure to norgestomet to arrest uterine gland development and produce an adult which has a uterus characterized by the lack of endometrial glands. Since endometrial glands in the sheep produce the lymphocyte-inhibitory protein, ovine uterine serpin (OvUS), an experiment was conducted with ewes of the UGKO phenotype to evaluate whether the inhibitory actions of progesterone on tissue rejection responses in utero are dependent upon the presence of endometrial glands. Control and UGKO ewes were ovariectomized and subsequently treated with either 100 mg/day progesterone or corn oil vehicle for 30 days. An autograft and allograft of skin were then placed in each uterine lumen and treatments were continued for an additional 30 days before grafts were examined for survival. All autografts survived and had a healthy appearance after histological analysis. Allografts were generally rejected in ewes treated with vehicle but were present for hormone-treated ewes, regardless of uterine phenotype. Analysis of the histology and protein synthetic capacity of the uterus revealed that progesterone induced differentiation of endometrial glands and synthesis and secretion of OvUS in UGKO ewes. The UGKO ewes had reduced density of CD45R<sup>+</sup> lymphocytes in the endometrial epithelium and there was a tendency for progesterone to reduce this effect in luminal epithelium. Taken together, results confirm the actions of progesterone to inhibit graft rejection response in utero. Responses of UGKO ewes to progesterone indicate that the hormone can induce de novo development and differentiation of endometrial glands, at least when skin grafts are in the uterus. *Mol. Reprod. Dev.* 71: 347–357, 2005. © 2005 Wiley-Liss, Inc.

**Key Words:** progesterone; uterine glands; immunosuppression; sheep

## INTRODUCTION

Among its many actions to maintain pregnancy, progesterone acts to inhibit uterine immune function and thereby prevent immunological rejection of the conceptus (Hansen, 1998). In sheep, for example, progesterone reduces numbers of specific populations of lymphocytes in the uterine endometrium (Gottshall and Hansen, 1992; Majewski and Hansen, 2002) and delays rejection or promotes survival of skin allografts (Hansen et al., 1986) and hybridoma xenografts (Majewski and Hansen, 2002) placed within the uterine lumen. The inhibitory effects of progesterone on uterine graft rejection are believed to be indirect because concentrations of progesterone required to directly inhibit lymphocytes are much higher than achieved in studies where progesterone inhibited uterine immune responses (Low and Hansen, 1988; Monterroso and Hansen, 1993). Rather, it has been hypothesized that immunosuppressive effects of progesterone in the uterus are mediated by secretion of a lymphocyte-inhibitory molecule produced by the uterus in response to progesterone. Indeed, treatment of ovariectomized ewes with progesterone results in the appearance of lymphocyte-inhibitory activity in uterine fluid (Stephenson et al., 1989a; Hansen and Skopets, 1992).

A likely candidate for the progesterone-induced immunosuppressive molecule in sheep is ovine uterine serpin. This protein, which is a member of the serine

Grant sponsor: USDA Cooperative Research, Education and Extension Service; Grant numbers: 2001-35204-10797, 2001-02259.

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Received 21 December 2004; Accepted 24 February 2005  
Published online 1 April 2005 in Wiley InterScience  
(www.interscience.wiley.com).

DOI 10.1002/mrd.20301

proteinase inhibitor superfamily (Ing and Roberts, 1989), is produced by uterine endometrium under the influence of progesterone (Moffatt et al., 1987; Ing et al., 1989; Leslie and Hansen, 1991; Spencer et al., 1999a). Related members of this family have been also reported in pregnant cows (Leslie et al., 1990; Mathialagan and Hansen, 1996), pigs (Malathy et al., 1990), and goats (Tekin et al., 2005). The major site of synthesis of OvUS is the glandular epithelium: expression of OvUS mRNA was limited to glandular epithelium through day 120 of pregnancy (Stewart et al., 2000) and OvUS protein was found in glandular epithelium but not luminal epithelium at day 60 of pregnancy (Stephenson et al., 1989a). By day 120–140 of pregnancy, however, immunoreactive OvUS was also detected in luminal epithelium (Moffatt et al., 1987; Stephenson et al., 1989b). A role of OvUS in inhibition of uterine immune responses is indicated by several observations. In vitro, OvUS inhibits lymphocyte activation and proliferation induced by T cell mitogens and interleukin-2 (Segerson et al., 1984; Skopets and Hansen, 1993; Skopets et al., 1995; Peltier et al., 2000) and natural killer cell activity (Liu and Hansen, 1993; Tekin and Hansen, 2002). In vivo, OvUS inhibits T-cell dependent antibody production in sheep (Skopets et al., 1995) and fetal loss induced by natural killer cell activation mediated by injection of poly(I) · poly(C) (Liu and Hansen, 1993).

Conclusive evidence that OvUS mediates the effects of progesterone on uterine immune function will be dependent upon demonstrating that progesterone is unable to regulate uterine immune function in sheep incapable of OvUS synthesis. While it is not practical to use homologous recombination to generate sheep without a functional *OvUS* gene, it is possible to produce epigenetic changes in ewes to lead to an animal without the presence of endometrial glands or the ability to produce glandular-derived OvUS. Changes in uterine morphology and function caused by the action of sex steroid hormones have been reported in many livestock animals (Spencer et al., 1993; Bartol et al., 1995; Carpenter et al., 2003; Tarlenton et al., 2003). Long-term exposure of lambs to norgestomet generates an adult that has either an absence of glands, slight glandular invaginations into the stroma, or limited numbers of small, cyst- or gland-like structures (Gray et al., 2000a,b, 2001a). There is no apparent effect of neonatal norgestomet on development of extrauterine reproductive tract structures (Gray et al., 2000b, 2001a). Ewes with the uterine gland knockout (UGKO) phenotype do not show disturbances in circulating concentrations of progesterone and retain the ability to respond to prostaglandin  $F_{2\alpha}$  (Gray et al., 2000a). The uteri of cyclic UGKO ewes displays normal expression patterns of progesterone, estrogen, and oxytocin receptors and several adhesion molecules on the uterine luminal epithelium (Gray et al., 2000a, 2002) and retains responsiveness to interferon- $\tau$  (Gray et al., 2002). However, UGKO ewes have altered estrous cycle lengths (Gray et al., 2000a) and exhibit pregnancy loss that involves inhibition of conceptus growth between days 9 and 14 of

pregnancy (Gray et al., 2000a, 2001a,b, 2002). Available evidence supports the hypothesis that one or more adhesion proteins are deficient in the secretions of the uterus that are required to support early conceptus survival and development (Gray et al., 2002).

In the present experiment, we tested the hypothesis that progesterone would be unable to prolong survival of skin allografts placed within the uterine lumen of UGKO ewes. An unexpected finding, that prolonged progesterone treatment induced the development and differentiation of endometrial glands in UGKO ewes, prevented testing of the role of OvUS but did result in evidence that one of the actions of progesterone in adult animals is to stimulate histogenesis of endometrial glands.

## MATERIALS AND METHODS

### Materials

Progesterone was obtained from Sigma-Aldrich (St. Louis, MO). Hybond ECL nitrocellulose membranes and ECL chemiluminescence Western blot kit were purchased from Amersham Bioscience (Piscataway, NJ). Precast ready gels, kaleidoscopic protein standards, 2-mercaptoethanol and gelatin were obtained from Bio-Rad (Hercules, CA). Hybridoma cells producing monoclonal antibody to CD45R<sup>+</sup> (clone 73B) were purchased from the European Collection of Cell Cultures (Salisbury, UK). Ascites fluid for CD45R<sup>+</sup> was produced by the Hybridoma Core Facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida. Monoclonal antibodies against OvUS (HL-218 and HL-708) were made as described previously (Leslie et al., 1990) and were prepared as hybridoma supernatants. Ovine uterine serpin was purified from crude uterine fluid of unilaterally-pregnant ewes as described elsewhere (Liu and Hansen, 1993).

### Experimental Design

A total of 23 Rambouillet crossbred ewes, 12 controls and 11 UGKO ewes, were used in the experiment. UGKO ewes were produced as described previously (Spencer et al., 1999b; Gray et al., 2000a) by implanting ewe lambs with a single Synchronate B<sup>®</sup> (Sanofi, Overland Park, KS) implant within 12 hr of birth and every 2 weeks thereafter for a total of 8 weeks. Implants were inserted subcutaneously in the periscapular area and released approximately 6 mg of norgestomet (17 $\alpha$ -acetoxy-11 $\beta$ -methyl-19-norpreg-4-ene-3,20-dione), a potent synthetic 19-norprogesterin, over a 14 day period (Bartol et al., 1988a). Control ewes did not receive implants. The control and UGKO ewes used in the present study were approximately 3 years of age.

Ewes were bilaterally ovariectomized via midventral laparotomy 30 days before the initiation of the experiment. Treatments were arranged according to a 2  $\times$  2 factorial design with main effect of type (control vs. UGKO) and hormone treatment (vehicle or progesterone). Ewes were randomly assigned within type to hormonal treatment so that eight control ewes and eight UGKO ewes received daily subcutaneous injections of

5 ml of 20 mg/ml progesterone (i.e., 100 mg/day) dissolved in a corn oil vehicle whereas four control ewes and three UGKO ewes received daily injections of 5 ml corn oil. On day 30 after the first injection, two skin grafts were placed in the uterus according to procedure described by Hansen et al. (1986). An autograft (a piece of skin of the abdominal area from the same ewe) was placed in one randomly-chosen uterine horn while an allograft (a piece of skin of the abdominal area from a different ewe) was placed into the other uterine horn. Daily injections were continued for an additional 30 days. On day 15 after graft placement, 10 ml blood samples were collected via jugular venipuncture at 2, 8, and 24 hr after injection to determine plasma concentrations of progesterone. On day 30 after graft placement, ewes were slaughtered by captive bolt stunning and exsanguination and reproductive tracts were recovered for examination of graft survival.

#### Collection of Tissues and Uterine Fluids

Uterine fluid was collected via aspiration using an 18 ga needle and syringe. The total amount of uterine fluid collected was recorded and the fluid centrifuged twice at 3,600g at 4°C for 20 min and the supernatant fraction stored at -20°C for further analysis. When uterine fluid was not noticeable, the uterus was flushed with 20 ml Dulbecco's phosphate buffered saline (DPBS) pH 7.3. After collection of fluid, the uterus was opened longitudinally and the survival of the skin grafts and their general appearance recorded. Pieces of surviving grafts were immediately preserved in a 10% (w/v) neutral buffered formalin solution. After graft collection, three tissue samples (3–4 mm<sup>3</sup>) of the intercaruncular endometrium were collected at random from each uterine horn and also preserved in neutral buffered formalin solution.

#### Skin Graft and Uterine Histology

Uterine and skin graft tissue sections were dehydrated, embedded in paraffin blocks, and 5 µm sections prepared and mounted on slides. Histological appearance was determined after staining with hematoxylin and eosin and examination under bright field with a Zeiss Axioplan microscope (Carl Zeiss, Inc., Göttingen, Germany). Photomicrographs were prepared using a Sony CD Mavica 400 digital camera (San Diego, CA).

#### Progesterone Radioimmunoassay

Blood samples collected via jugular venipuncture into heparinized tubes were placed on ice until they could be centrifuged at 2,000g for 20 min, and the plasma harvested and stored at -20°C until the day of the assay. Progesterone was measured using a solid-phase <sup>125</sup>I radioimmunoassay kit (Coat-A-Count<sup>®</sup>, Diagnostic Products Laboratory, Los Angeles, CA). Sensitivity of the assay (90% Bo) was 0.1 ng/ml and the intrassay and interassay CV were 11.42% and 4.13%, respectively. For statistical analysis, plasma samples with concentrations below the sensitivity of the assay were assigned a concentration equal to the sensitivity of the assay.

#### Determination of Protein Concentration in Uterine Fluid and Flushing

Protein concentration of uterine fluid was determined using the Bradford procedure (Bradford, 1976) with bovine serum albumin as standard. Total protein content of the uterine lumen was calculated as the protein concentration times either the volume of uterine fluid recovered, or for ewes in which flushing was performed, the volume of DPBS used for flushing.

#### Detection of OvUS in Uterine Fluid by Western Blotting

Samples of 0.5 µg of uterine proteins, purified OvUS, and purified ovalbumin (OVA) were separated under reducing conditions using one-dimensional discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 4%–15% (w/v) gradient polyacrylamide gels and Tris-HCl buffer. Proteins were transferred electrophoretically to Hybond ECL 0.2 µm nitrocellulose membranes. Membranes were blocked overnight in TBS-T [10 mM Tris pH 7.6, 0.9% (w/v) NaCl, and 0.3% (v/v) Tween-20] that also contained 1% (w/v) gelatin (TBS-TG), washed, and incubated for 1 hr at room temperature with a mouse monoclonal antibody recognizing OvUS (HL-218, 1:32,000 dilution of hybridoma supernatant in TBS-TG), washed, and then incubated for 1 hr at room temperature with horseradish peroxidase-conjugated sheep anti-mouse IgG (1:8,000 dilution in TBS-TG). After washing, blots were developed using the ECL Western blotting chemiluminescence substrate kit. Specificity of labeling was evaluated by including a negative control in which primary antibody was replaced with hybridoma cell culture medium.

#### Immunohistochemistry for OvUS and CD45R<sup>+</sup> Lymphocytes

Immunohistochemistry was performed using the HistoScan Universal Monoclonal Detector kit (Biomedica, Foster City, CA) that utilizes streptavidin–biotin peroxidase complex for detection. Primary monoclonal antibodies were specific for OvUS [HL-708, 1:800 dilution of hybridoma supernatant in PBS-GS (0.1 M sodium phosphate, pH 7.4 containing 0.9% (w/v) sodium chloride and 2% (v/v) donor goat serum)] and CD45R (clone 73B, 1:800 dilution of ascites fluid in PBS-GS). Negative controls were incubated with mouse ascites fluid, clone NS-1 (Sigma-Aldrich), at the same dilution as used for primary antibodies.

Slides were examined for staining using bright field with a Zeiss Axioplan microscope (Carl Zeiss, Inc., Göttingen, Germany). Photomicrographs were prepared using a Sony CD Mavica 400 digital camera (San Diego, CA). Presence of OvUS was evaluated qualitatively. CD45R<sup>+</sup> cells were evaluated by scoring the relative abundance in the luminal epithelium, glandular epithelium and stroma on a scale from 0 (no positive cells) to 4 (very dense accumulation of positive cells). One section per horn was evaluated for each sheep.

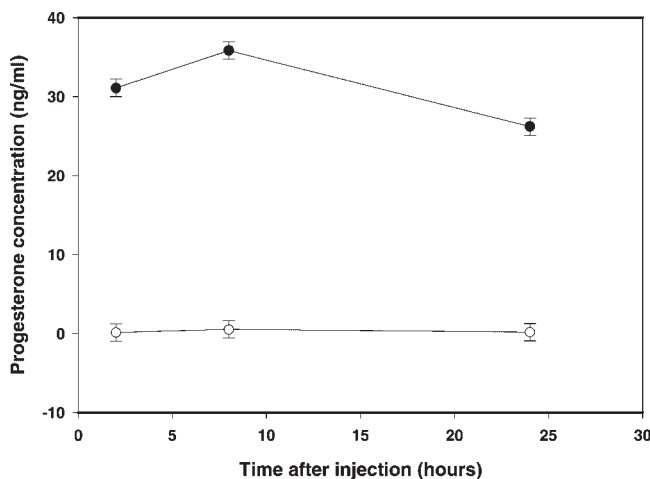
### Statistical Analysis

Data were analyzed by least-squares analysis of variance using the General Linear Models procedure of the Statistical Analysis System (SAS for Windows, Release 8.02, SAS Institute, Cary, NC). For repeated-measures data, ewe was considered a random effect and other main effects were considered fixed. Tests of significance were determined using error terms determined after calculation of the expected means squares. In general, the mathematical model considered main effects and all interactions. The one exception was for numbers of CD45R<sup>+</sup> cells in the glandular epithelium where the absence of glands in all but one UGKO ewe treated with corn oil required analyses with several models. For these data, various tests of subsets of data were performed to determine differences between control and UGKO ewes treated with corn oil and effects of progesterone and type of graft on control ewes. Data on total protein in the uterus exhibited heterogeneity of variance. Therefore, data were log-transformed before analysis and are presented as mean  $\pm$  individual SEM for each group. For other variables, heterogeneity was not apparent and data are reported using a pooled estimate of error.

## RESULTS

### Progesterone Concentration in Plasma

For both control and UGKO ewes, concentrations of progesterone were higher ( $P < 0.0001$ ) for ewes treated with 100 mg/day of the hormone than for ewes receiving corn oil vehicle (Fig. 1). Concentrations in the progesterone-treated ewes peaked at a concentration of 35.8 ng/ml at 8 hr after injection and then declined to a nadir of 26.2 ng/ml at 24 hr after injection (i.e., immediately before the subsequent progesterone



**Fig. 1.** Progesterone concentration in plasma (ng/ml) at 2, 8, and 24 hr after injection in ewes treated with corn oil vehicle (open circles) or progesterone (closed circles). Progesterone concentrations were determined at day 45 of progesterone treatment. Data represent least-squares mean  $\pm$  SEM. Progesterone concentrations differed between the two groups ( $P < 0.0001$ ).

injection). For ewes treated with the vehicle, values were generally below the limit of detection of the assay and in no case greater than 0.53 ng/ml.

### Gross Uterine Morphology

While uterine weights were not recorded, treatment with progesterone caused an increase in uterine size in control ewes (compare Fig. 2B showing a uterus from control ewe treated with corn oil vehicle with the uterus on the right of Fig. 2C that represents a uterus from a control ewe treated with progesterone). In contrast, there was not obvious increase in size of the uterus of UGKO ewes treated with progesterone as compared to UGKO ewes treated with the corn oil vehicle (compare Fig. 2A and the left-hand uterus in Fig. 2C). Moreover, the uterus of UGKO ewes treated with progesterone was much smaller than those of control ewes treated with the hormone (Fig. 2C).

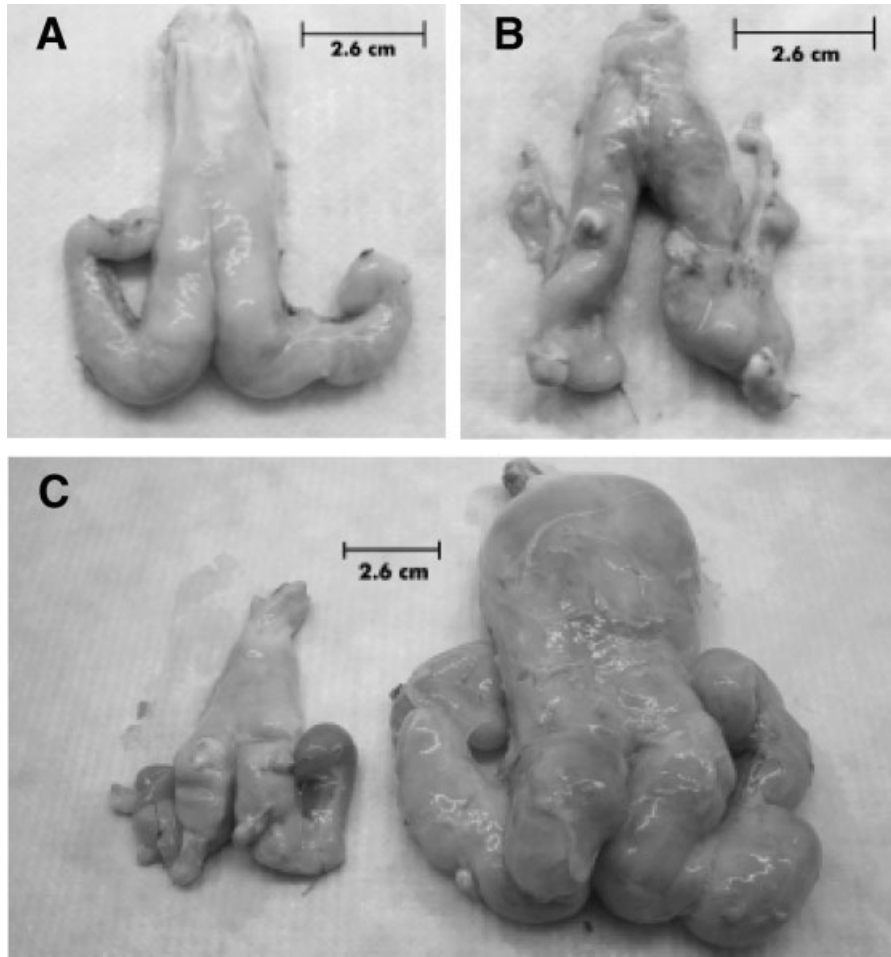
There were other characteristics of the uterus of the UGKO ewes that differed from the typical appearance of the sheep uterus. Often, the uterine wall was thin and appeared friable. The uterine lumen also usually contained a dark brown fluid—this was true for both vehicle-treated and progesterone-treated ewes. Finally, caruncles were almost totally absent on the endometrium of the vehicle-treated ewes. There were, however, prominent caruncles in most of the progesterone-treated ewes.

### Histological Analysis of Endometrium

The presence of uterine endometrial glands is summarized in Table 1. For control ewes, both luminal and glandular epithelia were present in all animals regardless of hormonal treatment. The chief difference between groups was the larger size of glands in the progesterone-treated animals (compare Fig. 3A,B). For UGKO ewes treated with corn oil vehicle, luminal epithelium was present in all cases, but glandular epithelium was absent or greatly reduced in two of three ewes. A few scattered cyst-like or primitive glands could be identified but otherwise uterine endometrium was composed of luminal epithelium and stroma (Fig. 3C). In contrast to this pattern, well-defined glandular epithelium was present in the remaining corn oil-treated ewe (Fig. 3D) and for all UGKO ewes treated with progesterone. For the latter case, glands were present in either one uterine horn ( $n = 4$ ; 2 on the autograft side and 2 on the allograft side; see Fig. 3E for an example) or in both uterine horns ( $n = 4$ ; see Fig. 3F for an example).

### Survival of Skin Grafts

Results of skin graft survival are summarized in Table 1. All autografts survived regardless of treatment. Grossly, the grafts appeared healthy and most were attached to the uterine endometrium (see Fig. 4 for examples). Histological analysis of the skin grafts confirmed the visual observations. Autografts were well organized and viable, with the presence of well defined epidermis (including a keratinized stratum corneum)



**Fig. 2.** Gross appearance of the uteri of control and uterine gland knockout (UGKO) ewes. **Panels A** and **B** illustrate uteri from a control (A) and UGKO ewe (B) that were treated with corn oil for 60 days. **Panel C** shows uteri of a UGKO (**left side**) and control ewe (**right side**) treated with progesterone for 60 days. Note the difference in size between the two uteri.

and dermis (Fig. 5A–C). Allograft survival, in contrast, depended upon treatment and, when present, allografts displayed signs of necrosis. For ewes receiving corn oil vehicle, allografts from four of four control ewes and two of three UGKO ewes had been adsorbed when examined 30 days after grafting; traces of wool were still in the

uterus but the skin tissue was gone (Fig. 4A,B). In the third UGKO ewe treated with vehicle, the allograft was present (Fig. 3C). The pattern of graft survival was altered by progesterone treatment. In this case allografts were present in eight of eight control ewes and, eight of eight UGKO ewes (Fig. 4D–F). The gross

**TABLE 1. Survival of Skin Grafts and Presence of Uterine Glands and Ovine Uterine Serpin (OvUS) in Control and Uterine Gland Knockout Ewes (UGKO) Treated With Corn Oil Vehicle (CO) or Progesterone (P4)\***

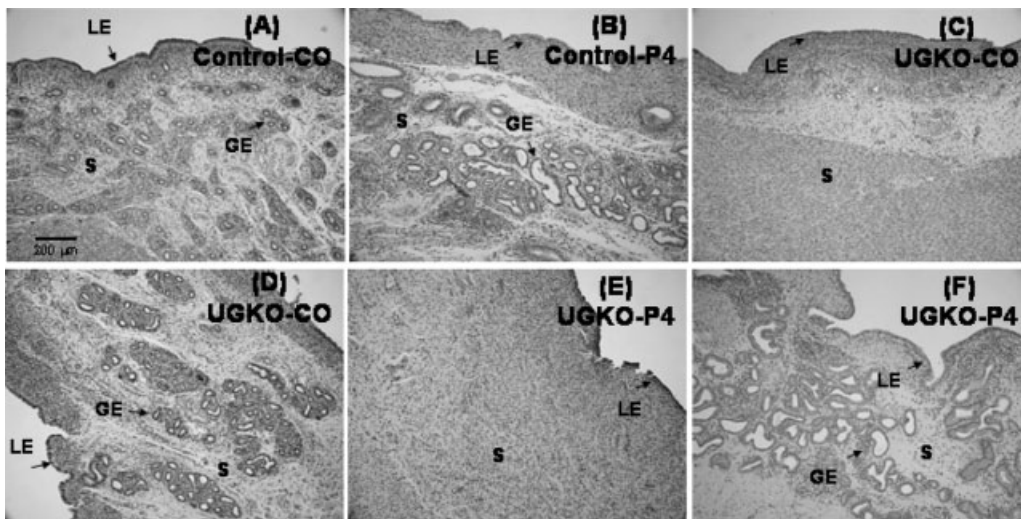
Ewe type	Treatment	Graft survival*		Histology <sup>a</sup>	Western blot <sup>b</sup>	IHC <sup>c</sup>
		Autograft	Allograft	Uterine glands	OvUS	OvUS
Control	CO	4/4	0/4	4/4	0/4	0/4
UGKO	CO	3/3	1/3	1/3	0/3	0/3
Control	P4	8/8	8/8	8/8	8/8	8/8
UGKO	P4	8/8	8/8	8/8	8/8	8/8

\*Results represent the fractions of graft surviving.

<sup>a</sup>Results are the fraction of ewes with uterine glands.

<sup>b</sup>Results are the proportion of ewes in which OvUS was detected in uterine fluid or flushings.

<sup>c</sup>Results are the proportion of ewes in which OvUS was immunolocalized to endometrium by immunohistochemistry.



**Fig. 3.** Endometrial histology for control and UGKO ewes treated with corn oil (C) or progesterone (P4) for 60 days. Sections were stained with hematoxylin and eosin. **Panels A and B** show uteri from control ewes with well defined luminal and glandular epithelium. **Panels C–F** show uteri from UGKO ewes treated with corn oil vehicle (C and D) and progesterone (E and F). Note the lack of glandular epithelium in C and E. Two of three UGKO corn-oil treated ewes had no glands (C), while

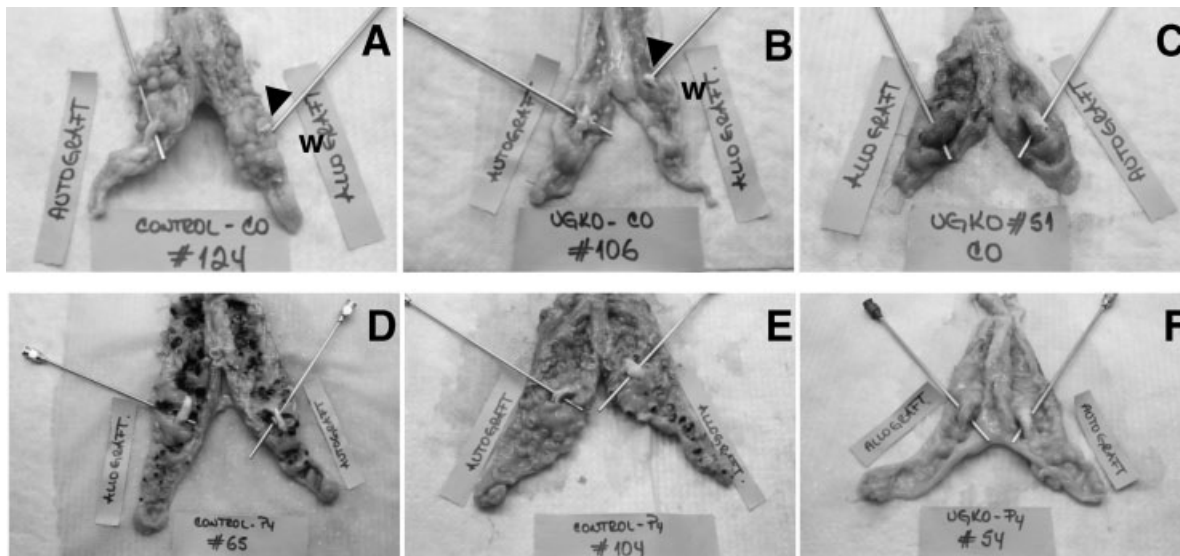
one ewe possessed glands (D). All UGKO ewes treated with progesterone had glands in one (n = 4) or both (n = 4) uterine horns. Examples of endometrium from a uterine horn from a progesterone-treated UGKO ewe without glands is in panel E while an example of endometrium from a uterine horn from a progesterone-treated UGKO ewe with glands is in panel F. LE, luminal epithelium; GE, glandular epithelium; S, stroma.

appearance of surviving allografts was often necrotic, however, with graft appearing brown and having a soft consistency. Histological examination of surviving grafts demonstrated that grafts were disorganized, lacked identifiable epidermis and were characterized by abundant infiltration of leukocytes (Fig. 5D–F).

**Total Protein Content in the Uterine Lumen**

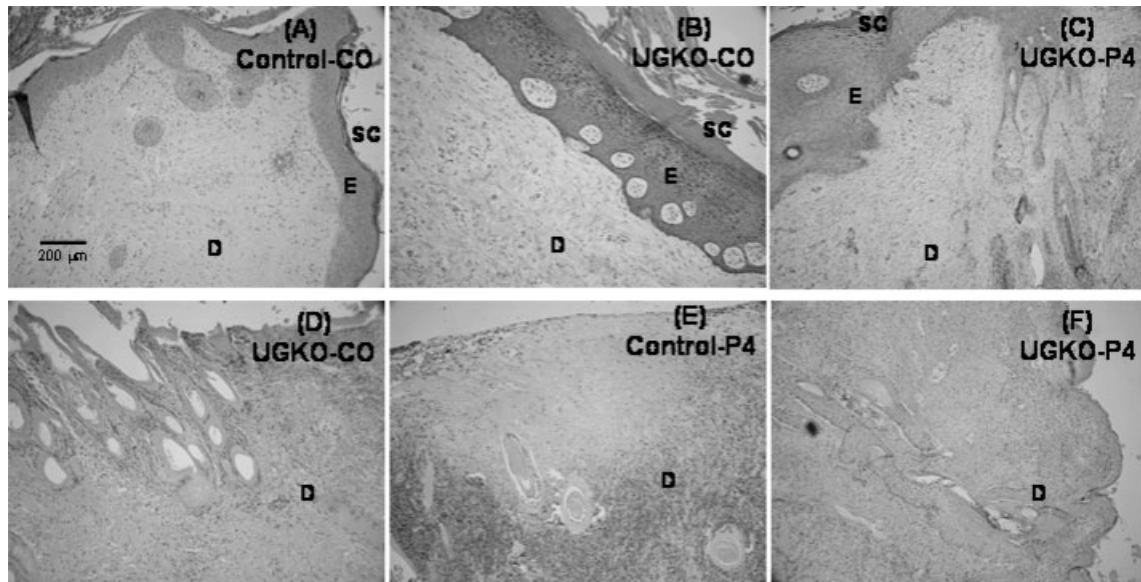
Total uterine protein content of the uterine lumen was greater for UGKO ewes than control ewes ( $P < 0.001$ )

and greater for progesterone-treated ewes than for ewes treated with corn oil vehicle ( $P < 0.01$ ) (Fig. 6). There was, however, an interaction between ewe type and treatment ( $P < 0.01$ ). Use of the pdiff mean separation test indicated that protein content was lower for control ewes receiving corn oil than protein content for the other three groups and that there was no significant difference among the three groups. Thus, progesterone caused a significant increase in protein content for control ewes but not for UGKO ewes.



**Fig. 4.** Gross appearance of surviving autografts and allografts placed into the uterus of control (A, D, E) and UGKO ewes (B, C, F) 30 days after surgery. Ewes were treated with corn oil (CO) (A–C) or progesterone (P4) (D–F). Note that autografts were present in all ewes and that allografts had been completely reabsorbed in all ewes treated

with corn oil [note the wool (w) in panels A and B; the tissue had been completely reabsorbed] except for one UGKO ewe (C). In contrast to the situation in ewes treated with corn oil, all allografts were present in ewes treated with progesterone; this was true for control (D and E) and UGKO ewes (F).



**Fig. 5.** Histology of autografts (A–C) and allografts (D–F) 30 days after grafting into the uterus of control and UGKO ewes. Ewes were treated with corn oil (CO) and progesterone (P4). Sections were stained with hematoxylin and eosin. **Panels A–C** show well organized skin tissue (autografts) with presence of epidermis (E) [including stratum corneum (SC)] and dermis (D). **Panels D–F** show allograft that were undergoing degeneration; note the lack of organized epidermis.

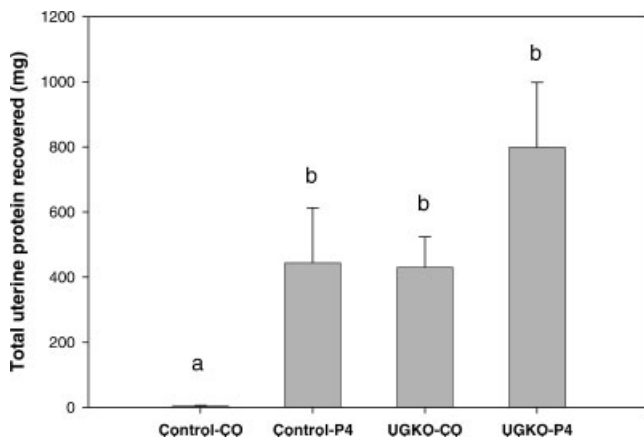
#### Presence of OvUS in Uterine Fluid

A representative immunoblot for the presence of OvUS in uterine fluid is shown in Figure 7 while a summary of the incidence of OvUS in uterine fluid is presented in Table 1. Immunoreactive OvUS was not detected in uterine fluids or flushings of control or UGKO ewes treated with corn oil vehicle. However, a single immunoreactive band at a molecular weight of 55,000–57,000 was detected in uterine fluids from all control and UGKO ewes treated with progesterone. The immunoreactive band seen using anti-OvUS was not

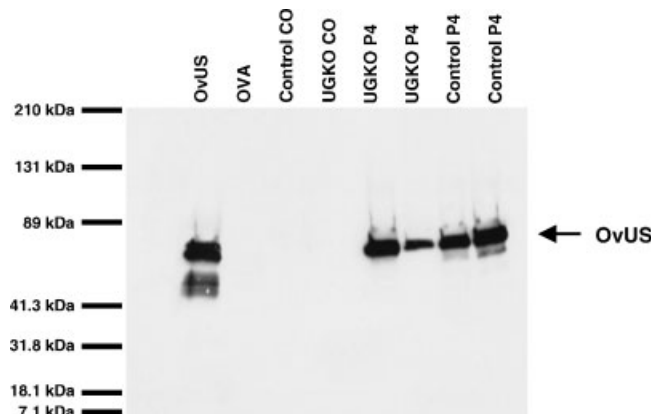
visible for negative control reactions in which culture medium replaced primary antibody (data not shown).

#### Immunochemical Localization of OvUS

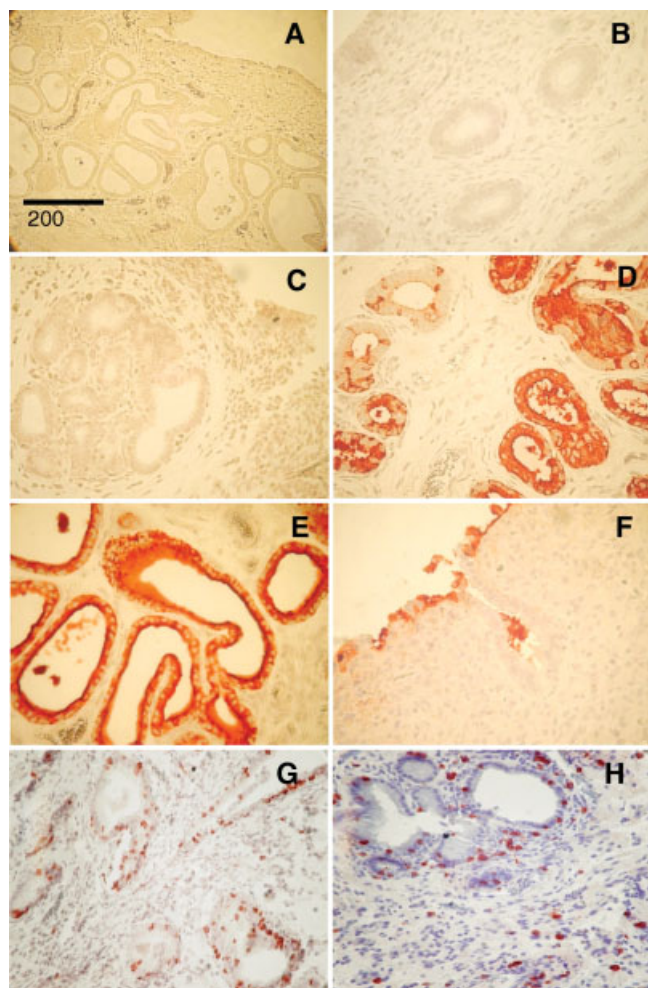
A summary of the presence of OvUS in uterine tissue sections is presented in Table 1. Immunoreactive OvUS was not detected in any sections of uterine endometrium from corn oil-treated control (Fig. 8B) or UGKO ewes (Fig. 8C). Immunoreactive OvUS was observed, however, in all endometrial sections from progesterone-treated ewes, whether from control (Fig. 8D) or UGKO (Fig. 8E,F) ewes. Immunoreactive OvUS was not detected in sections of the endometrium used as



**Fig. 6.** Total protein recovered from uterine fluid of control and UGKO ewes treated with corn oil vehicle (CO) or progesterone (P4) for a 60 days period. Data represents mean  $\pm$  SEM. Total protein was affected by ewe type (UGKO vs. control;  $P < 0.001$ ), progesterone treatment ( $P < 0.01$ ) and the interaction of ewe type with progesterone treatment ( $P < 0.01$ ). Bars with different superscripts differ significantly ( $P < 0.05$ ).



**Fig. 7.** Representative Western blots for detection of ovine uterine serpin (OvUS) in uterine fluid or flushings collected from control and UGKO ewes treated with corn oil (CO) or progesterone (P4) after 60 days. Purified OvUS and ovalbumin (OVA) were used as positive and negative control proteins, respectively.



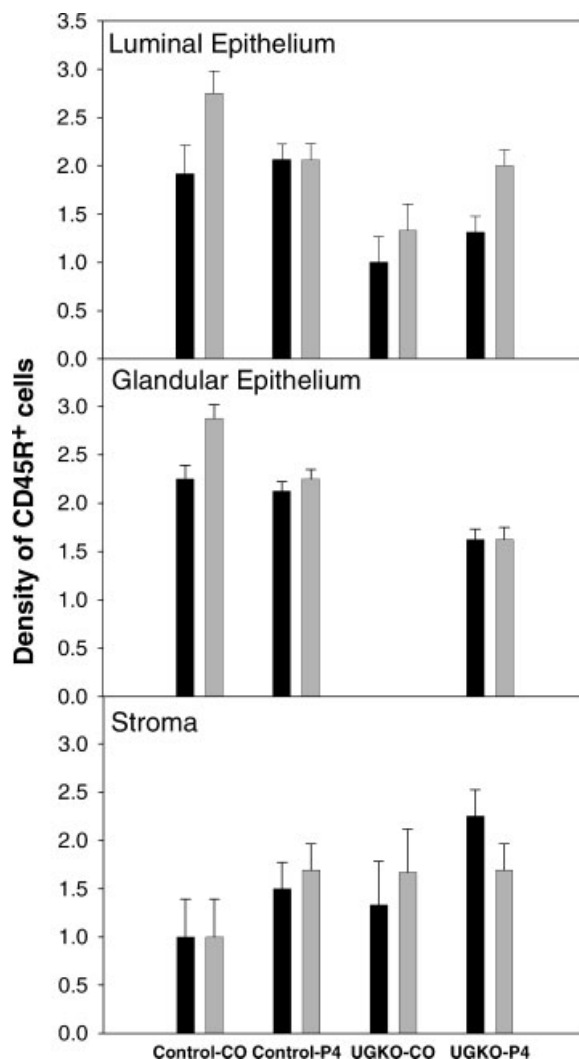
**Fig. 8.** Immunolocalization of OvUS (A–F) and CD45R<sup>+</sup> cells (G, H) in endometrium from control and UGKO ewes treated with corn oil vehicle (CO) and progesterone (P4) for 60 days. Panel A represents a negative control for OvUS. Panels B and C show the lack of immunoreactive OvUS in endometrium from control (B) and UGKO ewes (C) treated with corn. Panels D–F illustrate detection of immunoreactive OvUS in glandular (D and E) and luminal (F) epithelium of endometrium from control (D) and UGKO (E and F) ewes treated with progesterone. Panels G and H display represent immunoreactive CD45R<sup>+</sup> cells in the endometrium for control (G) and UGKO (H) ewes treated with progesterone (both are from the autograft side).

negative controls (Fig. 8A). The protein was immunolocalized to the glandular epithelium (Fig. 8D,E) and in some areas of the luminal epithelium (Fig. 8F). No positive reaction was detected in areas of the stroma.

**Immunolocalization of CD45R<sup>+</sup> lymphocytes**

Regardless of treatment, CD45R<sup>+</sup> cells in sections of the endometrium where autografts were present were mainly located in the luminal and glandular epithelium of control and UGKO ewes treated either with corn oil vehicle or progesterone. A reduced number of CD45R<sup>+</sup> cells were localized in the stroma area (Fig. 8G,H, respectively). The density of CD45R<sup>+</sup> cells was estimated by subjective scoring—results are shown in

Figure 9. For luminal epithelium, density of CD45R<sup>+</sup> cells was lower for UGKO ewes than for control ewes ( $P < 0.001$ ). In both types of ewes, the presence of allografts in the uterus produced an increase in of CD45R<sup>+</sup> cells in the luminal epithelium ( $P < 0.01$ ). There was a tendency for an interaction of type x treatment x graft ( $P = 0.08$ ). In particular, the presence of an allograft caused an increase in numbers of CD45R<sup>+</sup> cells for control ewes treated with corn oil. Progesterone blocked this increase. In the UGKO ewes, in contrast, the increase in numbers of CD45R<sup>+</sup> cells caused by



**Fig. 9.** Density score for CD45R<sup>+</sup> cells in uterine endometrium. Shown are data for endometrium from the side of the autograft (black bars) and allograft (gray bars) for control and UGKO ewes treated with corn oil vehicle (CO) or progesterone (P4) for 60 days. Data represents least-squares mean  $\pm$  SEM. In the luminal epithelium, the population of CD45R<sup>+</sup> cells was affected by type (UGKO vs. control;  $P < 0.001$ ), graft (allograft vs. autograft;  $P < 0.01$ ) and the interactions of type by treatment ( $P = 0.07$ ) and type  $\times$  treatment  $\times$  graft ( $P = 0.08$ ). Among control ewes, numbers of CD45R<sup>+</sup> cells in the glandular epithelium was affected by graft ( $P < 0.05$ ); treatment  $\times$  graft was  $P = 0.07$ . For progesterone-treated groups, density of CD45R<sup>+</sup> cells in the glandular epithelium was affected by type ( $P = 0.06$ ). There were no significant effects on numbers of CD45R<sup>+</sup> cells in the stroma.

the allograft was small and progesterone caused an increase in numbers of CD45R<sup>+</sup> cells in both uterine horns.

Among control ewes, numbers of CD45R<sup>+</sup> cells in the glandular epithelium were higher in the uterine horn with the allograft than for the horn bearing the autograft ( $P < 0.05$ ) and the difference between horns containing allografts and autografts tended to be reduced in the progesterone-treated ewes (treatment  $\times$  graft;  $P = 0.07$ ). For UGKO ewes treated with corn oil, the general absence of glands meant obviated analysis of numbers of CD45R<sup>+</sup> cells in that tissue. For UGKO ewes treated with progesterone, glands were present and the density of CD45R<sup>+</sup> cells in the progesterone-treated groups was lower for UGKO ewes than for control ewes treated with progesterone ( $P = 0.06$ ). For UGKO ewes treated with progesterone, moreover, there was no difference in density of CD45R<sup>+</sup> cells between horns with autografts and allografts. There were no significant effects of any treatment on intensity of staining of CD45R<sup>+</sup> cells in the stroma.

### DISCUSSION

Results from this experiment confirmed that progesterone delayed the rejection of allogeneic tissues placed into the uterine lumen. An unexpected finding was that prolonged progesterone treatment was capable of inducing development and differentiation of endometrial glands into functional cells capable of OvUS secretion. This effect of progesterone abrogated the UGKO phenotype, at least in part, and allowed progesterone to maintain skin graft survival through induction of OvUS synthesis or some other mechanism. The induction of functional endometrial glands made it impossible to answer the question posed at the beginning of the study, i.e., whether progesterone inhibits uterine immune function through mechanisms independent of induction of OvUS synthesis. Nonetheless, these results provide some novel insights into uterine biology including the conclusion that the adult uterus retains the ability to form endometrial glands and that the development processes causing differentiation of these cells into endometrial glands are under control of progesterone. Results also suggest the involvement of progesterone and, possibly, endometrial glands in homing of lymphocytes to the endometrium.

Other studies have shown that administration of progesterone at doses ranging from 50 to 200 mg/day maintains the presence of allogeneic and xenogeneic tissues in the sheep uterus (Hansen et al., 1986; Majewski and Hansen, 2002). It is clear that progesterone is not preventing graft rejection per se, but rather delaying rejection because surviving allografts were undergoing tissue disorganization and neutrophil invasion. Similar findings were reported by Hansen et al. (1986). This delay in tissue graft rejection is likely to reflect a decrease in function of effector lymphocytes or other leukocytes in the uterus. The role of T cells in rejection is illustrated in the present study by the observation that the endometrium in the uterine horn

containing the allograft had increased accumulation of CD45R<sup>+</sup> cells, which in the sheep uterus represent mostly T cells (Meeusen et al., 1993). Indeed, progesterone has been reported to decrease lymphocyte numbers in the endometrium (Gottshall and Hansen, 1992) and there was a slight decrease in the population of CD45R<sup>+</sup> cells caused by progesterone in control ewes in the present study. In particular, progesterone tended to reduce the increase in numbers of CD45R<sup>+</sup> cells caused by presence of the allograft.

The concentrations of progesterone causing a delay in graft rejection (in this case, a peak of 35.8 ng/ml) are below the concentrations of progesterone required to inhibit lymphocyte proliferation (Low and Hansen, 1988; Monterroso and Hansen, 1993). The hypothesis that has been put forward to explain the progesterone-induced delay in rejection of tissue grafts in the uterus is that the immunosuppressive protein OvUS mediates the effects of progesterone. A test of this hypothesis using the UGKO ewe was not possible, however, because progesterone induced appearance of endometrial glands in the UGKO ewe and these glands produced and secreted OvUS as indicated by results of immunohistochemistry and Western blotting. Thus, the newly-differentiated glandular epithelium in the UGKO ewe induced by progesterone treatment was functional with respect to OvUS secretion.

The actions of progesterone to induce new endometrial gland development and to cause these glands to differentiate into functional glands capable of secretion of the prototypical progesterone-induced protein in the sheep was an unexpected finding that casts light on the developmental processes controlling uterine differentiation and function. In ewes, the development of the glandular epithelium in the uterus is a postnatal event, starting between days 0 and 7, with bud formation from the luminal epithelium and proliferation into the stroma (Bartol et al., 1988b; Taylor et al., 2000). Tubular structures that start to branch and coil are found by day 21 after birth (Taylor et al., 2000) and the endometrial adenogenic process seems to be completed by day 56 of life when the histoarchitecture of the uterus resembles the adult ewe (Taylor et al., 2000). The process is under control of endocrine and paracrine regulators (Gray et al., 2001c). The existence of the UGKO phenotype indicates that neonatal exposure disrupts one or more of these regulatory systems to intercept the normal course of adenogenesis. Present results indicate that the endometrium retains the ability to initiate and complete glandular formation and that prolonged treatment with a high dose of progesterone can restore one or more of the components of the adenogenesis pathway that was disrupted by neonatal progestin treatment. There is evidence for the existence of stem cells for epithelial and stromal cells in endometrium from adult women (Chan et al., 2004; Cho et al., 2004) and it is possible that progesterone activates these cells to initiate glandular formation. Alternatively, existing luminal epithelial cells could be induced to undergo growth and differentiation into glandular structures.

Given the presence of skin grafts in all uteri, it is not clear whether the gland-inducing action of progesterone involves actions of progesterone alone or whether additional growth factors and cytokines from the skin grafts or activated lymphocytes provide important signals for glandular development.

A previously undescribed characteristic of the UGKO phenotype in the absence of progesterone is a large reduction in the population of CD45R<sup>+</sup> cells. Perhaps, the low numbers of these cells is the reason why the allograft was present in one UGKO ewe treated with corn oil. Reduction in the number of CD45R<sup>+</sup> cells in the UGKO ewe suggests that the lymphocyte homing mechanism is altered in this phenotype. One molecule involved in leukocyte extravasation, glycosylation-dependent cell adhesion molecule 1, is expressed in the endometrial epithelium of the sheep (Spencer et al., 1999c) and there are undoubtedly others. Neonatal progestin treatment may change endometrial function so that lymphocyte egress from the endometrium is hastened. It also cannot be excluded that changes in lymphocyte numbers may represent disruption of in situ differentiation of lymphocytes in the glandular epithelium. Recombinase genes (*RAG-1* and *RAG-2*) have been found expressed in human decidual mononuclear cells (Hayakawa et al., 1994). The fact that progesterone treatment of UGKO ewes tended to increase numbers of endometrial CD45R<sup>+</sup> cells may indicate that, as for its effects on endometrial gland morphogenesis, progesterone treatment of the adult can reverse actions of neonatal progestin exposure.

In conclusion, results confirm that progesterone delayed rejection of allogeneic tissue placed into the uterine lumen and showed that progesterone can reverse the effects of neonatal progestin exposure on endometrial gland morphogenesis. Among the differentiation events in the endometrium that are disrupted by neonatal progestin exposure are formation of the pool of CD45R<sup>+</sup> lymphocytes resident in the endometrial epithelium. Results provide some novel insights into uterine biology including the conclusion that the adult uterus retains the ability to form endometrial glands and that the development processes causing differentiation of these cells into endometrial glands are under influence of progesterone. Results demonstrate the potential of the UGKO ewe as a tool to study development of not only endometrial morphogenesis but uterine immune function as well.

#### ACKNOWLEDGMENTS

The authors thank all the members of the laboratory for help with surgery. Thanks are also extended to Dean Glicco for sheep management and the members of the meat laboratory in the Department of Animal Sciences, University of Florida for assistance with sheep slaughters; the University of Florida Diagnostic Referral Laboratories (UFDRL) for assistance for tissue preparation; and the Hybridoma Core Facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida, for production of monoclonal

antibodies. This is Journal Series No. R-10649 of the Florida Agricultural Experiment Station.

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