ENDOMETRIAL ADENOGENESIS AND UTERINE IMMUNE REGULATION IN SHEEP

By

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I dedicate this thesis to my mother, uncle, nanny, and siblings.

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LIST OF ABBREVIATIONS

CD	Cluster of Differentiation
CO	Corn Oil Vehicle
Con A	Concanavalin A
G-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HLA	Human Leukocyte Antigen
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IGF	Insulin Growth Factor
IL	Interleukin
IL-2R	Interleukin 2 Receptor
iNOS	Inducible Nitric Oxide Synthase
MCP	Monocyte Chemotactic Protein
MHC	Major Histocompatibility Complex
NK	Natural Killer
OvUS	Ovine Uterine Serpin
OVA	Ovalbumin
PHA	Phytohemagglutinin
PND	Post Natal Day
PolyI•PolyC	Polyinosinic-Polycytidylic Acid
P4	Progesterone

PHA	Phytohemagglutinin
RCL	Reactive Center Loop
TCR	T Cell Receptor
TNF	Tumor Necrosis Factor
TGF	Transforming Growth Factor
UGKO	Uterine Gland Knockout

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Progesterone is the hormone of pregnancy in mammals. Among its roles, it is involved in regulation of the maternal immune system to prevent rejection of the semi-allograft conceptus. In ewes, progesterone induces secretion of a 55-57 kDa member of the serine proteinase inhibitor superfamily called ovine uterine serpin (OvUS) by the endometrial glands in the uterus. OvUS exhibits a variety of immunosuppressive properties towards lymphoid cells and has been proposed to be a mediator of progesterone actions during pregnancy. To determine whether OvUS plays this role, an experiment was conducted to determine whether the immunosuppressive effects of progesterone occur in ewes that are treated hormonally to prevent the development of uterine glands. These so-called uterine gland knockout (UGKO) ewes are produced by exposure of the neonate to synthetic progestin for at least 8 weeks.

For this experiment, ovariectomized control and UGKO ewes were treated with 100 mg/day progesterone 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 the vehicle but were protected in hormone-treated ewes, regardless of uterine phenotype. Analysis of the histoarchitecture and protein synthetic capacity of the uterus revealed that progesterone induced differentiation of endometrial glands and synthesis and secretion of OvUS in UGKO ewes. In particular, endometrial glands were absent or greatly reduced in the UGKO ewes treated with the vehicle, but were present in UGKO ewes treated with progesterone. Indeed, OvUS was present in uterine fluid of UGKO ewes treated with progesterone and was localized by immunohistochemistry on both luminal and glandular epithelium. Progesterone also reduced the difference between control and UGKO ewes in the density of $CD45R^{+}$ lymphocytes residents in the uterine endometrium.

Taken together, the results confirm that progesterone delays graft response in uteri. The development of endometrial glands and induction of OvUS synthesis caused by progesterone treatment in the UGKO model limit conclusions regarding the role of endometrial glands in mediating the immunosuppressive action of progesterone. Nonetheless, responses of UGKO ewes to progesterone indicate that the hormone can induce *de novo* development and differentiation of endometrial glands and lymphocyte homing to the uterus. Use of the UGKO model should prove useful in further elucidating the molecular basis for progesterone-driven changes in uterine morphogenesis and immune function.

CHAPTER 1
REVIEW OF LITERATURE

Mechanisms Proposed to Allow Fetal Evasion of the Maternal Immune Response

The maternal immune system has long been recognized as a threat to the conceptus because the expression of paternal genes makes the conceptus an allograft to the mother. In 1953, Medawar proposed three possible mechanisms to explain how the conceptus survives this threat: (i) the presence of an anatomical barrier which separates the fetus and the mother, (ii) the antigenic immaturity of the conceptus, and (iii) the immunological tolerance of the mother. Medawar's landmark paper highlighting what he termed the "immunological paradox of pregnancy" has led to over 50 years of research into pregnancy immunology, mostly with mouse models, and the formulation of additional hypotheses to explain the survival of the fetus. Nonetheless, most current concepts can still be organized around Medawar's original three mechanisms.

Placenta as an Immunological Barrier

Medawar's idea that the placenta forms an immunological barrier between the mother and the conceptus is supported by observations that the placenta does inhibit the movement of antibodies and T cells from the mother to the conceptus (Chaouat et al., 1983). In species where there is transfer of antibodies across the placenta (for example, mouse), there is some evidence that antibodies against the fetal antigens are preferentially absorbed by placenta (Raghupathy et al., 1981).

Recent data suggest that binding of antibody to the placenta does not lead to cell lysis because of local downregulation of the complement system. In mouse, the gene

product Crry, which controls the deposition of activated complement proteins C3 and C4 on the surface of fetal cells, plays a key role in regulation of complement activation at the placenta because developing *Crry*$^{-/-}$ embryos had surface-deposited C3 in the trophoectoderm and the ectoplacental cone with an invasion of polymorphonuclear inflammatory granulocytes (Xu et al., 2000). Further studies demonstrated that the alternative pathway of the complement system is the primary contributor to the fetal loss, specifically the maternal complement component C3 (Mao et al., 2003).

T cell trafficking across the placenta is also limited. In mouse, the placenta acts as a barrier for passage of cells from the mother to the fetus and vice versa, which could otherwise lead to graft vs. host disease (Hunziker et al., 1984). In addition, it has been proposed that FasL of maternal and fetal origin could protect the placenta from maternal and fetal cell trafficking across the placenta (Hunt et al., 1997; Makrigiannakis et al., 2001). However, at least in humans, some fetal cells can pass through the placenta in normal pregnancies and these cells or their descendants can persist into the mother's circulation for decades (Lo et al., 2000; Bianchi and Lo, 2001). This process, known as microchimerism, could affect the maternal immune system and have been recently implicated in the development of autoimmune diseases in women (Nelson, 2002).

Antigenic Immaturity

Medawar's idea that the fetus is antigenically immature is wrong although he was correct in hypothesizing that the placenta would be of reduced antigenicity. In mouse, there is expression of paternal MHC antigens on the embryo as early as the 2-cell stage to the blastocyst stage (Searle et al., 1976; Webb et al., 1977; Fernandez et al., 1999). The definitive placenta also expresses MHC class I antigens. It is true, however, that MHC class I antigen expression is downregulated in those parts of the placenta in contact with

the maternal endometrium. In mice for example, MHC class I expression occurs on the spongiotrophoblast, but not on the labyrinthine trophoblast in contact with the maternal system (Billington and Bell, 1983). In human placenta, cytotrophoblast expresses MHC class I antigen, but the syncytiotrophoblast which is in contact with endometrium is MHC class I negative (Sunderland et al., 1981). Development of an immune response to fetal MHC class I antigens, which has been documented in mouse pregnancy (Kiger et al., 1985), could be due to shedding of MHC class I positive trophoblast or fetal tissue into the maternal circulation.

One implication of reduced antigenicity of the outer layers of trophoblast is that these tissues are potentially at risk for lysis by maternal natural killer (NK) cells. Natural killer cells recognize targets that do not express MHC class I antigen, which ordinarily interacts with an inhibitor receptor on the NK cell to block lysis (Ljunggreen and Karre, 1990). However, human and mouse trophoblasts are resistant to NK cell lysis despite the lack of MHC class I expression (Zuckermann and Head, 1987; King et al, 1990). In humans at least, partial protection against NK cell lysis is afforded by placental expression of a non-classical MHC class I called HLA-G (produced by HLA-class Ib genes) which has only limited antigenic variation between individuals, and can inhibit recognition of target cells by NK cells (Le Bouteiller and Mallet, 1997).

Mice deficient in NK cells by homologous recombination form anomalies at implantation site and decidual spiral arteries (Croy et al., 2002; 2003). It has been hypothesized that cytokine production by NK cells, especially IFN-γ, is involved in uterine vascular remodeling during pregnancy (Croy et al., 2002).

Immune Tolerance

Of all of Medawar's original hypotheses, most research has focused on the idea that there is either antigen-specific or nonspecific inhibition of immune responses against fetal antigens. There is evidence that both types of inhibition occur during pregnancy. Tafuri et al. (1995) proposed that maternal T cells undergo a transient tolerance to paternal alloantigens during pregnancy. This hypothesis was tested by producing mice that were transgenic for TCR specific for the paternal alloantigen H-2K^{b}. In this way, the numbers of T cells against paternal antigen could be monitored by flow cytometry. During midpregnancy, transgenic mice had reduced numbers of T cells of the same clonotype when conceptuses were K^{b} positive, but not when conceptuses were of other haplotypes. In addition, K^{b} tumor grafts were not rejected when they were placed in H-2^{kxd} TCR transgenic mice bearing a K^{b} positive conceptus, but rejection occurred for syngeneic and third–party allogeneic pregnancies (Tafuri et al., 1995). The ability for graft rejection was restored after parturition. In another model, it was shown that T cells specific for fetal H-Y antigens were decreased during pregnancy in transgenic mice expressing TCR specific for H-Y antigen (Jiang and Vacchio, 1998). Zhou and Mellor (1998) also showed that expression of paternally inherited MHC class I molecules (H-2K^{b}) by the trophoblast produced a reduction of CD8 expressed on the surface of maternal CD8^{+} T cells. Taken together, these experiments indicate specific inhibition of T cell populations that recognizes paternal antigens.

A host of molecules produced by the placenta and endometrium have also been proposed to act in a TCR-nonspecific manner to inhibit lymphocyte response. Among these molecules proposed for this role are transforming growth factor-β (TGF-β) (Arck et al., 1995), interleukin (IL)-10 (Chaouat et al., 1995), leukemia inhibitor factor and

macrophage-stimulating factor (M-CSF) (Clark et al., 1994; Piccini et al., 2001), prostaglandin E_2 (Low and Hansen, 1988; Parhar et al., 1989), indoleamine 2,3-dioxygenase (IDO) (Munn et al., 1998) and progesterone (Szekeres-Bartho et al., 1985; Hansen, 1986) among others.

In mouse, much emphasis recently has focused on the enzyme IDO which catabolizes tryptophan and is expressed in macrophages, specific subset of dendritic cells and in the human placenta (Mellor and Munn, 2001; Mellor et al., 2003). The expression of IDO is increased by IFN-γ and, in dendritic cells by IL-10 (Grohmann et al., 2003). Exposure of pregnant mice to 1-methyl-tryptophan, an inhibitor of IDO, induced rejection of allogeneic conceptuses but not syngeneic conceptuses (Munn et al., 1998). This result was interpreted to mean that IDO inhibits lymphocyte responses at the maternal-fetal interface by starving the lymphocytes of tryptophan while prevention of this depletion with an IDO inhibitor allows development of a maternal immune response against the conceptus (Munn et al., 1998). A similar role for IDO has been demonstrated in vitro when T cell proliferation was inhibited by monocytes differentiated into macrophages using M-CSF (Munn et al., 1999). Moreover, IDO is also involved in the regulation of the complement system since inflammation caused by C3 complement component deposition was detected at the maternal-fetal interface before fetal rejection of allogeneic pregnant mice exposed to the IDO inhibitor (Mellor et al., 2001).

T regulatory cells have also been implicated in the downregulation of the immune response at the maternal-fetal interface. These naturally occurring cells are characterized by the surface coexpression of $CD4^+$ and $CD25^+$. Although the inhibitory effects of these regulatory cells seems to be mediated by the production of immunosuppressive cytokines

such as IL-10 and TGF-β, recent experiments have shown that $CD4^+$ $CD25^+$ T regulatory cells can induce IDO production in dendritic cells via the cell surface marker cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (Fallarino et al., 2003; Wood and Sakaguchi, 2003). In human peripheral blood, T regulatory cells are found throughout pregnancy, peaking during the second trimester and decreasing at the postpartum period (Somerset et al., 2004). The best evidence that T regulatory cells regulate maternal immune system against the fetus is that T cell-deficient BALB/c nude (*nu/nu*) mice receiving a lymphocyte preparation depleted of $CD25^+$ lymphocytes did not sustain allogeneic pregnancies; fetal resorption was characterized by abnormal fetuses, hemorrhage and infiltration of $CD3^+$ T cells at the maternal-fetal interface (Aluvihare et al., 2004). Transfer of lymphocyte depleted of $CD25^+$ cells into females pregnant from syngeneic mating resulted in 50% of pregnancy success (Aluvihare et al., 2004).

T-cell derived cytokines seems to be an important contributor to the regulation of both fetal survival and fetal rejection. Activated $CD4^+$ T cells can be classified according to their cytokine production as type 1 $CD4^+$ T cells (Th1), which produce IL-2, tumor necrosis factor-β (TNF-β), and IFN-γ and which typically enhance cell-mediated immunity, and type 2 $CD4^+$ T cells (Th2), which produce IL-4, IL-5, IL-6, IL-10 and IL-13 and which typically promote B cell function (Saito, 2000). The production of cytokines by Th1 cells inhibits the Th2 subset and vice versa (Raghupathy, 2001). During murine normal pregnancy, the Th2 cytokines are constitutively present at the maternal-fetal interface, whereas IFN-γ is transient (Lin et al., 1993). Disruption of normal pregnancy in mouse was caused by injection of proinflammatory cytokines IFN-γ, TNF-α and IL-2 (Chaouat et al., 1990). In contrast, fetal resorption was prevented by

administration of IL-10 in abortion-prone mice (Chaouat et al., 1995). Defective cytokine production by Th2 cells subset, especially LIF, IL-4 and IL-10 was observed for decidual T cells of women with recurrent unexplained abortions and women undergoing abortions during the first trimester of pregnancy (Piccini et al., 2001).

Other studies have demonstrated that progesterone, the hormone most directly responsible for pregnancy, provides a transient state in which the uterus is able to support the allograft conceptus. Moriyama and Sugawa (1972) shown that progesterone allowed implantation and proliferation of different xenografts cells placed into the uterus of golden hamsters, but this effect was not achieved in those animals treated with estradiol alone (Moriyama and Sugawa, 1972). Progesterone also allows the survival of skin grafts and mouse hybridoma cells placed into uterine lumen of ovariectomized ewes (Hansen et al., 1986; Majewski and Hansen, 2002).

It is unclear whether the effect of progesterone on lymphocyte function is mediated directly through actions on the lymphocyte acting via progesterone receptor activation. Several authors have failed to identify progesterone receptors on lymphocytes (Mansour et al., 1994; Schuts et al., 1996; King et al., 1996) or inhibit effects of progesterone on lymphocyte function with progesterone receptor antagonists (Van Voorhis et al., 1989). In humans, Szekeres-Bartho et al. (1985) reported that progesterone receptors were induced in peripheral blood lymphocytes during pregnancy suggesting that progesterone has direct immunomodulatory effects on lymphocytes. In cows, however, there is no difference in lymphocyte sensitivity to progesterone between pregnant and non-pregnant cows (Monterroso and Hansen, 1993). Other studies have shown that the immunosuppressive actions of progesterone on lymphocytes are exerted by inhibition of

K^+ channels to depolarize the plasma membrane and inhibit Ca^{+2} signaling and subsequent IL-2 production (Ehring et al., 1998).

Perhaps progesterone exerts its effects by inducing the production of other immunossupresive molecules. It has been shown that Th2 cells are upregulated by progesterone (Piccini et al., 2001). Expression of IDO in most human cells are possibly regulated by progesterone (Mellor and Munn, unpublished observations cited in Mellor et al., 2001). Szekeres-Bartho et al. (2001) have proposed that human lymphocytes from pregnant individuals and peripheral blood lymphocytes from non-pregnant individuals exposed to progesterone produce a 34 kDa protein known as progesterone-induce blocking factor, which has inhibitory effects on NK cell activity and lymphocytes, actions on the cytokine balance (Th2 over Th1) and also reverse high resorption rates in the murine abortion system.

In summary, many mechanisms have been proposed to help the fetus to evade the maternal immune system during pregnancy. In the remainder of this chapter, relevant aspects of pregnancy in the ewe and possible mechanisms by which the allogeneic conceptus survive in the uterus of that species will be reviewed.

Pregnancy in Sheep

Pregnancy lasts approximately 147 days in ewes and is maintained by progesterone. During the first 50 days of gestation plasma concentration of progesterone range from 2-3 ng/ml at values similar to those found during the luteal phase of the estrous cycle (Bassett et al., 1969; Stabenfeldt et al., 1971). After day 50 of pregnancy, removal of the corpus luteum does not cause abortion (Casida and Warwick, 1945), suggesting that the placenta is a sufficient source of progesterone during the latter part of pregnancy. Indeed, there is a steady increase in progesterone concentration to concentrations about five times greater

(10-15 ng/ml) than values found during early pregnancy; concentrations start to decline again around two weeks before parturition (Bassett et al., 1969; Stabenfeldt et al., 1971).

Placentation

The placenta in sheep is of the epitheliochorial type, but it is generally referred to as synepitheliochorial because the uterine epithelium is modified by invasion and fusion of fetal trophectoderm binucleate cells to form a syncytium which lasts throughout pregnancy (Wooding 1982). The migration of binucleate cells towards the maternal tissue seems to be involved with the delivery of protein molecules such as placenta lactogen (Wooding 1982) and pregnancy-associated glycoprotein (Xie et al., 1991) to the maternal circulation. The most intimate attachment between placenta and endometrium, and the major site of gaseous and nutrient exchange, occurs at structure called placentomes (Davies and Wimsatt, 1966; Perry, 1981). These are formed by the combination of knob-like structures on the placenta called cotyledons and aglandular cup-like structures on the endometrium called caruncules (Davies and Wimsatt, 1966; Perry, 1981). The number of placentomes is around 60 to 100; they increase in size and number until day 90 of pregnancy when they start to shrink and possibly decrease in number (Davies and Wimsatt, 1966). Within the placentomes, at the base of the chorionic villi, maternal blood is leaked into spaces between the maternal and fetal tissues (Perry, 1981).

Unlike for placentomes, glandular epithelium is present in the interplacentomal endometrium (Perry, 1981). In this region, fetal and maternal epithelia are in contact and the luminal epithelium of the endometrium is infiltrated by fetal binucleate cells (Davies and Wimsatt 1966; Perry, 1981).

Major Histocompatibility Complex (MHC) Expression

MHC class I molecules were detected in most of the stromal cells and in the epithelium of the placentomes before day 19 of pregnancy when formation of the syncytium became apparent (Gogolin-Ewens et al., 1989). However, MHC class I molecules were not detected in the trophoblast or in the syncytial layer of the placentomes during latter stages of pregnancy (Gogolin-Ewens et al., 1989). Similarly, MHC class I mRNA or protein were not detected in the conceptus trophectoderm, even though β_2-microglobulin mRNA (but not protein) was found in the trophectoderm at day 20 of pregnancy (Choi et al., 2003). Thus, the absence of MHC class I expression may provide the trophoblast protection against T-cell cytotoxic activity. Similarly, placental tissues were negative for expression of MHC class II antigen expression (Gogolin-Ewens et al., 1989). In contrast, endometrial tissue does express MHC class I molecules on both placentomal and interplacentomal uterine epithelial cells (Gogolin-Ewens et al., 1989). In the interplacentomal regions, MHC class I was detected in the glandular epithelium and connective tissues at all later stages of pregnancy (Gogolin-Ewens et al., 1989). During days 10 and 12 of pregnancy, MHC class I and β_2-microglobulin mRNA and protein were detected in the luminal epithelium only (Choi et al., 2003). At days 14 to 20, these mRNAs and proteins were localized instead in the middle and deep glandular epithelium and stroma and there was a 3-fold increase in intensity of expression as compared to tissues from days 14 or 16 of the estrous cycle (Choi et al., 2003). Intrauterine infusion of interferon-τ (IFN-τ) increased the amount of mRNA for MHC class I and β_2-microglobulin in the glandular epithelium and stroma in ovariectomized, progesterone-treated ewes (Choi et al., 2003).

MHC class II molecules in the endometrium were found in the placentomal area throughout pregnancy with more positive cells during early stages (Gogolin-Ewens et al., 1989). In the interplacentomal regions, MHC class II molecules were localized in epithelium and stroma; numbers of positive cells decreased in the subepithelial stroma as pregnancy advanced (Gogolin-Ewens et al., 1989). In ovariectomized ewes, MHC class II positive cells were localized throughout the caruncular and intercaruncular endometrium with more intense staining in the luminal epithelium and subepithelial layers (Gottshall and Hansen, 1992). Treatment with progesterone for 60 days reduced the number of MHC class II positive cells (Gottshall and Hansen, 1992).

Uterine Leukocyte Populations in Sheep

In the uterus of cyclic ewes, lymphocytes are found in the caruncular and intercaruncular epithelium (Lee et al., 1988) but, during pregnancy, lymphocytes become nearly absent in the placentomes (Gogolin-Ewens et al., 1989). In the intercaruncular endometrium, lymphocytes are mainly localized in the luminal and glandular epithelium and in some areas of the stroma immediately beneath these epithelia (Lee et al., 1988; Gotshall and Hansen, 1992; Majewski et al., 2001). In the non-pregnant sheep uterus, around 50% of the intraepithelial lymphocyte population of the endometrium is composed of $CD8^+$ $CD45R^-$ $\gamma\delta$ TCR^- cells and the remaining population consists of equal numbers of $CD8^+$ $CD45R^+$ $\gamma\delta$ TCR^+ cells and $CD8^+$ $CD45R^+$ $\gamma\delta$ TCR^- cells (Meeusen et al., 1993). During the later stages of pregnancy, in contrast, the majority of intraepithelial lymphocytes are large granulated $CD8^+$ $CD45R^+$ $\gamma\delta$ TCR^+ cells (Meeusen et al., 1993). These cells contain the cytolytic molecule perforin (Fox and Meeusen, 1999). The proportion of $CD8^+$ $CD45R^+$ $\gamma\delta$ TCR^+ cells increase in number in the luminal epithelium of the interplacentomal areas during mid and late pregnancy (Lee et al., 1992;

Meeusen et al., 1993; Nasar et al., 2002). In contrast, this cell population remains nearly constant in numbers in the glandular epithelium throughout pregnancy (Lee et al., 1992; Nasar et al., 2002). During parturition, a significant decrease occurs in the percentage of both large granulated and non-granulated lymphocytes in the luminal and glandular epithelium (Nasar et al., 2002). Both lymphocyte populations remain constant at day 1 postpartum in both epithelia, but at postpartum day 3, there was an increase of large granulated lymphocytes in the luminal epithelium and in both types of lymphocytes in the glandular epithelium (Nasar et al., 2002).

While $CD8^+$ cells are present in the endometrium, there are very few $CD4^+$ T helper cells, mast cells, or B cells in the endometrium of cyclic or pregnant ewes (Lee et al., 1988; 1992, Gogolin-Ewens et al., 1989). Cells involved in innate immunity have been recently described in the ovine endometrium. Macrophages are mainly localized in the intercaruncular stroma, between the luminal and glandular epithelium, and their numbers increase greatly during pregnancy (Tekin and Hansen, 2004). Inducible nitric oxide synthase (iNOS), an enzyme required for activated macrophages to produce nitric oxide, is found in the luminal and glandular epithelium, and stromal cells of the intercaruncular endometrium, placentomes and intercotyledonary placenta of pregnant ewes (Zheng et al., 2000; Kwon et al., 2004). In the intercotyledonary placenta and intercaruncular endometrium, iNOS levels were high at day 60 of gestation, decline at day 80 and then increase again on days 100 and 120, respectively (Kwon et al., 2004). The highest levels of activity of iNOS were detected in the placentomes between days 100-120 of pregnancy (Kwon et al., 2004).

Activity characteristic of natural killer-like cells was found in endometrial epithelial cells (Tekin and Hansen, 2002). Moreover, there was intense immunolocalization of the cytolytic protein perforin in the glandular epithelium, less intense staining in the luminal epithelium and a few areas of staining in the endometrial stroma of pregnant and nonpregnant uterine horns of unilaterally-pregnant ewes (Tekin and Hansen, 2003). Also, eosinophils were localized in the stratum compactum stroma after day 11 in the pregnant ewe and cells expressed mRNA levels for the chemoattractants monocyte chemotactic protein (MCP)-1 and -2 (Asselin et al., 2001). Intrauterine infusion of IFN-τ in ovariectomized progesterone-treated ewes increased the abundance of endometrial MCP-1 and MCP-2 mRNAs (Asselin et al., 2001).

Messenger RNA for a variety of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-1α, IL-1β, IL-6, IL-8, IL-10, IFN-γ and TGF-β was found in the uterine epithelium of nonpregnant and pregnant ewes, although there was no differences in expression between the groups (Fox et al., 1998). In contrast, there was very low expression of IL-3 and granulocyte macrophage-colony stimulating factor (G-CSF) in the endometrium and no expression of IL-2 or IL-4 in the endometrium from nonpregnant or pregnant sheep (Fox et al., 1998).

There is evidence for local activation of endometrial lymphocytes by the conceptus. Expression of activation markers such as CD25, CD44, CD29 and L-selectin on γδ TCR^+ intraepithelial lymphocytes recovered from the pregnant horn of unilaterally-pregnant was higher than expression for lymphocytes from the non-pregnant horn or cyclic ewes (Liu et al., 1997). There is systemic regulation of endometrial leukocytes numbers as well, however. In particular, the increase in the number of γδ TCR^+ cells in the luminal

epithelium during mid and late pregnancy (Lee et al., 1992) is likely due to endocrine changes associated with pregnancy and not due to a local signal from the conceptus because numbers of γδ TCR^+ were higher in the luminal epithelium of both pregnant and nonpregnant uterine horns of unilaterally-pregnant ewes as compared to ovariectomized ewes (Majewski et al., 2001). Moreover, the difference in numbers between pregnant and non-pregnant horns was small and non-significant. Macrophage levels seem to be regulated by both systemic and local signals because the accumulation of macrophages in both horns of unilaterally-pregnant ewes was higher in the non-pregnant ewes and accumulation was greater in the pregnant uterine horn than in the non-pregnant horn (Tekin and Hansen, 2004).

Progesterone as Immunosuppressive Molecule in Sheep

The identity of pregnancy-associated systemic and local regulators of endometrial lymphocyte function is largely unknown. One molecule that has been well documented as a regulatory molecule for uterine immune function is progesterone. Progesterone can inhibit two aspects of the immune function – clearance of bacteria and rejection of allografts and xenografts. In cyclic ewes, intrauterine inoculations with *Actinomyces pyogenes* and *Escherichia coli* caused the establishment of uterine infections when inoculations were performed on day 7 of the estrous cycle, but not when inoculations were performed at day 0 of the cycle (Ramadan et al., 1997; Seals et al., 2003). Injections of progesterone for 20 days to ovariectomized-postpartum ewes resulted in uterine infections after intrauterine infusions of *A. pyogenes* and *E. coli* (Seals et al., 2002; Lewis 2003). Such uterine infections did not occur in animals treated with the vehicle (Ramadan et al., 1997; Seals et al., 2002; 2003; Lewis 2003). Daily injections of progesterone did not prevent rejection of skin grafts placed into the uterus when the

hormone was given three days before the placement of the grafts into the uterus (Reimers and Dziuk, 1974). However, long-term exposure to progesterone in ovariectomized ewes beginning 30 days before grafting prolonged survival of skin grafts (Hansen et al., 1986) and mouse hybridoma cells (xenografts) placed into uterine lumen of sheep (Majewski and Hansen, 2002).

Progesterone can also lead to changes in the population of endometrial lymphocytes. Treatment with progesterone for 60 days reduced the number of MHC class II^+ and $CD45^+$ cells in the intercaruncular endometrium of ovariectomized ewes (Gottshall and Hansen, 1992). In contrast, the number of macrophages was not affected by progesterone treatment in ovariectomized ewes (Tekin and Hansen, 2004) and expression of MCP-1 and MCP-2 mRNA in endometrial eosinophils in ovariectomized ewes was increased by progesterone treatment (Asselin et al., 2001).

The mechanisms by which progesterone inhibit uterine immune function are incompletely understood. It is unlikely that progesterone acts directly on uterine lymphocytes. High concentrations of progesterone (10^{-6} to 10^{-5} M) are required to suppress lymphocyte proliferation induced by mitogens (Staples et al., 1983; Low and Hansen, 1988; Monterroso and Hansen, 1993). These concentrations are higher than the K_D (10^{-10} M) of the progesterone receptor (Olea-Serrano et al., 1985) and higher than concentrations in circulations (~10^{-8} M). Probably, progesterone inhibits lymphocytes through a receptor-independent mechanism, because inhibition was not affected by the presence of the progesterone receptor antagonist RU 38486 (Monterroso and Hansen, 1993). It is possible that inhibitory actions of progesterone are exerted through the induction of synthesis and secretion of other molecules in the uterus that have

immunosuppressive properties. Treatment of ovariectomized ewes with progesterone induced the appearance of lymphocyte-inhibitory activity in uterine flushings or uterine fluid (Stephenson and Hansen, 1990; Hansen and Skopets, 1992). Moreover, uterine fluids from unilaterally-pregnant ewes contain a factor that can inhibit mitogen-induced lymphocyte proliferation (Stephenson et al., 1989a). This molecule has been identified as ovine uterine serpin (Skopets and Hansen, 1993).

Characteristics of Ovine Uterine Serpin

Relationship to Serpins

Ovine uterine serpin (OvUS) belongs to the serpin superfamily of serine proteinase inhibitors (Ing and Roberts, 1989), which also includes α-$_1$-antitrypsin, angiotensinogen, and ovalbumin among others. Uterine serpins are also found in uterine secretions from pregnant cows, sows and goats (Leslie et al., 1990; Malathy et al., 1990, Tekin et al., 2004). Ovine uterine serpin shows about 96% amino acid sequence identity to caprine uterine serpin (CaUS) (Tekin et al., 2004), 72% identity to bovine uterine serpin (BoUS) but only about 50% and 56% identity, respectively, to two distinct porcine uterine serpins (PoUS-1 and PoUS-2) (Mathialagan and Hansen, 1996).

The structure of serpins is characterized by three β-sheets, nine α-helices and the presence of a reactive center loop (RCL) that is exposed for interaction with the proteinase (Irving et al., 2000; Silverman et al., 2001; van Gent et al., 2003). Binding of proteinase and serpin leads to a cleavage and inactivation of the serpin and a conformational change that makes the protein more thermodynamically stable (Irving et al., 2000; Silverman et al., 2001; van Gent et al., 2003). Not all the members of the serpin superfamily function as proteinase inhibitors. Among the non-inhibitory serpins are the molecular chaperone heat shock protein 47, the hormone transport proteins

corticosteroid binding globulin and thyroxine binding globulin, and proteins without a well-understood function like ovalbumin (Irving et al., 2000; van Gent et al., 2003).

Ovine uterine serpin also appears to be an inactive proteinase. The protein does have some inhibitory activity to the aspartic proteinases pepsin A and pepsin C (Mathialagan and Hansen, 1996; Peltier et al., 2000a), but the concentration required to inhibit pepsin is too high using a serpin-like inhibitory mechanism. Also, OvUS did not inhibit a wide range of serine proteinases (Ing and Roberts, 1989). The tertiary structure of OvUS appears to be different from a prototypical serpin since, unlike a typical serpin, limited proteolysis with trypsin did not cleave its RCL or affect its secondary structure, thermal stability or biological activity (Peltier et al., 2000a).

Biochemical Properties

Ovine uterine serpin exists in uterine fluid as a pair of basic glycoproteins with molecular weights of 55,000 and 57,000, derived from a single 54,000 precursor (Moffatt et al., 1987; Hansen et al., 1987). The isoelectric point is 9.2 (Hansen et al., 1987). Amino acid sequence of OvUS indicates the presence of two N-linked glycosylation sites, indicating that the two major forms of OvUS may differ in the number of carbohydrate chains they possess after post- translational modification (Hansen et al., 1987; Ing and Roberts, 1989). The carbohydrate content of OvUS consists of 2.8% neutral sugars, 2.5% amino sugars, and 0.3% sialic acid (Hansen et al., 1987).

Ovine uterine serpin can bind the pregnancy-associated glycoproteins which are inactive members of the aspartic proteinase family produced by the ovine trophoblast (Mathialagan and Hansen, 1996). It also binds activin A present in allantoic fluids (McFarlane et al., 1999) and the immunoglobulins IgA and IgM, but not IgG (Hansen and Newton, 1988). Thus, OvUS may act as a carrier serpin for other proteins.

Similarly, PoUS was found to mediated iron transfer across the pig placenta by binding to the endometrial iron-binding protein uteroferrin (Renegar et al., 1982)

Endometrial Secretion

Ovine uterine serpin is the major protein produced by the sheep uterus during most of pregnancy (Bazer et al., 1979; Moffatt et al., 1987). It is also transported across the placenta and can be found in amniotic and allantoic fluids (Newton et al., 1989). Large amounts of the protein are present in the uterus – at day 140 for example, the total protein present in the uterine fluid recovered from unilaterally-pregnant ewes is around 15 g and most of this, as identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), is OvUS (Bazer et al., 1979; Moffat et al., 1987).

Ovine uterine serpin mRNA can first be detected around Days 13-16 of the estrous cycle and at Days 13-15 of pregnancy (Ing et al., 1989; Stewart et al., 2000). In the intercaruncular endometrium, a 3-fold increase of steady-state levels of OvUS mRNA occurs between Days 20 and 60, another 3-fold between Days 60 and 80, and a decline at Day 120 of pregnancy (Stewart et al., 2000). Between days 20 and 50 of gestation, the expression of OvUS mRNA is lower in the deep endometrial glandular epithelium than in the upper glandular epithelium of the stratum spongiosum (Stewart et al., 2000). There was however, no difference in mRNA expression between the upper and lower glandular epithelium of the stratum spongiosum between days 50 and 60 of pregnancy (Stewart et al., 2000). OvUS mRNA was expressed at high levels in all glandular epithelium in the stratum spongiosum between days 60 and 120 of gestation (Stewart et al., 2000). On postpartum day 1, OvUS mRNA was still detected in the stratum spongiosum of the glandular epithelium, but not in the superficial glandular epithelium (stratum compactum)

(Gray et al., 2003). At postpartum days 7 and 28, OvUS mRNA was not detected in the endometrial glands (Gray et al., 2003).

The secretion of OvUS is under influence of progesterone (Moffatt et al., 1987; Ing et al., 1989; Leslie and Hansen, 1991). In ovariectomized ewes, ovine uterine serpin can be detected after 6 days of progesterone therapy (Ing et al., 1989). A large increase in the secretion of OvUS is observed by 14-30 days of progesterone treatment (Ing et al., 1989; Leslie and Hansen, 1991). Levels of OvUS mRNA were not affected by uterine infusion of placental lactogen and growth hormone in progesterone-treated ovariectomized ewes (Spencer et al., 1999a), but was increased in the glandular epithelium by uterine infusion of placental lactogen and/or growth hormone combined with IFN-τ infusions (Spencer et al., 1999a; Noel et al., 2003). A decrease of OvUS mRNA in the glandular epithelium of ovariectomized ewes was induced by co-administration of estradiol with progesterone, which up-regulates the expression of progesterone receptors in the endometrium (Spencer et al., 1999a).

It appears that OvUS is initially produced only by the uterine glands and then its expression spreads to the luminal epithelium. Although OvUS protein was found only in glandular epithelium at day 60 of pregnancy, it was immunolocalized in the luminal and glandular epithelium of the intercaruncular endometrium by days 120 to 140 of pregnancy (Moffatt et al., 1987; Stephenson et al., 1989b).

Immunosuppressive Properties

Immunosuppressive actions of OvUS have been broadly tested. Purified OvUS inhibited lymphocyte proliferation produced by exposure to the antigen *Candida albicans*, phytohemagglutin (PHA), concanavalin A (Con A), and in the mixed lymphocyte reaction (Segerson et al., 1984; Stephenson et al., 1989a; Skopets and

Hansen, 1993; Skopets et al., 1995). Although OvUS inhibited T cell-dependent lymphocyte proliferation, it did not cause any immunosuppressive activity against lymphocytes activated by the T and B cell mitogen pokeweed (PWM) (Skopets and Hansen, 1993). OvUS also reduced the antibody titer in ewes immunized against the T-cell dependent antigen ovalbumin (OVA) (Skopets et al., 1995).

Ovine uterine serpin had no effect on the reduction of skin-fold thickness caused by *Mycobacterium tuberculosis* in sheep (Skopets et al., 1995) and failed to inhibit expression of CD25 on $\gamma\delta$-T^+ cells induced by Con A (Peltier et al., 2000b). Perhaps, the failure of OvUS to suppress proliferation of $\gamma\delta$-T^+ cells reflects the increase in numbers of these cells in the endometrial epithelium during mid and late pregnancy (Lee et al., 1992) when OvUS is produced at high concentrations in the uterus.

Ovine uterine serpin also inhibits NK cells. This was first demonstrated by Liu and Hansen (1993) who found OvUS inhibited NK-like activity in sheep lymphocytes and mouse splenocytes against K562 and YAC-1 target cells (Liu and Hansen, 1993). OvUS also inhibited lytic activity of NK-like cells in peripheral blood lymphocytes and endometrial epithelium against D-17 cells infected with bovine herpes virus-1 (Tekin and Hansen, 2002). In vivo, OvUS blocked abortion induced by poly(I)•poly(C) in pregnant mice (an NK-cell mediated phenomenon; Kinsky et al., 1990), and reduced basal splenocyte NK cell activity (Liu and Hansen, 1993).

Binding of OvUS to lymphocytes is specific, dose dependent and saturable (Liu et al., 1999). OvUS contains several phosphorylation sites such as tyrosine kinase, protein kinase C, and cyclic adenosine monophosphate (Peltier et al., 2000c). Although the exact mechanism by which OvUS inhibits lymphocyte proliferation remains unknown, the

protein does block IL-2 induced proliferation and reduced expression of CD25 (IL-2Rα chain) (Peltier et al., 2000b). OvUS does not inhibit the costimulatory effect of CD26 on PHA-stimulated lymphocytes (Liu and Hansen, 1995) and does not block the increase in IL-2 mRNA caused by Con A (Peltier et al., 2000b). The immunosuppressive effect of OvUS is not blocked by addition of neutralizing antibody to transforming growth factor-β (Skopets and Hansen, 1993).

Ovine Uterine Morphogenesis

In sheep, uterine morphogenesis occurs during fetal and neonatal life. Uterine horns are already fused and slightly curved on gestational days 55 – 60 and the mesenchyme is already differentiated into endometrium and myometrium (Wiley et al., 1987). Fetal uteri at day 90 – 100 have curved uterine horns, characteristic of the adult uterus, with clearly defined nodular (aglandular) and internodular (glandular) areas (Wiley et al., 1987). Although uterine glands are not present during fetal development, small invaginations in the mucosal epithelial are observed in the internodular areas on days 135 to 150 of fetal life (Wiley et al., 1987). Endometrial glands are still absent in the neonate at postnatal Day 0 – 1 (PND 0 - 1) (Bartol et al., 1988ab; Taylor et al., 2000). Adenogenesis starts between PND 0 – 7 when shallow invaginations appear in the luminal epithelium; tubular structures that branch and coil into the stroma are seen on PND 7 – 14 (Wiley, 1987; Bartol et al., 1988ab; Taylor et al., 2000). Extensive uterine gland development that advances to the myometrium is reached on PND 21 – 28 (Wiley et al., 1987; Bartol et al., 1988b; Taylor et al., 2000). Complex, coiled and branched tubular glands are present throughout the stroma on PND 42 – 56 that appear very similar to the adult uterus (Taylor et al., 2000).

The process of adenogenesis requires an increase in cell proliferation of epithelial cells of the luminal and glandular epithelium (Bartol et al., 1988b; Taylor et al., 2000). The initiation of endometrial gland formation is an ovary-independent event since gland development on PND 14 was not affected by ovariectomy at birth (Bartol et al 1988a; Carpenter et al., 2003a). Although ovariectomy on PND 7 did not affect the number of superficial ductal invaginations of the glandular epithelium from the luminal epithelium or the density of endometrial glands in the stratum compactum area of the stroma on PND 56, it reduced the total number of endometrial glands and the density of endometrial glands in the stratum spongiosum area of the stroma, suggesting that some ovarian derived factors regulate in part the process of coiling and branching between PND 14 and 56 (Carpenter et al., 2003a). In addition, reduction in mRNAs expression for follistatin, activin subunit βA, activin receptor types IA and II and an increase in activin subunit βB expression were detected by in situ hybridization on PND 56 in ewes ovariectomized on PND 7 (Carpenter et al., 2003a; Hayashi et al., 2003).

Taylor et al. (2001) suggests that uterine gland proliferation may be promoted by the action of stromal insulin-like growth factor-I (IGF-I) and IGF-II acting through IGF-I epithelial receptor. IGF-I and IGF-II mRNAs expression were localized abundantly in the intercaruncular stroma underlying proliferating and differentiating endometrial glands on PND 7 to 56 and PND 21 to 42, respectively (Taylor et al., 2001). In contrast, IGF-I receptor mRNA expression was particularly abundant in the luminal epithelium on PND 1 and also in the nascent and proliferating glands on PND 21 to 56 (Taylor et al., 2001). In addition, fibroblast growth factor-7 and hepatocyte growth factor may be involved in the process of coiling and branching of gland morphogenesis because there was an

increase in expression of their mRNA for these growth factors in the intercaruncular endometrium after PND 21 (Taylor et al., 2001).

Activation of the estrogen receptor (ER) seems to regulate in part the process of gland formation in the intercaruncular endometrium between PND 14 and 56. The initial stages of the adenogenesis process between birth and PND 14, which involves budding differentiation and formation of tubules, were not affected by treatment of neonatal ewes with an antagonist of ERα and ERβ, but the antagonist retarded the coiling and branching processes of the gland formation that occur between PND 14 and 56 (Carpenter et al., 2003c).

In recent studies, endometrial adenogenesis was down-regulated by hypoprolactinemia and up-regulated by hyperprolactinemia, demonstrating that prolactin is implicated in uterine gland formation (Carpenter et al., 2003b). In addition, signal transducers and activators of transcriptions (STAT) 1, 3 and 5 were expressed in the nascent glandular epithelium and prolactin increased phosphorylation of STATs 1 and 5 in uterine explants (Carpenter et al., 2003b).

Uterine Gland Knockout Model

In ewes, administration of a potent synthetic progestin to the neonate inhibits endometrial adenogenesis to generate an adult uterine gland knockout (UGKO) phenotype (Spencer et al., 1999b; Gray et al., 2000ab; 2001ab). Depending upon the animal, the intercaruncular endometrial areas contain ruffled luminal epithelium and compact stroma with either complete absence of glands, slight glandular invaginations or infrequent cyst and gland-like structures in the stroma (Gray et al., 2000ab; 2001a). Development of the UGKO phenotype requires about eight weeks of progestin exposure; neonatal administration for 13 days did not induce a complete ablation of uterine glands

(Bartol et al., 1988b; Gray et al., 2000a). Exposure of lambs to estradiol valerate also generates an UGKO adult phenotype (Carpenter et al., 2003c; Hayashi et al., 2004). Uterine morphology and function is disrupted by long term-exposure of neonatal pigs to estradiol valerate (Spencer et al., 1993; Tarlenton et al., 2003). Treatments with progesterone and estradiol produced partial to complete uterine gland knockout phenotype in cows (Bartol et al., 1995). The mechanism by which neonatal exposure to progestin blocks development of endometrial glands is not known, but the inhibition of adenogenesis is not produced by direct inhibition of endometrial cell proliferation (Gray et al., 2000b).

Although UGKO ewes exhibit variability in the length of the estrous cycle, they have normal progesterone concentrations in plasma and respond to prostaglandin $F_{2\alpha}$ by undergoing luteinization (Gray et al., 2000a). Histoarchitectural evaluation of UGKO ewes showed normal ovaries, oviductal ampullar and isthmus, cervix, vagina and uterine myometrium (Gray et al., 2000b; 2001a). Ewes with the UGKO phenotype also have normal expression of endometrial progesterone, estrogen and oxytocin receptors (Gray et al., 2000a).

UGKO ewes are not able to support pregnancy to day 25 (Gray et al., 2000a; 2001ab; 2002). Immunoreactive IFN-τ in uterine flushes from UGKO ewes was present in very low amounts or was undetectable and ewes either had no conceptus, degenerating tubular conceptus or fragmenting filamentous conceptus (Gray et al., 2001b; 2002). In addition, other molecules involved in cell-cell adhesions such as osteopontin and GlyCAM-1 were undetectable or present in low levels in uterine flushes of UGKO ewes at 14 days after mating (Gray et al., 2002). In contrast, normal patterns of

immunoreactive Muc-1 and integrins were present in the luminal epithelium of the endometrium of UGKO ewes (Gray et al., 2002) and expression of IFN-τ dependent genes in the endometrium could be induced by intra-uterine injections of recombinant ovine IFN-τ (Gray et al., 2002).

Synopsis and Hypothesis

Based on this literature, there is evidence to support the idea that OvUS inhibits lymphocyte proliferation and is a mediator of immunosuppressive effects of progesterone in the ewe. The definitive test to determine whether OvUS plays such a role is to determine whether removal of OvUS (usually performed homologous recombination) blocks the effect of progesterone on uterine immune function. Since it is not feasible to use the transgenic model in sheep, the UGKO ewe is an alternative model to test the effects of OvUS on uterine graft rejection because the absence of uterine endometrial glands should result in a progesterone-treated ewe without the capacity for OvUS synthesis. Therefore, the objective presented in this dissertation is to use the UGKO model to evaluate the role of endometrial glands and by inference, OvUS in regulation of uterine immune function by progesterone (Figure 1-1).

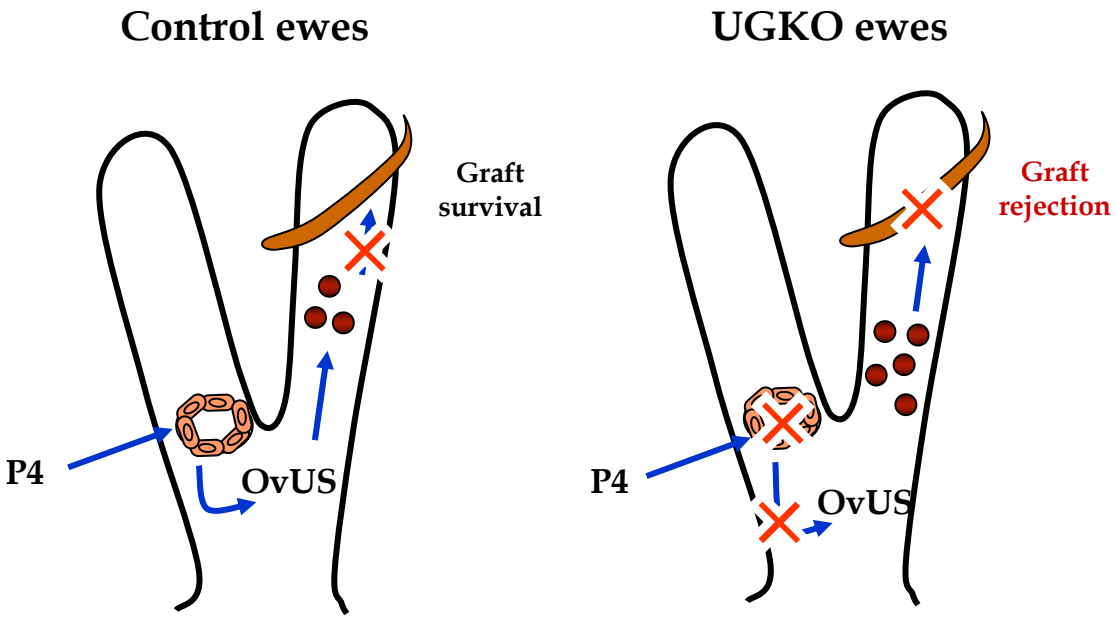

Figure 1-1. Schematic illustration of the hypothesis. For control ewes, progesterone (P4) induces the secretion of OvUS from the uterine endometrial glands, and OvUS in turn decreases activation of lymphocytes in response to the skin allograft placed within the uterus. As a result, the allograft survives within the uterus. For the uterine gland knockout ewes (UGKO) ewes, progesterone will not induce the synthesis of OvUS because uterine endometrial glands are absent. As a result, lymphocytes against the allograft will become

CHAPTER 2
ACTIONS OF PROGESTERONE ON UTERINE IMMUNOSUPPRESSION AND ENDOMETRIAL GLAND DEVELOPMENT IN THE UTERINE GLAND KNOCKOUT (UGKO) EWE

Introduction

Among its many actions to maintain pregnancy, progesterone acts to inhibit uterine immune function which may 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 response (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 OvUS, also known an ovine uterine milk protein. This protein is a member of the serine proteinase inhibitor superfamily (Ing and Roberts, 1989) and produced by uterine endometrium under the influence of progesterone (Moffat et al., 1987; 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) and pigs (Malathy et al., 1989). The major site of synthesis of OvUS is the glandular epithelium on the endometrium. Expression of OvUS mRNA was limited to glandular epithelium between days 17 and 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., 1989b). By days 120 to 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., 2000b) 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; Tarlenton et al., 2003; Carpenter et al., 2003c). 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 cyst- or gland-like structures (Gray et al., 2000ab; 2001a), without apparent effects on development of other extrauterine reproductive tract structures or the ovary (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 a normal response to interferon-τ (Gray et al., 2002). However, UGKO ewes exhibit a recurrent pregnancy loss that involves loss of the elongation conceptus between days 9 and 14 of pregnancy (Gray et al., 2000a; 2001ab; 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 is 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 also provide 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, kaleidoscope protein standard, 2-mercaptoethanol and gelatin were obtained from Bio–Rad (Hercules, CA). Hybridoma cells producing monoclonal antibody to $CD45R^+$ (clone 73B) were purchased from the European Collection of Cell Cultures (Salisbury, UK). Ascites fluid for $CD45R^+$ 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, 1995).

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 crossbred Rambouillet ewe lambs with a single Synchromate B® (Sanofi, Overland Park, KS) implant within 12 hours of birth and every two weeks thereafter for a total of 8 weeks. Implants were inserted subcutaneously in the periscapular area and released approximately 6 mg of norgestomet (17α-acetoxy-11β-methyl-19-norpreg-4-ene-3,20-dione), a potent synthetic 19-norprogestin, over a 14

day period (Bartol et al., 1988b). Normal control ewes did not receive implants. The normal control and UGKO ewes used in the present study were approximately three years of age.

All ewes were bilaterally ovariectomized via midventral laparatomy 30 days before the initiation of the experiment. Treatments were arranged according to a 2 x 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 8 control ewes and 8 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 4 control ewes and 3 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 hours 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

Visible 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 3600 x g at 4^{o}C for 20 minutes and the supernatant fraction stored at – 20^{o}C for further analysis. When visible uterine fluid was not present, the uterus was flushed with 20 ml of

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^3) of the intercaruncular endometrium were collected at random from each uterine horn, near the area where the graft was placed, 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 5µm 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, USA).

Progesterone Radioimmunoassay

Blood samples collected via jugular venipuncture into heparinized tubes were placed on ice until they could be centrifuged at 2000 x g for 20 minutes, and the plasma harvested and stored at -20^{o}C until the day of the assay. Progesterone was measured using a solid-phase ^{125}I radioimmunoassay kit (Coat-A-Count® Progesterone Diagnostic Products Laboratory, Los Angeles). 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

Aliquants of 1 µg uterine protein diluted in a total volume of 20 µl with DPBS were mixed 1:1 (v/v) with loading buffer [0.125 M Tris - HCl pH 6.8 containing 20% (v/v) sucrose, 10% (w/v) SDS, a trace amount of bromephenol blue and 5% (v/v) 2-mercaptoethanol] and boiled for 3 minutes. Samples of 0.5 µg of uterine protein, as well as samples of purified OvUS and OVA were then separated according to molecular weight 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. Conditions for transfer were 200 mA for 1 hour at room temperature using a degassed buffer of 25 mM Tris, 193 mM glycine and 20% (v/v) methanol. 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). Membranes were rinsed four times with TBS-T and then incubated for 1 hour at room temperature with a mouse monoclonal antibody recognizing OvUS (HL–218, 1:32,000 dilution of hybridoma supernatant in TBS-TG) and then washed as described before. Membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated sheep anti-mouse IgG (1:8000 dilution in TBS-TG) and washed as previously

described. Blots were developed using the ECL Western blotting chemiluminescence substrate kit for 1 minute. 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$^+$ Lymphocytes

Immunohistochemistry was performed using the HistoScan Universal Monoclonal Detector kit (Biomeda, Foster City, CA) that utilizes strepavidin-biotin peroxidase complex for detection. The procedure was performed on 5 µm-thick formalin-fixed, paraffin-embedded sections placed on poly-L-lysine-coated slides. After deparaffinization and rehydration slides were microwaved while immersed with 10 mM citrate pH 6.0. The procedure was performed three times for 2 minutes each and specimens were allowed to cool between procedures and finally for 20 minutes. Slides were then washed twice in deionized water (5 minutes each), once in phosphate buffered saline [PBS; 0.1 M sodium phosphate, pH 7.4 containing 0.9% (w/v) sodium chloride] containing 2% (v/v) hydrogen peroxide for 5 minutes and once in PBS for 5 minutes. All further steps were performed in a humidity chamber at room temperature and slides were washed for 3 minutes between each step with PBS-GS [PBS containing 2% (v/v) donor goat serum]. After application of the tissue conditioner provided in the kit for 5 minutes, sections were incubated for 30 minutes with the primary antibody (for OvUS, HL-708, 1:800 dilution of hybridoma supernatant in PBS-GS; for 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, St. Louis, MO), at the same dilution as used for primary antibodies. Reagents provided in the kit were used for the other steps as recommended by the manufacturer. Incubation with biotin-conjugated goat anti-mouse IgG and streptavidin-peroxidase were for 30 minutes each, incubation with the

chromogen reagent, 3-amino-9-ethylcarbazole was for 15 minutes and counterstaining with hematoxylin was for 3 minutes. Slides were then rinsed with deionized water for several minutes and blotted before applying Crystal/Mount medium (Biomeda, Foster City, CA) and coverslips.

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, USA). Presence of OvUS was evaluated qualitatively. $CD45R^+$ 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-square analysis of variance using the General Linear Models procedure of the Statistical Analysis System (SAS for Windows, Release 8.02, SAS Institute, Cary, NC, USA). For repeated-measures data (progesterone concentrations and numbers of $CD45R^+$ cells), 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 $CD45R^+$ 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 means ± 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 on day 15 of treatment, concentrations of progesterone were higher ($p<0.001$) for ewes treated with 100 mg/day of the hormone than for ewes receiving corn oil vehicle (Figure 2-1). Concentrations in the progesterone-treated ewes peaked at a concentration of 17.9 ng/ml at 8 hours after injection and then decline to a nadir of 13.1 ng/ml at 24 hours after injection (i.e., immediately before the subsequent progesterone 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 Figure 2-2B showing a uterus from control ewe treated with corn oil vehicle with the uterus on the right of Figure 2-2C that represents a uterus from a control ewe treated with progesterone). In contrast, there was no 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 Figures 2-2A and the left-hand uterus in Figure 2-2C). Moreover, the uterus of UGKO ewes treated with progesterone was much smaller than those of control ewes treated with the hormone (Figure 2-2C).

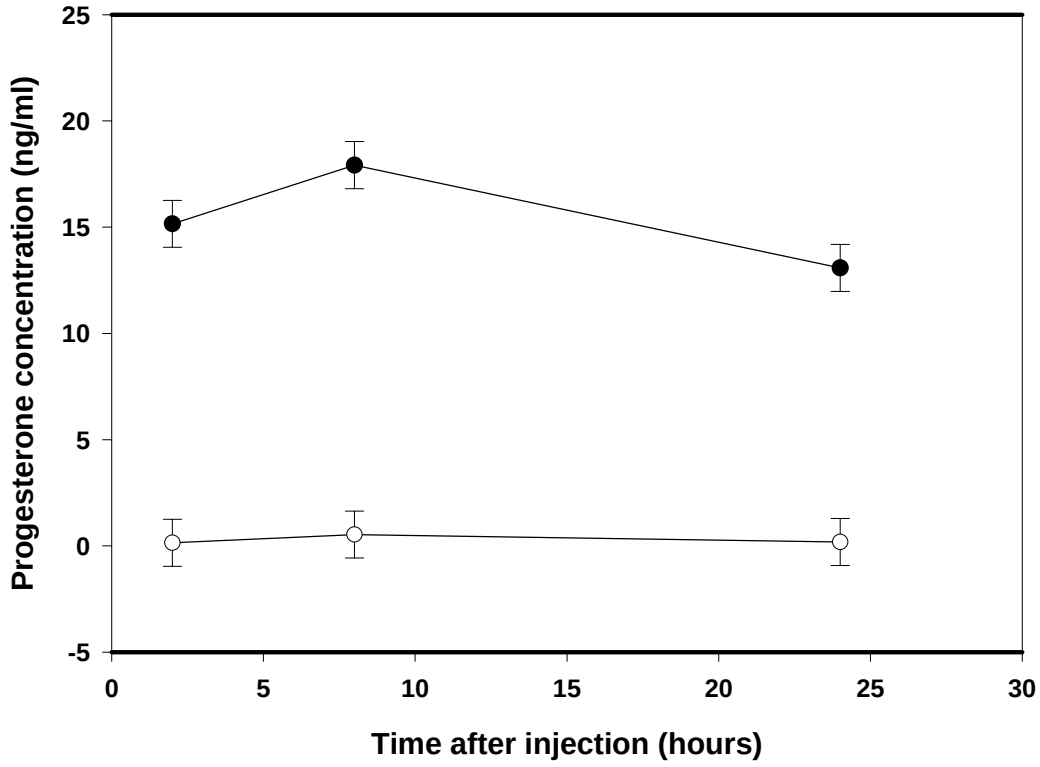

Figure 2-1. Progesterone concentration in plasma (ng/ml) 2, 8 and 24 hours after injection in ewes treated with corn oil vehicle (open circles) or progesterone (closed circles) over a 45 days period. Data represent least square means ± SEM. Progesterone concentrations differed between the two groups ($p<0.001$).

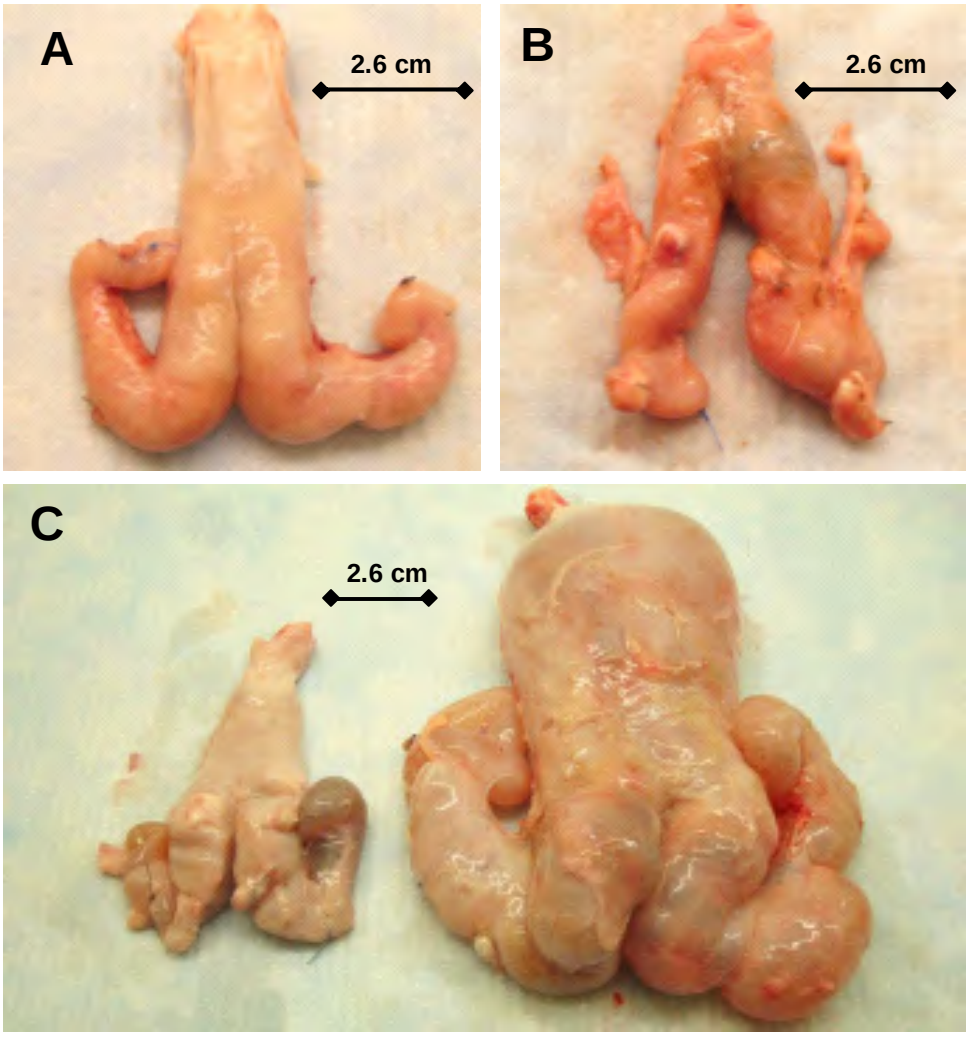

Figure 2-2. Gross appearance of the uteri. Panels A and B illustrate uteri from a control (A) and uterine gland knockout (UGKO) ewe (B) that were treated with corn oil for 60 days. Panel C shows uteri of a UGKO (left side) and control ewe (right side) treated with progesterone for 60 days. Note the difference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

There were other characteristics of the uterus of the UGKO ewes that differed from the typical appearance of the sheep uterus. Oftentimes, 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, caruncules were almost totally absent on the endometrium of the vehicle-treated ewes. There were, however, prominent caruncules in most of the progesterone-treated ewes.

Histological Analysis of Endometrium

The presence of uterine endometrial glands is summarized in Table 2-1. 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 Figures 2-3A and 2-3B). For UGKO ewes treated with corn oil vehicle, luminal epithelium was present in all cases, but glandular epithelium was absent or greatly reduced in 2 of 3 ewes. A few scattered cyst-like or primitive glands could be identified but otherwise uterine endometrium was composed of luminal epithelium and stroma (Figure 2-3C). In contrast to this pattern, well-defined glandular epithelium was present in the remaining corn oil-treated ewe (Figure 2-3D) 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) or in both uterine horns (n=4) (Figure 2-3E and 2-3F respectively).

Survival of Skin Grafts

Results of skin graft survival are summarized in Table 2-1. All autografts survived regardless of treatment. Grossly, the grafts appeared healthy and most were attached to

Table 2-1. 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).[1]

Ewe type	Treatment	Graft survival		Histology	Western blot	IHC
		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

[1] Results are fraction of graft surviving (graft survival), the fraction of ewes with uterine glands (Histology), the proportion of ewes in which OvUS was detected in uterine fluid or flushings (Western blot) and the proportion of ewes in which OvUS was immunolocalized to endometrium by immunohistochemistry (IHC).

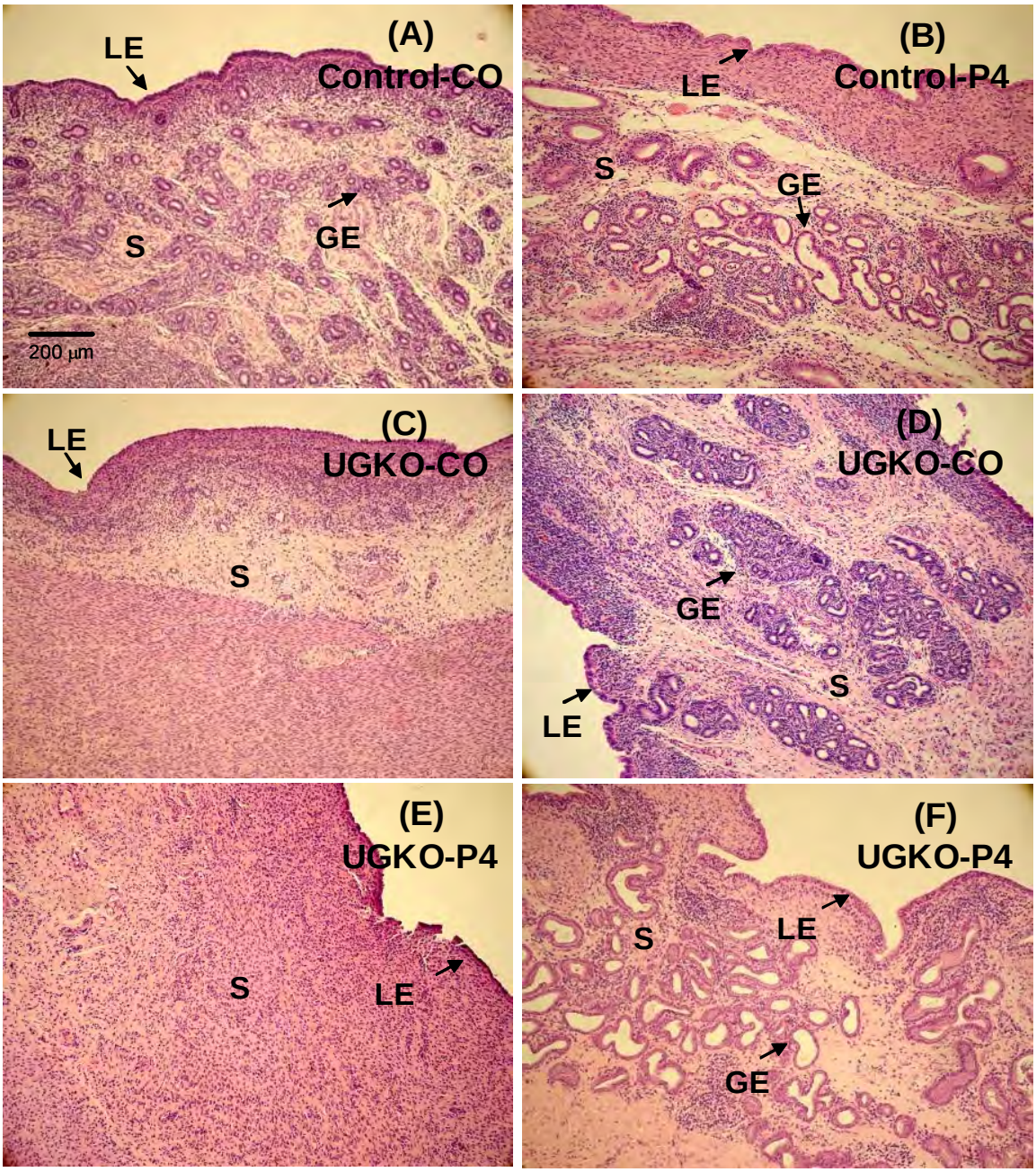

Figure 2-3. Endometrial histology for control and uterine gland knockout (UGKO) ewes treated with corn oil (CO) or progesterone (P4) for 60 days. Sections were stained with hematoxylin and eosin. Panels A and B show uteri from control ewes with well defined luminal and glandular epithelium. Panels C, D, E and 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 uterine horn (n=4) or both (n=4) uterine horns. LE, luminal epithelium; GE, glandular epithelium; S, stroma.

the uterine endometrium (see Figure 2-4 for examples). Histological analysis of the skin grafts confirmed the visual observations. Autografts were well organized and viable, with the presence of well defined keratin, epidermis and dermis (Figure 2-5A to C). Allograft survival, in contrast, depended upon treatment and, when present, allograft displayed signs of necrosis. For ewes receiving corn oil vehicle allografts from 4 of 4 control ewes and 2 of 3 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 (Figure 2-4A and 2-4B). In the third UGKO ewe treated with vehicle, the allograft was present (Figure 2-4C). The pattern of graft survival was altered by progesterone treatment. In this case allografts were present in 8 of 8 control ewes and, 8 of 8 UGKO ewes (Figures 2-4D, E and F). The gross 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 keratin and epidermis and were characterized by abundant infiltration of leukocytes (Figures 2-5D, E and F).

Total Protein Content in the Uterine Lumen

Total uterine protein content of the uterine lumen was greater for UGKO ewes than control ewes ($p<0.001$). For both groups, total protein content was higher for progesterone-treated ewes than for ewes treated with corn oil vehicle ($p<0.01$), but there was an interaction between ewe type and treatment ($p<0.01$) that reflects the fact that total protein content was increased by progesterone treatment to a greater extent for UGKO ewes than control ewes (Figure 2-6).

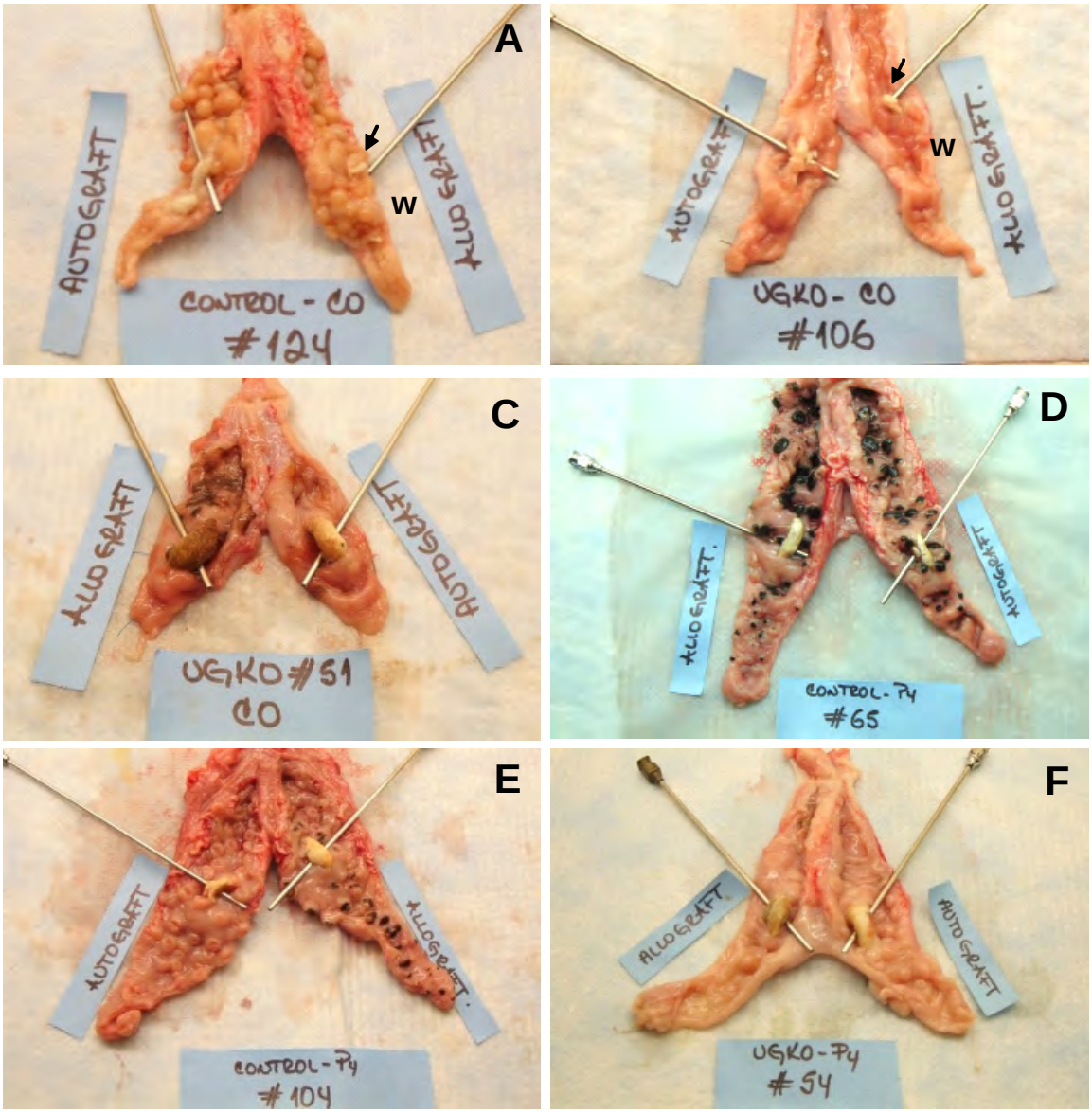

Figure 2-4. Gross appearance of surviving autografts and allografts placed into the uterus of control (A, D, E) and uterine gland knockout (UGKO) ewes (B, C, F) 30 days after surgery. Ewes were treated with corn oil (CO) (A-C) or progesterone (P4) (D-F). Note that autografts were present in all ewes 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).

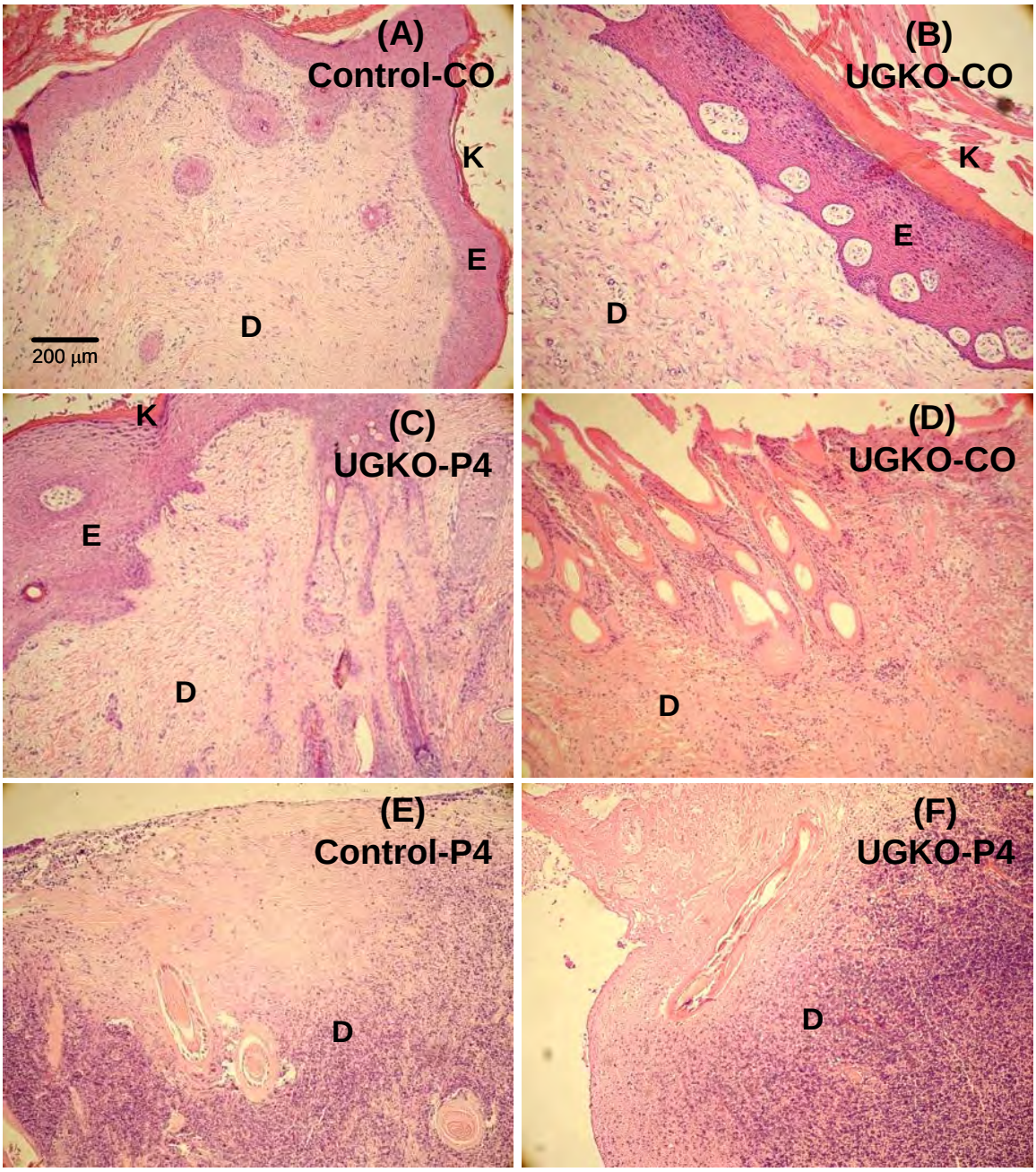

Figure 2-5. Histology of autografts (A-C) and allografts (D-F) 30 days after grafting into the uterus of control and uterine gland knockout (UGKO) ewes. Ewes were treated with corn oil (CO) or progesterone (P4). Sections were stained with hematoxylin and eosin. Panels A-C show well organized skin tissue (autografts) with presence of keratin (K), epidermis (E) and dermis (D). Panels D-F show allografts that were undergoing degeneration; note the lack of keratin and epidermis

Presence of OvUS in Uterine Fluid

A representative immunoblot for the presence of OvUS in uterine fluid is shown in Figure 2-7 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 bands seen using anti-OvUS were 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 2-1. Immunoreactive OvUS was not detected in any sections of uterine endometrium from corn oil-treated control (Figure 2-8B) or UGKO ewes (Figure 2-8C). Immunoreactive OvUS was observed, however, in all endometrial sections from progesterone-treated ewes, whether from control (Figure 2-8D) or UGKO (Figures 2-8E and 2-8F). Immunoreactive OvUS was not detected in sections of the endometrium used as negative controls (Figure 2-8A). The protein was immunolocalized to the glandular epithelium (Figures 2-8D and 2-8E) and in some areas of the luminal epithelium (Figure 2-8F). No positive reaction was detected in areas of the stroma.

Immunolocalization of CD45R$^+$ Lymphocytes

Regardless of treatment, CD45R$^+$ 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. Some CD45R$^+$ cells were localized in the stroma area (Figures 2-8G and 2-8H respectively).

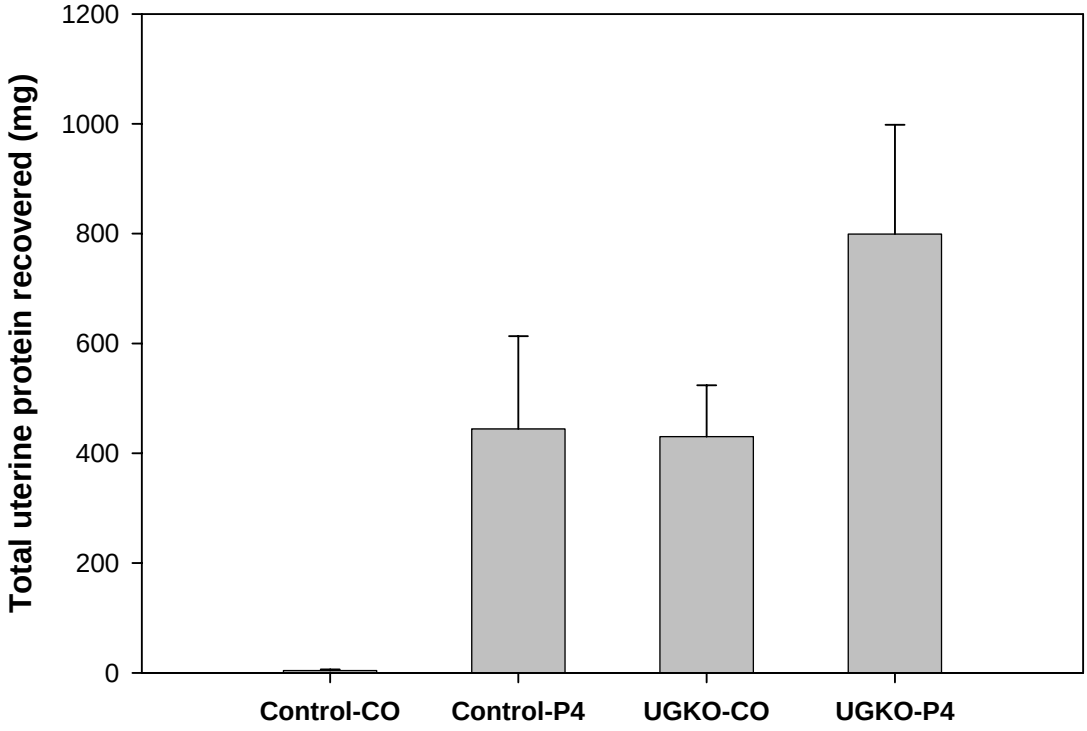

Figure 2-6. Total protein recovered from uterine fluid of control and uterine gland knockout (UGKO) ewes treated with corn oil vehicle (CO) or progesterone (P4) for a 60 days period. Data represents means ± SEM. Total protein was affected by ewe type (UGKO vs control; $p<0.001$), progesterone treatment ($p<0.01$) and the interaction of ewe type with progesterone treatment ($p<0.01$).

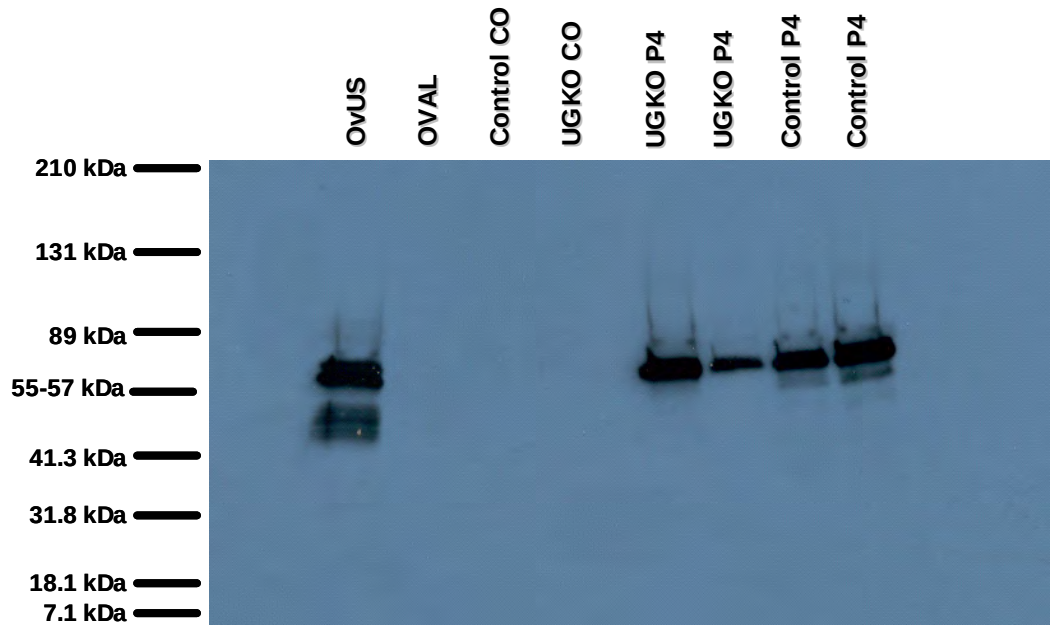

Figure 2-7. Representative western blot for detection of ovine uterine serpin (OvUS) in uterine fluid or flushings collected from control and uterine gland knockout (UGKO) ewes treated with corn oil (CO) or progesterone (P4) after 60 days. Purified OvUS and ovalbumin (OVA) were used as positive and negative control proteins respectively.

Positive cells were also detected in the luminal and glandular epithelium of the uterine endometrium where allografts were present of corn oil-treated ewes (Figure 2-8I) and in hormone-treated ewes but abundant infiltration of cells was observed into the stroma was also apparent (Figures 2-8K and L respectively). Few immunoreactive $CD45R^+$ cells were detected in the luminal epithelium and stroma of UGKO corn oil-treated ewes (Figure 2-8J).

The density of $CD45R^+$ cells was estimated by subjective scoring of each section of endometrium examined – results are shown in Figure 2-9. For luminal epithelium, density of $CD45R^+$ cells was lower for UGKO ewes than for control ewes ($p<0.01$). In both types of ewes, the presence of allografts in the uterus produced an increase of $CD45R^+$ cells in the luminal epithelium ($p<0.01$). There was a type x treatment x graft interaction ($p=0.08$). In particular, the presence of an allograft caused an increase in numbers of $CD45R^+$ cells for control ewes treated with corn oil. Progesterone blocked this increase. In the UGKO ewes in contrast, the increase in numbers of $CD45R^+$ cells caused by the allograft was small and progesterone caused an increase in numbers of $CD45R^+$ cells in both uterine horns.

Among control ewes, numbers of $CD45R^+$ 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 x graft; $p=0.07$). For glandular epithelium, density of $CD45R^+$ cells in the progesterone-treated groups was lower for UGKO ewes than for control ewes ($p=0.06$). For the UGKO ewes treated with

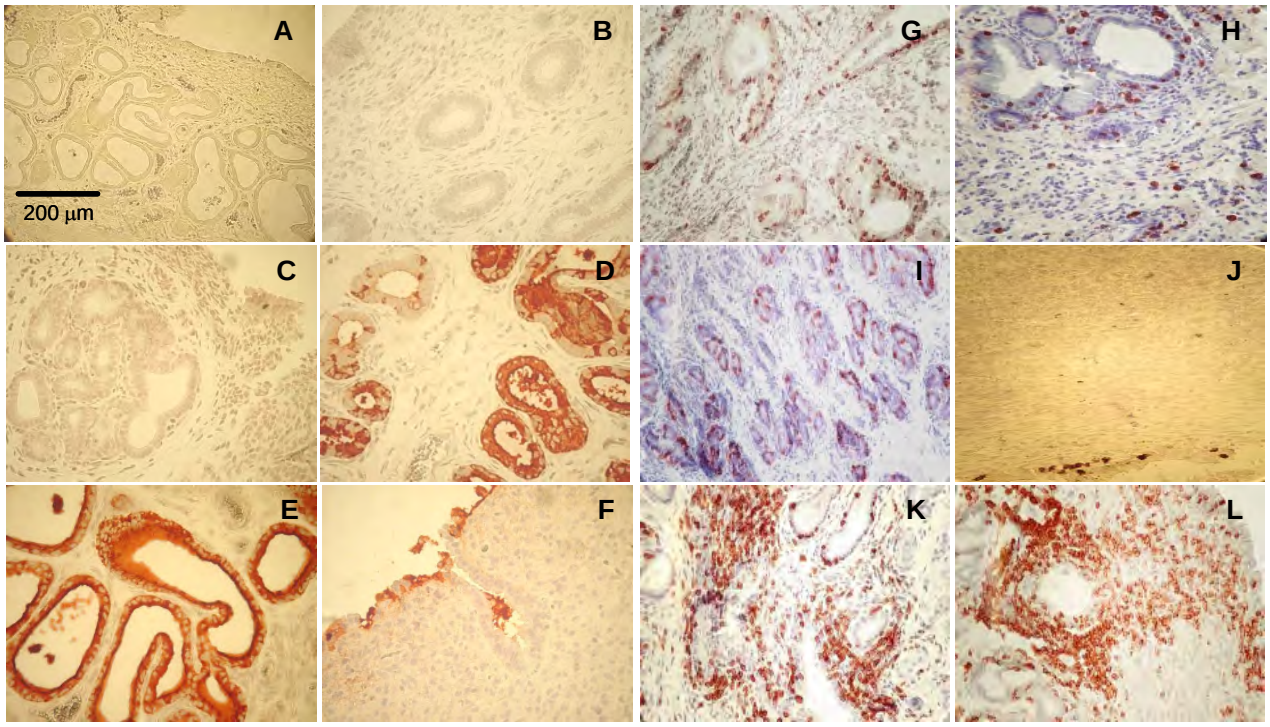

Figure 2-8. Immunolocalization of OvUS (A-F) and CD45R^{+} cells (G-L) in endometrium from control and uterine gland knockout (UGKO) ewes treated with corn oil vehicle (CO) or progesterone (P4) for 60 days. Panel A represents a negative control for OvUS. Panels B and C the lack of immunoreactive OvUS in endometrium from control (B) and UGKO ewes treated with corn oil. Panels D, E and F illustrate detection of immunoreactive OvUS in the glandular (D and E) and luminal (F) epithelium of endometrium from control (D) and UGKO (E and F) ewes treated with progesterone. Panels G-H represent immunoreactive CD45R^{+} cells in the endometrium on the side of the autograft for control (G) and UGKO (H) ewes treated with progesterone. Panels I-L represent immunoreactive CD45R^{+} cells in the endometrium on the side of the allograft of control (I) and UGKO (J) ewes treated with the vehicle; and for UGKO (K) and control (L) ewes treated with progesterone.

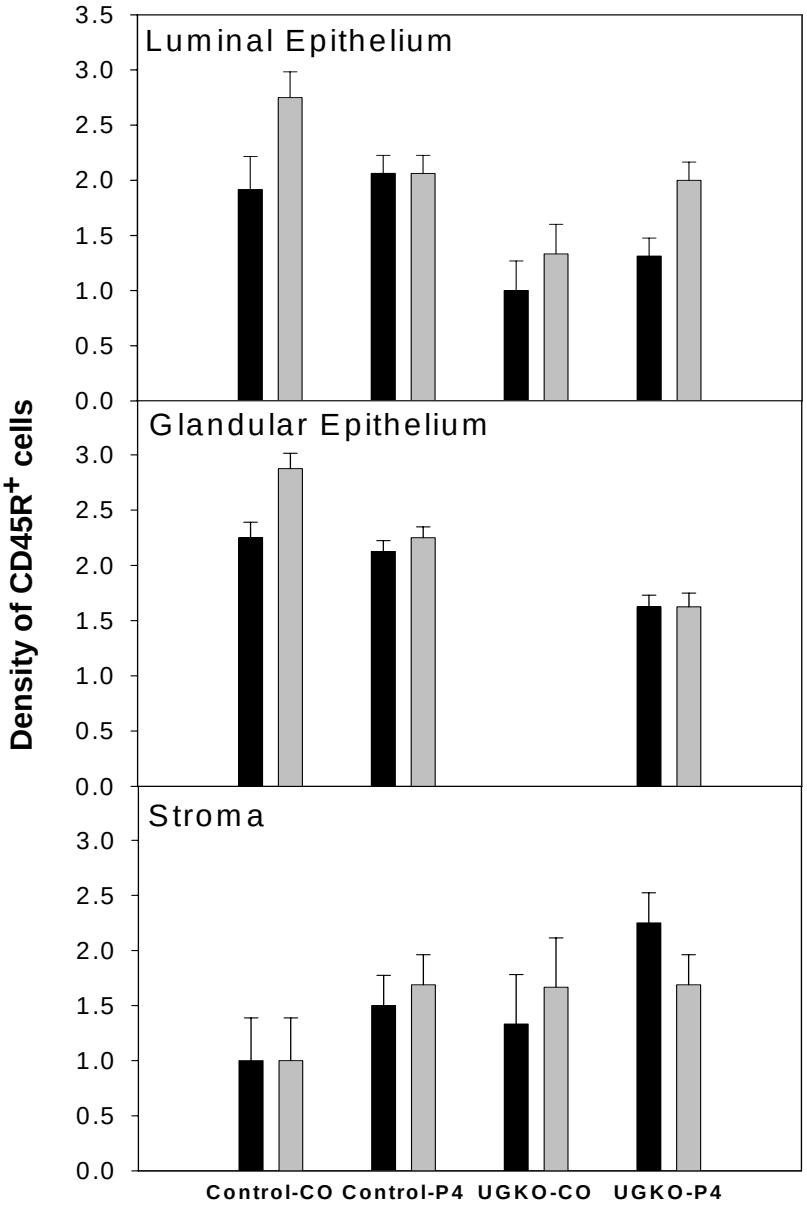

Figure 2-9. Density of $CD45R^+$ cells in different areas of the uterine endometrium according to the presence of autografts (black bars) and allografts (grey bars) for control and uterine gland knockout (UGKO) ewes treated with corn oil vehicle (CO) or progesterone (P4) for 60 days. Data represents least square means ± SEM. In the luminal epithelium, the population of $CD45R^+$ 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 x treatment x graft ($p=0.08$). Among control ewes, numbers of $CD45R^+$ cells in the glandular epithelium was affected by graft ($p<0.05$); treatment x graft was $p=0.07$. For progesterone-treated groups, density of $CD45R^+$ cells in the glandular epithelium was affected by type ($p=0.06$). There were no significant effects on numbers of $CD45R^+$ cells in stroma.

progesterone, moreover, there was no difference in density of $CD45R^+$ cells between horns with autografts or allografts. In the stroma, there was an increase of $CD45R^+$ cells regardless of the type, but it was not significant.

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 may have abrogated the UGKO phenotype 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 is 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 maintain 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 $CD45R^+$ cells, which in the sheep uterus represent mostly T cells (Meussen et al., 1993). Indeed, progesterone has been reported to decrease lymphocyte numbers in the endometrium (Gottshall and Hansen, 1992) and progesterone blocked the increase in numbers of $CD45R^+$ cells in luminal and glandular epithelium caused by the local presence of the allograft in control ewes from the present study.

The concentrations of progesterone causing a delay in graft rejection (in this case, a peak of 17.9 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 to 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., 1988ab; 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 processes involved in gland formation, which include invasion of endometrial cells into the stroma and their proliferation and organization into branched and coiled glands, requires remodeling of the stroma extracellular matrix, epithelial-epithelial and epithelial-stromal cell interactions, and other cellular and biochemical events. Undoubtedly, the process is under control of endocrine and paracrine regulators (Gray et al., 2001c). Clearly, 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. What the present results indicate is that the endometrium retains the ability to initiate and complete glandular formation and that prolonged treatment with a high dose of progesterone restores 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). Present results suggest cells (probably luminal epithelial cells) with the capacity for differentiating into glandular epithelium persist in the UGKO ewe and can be activated by prolonged progestin treatment.

A previously undescribed characteristic of the UGKO phenotype in the absence of progesterone is the large reduction in the population of $CD45R^+$ cells. Perhaps, it is low numbers of these cells that led to one allograft being present in a UGKO ewe treated with corn oil. Reduction in the number of $CD45R^+$ in the UGKO ewe suggests that the lymphocyte homing mechanism is altered in this phenotype. One molecule involved in leukocytes 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. Perhaps, neonatal progestin treatment disrupts the normal pattern of expression of lymphocyte homing receptors. Alternately, progestin treatment may change endometrial function so that lymphocyte egress from the endometrium is hastened. It can also not be excluded that changes in lymphocytes numbers may not represent a disruption in lymphocyte trafficking in the UGKO ewe but rather 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 increased numbers of endometrial $CD45R^+$ cells means that, as for its effects on endometrial gland morphogenesis, progesterone treatment of the adult can reverse actions of neonatal progestin exposure. It is possible that the two effects of progesterone in the adult UGKO ewe, inducing gland formation and increasing numbers of epithelial lymphocytes induction are unrelated. Alternatively, however, it is possible that the induction of new gland development is functionally related to restoration of homing of $CD45R^+$ lymphocytes to the uterus, i.e., that migration of lymphocytes to the endometrium or their

retention in the endometrium depends upon the glandular epithelium. One possibility is that some $CD45R^+$ lymphocytes enter the endometrium through homing to the glandular epithelium and then traverse to the luminal epithelium.

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 to induce appearance of functional glands capable of OvUS synthesis. Among the differentiation events in the endometrium that are disrupted by neonatal progestin exposure are formation of the pool of $CD45R^+$ lymphocytes resident in the endometrial epithelium. This effect of progestin treatment, too, could be reversed by progesterone exposure during adulthood and it is possible than the induction of glandular development induced by progesterone is functionally related to the increase in lymphocyte numbers in the endometrium. 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 influence of progesterone. Results also suggest the involvement of progesterone and, possibly endometrial glands, in homing of lymphocytes to the endometrium. Overall, results of the present study demonstrate the potential of the UGKO ewe as a tool to study uterine morphogenesis and immune function.

CHAPTER 3
GENERAL DISCUSSION

The main goal of this dissertation was to understand the possible role of OvUS in the survival of the allogeneic conceptus during pregnancy in ewes. As stated before, the specific hypothesis for the experiment was to test the role of OvUS in the rejection of allografts placed into the uterine lumen using the UGKO ewe as a model. Results of the research described in this thesis could not adequately test this hypothesis because of the unexpected result that progesterone induced the appearance of endometrial glands in the UGKO ewes and because this newly-differentiated glandular epithelium was functionally active as indicated by immunolocalization of OvUS in uterine fluids and endometrial tissues of ewes treated with progesterone. This result is revealing because it implicates progesterone in the process of endometrial adenogenesis in adult ewes and the possible link between endometrial glands and homing of lymphocytes into the uterus.

An unanswered question from this thesis and one that could be subject to additional research is how progesterone can initiate endometrial gland formation in the adult uterus. Possible mechanisms for such an action of progesterone on the adult ewe are illustrated in Figure 3-1. As a result of exposure to progestin of lambs at birth, the adult uterine endometrium of UGKO ewes can have a ruffled luminal epithelium and compact stroma with no glands, small glandular invaginations, or occasional cyst and gland-like structures in the stoma (Gray et al., 2000ab; 2001a). Progesterone could induce the reinitiation of the glandular epithelium by either inducing invagination of the luminal epithelium or by causing further differentiation of the cyst-like structures in the stroma.

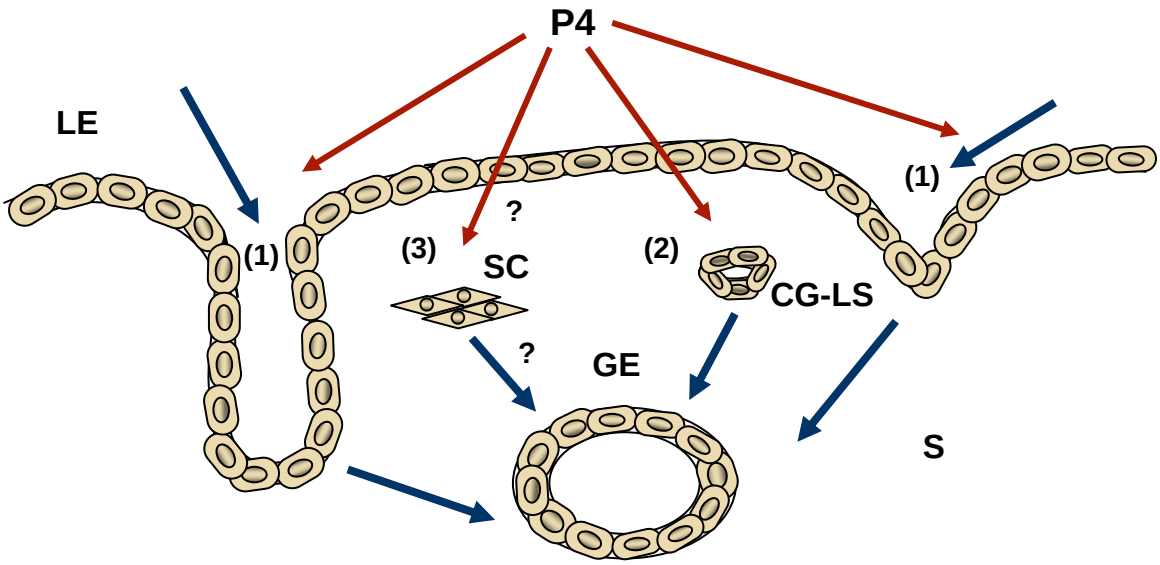

Figure 3-1. Proposed model for the process of endometrial gland formation in the uterus of the adult ewe. In the neonate, adenogenesis is initiated with shallow invaginations in the luminal epithelium (LE). It is proposed that progesterone (P4) can initiate this process in adult ewes (1). In addition, progesterone may act to induce gland formation causing differentiation of cyst and gland-like structures (CG-LS) present in the stroma (S) (2) or from stem cells (SC) resident in the luminal epithelium and stroma (3).

Of the two possibilities, the first is more likely because of previous data suggesting that glands form by invaginations of the luminal epithelium (Wiley, 1987; Bartol et al., 1988ab; Taylor et al., 2000). There is also a possibility that the actions of progesterone on the adenogenesis process are the result of actions on stem cells present in the endometrium. Such cells have been detected in epithelial and stromal cells of the human endometrium (Chan et al., 2004; Cho et al., 2004). Progesterone may also have induced the release of growth factors from keratinocytes present in the skin graft placed into the uterus which could have promoted the development of endometrial glands. An important question to address is whether the ability of progesterone to induce the formation of new glands is a phenomenon limited to UGKO ewes, which have abnormal gland formation, or whether if it also occurs in normal ewes.

In addition to the effects of progesterone on the induction of endometrial gland morphogenesis, results presented in this dissertation also shown that progesterone could restore the population of lymphocytes present in the uterine endometrium and that this effect may be related with the development of the glandular epithelium caused by progesterone. In the sheep uterus, lymphocytes are localized in the luminal and glandular epithelium of the intercaruncular endometrium (Lee et al., 1998; Gotshall and Hansen, 1992; Majewski et al., 2001). The lymphocyte population that resides in the pregnant uterus is mainly composed of $CD8^+$ $CD45R^+$ $\gamma\delta TCR^+$ cells and these increase in number in the luminal epithelium during mid and late pregnancy (Lee et al., 1992; Meussen et al., 1993; Nasar et al., 2002). Results presented in this dissertation shown that $CD45R^+$ cells were greatly reduced in the luminal epithelium of UGKO ewes treated with the vehicle and this population of cells seems to be restored in the luminal epithelium by

progesterone. Perhaps the restoration of lymphocyte numbers in the epithelium and development of endometrial glands are functionally linked. The increase in the numbers of lymphocytes in the endometrium may have beneficial effects in the formation of new uterine glands due to the increase in cytokines released by these cells. A proposed model that illustrates the possible relationship between gland morphogenesis and homing of lymphocytes in the uterine endometrium in the adult ewe is shown in Figure 3-2. Initially, $CD45R^{+}$ cells could migrate from the capillary network to the luminal and glandular epithelium of the intercaruncular endometrium and from the glandular epithelium some cells migrate to the luminal epithelium. In the absence of endometrial glands, $CD45R^{+}$ cells can only migrate from the capillary network to the luminal epithelium and the traffic of these cells from the glandular to the luminal epithelium would be eliminated. If so, progesterone could restore numbers of $CD45R^{+}$ cells in the luminal epithelium by replacing the glandular epithelium. Another possibility is that UGKO ewes have a deficiency in chemokines that attract lymphocytes because the glandular source for these molecules is eliminated.

Given the inadequacy of the UGKO model, an important question is how the hypothesis about the effect of OvUS on allograft rejection could be tested in the future using the sheep as a model of experimentation. One possibility could be to produce UGKO ewes in which elimination of glands was irreversible, for example by extending the period of neonatal treatment with progestin. Another approach could be to test whether infusion of purified OvUS (purified from pregnant uterine fluid or recombinant OvUS) into the uterine horn would block allograft rejection. The concentration of OvUS to be infused should be at the same concentration used to inhibit lymphocyte proliferation

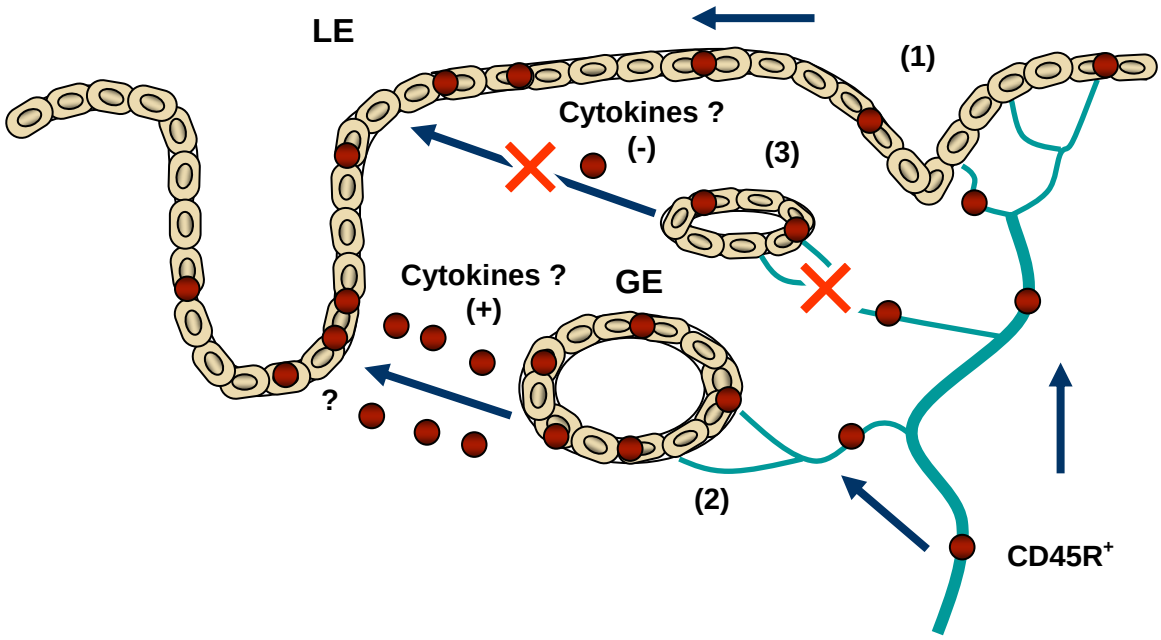

Figure 3-2. Proposed model for homing lymphocytes in the luminal (LE) and glandular epithelium (GE) in the uterine endometrium of adult ewes. In the initial process, $CD45R^{+}$ lymphocytes (red circles) could migrate from the capillaries to the luminal (1) and glandular epithelium (2). Perhaps lymphocytes resident in endometrial glands traffic to the luminal epithelium. In the absence of endometrial glands, this route of delivery of $CD45R^{+}$ lymphocytes to the luminal epithelium is blocked (3).

in vitro (1 mg/ml) (Liu and Hansen, 1993). There is also the possibility of using small interfering RNA molecules to inhibit or silence the OvUS gene and block the synthesis of the protein in the uterine endometrium. Although this novel technique has had good results in in-vitro models, it is still a challenge for in vivo studies because of lack of a good delivery system that mediate efficient their cellular uptake and release (Wang et al., 2003).

In summary, progesterone delayed skin graft rejection placed into the uterine lumen of UGKO ewes and this process was in conjunction with the development of functional endometrial glands that were able to synthesize OvUS. Results also indicate a possible connection between progesterone and gland formation in regulation of the lymphocyte population of the uterus.

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BIOGRAPHICAL SKETCH

Maria Beatriz Padua was born in 1968 in Caracas, Venezuela. She graduated from El Carmelo High School in the same city in 1985 and enrolled the next year in the School of Agronomy at the University Centroccidental Lisandro Alvarado in Barquisimeto, Lara State, Venezuela, where she received her Bachelor of Science degree in agricultural engineering in 1994. In 1995, she worked as a sales representative for the veterinary health supply firm, Grupo Catalina C.A., in Caracas, Venezuela. From 1996 to 2000, she was a pharmacist assistant for Celbefar C.A. Farmacia El Roble in the same city. She enrolled in an English language program at the University of Florida in 2001 and she enrolled in the Animal Molecular and Cell Biology Graduate Program of the Department of Animal Sciences at the University of Florida under the supervision of Dr. Peter J. Hansen in April, 2002. She is currently a Master of Science candidate. Upon completion of her degree, she will pursue a Doctor of Philosophy degree at the University of Florida under Dr. Hansen.