ABSTRACT

Endometrial glands secrete molecules hypothesized to support conceptus growth and development. In sheep, endometrial gland morphogenesis occurs postnatally and can be epigenetically ablated by neonatal progesterin exposure. The resulting stable adult uterine gland knockout (UGKO) phenotype was used here to test the hypothesis that endometrial glands are required for successful pregnancy. Mature UGKO ewes were bred repeatedly to fertile rams, but no pregnancies were detected by ultrasond on Day 25. Day 7 blastocysts from normal superovulated ewes were then transferred synchronously into Day 7 control or UGKO ewes. Ultrasound on Days 25–65 postmating indicated that pregnancy was established in control, but not in UGKO ewes. To examine early uterine-embryo interactions, four control and eight UGKO ewes were bred to fertile rams. On Day 14, their uteri were flushed. The uterus of each control ewe contained two filamentous conceptuses of normal length. Uteri from four UGKO ewes contained no conceptus. Uteri of three UGKO ewes contained a single severely growth-retarded tubular conceptus, whereas the remaining ewe contained a single filamentous conceptus. Histological analyses of these uteri revealed that endometrial gland density was directly related to conceptus survival and developmental state. Day 14 UGKO uteri with moderate gland development contained a filamentous conceptus. Collectively, these results demonstrate that endometrial glands and, by inference, their secretions are required for pre-implantation conceptus survival and development.

INTRODUCTION

Endometrial glands are present in all mammalian uteri and develop after birth in sheep [1–5], pigs [6, 7], rats [8], and mice [9]. Uterine glands secrete a variety of molecules, collectively termed histotroph, that are hypothesized to be essential for maternal support of conceptus (embryo/fetus and associated extraembryonic placental membranes) survival and growth in mammals [10–15]. Available evidence supports the idea that secretions of endometrial epithelia influence conceptus development, onset of pregnancy recognition signals, and conceptus growth in species that display synepitheliochorial placentation [16, 17]. Components of uterine histotroph include enzymes, cytokines, growth factors, ions, hormones, glucose, transport proteins, and adhesion molecules [14, 18]. Specific functional roles for histotroph components in ovine conceptus development are not known. However, two endometrial gland secretory products, leukemia inhibitory factor (LIF) and calcitonin, are required for implantation in rodents [19, 20].

Establishment of pregnancy requires that the preimplantation ovine conceptus enter a progesterone-stimulated uterus and develop sufficiently to synthesize and release interferon tau (IFNτ), the pregnancy recognition signal [21]. After pregnancy recognition, maintenance of pregnancy requires reciprocal communication between the conceptus and endometrium during implantation and synepitheliochorial placentation [22]. Throughout gestation, both conceptus and maternal endometrial secretions affect uterine fluid composition. Prior to implantation, these secretions are hypothesized to influence conceptus survival, development and production of IFNτ [16, 23]. Specific components, such as mitogenic uterine-derived growth factors, can directly induce proliferation of ovine trophoblast and myoblast cells in vitro [24]. Species that display delayed implantation serve to illustrate the importance of uterine histotroph in conceptus development [25–27]. Delayed implantation extends gestation by arresting conceptus development at the blastocyst stage. In marsupials, the end of this developmental arrest is marked by increased endometrial secretory activity that directly promotes conceptus development [26, 28, 29]. In ruminants, similar direct embryotrophic effects of endometrial secretions during early pregnancy are not well defined.

Ovine uterine glandular development can be inhibited epigenetically by neonatal progesterin exposure for 8 wk from birth, resulting in adult ewes that lack endometrial glands and display the uterine gland knockout (UGKO) phenotype [17, 30, 31]. Mature UGKO ewes cannot support pregnancy [17] and do not express a number of glandular epithelial genes that may be required for conceptus development and growth [31]. We hypothesize endometrial glands and their secretions are essential for normal concep-
tus development. Therefore, these studies were designed to identify the stage at which early pregnancy loss occurs in UGKO ewes and to begin elucidation of uterine factors and conditions responsible for pregnancy failure.

MATERIALS AND METHODS

Animals

Experimental and surgical procedures complied with the Guide for Care and Use of Agriculture Animals and were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University (Animal Use Protocol 7-286).

The UGKO ewes were produced by implanting Rambouillet ewe lambs with a single Synchromate B (Sanofi, Overland Park, KS) implant within 12 h of birth and every 2 wk thereafter for a total of 8 wk. Implants were inserted s.c. 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-norprogesterin, over a 14-day period [4]. Control ewes did not receive implants.

Natural Breeding

At approximately 17 mo of age, adult UGKO (n = 18) ewes were given two i.m. injections (0700 h and 1700 h) of 10 mg prostaglandin F₂α (Lutalyse; Upjohn, Kalamazoo, MI). These injections were repeated 7 days later to synchronize estrus. All ewes were bred at estrus (Day 0) and at 12 h and 24 h postestrus by intact rams of proven fertility. Ewes were monitored daily for estrous behavior using vasectomized rams. Ewes not returning to estrus were subjected to transabdominal ultrasound on Days 25 and 35 postmating to confirm pregnancy by detection of the conceptus or placentome formation. This experiment was repeated three times.

Embryo Transfer

Donor ewes (n = 5) were synchronized to estrus by administering controlled internal drug releasing (CIDR) progesterone pessaries (InterAg, New Zealand). The CIDRs were inserted on Day 0 and replaced on Day 7. Donor ewes were superovulated using FSH by administering 2.5 ml Folltropin-V (Vetrapham Canada Inc., London, ON, Canada) i.m. on Day 8 (0700 h), followed by 1.25 ml Folltropin-V twice daily (0700 h and 1700 h) for 3 days (Day 8, 1700 h; Day 10, 1700 h). On Day 8 (0700 h), donor ewes received 250 IU (i.m.) Pregnecol (Horizon Technology Pty Limited, North Ryde, Australia) and 10 mg Lutalyse i.m. (1700 h). On Days 11–12 (1700–0700 h), donor ewes were mated by fertile rams. Recipient ewes were also synchronized using CIDRs inserted on Day 0 and removed on Day 9. On Day 8 (1700 h), recipient ewes received 10 mg Lutalyse i.m., followed by 400 IU Pregnecol (Horizon Technology Pty Limited) i.m. on Day 9 (1700 h).

On Day 7 postmating, the uteri of donor ewes were flushed with 20 ml Medium 199 with Hanks salts, L-glutamine, and 25 mM Hepes buffer (Gibco BRL, Grand Island, NY) to obtain blastocysts. Blastocysts (grade 1 or 2) were transferred into recipient control (n = 7) and UGKO (n = 5) ewes by laparoscopy. Control ewes received one blastocyst and UGKO ewes received two blastocysts. In addition to daily observation for estrous behavior using vasectomized rams, ultrasonography was performed every 5–7 days from Day 25 to approximately Day 65 postmating to determine pregnancy status.

Day 14 Postmating Uterine Flush

The UGKO (n = 8) and normal control (n = 4) ewes were given two i.m. injections (0700 h and 1700 h) of 10 mg Lutalyse. Injections were repeated 9 days later to synchronize estrus. All ewes were bred at estrus and at 12 h and 24 h postestrus by fertile rams. On Day 14 postmating, the uterine lumen of each ewe was flushed with 20 ml Dulbecco minimum essential medium/F-12 (Sigma, St. Louis, MO) medium. Conceptus morphology was analyzed using a dissecting microscope, and length of conceptus was measured. All ewes were then ovariohysterectomized. Both ovaries and a portion (~1.0 cm) from the middle region of each uterine horn were fixed in 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). Tissues were sectioned (4–6 μm) and stained with hematoxylin and eosin as described previously [17]. Endometrial gland density was determined by counting the number of gland cross sections present per uterine cross section for both UGKO (n = 8) and control (n = 4) ewes. Multiple uterine cross sections (n = 4) were counted per ewe.

Western Blot Analysis

Uterine flushes (2 ml aliquant) obtained from normal control and UGKO ewes on Day 14 postmating were concentrated using Centricon-3 columns (Amicon, Inc., Beverly, MA). Protein content was determined using a Bradford protein assay (Bio-Rad, Hercules, CA) with BSA as the standard. Uterine flush proteins (20 μg) were denatured, separated on a 12% SDS-PAGE gel, and transferred to nitrocellulose membranes as described previously [32]. Membranes were blocked for 1 h at room temperature with 5% milk-Tris-buffered saline with Tween-20 (TBST) and then incubated with mouse monoclonal antiovine IFNγ (HL129) [33] as primary antibody or mouse IgG at 20 μg/ml, as a negative control, overnight at 4°C. Following primary antibody incubation, membranes were rinsed for 30 min with TBST and then incubated with goat antimouse IgG-horse-radish peroxidase-conjugated secondary antibody (KPL, Bethesda, MD) for 1 h at room temperature. Membranes were again rinsed with TBST for 30 min before detection by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) and Kodak X-OMAT AR film.

Photomicroscopy

Photomicrographs of hematoxylin and eosin-stained tissues were taken using a Zeiss Axiosplan 2 photomicroscope (New York, NY) fitted with a Hamamatsu chilled 3CCD color camera (Hamamatsu, Japan). Digital images were captured and assembled using Adobe Photoshop 4.0 (Adobe Systems, Seattle, WA) and a MacIntosh PowerMac G3 computer (Apple Computer, Cupertino, CA). Black-and-white prints were made using a Kodak DS8650 color printer.

Statistical Analyses

Data for pregnancy rate, conceptus length, and endometrial gland density were subjected to one-way least-squares ANOVA, using general linear models procedures of the Statistical Analysis System [34]. Data are presented as least square means with SEM.
RESULTS

Pregnancy was not detected in any of the UGKO ewes following repeated matings with rams of proven fertility. The same ewes were then used to determine the ability of UGKO ewes to support an embryo transferred from a normal control ewe and to eliminate the possibility of a fertilization or embryo transport defect, Day 7 sheep embryos were transferred from normal, superovulated donor ewes into synchronized UGKO and control ewes on Day 7 after onset of estrus. Ultrasonography of ewes on Days 25–65 postmating indicated that five of seven control ewes established and maintained pregnancy. Pregnancy was detected in zero of five UGKO ewes. Indeed, pregnancy rate was higher (P < 0.01) in normal control as compared to UGKO ewes.

Control (n = 4) and UGKO (n = 8) ewes were then bred at estrus and their uteri flushed on Day 14. The uterine flush from each control ewe contained two filamentous conceptuses of normal length (97 ± 15 mm; Table 1). Uterine flushes from four of the UGKO ewes did not contain a conceptus. Uterine flushes from the other four UGKO ewes contained either a filamentous conceptus of approximately normal length (82 mm; n = 1) or severely growth-retarded tubular conceptuses (7 ± 3 mm length; n = 3). Indeed, conceptus length was greater (P < 0.01) in uterine flushes recovered from normal control as compared to UGKO ewes.

Immunoreactive IFNα was detected in uterine flushes obtained from all control ewes on Day 14 postmating (Fig. 1). The IFNα was also present in the uterine flush from the UGKO ewe that contained a filamentous conceptus. However, IFNα was not detected in uterine flushes from UGKO ewes lacking a conceptus or containing a tubular conceptus.

Uteri of all control ewes exhibited extensive, normal glandular development in the intercaruncular endometrium (Fig. 2a). As expected, none of the UGKO ewes displayed uterine glandular development characteristic of controls. However, among UGKO ewes, uterine glands were most often either absent (Fig. 2b) or sporadically distributed at very low density (Fig. 2c) or were distributed somewhat more regularly, albeit at lower density than in controls, as observed in the one UGKO ewe in which a filamentous conceptus was found (Fig. 2d). Endometrial glandular density was directly related to state of conceptus development on Day 14 postmating (Table 1). Uteri of UGKO ewes with the more glandular endometria contained more developmentally advanced conceptuses. Endometrial glandular density was greater (P < 0.10) in uteri of control ewes than that of UGKO ewes containing either a tubular conceptus or no conceptus. However, endometrial glandular density was not different (P > 0.10) in uteri of control ewes as compared to the one UGKO uterus from which a filamentous conceptus was recovered.

DISCUSSION

Results of the present study provide the first direct evidence to support the hypothesis that endometrial glands and, by extension, their secretions are required for conceptus survival and growth. Together, control and UGKO ewes presented a continuum of endometrial phenotypes, ranging from intensely glandular to effectively aglandular. Thus, a novel opportunity to establish relationships between state of conceptus development and endometrial histoarchitecture was created using the ovine UGKO model. Uterine gland density was positively related to conceptus survival and state of conceptus development on Day 14 postmating. However, regardless of endometrial phenotype, none of the UGKO ewes were able to support pregnancy to Day 25. Data indicate that the UGKO phenotype, characterized by extreme reduction in or absence of endometrial glands, constitutes a uterine lesion that compromises periimplantation conceptus growth and survival.

Results of embryo transfer experiments indicate that early stages of ovine conceptus development, i.e., prior to Day 11, may not be entirely dependent upon the uterine envi-
ronment. Averill et al. [35] transferred 2-cell- through morula-stage ovine embryos into the oviducts of pseudopregnant rabbit does. Five days later, morula- and blastocyst-stage conceptuses were recovered and transferred into the uteri of Day 6 nonpregnant ewes, wherein they developed normally [35]. Consistently, uterine flushes obtained from UGKO ewes on Days 6 and 9 postmating contained developmentally normal blastocysts (unpublished results). This suggests that compaction and blastocyst differentiation by ovine conceptuses are not dependent upon histotroph in the ovine uterine environment. In addition, preliminary studies in our laboratory indicate that conceptuses recovered from UGKO ewes are capable of implanting in the uteri of normal recipient control ewes, supporting the hypothesis that the pregnancy loss defect in UGKO ewes occurs due to an inappropriate uterine environment rather than a developmental defect in the conceptus (unpublished results).

In sheep, blastocysts hatch from the zona pellucida on Days 8–9 and then begin to expand and grow to tubular form by Day 11. Tubular conceptuses elongate rapidly on Day 13 to the filamentous state, reaching 150–190 mm in length by Day 15 [36–38]. During the preimplantation period from Days 4 to 15 [39, 40] conceptuses are free-floating in the uterine lumen where they are bathed in and presumed to be supported by uterine histotroph [41]. Fléchon et al. [42] demonstrated that trophoblastic vesicles derived from Day 12 conceptuses survived but were unable to undergo elongation in vitro. However, when these vesicles were transferred into the uterus, they elongated and produced IFNγ over the ensuing 5-day period [42]. In the present study, distinct differences in conceptus development were detected between UGKO and control ewes by Day 14 postmating, suggesting that a critical window of conceptus development occurs during the periimplantation period that requires endometrial secretions. Growth-retarded conceptuses recovered from uteri of UGKO ewes were equivalent developmentally to tubular conceptuses normally found on Days 11–12. This suggests that endometrial secretions begin to impact conceptus growth and development on Day 11 of pregnancy.

The amount of IFNγ secreted by the ovine trophectoderm is directly related to stage of conceptus development but is not dependent upon a synchronous uterine environment [16]. Filamentous in vivo-derived ovine conceptuses produced approximately 16 times more IFNγ than normal tubular stage conceptuses [16]. Results from the present study support these findings. The IFNγ was easily detected in the uterine flushes obtained from Day 14 pregnant control and UGKO ewes with elongated conceptuses, whereas IFNγ was not detected in uterine flushes containing growth-retarded tubular conceptuses. The IFNγ may be present in uterine flushes containing tubular conceptuses but at levels below detectable limits of the antibody. This suggests that the filamentous conceptus present in the one UGKO ewe was functionally equivalent to normal control conceptuses at the same developmental stage.

In addition to providing nutrition for the conceptus, uterine histotroph contains growth factors and cytokines that promote cell division, proliferation, morphogenesis, and differentiation [18]. The endometrium also expresses proteins that may be important mediators of conceptus-maternal interactions and implantation. For example, osteopontin (OPN), a 70-kDa acidic component of the extracellular matrix [43], and glycosylated cell adhesion molecule-1 (GlyCAM-1), a sulfated glycoprotein in the mucin family [32], are both expressed by the endometrial glands and present in uterine luminal fluid on and after Day 13 of pregnancy that represents the onset of implantation in sheep [39]. By interacting with or modulating activation of endometrial cell surface integrins, such as the αvβ3 heterodimer thought to mediate critical cell-cell interactions in human implantation [44] and serving as a selectin receptor ligand, these uterine proteins may promote conceptus elongation and stabilize interactions between conceptus trophectoderm and endometrial luminal epithelium that serve as essential for implantation [32, 43]. Thus, patterns of conceptus development and the process of implantation might be
expected to be compromised in the UGKO uterus should OPN, GlyCAM-1, or other adhesion-promoting molecules fail to be expressed appropriately.

This study demonstrates that the UGKO ewe is an excellent model for understanding developmental aspects of uterine biology and uterine function in adults. In humans, endometrial gland morphogenesis occurs with each menstrual cycle, thereby increasing the opportunity for dysgenesis, dysplasia, and dysfunction. Survival of the human embryo prior to implantation appears to depend upon endometrial gland secretions [15]. It is likely that the human conceptus does not receive direct hematotrophic nutrition from the maternal circulation until the end of the first trimester of pregnancy [45]. Therefore, alterations in human endometrial gland formation and function may lead to infertility and early pregnancy loss. Decreased expression of cell-surface and secretory proteins within the uterine environment are correlated with abnormal uterine gland morphology that adversely affect uterine receptivity during the peri-implantation and early placentation stages of gestation [44, 46, 47]. Perhaps these documented human reproductive defects can be related to defects in UGKO ewes wherein the absence of epithelial-specific gene expression [31] and histotroph production results in failure of conceptus development beyond the peri-implantation period.

The present study confirms that administration of a synthetic 19-norprogestin to neonatal ewe lambs disrupts normal endometrial gland development and results in adult ewes that are unable to establish or maintain pregnancy [17]. The absence of endometrial glands results in 1) defects in conceptus development during elongation and 2) an inability of ewes to maintain pregnancy beyond the pre-implantation period. Variations in UGKO phenotype observed for some ewes offer a unique opportunity to investigate the role of endometrial glands and their secretions during various stages of gestation and to define specific components of histotroph required for normal pre-implantation conceptus development in sheep and, perhaps, other mammals. In future studies, techniques of genomics and proteomics will be employed to compare differences in endometrial gene expression and histotroph production between control and UGKO ewes.

ACKNOWLEDGMENT

Thanks are due to Mr. Todd Taylor of the Texas A&M Sheep and Goat Center for animal husbandry and assistance with embryo transfer.

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