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Ovine Placental Lactogen Specifically Binds to Endometrial Glands of the Ovine Uterus[1]

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ABSTRACT

A hormonal servomechanism has been proposed to regulate differentiation and function of the endometrial glandular epithelium (GE) in the ovine uterus during pregnancy. This mechanism involves sequential actions of estrogen, progesterone, ovine interferon τ (IFNτ), placental lactogen (oPL), and placental growth hormone (oGH). The biological actions of oPL in vitro are mediated by homodimerization of the prolactin receptor (oPRLR) and heterodimerization of the oPRLR and oGH receptor. The objectives of the study were to determine the effects of intrauterine oPL, oGH, and their combination on endometrial histoarchitecture and gene expression and to localize and characterize binding sites for oPL in the ovine uterus in vivo using an in situ ligand binding assay. Intrauterine infusion of oPL and/or oGH following IFNτ into ovariectomized ewes treated with progesterone daily differentially affected endometrial gland number and expression of uterine milk proteins and osteopontin. However, neither hormone affected PRLR, insulin-like growth factor (IGF)-I, or IGF-II mRNA levels in the endometrium. A chimeric protein of placental secretory alkaline phosphatase (SEAP) and oPL was used to identify and characterize binding sites for oPL in frozen sections of interplacentomal endometrium from pregnant ewes. Specific binding of SEAP-oPL was detected in the endometrial GE on Days 30, 60, 90, and 120 of pregnancy. In Day 90 endometrium, SEAP-oPL binding to the endometrial GE was displaced completely by oPL and prolactin (oPRL) but only partially by oGH. Binding experiments using the extracellular domain of the oPRLR also showed that iodinated oPL binding sites could be competed for by oPRL and oPL but not by oGH. Collectively, results indicate that oPL binds to receptors in the endometrial glands and that oPRL is more effective than oGH in competing for these binding sites. Thus, effects of oPL on the endometrial glands may be mediated by receptors for oPRL and oGH.

growth hormone, mechanisms of hormone action, pregnancy, prolactin receptor, uterus

INTRODUCTION

In ungulates, establishment and maintenance of pregnancy requires integration of endocrine and paracrine signals from the ovary, conceptus (embryo/fetus and associated membranes), and uterus (see [1] for review). Establishment of pregnancy requires that the preimplantation ovine conceptus enter a progestinized uterus and develop sufficiently to synthesize and release interferon τ (IFNτ), the pregnancy recognition signal (see [2, 3] for review). After pregnancy recognition on Day 13 postmating, maintenance of pregnancy requires reciprocal communication between the conceptus and endometrium during implantation and synepitheliochorial placentation (see [1] for review). In sheep, superficial implantation and placentation is a lengthy process that begins on Days 15–16 but is not completed until Days 50–60 of pregnancy [4, 5]. During this period, the uterus grows and remodels substantially to accommodate rapid conceptus development and growth in the latter two-thirds of pregnancy. In addition to placentomal development in the caruncular areas of the endometrium and changes in uterine vascularity, the intercaruncular endometrial glands grow substantially in length (4-fold) and width (10-fold) during pregnancy [4]. During gestation, endometrial gland hyperplasia occurs between Days 15 and 50. The uterine glands synthesize and secrete or transport a variety of enzymes, growth factors, cytokines, lymphokines, hormones, transport proteins, and other substances that are collectively termed histotroph (see [7–9] for review). After Day 60, the uterine glands become hypertrophic and maximal production of histotroph occurs [6]. Available evidence strongly suggests that histotrophic nutrition complements hematotrophic nutrition and influences conceptus development, onset of pregnancy recognition signals, and growth of the fetus and placenta in ungulates and humans [10, 11].

The hormonal, cellular, and molecular mechanisms regulating endometrial gland morphogenesis and function during pregnancy are not fully understood (see [9] for review). During pregnancy, the ovine uterus is exposed sequentially to estrogen, progesterone, ovine IFNτ (oIFNτ), ovine placental lactogen (oPL), and ovine placental growth hormone (oGH), which appear to initiate and maintain endometrial gland morphogenesis and differentiated secretory function [6, 12]. The placentae of a number of species, including rodents, humans, nonhuman primates, and sheep, secrete hormones structurally related to pituitary GH and prolactin (PRL) that are termed PLs [13–15]. Ovine PL is a nonglycosylated single-chain 23-kDa protein [16, 17] produced by binucleate cells of the conceptus trophectoderm beginning on Day 16 of pregnancy [18]. The onset of PL production

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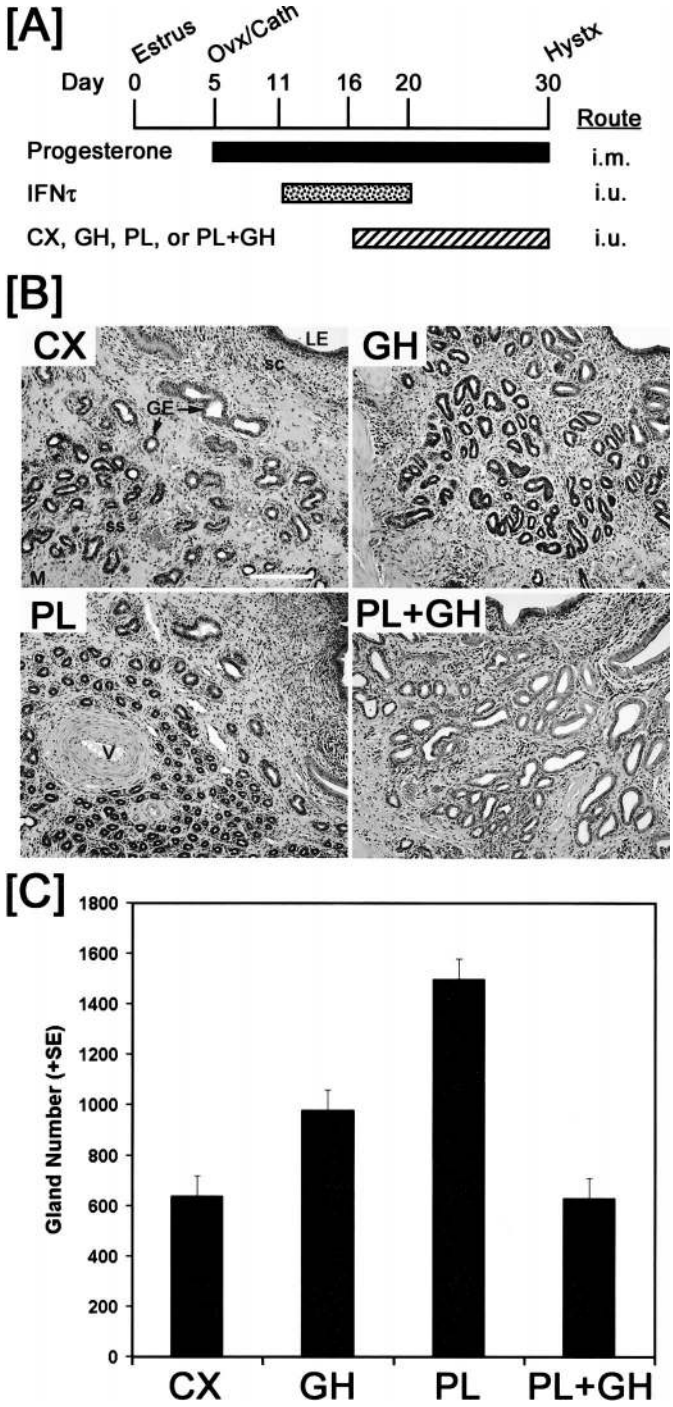

FIG. 1. Effects of i.u. infusion of somatolactogenic hormones on endometrial histoarchitecture and gland number. **A**) Experimental design. Cath, Uterine catheterization; Hystx, hysterectomy; Ovx, ovariectomy. **B**) Uterine endometrium from treated ewes. All photomicrographs are shown at the same magnification. Bar = 150 μm. LE, Lumenal epithelium; M, myometrium; sc, stratum compactum; ss, stratum spongiosum; V, blood vessel. **C**) Endometrial gland density in uteri of treated ewes. Data are presented as mean gland number (LSM) per uterine cross section (with SEM).

on Day 16 is concomitant with the initiation of uterine milk protein (UTMP) gene expression in the endometrial GE [6]. UTMPs are members of the serpin family of serine protease inhibitors [19] and serve as excellent markers for endometrial gland differentiation and secretory capacity during pregnancy in the sheep [6, 12, 20, 21]. In maternal serum, oPL can be detected as early as Day 50 and peaks between Days 120 and 130 of gestation [22–24]. The temporal changes in circulating levels of oPL can be correlated with endometrial gland hyperplasia and hypertrophy and increases in production of UTMPs [6]. The ovine placenta also expresses oGH between Days 35 and 70 of gestation [25]. In the intercaruncular endometrium, oGH receptor (oGHR) mRNA was detected at low levels between Days 8 and 120 of pregnancy [26]. Expression of placental oGH is correlated with onset of glandular epithelium (GE) hypertrophy and maximal increases in UTMP production by the endometrium [6]. In the ovine uterus, intrauterine infusion of oPL or oGH following oIFNτ in progesterone-treated ovariectomized ewes increased endometrial gland proliferation and production of UTMP [12]. These findings support the hypothesis that oPL initially acts in a paracrine manner on the endometrium to stimulate GE hyperplasia and is augmented by placental oGH, which stimulates GE hypertrophy to increase production of histotroph presumably required for nutrition of the developing fetus [6, 12]. However, the cellular and molecular mechanisms mediating the effects of oPL and oGH on endometrial morphogenesis and function remain to be determined.

In several radioreceptor and bioassays, PLs exhibited PRL-like (lactogenic) activity [14]. In the sheep uterus, oPRL receptor (oPRLR) expression is specifically restricted to the GE and increases during pregnancy [6, 27]. This receptor transduces signals by oPRL and oPL because oPL homodimerizes the oPRLR and activates signaling pathways [28]. Comparative binding studies and in vitro bioassays using heterologous receptors prompted several research groups to suggest that oPL induces somatogenic effect through oGHRs (see [17] for review). Recent evidence indicates that conclusions drawn from heterologous interactions may be misleading [17, 29, 30]. Ovine PL appears to be an oGHR antagonist [29]. Moreover, homologous in vitro assays indicated that oPL can bind to a homodimer of the long form of the oPRLR and to a heterodimer of oPRLR and oGHR [28, 30]. However, the existence and role of these receptors for oPL in vivo has not been determined. Therefore, the objectives of the present studies were to determine the effects of intrauterine infusion of oPL, oGH, and their combination on endometrial histoarchitecture and gene expression and to localize binding sites for oPL and oGH using an in situ ligand binding assay.

MATERIALS AND METHODS

Animals

Mature Suffolk cross-bred ewes were observed daily for estrus (using vasectomized rams) and were assigned to treatments after exhibiting at least two estrous cycles of normal duration (16–18 days). 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.

Study 1: Intrauterine Infusion of oPL, oGH, and oPL plus oGH

Twenty cyclic ewes were ovariectomized and fitted with uterine catheters on Day 5 of the estrous cycle (Day 0 = estrus) (Fig. 1A). The ewes received daily i.m. injections of 50 mg progesterone from Day 5 to Day 30, daily intrauterine (i.u.) injections of recombinant oIFNτ (2×10^7 antiviral units/day) from Day 11 to Day 20, and daily i.u. injections from Day 16 to Day 30 (n = 5 ewes/treatment) of 1) serum proteins from a Day 5 cyclic ewe as a control (CX; 200 μg), 2) recombinant oPL (PL; 200 μg), 3) recombinant oGH (GH; 200 μg), or 4) recombinant oPL and oGH (PL+GH). Progesterone was administered at 0700 h in a total volume of 1 ml corn oil vehicle. The uterine horns of each ewe received injections (50 μg protein/horn) in 1 ml sterile saline at 0700 h and 1900

h. Therefore, the uterus of each ewe received a cumulative dose of 200 μg protein/day. All ewes were hysterectomized on Day 30.

At hysterectomy, portions (~1.0 cm) from the middle region of each uterine horn were fixed in fresh 4% paraformaldehyde in PBS (pH 7.0) for 24 h and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). From the remainder of each uterine horn, endometrium was dissected from myometrium, frozen separately in liquid nitrogen, and stored at −180°C.

Study 2: Binding Sites for oPL and oGH

Experimental design. Ewes were bred to intact rams at estrus (Day 0) and then assigned randomly to be hysterectomized (n = 3 ewes/day) on Days 30, 60, 90, or 120 of pregnancy. At hysterectomy, placentomal and interplacentomal areas of the uterus were separated, placed in Tissue-Tek OCT compound (OCT; Miles Inc., Oneonta, NY), frozen in liquid nitrogen, and stored at −180°C.

Preparation of recombinant oIFNτ, oPL, and oGH and i.u. protein injections. Recombinant oIFNτ was produced from a synthetic gene construct in *Pichia pastoris* and purified at the Fermentation Core Facility (Department of Food Science, University of Nebraska, Lincoln, NE) as described previously [31, 32]. Recombinant oPL and oGH were produced in *Escherichia coli* and purified as described previously [33]. Intrauterine protein injections were prepared as described previously [12].

Histology and morphometry. Embedded tissues were sectioned (5–7 μm), deparaffinized, and stained with Mayer hematoxylin and eosin for general histomorphological evaluation as described previously [12]. The total number of uterine glands in a cross section of the uterus was counted. The observation of a gland cross section with an open lumen was counted as a gland. Gland number was determined for at least six nonsequential sections from each uterine horn of each ewe. Intra- and intersection repeatability estimates for determination of gland number by a single observer were 0.8 and 0.9, respectively. Data are presented as gland numbers per cross section of the uterus.

RNA isolation and analyses. Total cellular RNA was isolated from frozen endometrium using the Trizol reagent (Gibco-BRL, Grand Island, NY). For each ewe, denatured total cellular RNA (20 μg) was analyzed by slot blot hybridization using radiolabeled antisense cRNA probes generated by in vitro transcription with [α-^{32}P]UTP (Amersham, Piscataway, NJ) as described previously [12]. Plasmid templates containing cDNAs for the bovine PRLR [34], ovine UTMP [19], ovine osteopontin (OPN) [35], ovine insulin-like growth factor (IGF) I [36], ovine IGF-II [36], and 18S rRNA (pT718S; Ambion, Austin, TX) were used to produce radiolabeled cRNA probes. Slot blots were quantitated by electronic autoradiography using an Instant Imager (Packard, Meriden, CT).

Cell culture and reagents. The Cos-7 and HEK-293 cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in basal medium containing Dulbecco modified Eagle culture medium with F-12 salts (DMEM-F12; Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin/amphotericin B (Gibco-BRL, Rockville, MD). All other chemicals or reagents were purchased from Sigma Aldrich unless otherwise noted. Recombinant oPL, oGH, oPRL, and oGHR extracellular domain (oGHR-ECD) were prepared and assayed for biological activity as described previously [28, 33, 37].

Construction of the SEAP-oPL expression vector. Identification of oPL receptors in the ovine uterus was performed using a fusion protein of heat-stable secreted alkaline phosphatase (SEAP) [38] with oPL (SEAP-oPL). The oPL cDNA was amplified from an existing oPL cDNA using polymerase chain reaction (PCR) techniques [33]. The 5′ primer (5′-TAC TCT AGA GGC GGC GGC gca cag cat cca cca tac tgt cga aac ca-3′) included an *Xba*I restriction site and was used to eliminate the initiation codon and to introduce three consecutive glycine residues and one alanine residue that served as a flexible linker. The 3′ primer (3′-taa gtg ccg att gac ctc atg cga aac cta gTC TAG ATG C-5′) was used to introduce an *Xba*I restriction site downstream of the stop codon. The PCR product was ligated into the *Xba*I site of modified pCMV-SEAP [39] (Dr. Michael Soares, University of Kansas Medical Center, Kansas City, KS), to produce a cytomegalovirus-driven vector encoding the SEAP-oPL fusion protein. Colonies containing vector with insert in the desired orientation were verified using restriction digestion with *Apa*I and *Afl*III. DNA sequencing of the insert was performed to verify the accuracy of the PCR amplification (data not shown).

Western blot analysis of SEAP-oPL. The HEK-293 cell line was transiently transfected with pCMV-SEAP-oPL or an unmodified pCMV-SEAP vector (SEAP) using methods described previously [39, 40]. After 24 h, transfected HEK-293 cells were washed, and the medium was changed to serum-free DMEM. After 72 h, conditioned medium was collected, clarified by centrifugation, sterile filtered (0.22 μm), and stored at 4°C. SEAP activity in conditioned medium was determined using a colorimetric assay (Fast P-nitrophenylphosphate tablet sets; Sigma) according to the manufacturer's instructions.

Proteins in samples of culture medium, concentrated or unconcentrated, were separated by PAGE in 10% gels under reducing conditions. Proteins from the gels were electrophoretically transferred to nitrocellulose in a Trans-Blot Cell (Bio-Rad, Hercules, CA). Polyclonal rabbit antibody generated against recombinant oPL was used in the Western blot analyses [41]. The blots were stained by the immunoperoxidase method, and bands were detected using enhanced chemiluminescence.

Alkaline phosphatase in situ ligand binding assay. Cos-7 cells were transfected with pCMV-SEAP or pCMV-SEAP-oPL, and stable expressing cell lines were selected using G418 according to methods described by Muller et al. [39]. For the production of conditioned medium for the in situ binding assays, Cos-7 cells were cultured in modified Eagle medium (MEM) supplemented with 20 mM Hepes, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FBS in an atmosphere of 5% CO_2/95% air at 37°C in a humidified incubator. After the cells reached confluence, the medium was changed to serum-free MEM with Hepes and was further conditioned for 72 h. The medium was then collected, clarified by centrifugation, concentrated using a Vivaspin Concentrator (5000 MWCO; Sartorius, Goettingen, Germany), sterile filtered (0.22 μm), and stored at 4°C. SEAP activity was measured in conditioned medium using a colorimetric assay. To determine specific SEAP activity, 800 μl of 1 M diethanolamine buffer with 0.5 mM magnesium chloride (pH 9.8) and 200 μl of p-nitrophenyl phosphate solution was added to 2 ml of conditioned medium, mixed, and incubated at 37°C for 5 min. The reaction was terminated with 50 μl of 3 M NaOH, and absorbance at 405 nm was determined using a spectrophotometer to calculate activity in milliunits (mU).

The in situ ligand binding assay was conducted using methods described previously [39]. Frozen sections (7–10 μm) of uterine tissues from each ewe were embedded in OCT compound, cut with a cryostat, and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Sections were then washed in a modified Hank balanced salt solution (HBSS; Sigma) containing 20 mM Hepes, 0.5 mg/ml BSA, and 0.1% NaN_3 (HBHA). The sections were then incubated with SEAP or SEAP-oPL in HBHA in a humidified chamber for at least 16 h at 4°C. The SEAP and SEAP-oPL fusion proteins were used at a concentration of 1000 mU/ml. For competition studies, specific binding was characterized by coincubation of uterine tissues with various concentrations of recombinant oPL, oPRL, or oGH along with SEAP-oPL. After incubation, the sections were washed with HBHA supplemented with 0.1% Tween 20 and fixed for 2 min in 20 mM Hepes buffer containing acetone (60%) and formaldehyde (3%). The fixed sections were then washed three times with HBSS (3×), heated at 65°C for 30 min in HBSS to inactivate endogenous tissue alkaline phosphatase (AP), and then processed for detection of the heat-stable SEAP activity associated with the fusion proteins. Coverslips were affixed to slides using Permount (Fisher Scientific, Fairlawn, NJ).

In situ ligand binding assay experiments were conducted in triplicate, with multiple sections of uteri from each ewe in an experiment. Competition experiments were conducted in triplicate with multiple sections of uteri from each Day 90 pregnant ewe. Stained tissues were photographed using a Zeiss Axioplan2 photomicroscope (New York, NY) fitted with a Hamamatsu chilled 3CCD color camera (Hamamatsu, Japan). Digital images were captured and assembled using Adobe Photoshop 5.0 (Adobe Systems, Seattle, WA).

Construction of oPRLR-ECD expression vectors and preparation of the recombinant oPRLR-ECD protein. The full-length cDNA of the oPRLR (GenBank AF014978) was kindly provided by Dr. Jean Djiane (INRA, Jouy-en-Josas, France). The forward primer (5′-CCC ACA TGT AAT GCA GTC ACC TCC TGA AAA ACC CAA ACT TAT-3′) introduced an initiator methionine codon and an *Afl*IIII restriction enzyme site and eliminated a *Hin*dIII site. The reverse primer (5′-GGG GCA AGC TTT AAT CCT TCA CTG GGA AGT CA-3′) introduced a stop codon immediately after the final codon and a *Hin*dIII restriction site downstream from the stop codon. The PCR was conducted using *Taq* polymerase in a capillary thermal cycler apparatus (Idaho Technology, Salt Lake City, UT) as follows: 2 min at 94°C; 30 cycles of 30 sec at 94°C, 60 sec at 60°C, 45 sec at 72°C; and 2 min at 72°C. The PCR product was digested with *Afl*III and *Hin*dIII restriction enzymes and after heat inactivation of the enzymes was ligated to parental vector pMON34Ol [42] linearized with *Nco*I and *Hin*dIII restriction enzymes. The ligation product was transfected to JM-109 *E. coli* cells prior to transformation of MON 105 cells. Automatic DNA sequencing confirmed the proper sequence. One of the expressing clones was chosen for large-scale expression.

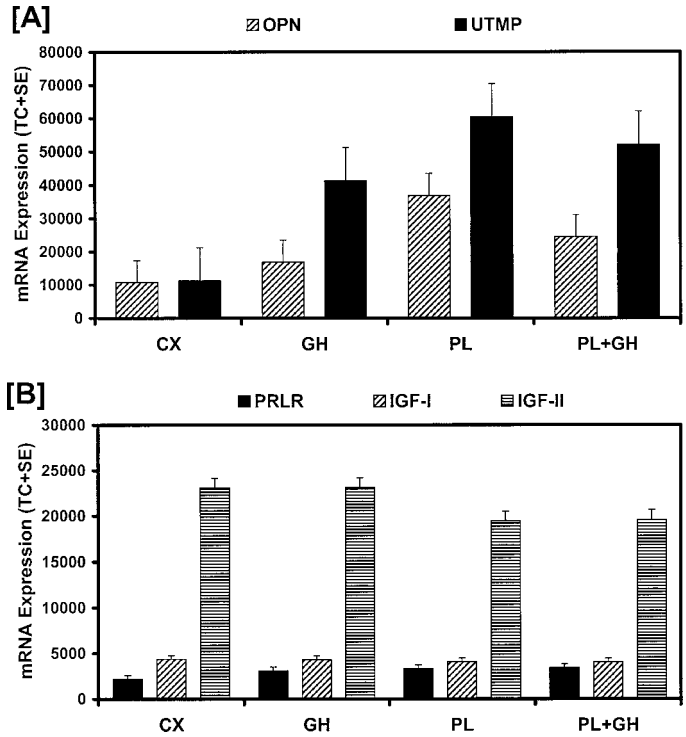

FIG. 2. Effects of i.u. infusion of somatolactogenic hormones on steady-state levels of endometrial mRNA. **A**) Steady-state levels of endometrial mRNAs for OPN and UTMP. **B**) Steady-state levels of endometrial mRNAs for PRLR, IGF-I, and IGF-II. Data are presented as total counts (CPM) with SEM.

Preparation of inclusion bodies and the refolding procedure for o-PRLR-ECD has been described previously [28, 29]. Cells were collected by centrifugation and then lysed, and the insoluble fraction was collected by repeated cycles of sonication and centrifugation. After solublization in 4.5 M urea, the solution was stirred at 4°C for 1 h, dialyzed against 10 mM Tris-HCl buffer (pH 9.0), and purified on a Q-Sepharose column (2.6 times 7 cm) that was preequilibrated with the same buffer. The monomeric fraction was eluted with 150 mM NaCl. Immoblization to Affigel (Bio-Rad) was performed according to the manufacturer's instructions.

Binding experiments. Binding to soluble oPRLR-ECDs was carried out as described previously [29, 33, 43]. The ligand was ^{125}I-oPL, and the competitors were recombinant oPL, oPRL, oGH, and human GH (hGH). Iodination of oPL was performed as described previously [44].

Statistical Analyses

All quantitative data were subjected to least-squares ANOVA (LS-ANOVA) using the general linear models (GLM) procedures of the Statistical Analysis System [45]. Analyses of steady-state levels of endometrial mRNA measured by slot blot hybridization included the 18S rRNA data as a covariate in LS-ANOVA to correct for differences in sample loading. For study 1, statistical models for analysis of gland number data included main effects of treatment (CX, PL, GH, PL+GH), ewe within treatment, uterine horn, and tissue section. Initial analyses indicated that uterine horn and tissue section were not significant sources of variation. In all analyses, error terms used in tests of significance were identified according to the expectation of the mean squares for error [46]. In all experiments, preplanned comparisons were used to determine treatment effects. Data are presented as least-square means (LSM) with overall standard errors (SE).

RESULTS

Study 1

Effects of i.u. infusion of oPL, oGH, and oPL plus oGH on endometrial histoarchitecture and gland density. The intercaruncular endometrium of CX ewes contained numerous endometrial glands in the stratum compactum and stratum spongiosum near the myometrium (Fig. 1B). In uteri of GH ewes, the diameter of the endometrial glands appeared greater than that in CX ewes. Endometrial gland density was increased ($P < 0.10$) approximately 1.5-fold in the endometrium of GH ewes compared with that of CX ewes (Fig. 1C). Likewise, the intercaruncular endometrium of PL ewes appeared to contain more endometrial glands. Although the diameter of the endometrial gland appeared smaller, i.u. infusion of PL increased endometrial gland density ($P < 0.01$) approximately 2.4-fold compared with that of CX ewes. In ewes infused with the PL+GH combination, the intercaruncular endometrium contained glands that appeared larger in diameter than those observed in CX, GH, or PL ewes. In contrast to ewes infused with either GH or PL, ewes infused with the PL+GH combination had endometrial gland density that was not different from that in CX ewes ($P > 0.10$).

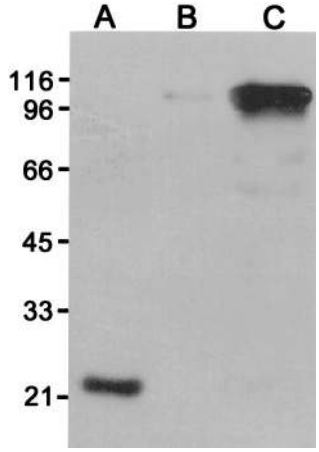

FIG. 3. Western blot analysis using polyclonal antibodies generated against oPL. Lane A: oPL (1 ng); lane B: conditioned medium from cultured HEK-293 cells transfected with the SEAP-oPL vector; lane C: conditioned medium from cultured HEK-293 cells transfected with the SEAP-oPL vector and concentrated 100-fold. Molecular mass standards (kDa) are denoted on the left.

Effects of i.u. infusion of oPL, oGH, and oPL plus oGH on endometrial gene expression. Intrauterine infusion of GH did not affect steady-state levels of endometrial OPN mRNA ($P > 0.10$) but did increase UTMP mRNA compared with the levels ($P < 0.01$) in ewes receiving CX proteins (Fig. 2A). In contrast, i.u. infusion of PL increased endometrial levels of OPN ($P < 0.05$) and UTMP ($P < 0.01$) mRNAs compared with levels in CX ewes. Steady-state levels of endometrial OPN mRNA were not affected by infusion of both oPL and oGH compared with ewes receiving CX proteins, but endometrial UTMP mRNA was increased ($P < 0.01$) by this treatment. However, infusion of both oPL and oGH did not have an additive or synergistic effect on endometrial OPN or UTMP expression (PL vs. PL+GH and GH vs. PL+GH, $P > 0.10$).

Steady-state levels of PRLR and IGF-I mRNAs in the endometrium were not affected ($P > 0.10$) by i.u. infusion of GH, PL, or PL+GH (Fig. 2B). Levels of IGF-II mRNA were not affected ($P > 0.10$) by infusion of GH. However, infusion of PL and the PL+GH combination did slightly decrease endometrial IGF-II mRNA levels ($P < 0.05$).

Study 2

Generation and characterization of SEAP-oPL fusion protein. To identify and characterize binding sites for oPL

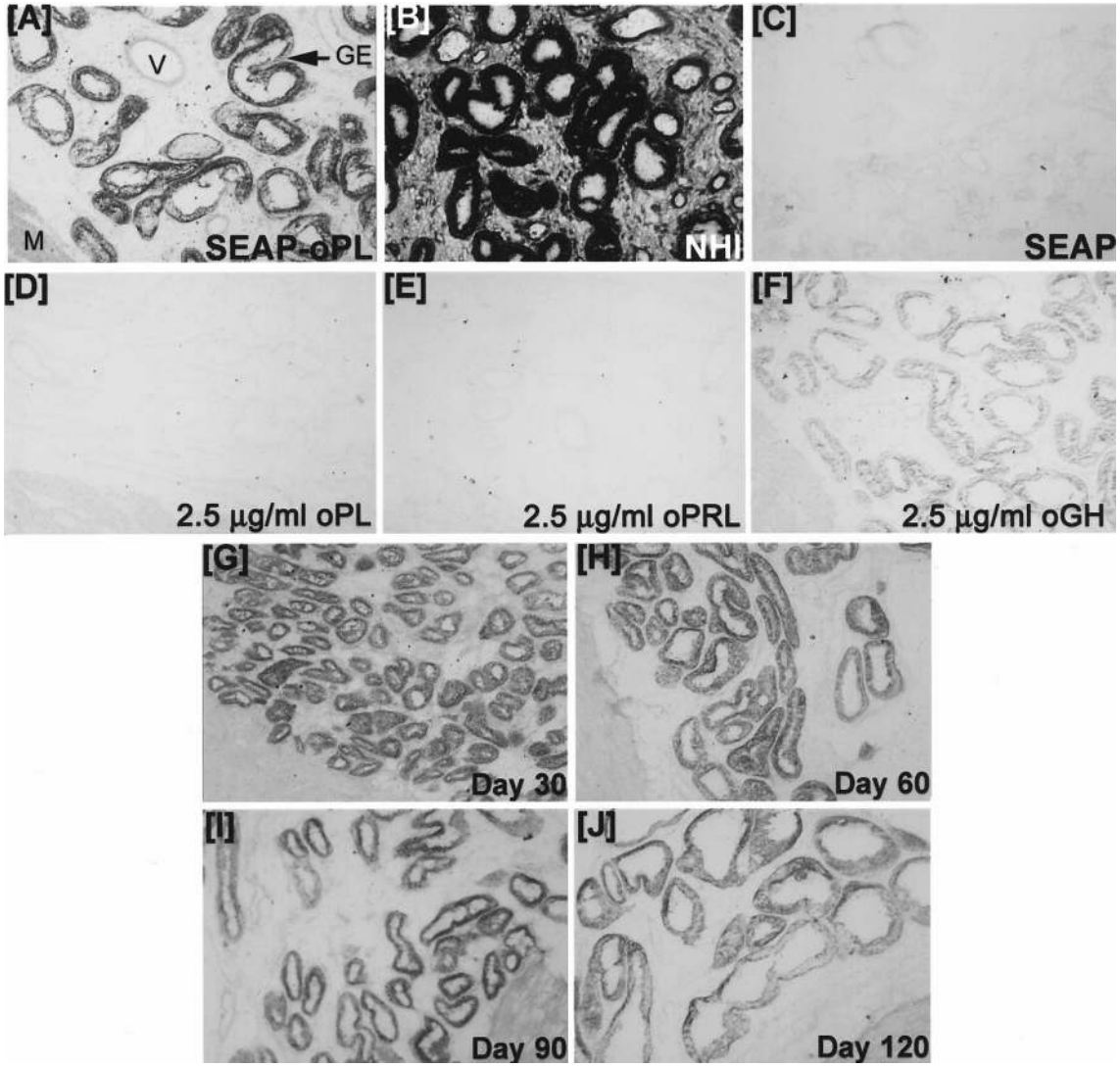

FIG. 4. Localization of PL binding sites in the interplacentomal regions of the ovine uterus from pregnant ewes using an in situ ligand binding assay. **A**–**F**) Uterine wall from a Day 90 pregnant ewe. **A**) SEAP-oPL. Note specific binding to the endometrial GE but not the stroma, blood vessels (V), or myometrium (M). **B**) As **A** but endogenous AP activity was not heat inactivated (NHI). Note the high level of endogenous AP activity in all cell types in the endometrium. **C**) SEAP alone. **D**) SEAP-oPL and 2.5 μg/ml oPL added as a competitor. **E**) SEAP-oPL and 2.5 μg/ml oPRL added as a competitor. **F**) SEAP-oPL and 2.5 μg/ml oGH added as a competitor. **G**–**J**) Uterine wall from Days 30, 60, 90, and 120 of pregnancy probed with SEAP-oPL.

in ovine uterine tissues, a chimeric protein of oPL fused to SEAP was generated by insertion of the oPL cDNA into the pCMV-SEAP vector downstream from SEAP. The SEAP-oPL vector and the unmodified SEAP parental vector were then transfected into HEK-293 cells. The presence of SEAP activity in the conditioned medium of SEAP- and SEAP-oPL-expressing cells was detected using colorimetric assay after heat inactivation of endogenous AP. Western blot analyses revealed that rabbit anti-oPL antibody could specifically recognize native oPL (Fig. 3, lane A) and SEAP-oPL in both undiluted and concentrated (100-fold) conditioned medium from transfected cells (Fig. 3, lanes B and C). The apparent molecular weight of the immunoreactive SEAP-oPL was approximately 100 000, which is consistent with the calculated molecular weight of the fused protein. The anti-oPL antibodies did not recognize SEAP itself (data not shown).

In a binding assay, concentrated conditioned medium containing either SEAP or SEAP-oPL was applied to a Affigel column containing immobilized oGHR-ECD. An excess amount of oPL was used to elute the bound protein, and SEAP activity was found only in the eluent of the column loaded with conditioned medium containing SEAP-oPL (data not shown). Thus, the fusion protein retained characteristic binding activity of oPL to oGHR-ECD in vitro as reported previously [28].

In situ localization of oPL receptors in the ovine uterus. Specific binding of SEAP-oPL was observed in the GE of the interplacentomal endometrium on Days 30, 60, 90, and 120 of pregnancy (Fig. 4, A and G–J). No significant binding of SEAP-oPL was detected in the placenta, endometrial stroma, blood vessels, myometrium, or caruncular and cotyledonary areas of the placentomes (data not shown). In sections not heat inactivated (Fig. 4B), high levels of endogenous AP activity were observed in the endometrial GE, stroma, intraepithelial and stromal immune cells, and blood vessels (Fig. 4B) and in the conceptus trophectoderm (data not shown). However, the activity of the endogenous AP was almost completely eliminated by heat inactivation as observed in the SEAP control (Fig. 4C). Sections treated with the control SEAP alone (Fig. 4C) showed no specific binding compared with SEAP-oPL sections (Fig. 4A).

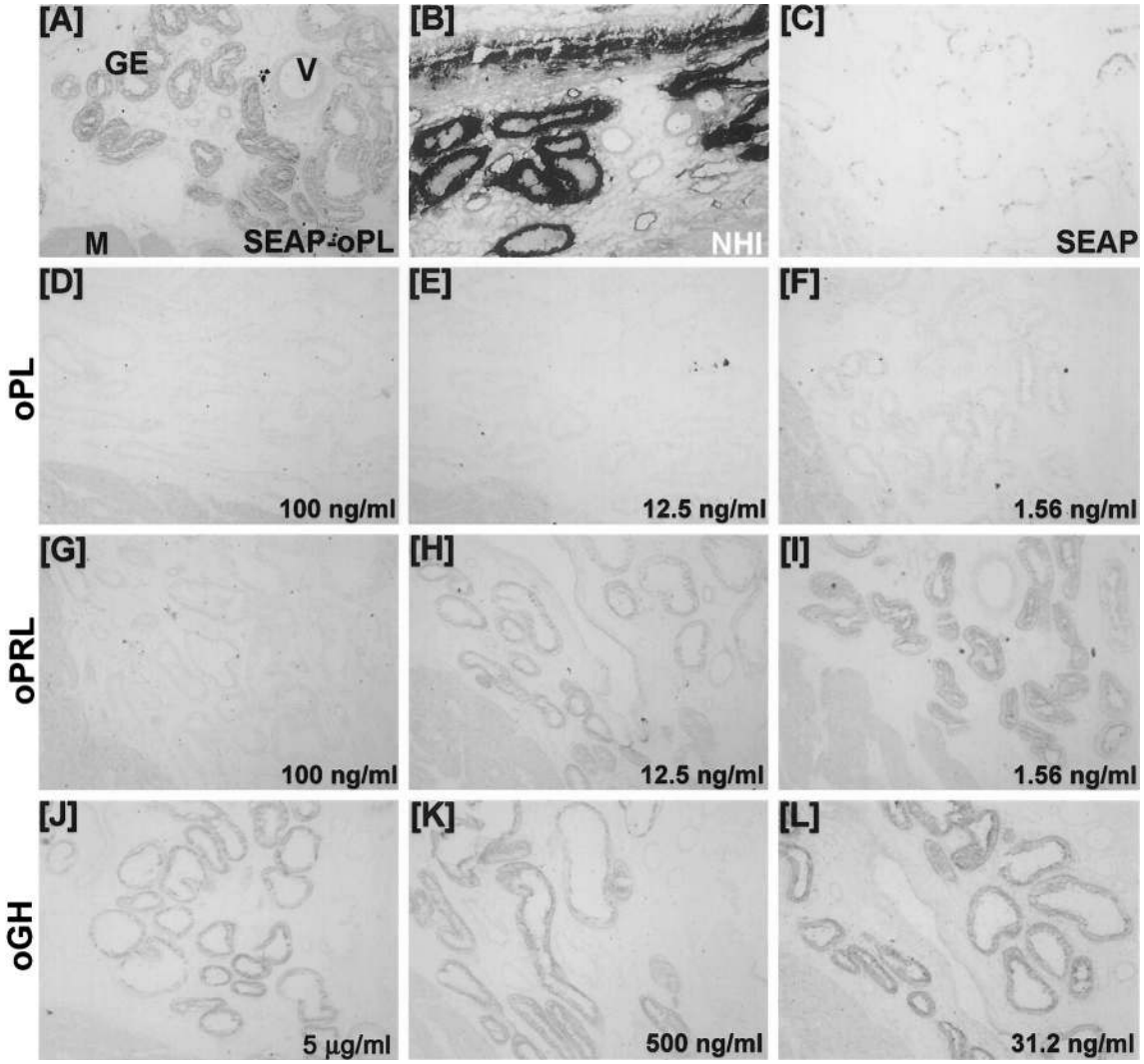

FIG. 5. Characterization of oPL binding sites in the the interplacentomal regions of the uterus from Day 90 pregnant ewes using an in situ ligand binding competition assay. **A**–**L**) Uterine wall from a Day 90 pregnant ewe. **A**) SEAP-oPL. Note specific binding to the endometrial GE but not the stroma, blood vessels (V), or myometrium (M). **B**) SEAP-oPL, but endogenous AP activity was not heat inactivated (NHI). Note the high level of endogenous AP activity in all cell types in the endometrium. **C**) SEAP alone. **D**–**F**) SEAP-oPL and 100, 12.5, or 1.56 ng/ml oPL added as a competitor. **G**–**I**) SEAP-oPL and 100, 12.5, or 1.56 ng/ml oPRL added as a competitor. **J**–**L**) SEAP-oPL and 5 μg/ml, 500 ng/ml, or 31.2 ng/ml oGH added as a competitor.

Some nonspecific background was observed in the myometrium.

SEAP-oPL binding was effectively outcompeted by coincubation with 2.5 μg/ml oPL (Fig. 4D) or oPRL (Fig. 4E). In contrast, SEAP-oPL binding was only partially outcompeted by addition of 2.5 μg/ml oGH (Fig. 4F). The relative amount of SEAP-oPL binding to the endometrial glands was not affected by day of pregnancy between Days 30 and 120 (Fig. 4, G–J). The slight background reaction observed in the myometrium was not specific binding of SEAP-oPL because the reaction was also observed in sections probed with control SEAP and in sections coincubated with SEAP-oPL and an excess of oPL (Fig. 4D).

Displacement of SEAP-oPL binding by oPL, oPRL, and oGH. A series of competition studies were conducted on interplacentomal endometrium from Day 90 pregnant ewes to determine the nature of oPL binding sites in the GE. As observed previously, SEAP-oPL binding was specific to the endometrial GE (Fig. 5A). In some sections, residual endogenous AP activity was detected in intraepithelial cells present in endometrial glands that appeared to be immune cells, based on morphology (Fig. 5C). Coincubation with an unrelated hormone, recombinant oIFNτ, did not produce competition for SEAP-oPL binding to the endometrial GE (data not shown). SEAP-oPL binding could be outcompeted by recombinant oPL, oPRL, and oGH (Fig. 5). SEAP-oPL (~17 ng/ml) binding was blocked completely in the presence of 2.5 μg/ml (data not shown) to 12.5 ng/ml (Fig. 5, D and E) oPL. A small amount of binding was observed in sections coincubated with oPL at 1.56 ng/ml (Fig. 5F).

Similar to oPL, coincubation of uterine tissue with oPRL along with SEAP-oPL effectively prevented detectable SEAP-oPL binding to GE with higher concentrations of oPRL at 2.5 μg/ml (data not shown) to 100 ng/ml (Fig. 5G) of oPRL but not at lower concentrations (12.5-1.56 ng/ml) of unlabeled oPRL (Fig. 5, G–I). In contrast, coincubation of endometrial tissue with oGH and SEAP-oPL only partially outcompeted SEAP-oPL binding to GE with higher concentrations of oGH at 5 μg/ml to 500 ng/ml (Fig. 5, J and K). An increase in SEAP-oPL binding was observed with lower concentrations of oGH at 250 ng/ml (data not shown) to 31.2 ng/ml (Fig. 5, K and L).

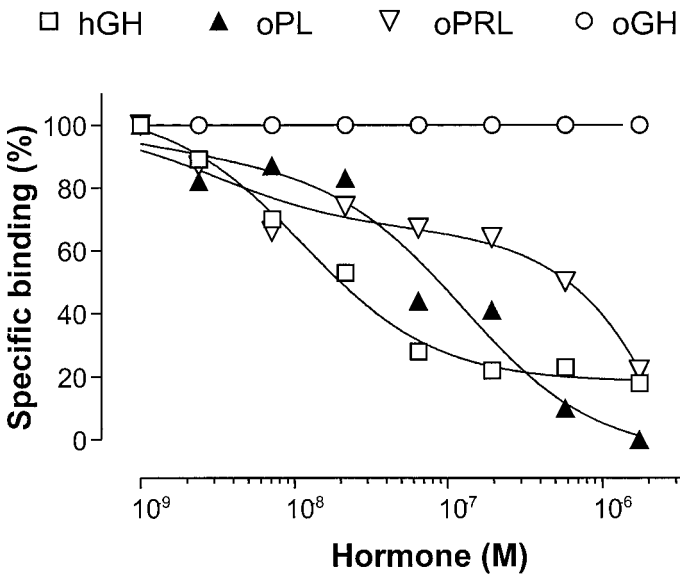

FIG. 6. Competition of unlabeled hGH (□), oPL (▲), oPRL (▽), and oGH (○) with the binding of ^{125}I-oPL to oPRLR-ECD. Results represent percentage of specific binding; 100% is the specific binding in the absence of competitor. ^{125}I-oPL binding could not be blocked by oGH.

The collective results of several independent qualitative competition experiments performed using uterine tissue sections from three Day 90 pregnant ewes were used to deduce the concentration of hormones needed for partial and complete displacement of SEAP-oPL. Only 1.5 or 3 ng/ml of oPL or oPRL, respectively, were needed to achieve a detectable reduction in SEAP-oPL binding, whereas concentrations about 8-fold higher could completely displace SEAP-oPL. However, 62.5 ng/ml of oGH was necessary to reduce SEAP-oPL binding, but complete displacement of SEAP-oPL binding by oGH was not detected even at 5 μg/ml. The displacement of SEAP-oPL by these recombinant homologous hormones in a dose-response manner indicates the specific nature of this binding; however, these binding assays are qualitative rather than quantitative.

Binding to oPRLR-ECD. To eliminate the possibility that oGH can weakly bind oPRLR and thus displace SEAP-oPL binding at high concentrations, oPRLR-ECD was prepared. The renatured and purified protein appeared as a monomeric 28-kDa protein that was able to form a complex with oPRL or oPL in a gel filtration assay (data not shown). A comparative binding assay was conducted to test the ability of recombinant hGH, oPRL, oPL, and oGH to compete with ^{125}I-oPL binding to oPRLR-ECD (Fig. 6). The three lactogenic hormones (hGH, oPRL, and oPL) were able to compete with ^{125}I-oPL, with respective IC_{50} values of 11.4, 700, and 100 nM. In contrast, ^{125}I-oPL binding could not be outcompeted by oGH even at the high concentration of 2 μM.

DISCUSSION

Endometrial gland morphogenesis and the secretory capacity of the uterus, indicated by temporal changes in gene expression and secretion of UTMP, are correlated with production of PL and GH by the ovine placenta [4, 6, 21]. Results from study 1 indicate that PL and GH have specific paracrine actions on the endometrial GE and stimulate proliferation, remodeling, and differentiated function in terms of secretory protein gene expression. Intrauterine infusion of oPL or oGH alone increased endometrial gland density and expression of UTMP mRNA. Ovine PL is produced by binucleate trophectoderm cells as early as Day 16 of pregnancy and can be detected in the maternal circulation from Day 50 until near the end of pregnancy [18, 22, 24]. In contrast, expression of GH by the ovine placenta is restricted to Days 35–70 of gestation [25]. These temporal differences in oPL and oGH expression by the placenta may determine, in part, the specific roles that each plays with respect to endometrial remodeling and function. In the present study, i.u. infusion of ewes with the combination of oPL plus oGH elicited an increase in uterine gland hypertrophy that was not observed in ewes infused with either oPL or oGH alone. During pregnancy, the production of GH by the placenta from Day 35 to Day 70 is accompanied by increasing production of oPL by the placenta and is correlated with the onset of uterine gland hypertrophy and a large increase in expression of UTMP [6]. Results of the present study support the idea that oGH from the placenta stimulates uterine gland hypertrophy, which occurs between Day 50 and Day 60 of gestation [4, 6].

In study 1 and as observed previously [12], i.u. infusion of oPL and oPL plus oGH increased expression of both UTMP and OPN mRNAs in endometrium, whereas oGH alone only increased expression of UTMP mRNA. Although both oPL and oGH activate the JAK2-STAT5 and mitogen-activated protein kinase (MAPK) signal transduction pathways in other model systems [47, 48], individual genes, such as UTMP and OPN, may be regulated differentially by these placental hormones. It is difficult to determine whether PL and GH acted directly on transcription of UTMP and OPN genes or whether increases in UTMP and OPN mRNA were the result of the effects of oPL, oGH, or oPL plus oGH on endometrial gland density and size. Further research is needed to assess the apparently diverse effects of PL and GH on GE morphogenesis and secretory protein gene expression. In other epitheliomesenchymal organs, PRL induces mitogenesis and alters postdifferentiation gene expression programs. For instance, in the pigeon crop sac, PRL stimulates proliferation of mitogenically competent germinal layer cells and causes the differentiated cells to enter an altered program of gene expression and phenotypic differentiation leading to production of large volumes of crop milk [47, 49]. Hyperprolactinemia causes endometrial hypertrophy and glandular differentiation and alters secretions found in the uterine lumen of both the rabbit and pig [50–53]. In ruminants, PL may act on the endometrial GE to stimulate morphogenesis and differentiated functions in a manner similar to effects of PRL on the pigeon crop sac [49] and mouse mammary gland [54].

The second experimental objective was to locate the binding sites of oPL in the ovine uterus and to identify the receptor(s) involved in this binding. Using an in situ ligand binding assay method with SEAP-oPL fusion protein, specific oPL binding was detected only in the endometrial GE. Ovine PL in vitro can heterodimerize the extracellular domains of oGHR and oPRLR, and if this heterodimerization were to occur in a living cell it could activate signal transduction [28]. In the ovine uterus, oPRLR expression is abundant and restricted to the endometrial GE [6, 27], whereas oGHR expression is relatively much lower and detectable by reverse transcription PCR in most uterine cell types, including the endometrial GE [26] (unpublished results). In the present study, SEAP-oPL binding to endometrial GE was completely displaced by oPL and oPRL,

whereas oGH only partly displaced binding of SEAP-oPL to the endometrial GE. Results of the competition in situ ligand binding assays indicated a difference in SEAP-oPL and recombinant oPL for binding to the endometrial glands. The affinities of SEAP-oPL and oPL for their receptors on the endometrial glands are different. These differences may result from production and purification of SEAP-oPL by a mammalian cell compared with that achieved by recombinant oPL from bacteria that had to be renatured. Further, the reduced affinity of SEAP-oPL may be due to the fact that it is a fusion protein and may cause minor interference when approaching the receptor. The amount of SEAP-oPL was estimated using the biological activity of the SEAP along with the calculated mass of the fusion protein, a method that is not entirely quantitative. Mutations impairing the ability of oPL or bovine PL to form stable complexes with lactogenic receptors do not necessarily lead to a decrease in the biological activity because the transient existence of the homodimeric complex is still sufficient to initiate signal transduction [30]. Collectively, available results can be interpreted to indicate that the majority of oPL binding sites in the endometrial GE consist of a homodimer of two oPRLRs and the minority of oPL binding sites are a heterodimer of the oPRLR and oGHR. However, the existence of a unique oPL receptor different from a heterodimer of the oPRLR and oGHR cannot be ruled out.

Binding studies indicated that oPL binding to the oGHR-ECD could be outcompeted by oGH, but oPL binding to the oPRLR-ECD could not be displaced by oGH. The exact mechanism whereby oPL initiates heterodimerization of oPRLR and oGHR is not clear. The finding that oPRL could completely displace the binding of SEAP-oPL invalidates the explanation that oPL is independently bound to oPRLR and oGHR. However, in vitro gel filtration studies indicated that oPL first binds oGHR at binding site I and then binds oPRLR at binding site II [28]. Results from the comparative binding assays of oPL binding to the oPRLR-ECD indicated that all three lactogenic hormones (hGH, Oprl, and oPL) were able to compete with oPL with respective IC_{50} values of 11.4, 700, and 100 nM. Although this is the first report of binding assays of the oPRLR-ECD using a homologous system based on recombinant proteins, these values are higher than those of some studies utilizing receptors purified from tissues and heterologous proteins. These discrepancies may result from binding assays performed in a heterologous rather than a homologous manner (see [17] for review). Furthermore, one concern with the use of recombinant proteins, especially bacterially derived proteins, is proper renaturation and folding. The discrepancies between the apparent affinities of oPL and oPRL for the recombinant oPRLR-ECD and those determined for oPL and oPRL for tissue oPRLR may be a function of how correctly the oPRLR-ECD was renatured.

Results from study 2 strongly support the idea that the paracrine effects of oPL on the ovine uterus are specific to the endometrial glands. PLs may act as unique fetal GHs [16, 17], affecting responses that include stimulation of glycogen synthesis, amino acid transport, cellular proliferation, and IGF-I synthesis. These biological effects in the fetus are only slightly, if at all, affected by oGH, hGH, or oPRL, suggesting potentially unique and specific effects of oPL (for review, see [16, 17]). Results from study 1 indicate that both oPL and placental oGH have overlapping and specific effects on the endometrial glands. However, these effects appear to not be mediated by IGF-I or IGF-II because neither hormone affected expression of these mRNAs in the endometrium, as assessed by slot-blot hybridization analyses. One limitation of slot-blot analyses of total RNA is the inability to detect changes in specific transcripts of IGF-I and IGF-II, which are known to vary with tissue type and physiological state. Therefore, the present results do not preclude an effect of i.u. oPL or oGH on specific IGF-I or IGF-II transcripts expressed in the endometrial stroma. In the adult ovine uterus, IGF-I and IGF-II expression is confined to the endometrial stroma and myometrium [55, 56]. Therefore, the lack of effects of oPL infusion on endometrial IGF-I and IGF-II mRNAs was not unexpected because of the finding that oPL binding is unique to endometrial GE.

Results from the present study confirm and extend our working hypothesis that members of the lactogenic and somatogenic hormone family play key roles in stimulating endometrial gland morphogenesis and differentiated function during pregnancy to facilitate conceptus growth and development during implantation and placentation [9, 12]. The pregnant ovine endometrium is sequentially exposed to estrogen, progesterone, IFNτ, PL, and placental GH, and available results support the idea that these hormones constitute a servomechanism that activates and maintains endometrial remodeling, secretory function, and uterine growth during gestation. In future experiments, we will examine the precise cellular and molecular signaling pathways of oPL and placental oGH and differences in oPL signaling through the oPRLR homodimer and oPRLR and oGHR heterodimer. These experiments are necessary to understand shared and hormone-specific effects of oPL and placental oGH on endometrial morphogenesis and function in the ewe.

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