Insulin-Like Growth Factor Binding Protein-1 in the Ruminant Uterus: Potential Endometrial Marker and Regulator of Conceptus Elongation

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Establishment of pregnancy in ruminants requires conceptus elongation and production of interferon-τ (IFNT), the pregnancy recognition signal that maintains ovarian progesterone (P4) production. These studies determined temporal and spatial alterations in IGF binding protein (IGFBP)-1 and IGFBP3 in the ovine and bovine uterus; effects of P4 and IFNT on their expression in the ovine uterus; and effects of IGFBP1 on ovine trophectoderm cell proliferation, migration, and attachment. IGFBP1 and IGFBP3 were studied because they are the only IGFBPs specifically expressed by the endometrial luminal epithelia in sheep. In sheep, IGFBP1 and IGFBP3 expression was coordinate with the period of conceptus elongation, whereas only IGFBP1 expression was coordinate with conceptus elongation in cattle. IGFBP1 mRNA in the ovine endometria was between 5- and 29-fold more abundant between d 12 and 16 of pregnancy compared with the estrous cycle and greater on d 16 of pregnancy than nonpregnancy in the bovine uterus. In sheep, P4 induced and IFNT stimulated expression of IGFBP1 but not IGFBP3; however, the effect of IFNT did not mimic the abundant increase observed in pregnant ewes. Therefore, IGFBP1 expression in the endometrium is regulated by another factor from the conceptus. IGFBP1 did not affect the proliferation of ovine trophectoderm cells *in vitro* but did stimulate their migration and mediate their attachment. These studies reveal that IGFBP1 is a common endometrial marker of conceptus elongation in sheep and cattle and most likely regulates conceptus elongation by stimulating migration and attachment of the trophectoderm. **(*Endocrinology* 150: 4295–4305, 2009)**

Maternal support of blastocyst growth and development into an elongated conceptus (embryo/fetus and associated membranes) is critical for pregnancy recognition signaling and implantation in ruminants (1, 2). After hatching from the zona pellucida on d 8 (sheep) or d 9–10 (cattle), the blastocyst develops into an ovoid or tubular form by d 11 (sheep) or d 13 (cattle) and is termed a conceptus (1, 3, 4). The ovoid conceptus begins to elongate on d 12 (sheep) or d 14 (cattle) and forms a filamentous conceptus of 14 cm or more in length by d 16 (sheep) or d 19 (cattle). Conceptus elongation involves exponential increases in length and weight of the trophectoderm as well as differentiation of the extraembryonic membranes (5) and requires substances secreted from the endometrial luminal (LE) and glandular epithelia (GE) (6, 7). During early pregnancy in ruminants, endometrial functions are regulated primarily by progesterone (P4) from the corpus luteum (CL) and secreted cytokines and hormones from the trophectoderm/chorion including interferon-τ (IFNT) (8–10). IFNT, produced during conceptus elongation, exerts antiluteolytic effects on the endometrium to maintain CL function and ensure continual production of P4 that, in turn, stimulates and maintains uterine endometrial functions necessary for conceptus growth, implantation, placentation, and successful development of the fetus to term (8). Additionally, IFNT acts on the endometrium to

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Abbreviations: CL, Corpus luteum; CX, control; FN, fibronectin; GE, glandular epithelia; IFNT, interferon-τ; IGFBP, IGF binding protein; LE, luminal epithelia; LSM, least squares means; P4, progesterone; PGR, progesterone receptor; RGD, Arg-Gly-Asp; RU, RU486; sGE, superficial GE.

induce or increase expression of many genes that potentially regulate conceptus growth and development (for review see Refs. 2, 10, and 11).

We recently reported results from an ovine model of accelerated blastocyst growth and conceptus development elicited by advancing the postovulatory rise in circulating levels of P4 during metestrus (12). That model was used to identify a number of candidate P4-regulated genes encoding secreted proteins (*galectin-15* or *LGALS15*, *gastrin-releasing peptide* or *GRP*, *insulin-like growth factor one* or *IGFBP1*, and *IGFBP3*) implicated in periimplantation conceptus elongation (12–14). *IGFBP1* is expressed exclusively in the LE/superficial GE (sGE) of the endometrium of both sheep and cattle (15, 16). *IGFBP3* is expressed predominantly in the LE/sGE of sheep endometria (17) but is expressed in stroma and GE of bovine endometria (15). Limited or no information is available on effects of the conceptus, P4, or IFNT on *IGFBP1* and *IGFBP3* expression in endometria of sheep and cattle. IGF binding protein (IGFBP)-1 and IGFBP3 are among the 16 known members of the IGFBP superfamily that regulate IGF bioavailability and cellular actions (for reviews see Refs. 18–21). IGF-I and IGF-II possess both mitogenic and differentiative properties and are implicated in early embryonic and placental development in many species including sheep and cattle (22–24). IGFBPs can both enhance and retard IGF actions (25, 26). IGFBP1 is a unique IGFBP because it contains a functional Arg-Gly-Asp (RGD) integrin recognition domain (27). Integrins expressed constitutively on both the conceptus trophectoderm and endometrial LE in sheep and cattle (28, 29) and are essential for blastocyst implantation but require functional binding and cross-linking to regulate implantation (30, 31). The biological functions of IGFBP1 include stimulation of trophoblast cell migration (32, 33) and inhibition of trophoblast invasiveness (27). IGFBP3 has a very high affinity for IGF-I and IGF-II, prolongs their half-life in serum, alters their interaction with cell surface receptors, and may have IGF-independent actions to control the cell cycle and apoptosis (34, 35).

The reported biological roles for IGFBP1 and IGFBP3 make these molecules excellent candidates to influence trophectoderm proliferation, migration, and attachment to uterine LE that are essential processes modulating periimplantation ruminant conceptus growth and development (1–3). Thus, the working hypothesis for the present study is that IGFBP1 and IGFBP3 have biological roles in periimplantation conceptus growth and development in ruminants. As a first step in testing this hypothesis, these studies determined: 1) effects of the estrous cycle and early pregnancy on *IGFBP1* and *IGFBP3* expression in ovine and bovine uteri; 2) effects of P4 and IFNT on *IGFBP1* and *IGFBP3* expression in the ovine uterus; and 3) effects of IGFBP1 on ovine trophectoderm cell proliferation, migration, and attachment.

Materials and Methods

Experimental design

All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Study 1

At onset of estrus (d 0), ewes were mated to either an intact or vasectomized ram and then hysterectomized (n = 5 ewes/d) on d 3, 6, 10, 12, 14, or 16 of the estrous cycle or d 10, 12, 14, 16, 18, or 20 of pregnancy. Uterine and/or conceptus tissues processed as described previously (36). At hysterectomy, several sections (~0.5 cm) from the midportion of each uterine horn ipsilateral to the CL were fixed in fresh 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). The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at −80 C for subsequent RNA extraction. In monovulatory pregnant ewes, uterine tissue samples were only from the ipsilateral uterine horn to the ovary bearing the CL. The uterine lumen was flushed with 20 ml of sterile 10 mM Tris buffer (pH 7.2) on d 10–16 of pregnancy and the estrous cycle, and in pregnant ewes, the flushing was examined for the presence of a morphologically normal conceptus.

Study 2

Cross-bred nulliparous heifers were artificially inseminated with semen from a single bull after a timed artificially inseminated synchronization protocol (37) and then slaughtered on d 10, 13, 16, or 19 after mating. The uterus was flushed with 20 ml of sterile 10 mM Tris buffer (pH 7.2). Heifers were classified as pregnant if the uterine flush contained a blastocyst/conceptus of the correct morphology and size or nonpregnant if the uterine flush did not contain a blastocyst/conceptus. The ipsilateral horn of the uterus was processed as described for study 1. Uterine tissues were collected from nonpregnant heifers on d 10, 13, 16, and 19 (n = 6/d) and pregnant heifers on d 13, 16, and 19 (n = 6/d).

Study 3

Cyclic ewes (n = 20) were checked daily for estrus and then ovariectomized and fitted with indwelling uterine catheters on d 5. Ewes were then assigned randomly (n = 5 per treatment) to receive daily im injections of P4 and/or a progesterone receptor (PGR) antagonist [mifepristone or RU486 (RU); Sigma Chemical Co., St. Louis, MO] and intrauterine infusions of either control (CX) serum proteins and/or recombinant ovine IFNT as follows: 1) 50 mg P4 (d 5–16) and 200 μg serum proteins (d 11–16) (P4+CX); 2) P4 and 75 mg RU486 (d 11–16) and serum proteins (P4+RU+CX); 3) P4 and IFNT (2×10^7 antiviral units, d 11–16) (P4+IFN); or 4) P4 and RU and IFNT (P4+RU+IFN). Steroids were administered im daily in corn oil

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vehicle. Both uterine horns of each ewe received twice-daily injections of either CX serum proteins (50 μg/horn per injection) or recombinant IFNT (5×10^6 antiviral units/horn per injection). Recombinant ovine IFNT was produced in *Pichia pastoris* and purified as described previously (38). Serum proteins and IFNT were prepared for intrauterine injections as described previously (39). This regimen of P4 and IFNT mimics the effects of P4 and IFNT from the CL and conceptus, respectively, on endometrial expression of hormone receptors and IFNT-stimulated genes during early pregnancy in ewes (40). All ewes were hysterectomized on d 17 and uteri processed as described for study 1.

Slot blot hybridization analysis

Total cellular RNA was isolated from frozen ipsilateral endometrium (studies 1 and 2) using Trizol reagent (Life Technologies, Inc.-BRL, Bethesda, MD) according to the manufacturer's instructions. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively. Steady-state levels of *IGFBP1* and *IGFBP3* mRNAs in endometria were assessed by slot blot hybridization using radiolabeled antisense *IGFBP1*, *IGFBP3*, or *18S* cRNA probes as described previously (13, 41). Radioactivity associated with slots was quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ). Data are expressed as relative units.

In situ hybridization analysis

Cell-specific expression of *IGFBP1* and *IGFBP3* mRNAs in ovine and bovine uteri was determined using radioactive *in situ* hybridization analysis methods described previously (13, 41). All slides for each respective gene were exposed to photographic emulsion for the same period of time. Images of representative fields were recorded under bright- or dark-field illumination using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX) fitted with a Nikon DXM1200 digital camera.

Cell proliferation assay

An ovine trophectoderm cell line (oTr1) from a d 15 conceptus reported previously (42) was used to conduct trophectoderm proliferation assays as described previously (42, 43). Briefly, oTr1 cells were subcultured into 12-well plates (no. 3513; Corning Costar, Corning, NY) to about 50% confluency in trophoblast growth medium for 6–8 h and then switched to serum and insulin-free DMEM for 24 h. In some experiments, cells were cultured under low serum (1%) or full serum (10%). After 24 h, the wells (n = 4 per treatment) were treated with either increasing amounts of purified human IGFBP1 (catalog 8-IGFBP; Advanced Immunochemical Inc., Long Beach, CA) in serum and insulin-free DMEM, trophoblast growth medium containing serum and insulin as a positive control, or DMEM alone as a negative control. After 48 h of culture, cell numbers were determined as described previously (44). The entire experiment was repeated at least three times with different passages of oTr1 cells.

Cell migration assay

The oTr1 cells (100,000 per 100 μl serum free DMEM) were seeded in a confluent layer on 8-μm pore transwell inserts (Corning Costar). Purified human IGFBP1 (Advanced Immunochemical) or BSA (Sigma) was then added to separate wells in serum-free DMEM-F12 at 1, 10, 100, or 1000 ng/ml (n = 3 replicates/treatment). After 12 h, cells remaining on the top portion of the membrane were removed by scraping with a cotton swab and membranes were fixed in −20 C methanol for 10 min. Membranes were removed, placed on slides, and stained with 4′,6′-diamidino-2-phenylindole (Invitrogen, Hercules, CA). Cells that migrated to the bottom surface of the membrane were counted in five nonoverlapping sections of each membrane, which accounted for approximately 70% of the membrane area, using a Axioplan 2 fluorescence microscope (Zeiss, New York, NY) with an Axiocam HR digital camera and Axiovision 4.3 software. Cells incubated in DMEM-F12 containing 10% fetal bovine serum served as a positive control for migration.

Cell attachment assay

Cell attachment assays were conducted with oTr1 cells as described previously (45). Polystyrene microwells (Corning Costar) were coated overnight at 4 C with 2-fold serial dilutions (10 μg/ml to 20 ng/ml) of the following proteins (50 μl) in PBS (n = 3 replicates/treatment): full-length recombinant human fibronectin (FN; Sigma), purified human IGFBP1 (Advanced Immunochemical); or BSA (Sigma). After blocking each well with 10 mg/ml BSA in PBS (100 μl), oTr1 cells (n = 50,000) were added to the well and allowed to attach for 1 h (37 C, 5% CO_2). Nonadherent cells were removed by washing in isotonic saline, and attached cells were fixed using 10% formalin. Plates were stained with 0.1% Amido black for 15 min, rinsed, and solubilized with 2 N NaOH to obtain an absorbance reading at 595 nm, which directly correlated with the number of cells stained in each well (46).

Statistical analyses

Data from slot blot hybridization analyses were subjected to least-squares ANOVA using the general linear models procedures of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Slot blot hybridization data were corrected for differences in sample loading by using the *18S rRNA* mRNA data as a covariate. In study 3, preplanned orthogonal contrasts were used to determine effects of treatment (P4+CX *vs.* P4+RU+CX, P4+CX *vs.* P4+IFNT, and P4+RU+CX *vs.* P4+RU+IFNT). In all analyses, error terms used in tests of significance were identified according to the expectation of the mean squares for error. Significance ($P < 0.05$) was determined by probability differences of least squares means (LSM). Data are presented as LSM with overall SE.

Results

IGFBP1 and IGFBP3 in the ovine uterus

Steady-state levels of *IGFBP1* mRNA in ovine uterine endometria are presented in Fig. 1A. In cyclic ewes, endometrial *IGFBP1* mRNA levels were low to undetectable on d 3, 6, and 10 but increased 29-fold on d 12 and then declined on d 16 (cubic effect of day, $P < 0.01$). Between d 12 and 16, endometrial *IGFBP1* mRNA levels were greater in pregnant than cyclic ewes (day × status, $P < 0.01$). Indeed, *IGFBP1* mRNA levels were about 4-fold higher on d 12 and about 50-fold higher on d 16 of pregnancy than for the same days of the estrous cycle. In preg-

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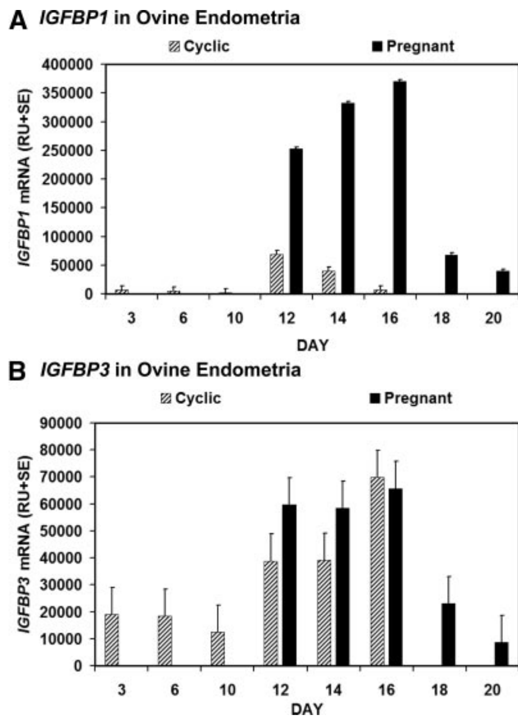

FIG. 1. Steady-state levels of *IGFBP1* (A) and *IGFBP3* (B) mRNA in endometria of cyclic and pregnant ewes. Endometrial mRNA abundance was determined by slot blot hybridization analyses (see Materials and Methods). Data are presented as LSM with SE.

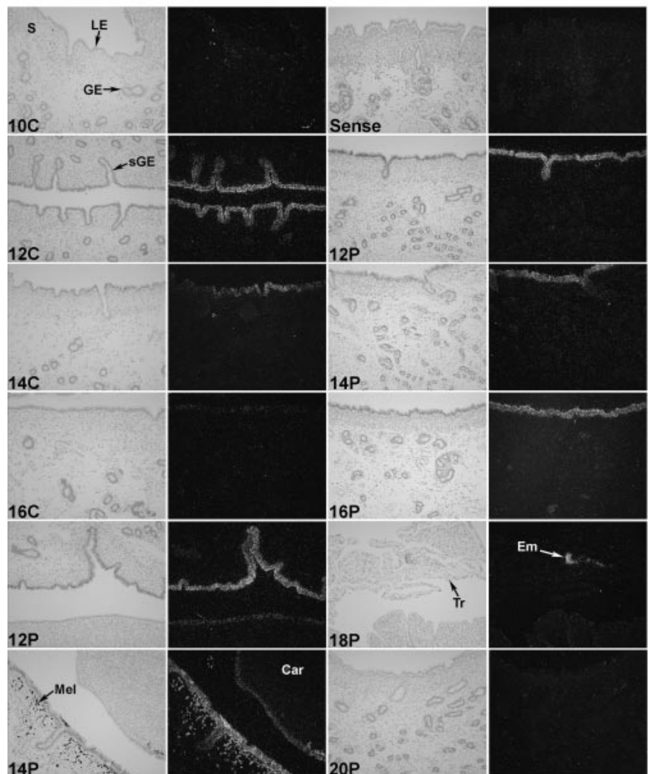

FIG. 2. *In situ* hybridization analysis of *IGFBP1* mRNA in uteri of cyclic and pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radiolabeled antisense or sense ovine *IGFBP1* cRNAs. Note that *IGFBP1* mRNA is most abundant in the endometrial LE and sGE. Car, Caruncle; Em, embryo; Mel, melanocyte; S, stroma. All photomicrographs are displayed at the same width of field (560 μm).

nant ewes, endometrial *IGFBP1* mRNA levels increased from d 12 to 16 and declined substantially to d 18 and 20 (quadratic effect of day, $P < 0.01$). *In situ* hybridization analysis found that *IGFBP1* mRNA was present specifically in endometrial LE and sGE of both cyclic and pregnant ewes and in the d 18 embryo (Fig. 2). Interestingly, *IGFBP1* mRNA appeared to be more abundant in the intercaruncular endometrial LE and sGE compared with LE covering the caruncles.

Steady-state levels of *IGFBP3* mRNA in ovine endometria are illustrated in Fig. 1B. In cyclic ewes, endometrial *IGFBP3* mRNA levels were lowest on d 3–10, increased about 2.5-fold on d 12 and increased further between d 14 and 16 (cubic effect of day, $P < 0.01$). On d 12–16, endometrial *IGFBP1* mRNA levels were not different between cyclic and pregnant ewes (day $\times$ status, $P <$ 0.10). In pregnant ewes, endometrial *IGFBP3* mRNA levels were highest between d 12 and 16 and then declined substantially on d 20 (quadratic effect of day, $P < 0.05$). *In situ* hybridization analysis found that *IGFBP3* mRNA was most abundant in the endometrial LE and sGE of both cyclic and pregnant ewes and was also present in the endothelium of blood vessels (Fig. 3).

IGFBP1 and IGFBP3 in the bovine uterus

Steady-state levels of *IGFBP1* mRNA in bovine endometria are illustrated in Fig. 4A. In nonpregnant heifers, endometrial *IGFBP1* mRNA levels were very low or undetectable on d 10 but increased about 228-fold on d 13 and then declined about 5-fold on d 19 (cubic effect of day, $P < 0.01$). Between d 13 and 19, endometrial *IGFBP1* mRNA levels were affected by pregnancy (day $\times$ status, $P < 0.01$) in that they were not different on d 13 but were 3.3-fold higher on d 16 in pregnant than nonpregnant heifers. In pregnant heifers, *IGFBP1* mRNA levels increased between d 13 and 16, were maximal on d 16, and then declined substantially on d 19 (cubic effect of day, $P < 0.01$). *In situ* hybridization analysis found that *IGFBP1* mRNA was present specifically in the endometrial LE and sGE of both nonpregnant and pregnant heifers on d 13 and 16 and also in the middle GE on d 19 in pregnant heifers (Fig. 4B).

Steady-state levels of *IGFBP3* mRNA in bovine endometria are illustrated in Fig. 4C. In nonpregnant heifers, endometrial *IGFBP3* mRNA levels did not change ($P >$ 0.10) between d 10 and 19. Between d 13 and 19, endometrial *IGFBP3* mRNA levels were not affected by pregnancy (day $\times$ status, $P < 0.10$). Moreover, *IGFBP3* mRNA levels were not different ($P > 0.10$) between d 13

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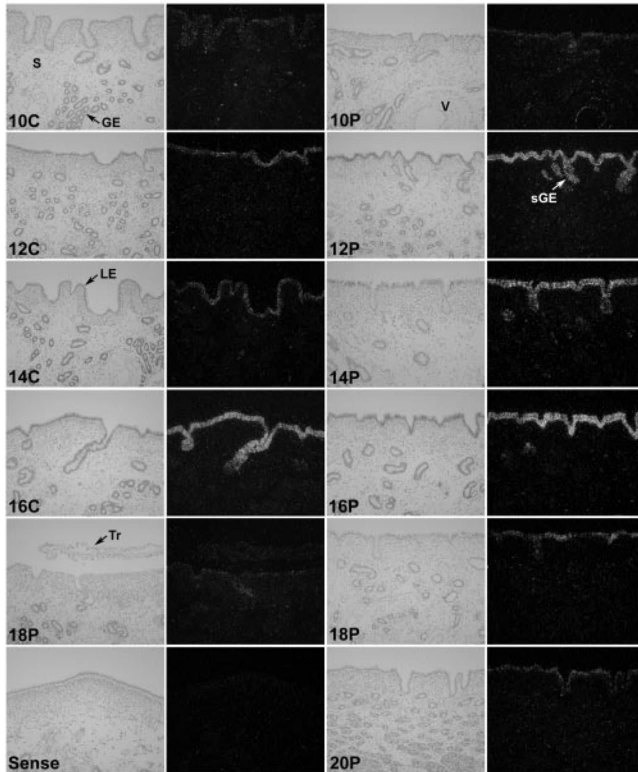

FIG. 3. *In situ* hybridization analysis of *IGFBP3* mRNA in uteri of cyclic and pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radiolabeled antisense or sense ovine *IGFBP3* cRNAs. Note that *IGFBP3* mRNA is most abundant in endometrial LE and sGE. S, Stroma; Tr, trophectoderm; V, blood vessel. All photomicrographs are displayed at the same width of field (560 μm).

and 19 in pregnant heifers. *In situ* hybridization analysis was not conducted due to the lack of effects of day and pregnancy status on endometrial *IGFBP3* expression in heifers.

IGFBP1 is induced by P4 and stimulated by IFNT

The temporal changes in endometrial *IGFBP1* and *IGFBP3* mRNAs in uterine LE/sGE of cyclic ewes suggested that the *IGFBP1* and *IGFBP3* genes are regulated by ovarian P4, whereas the pregnancy-specific increase in endometrial *IGFBP1* mRNA in ovine and bovine uteri suggested regulation by a factor from the conceptus such as IFNT. Therefore, the effects of P4, RU, and IFNT on endometrial *IGFBP1* and *IGFBP3* expression were studied in the ovine uterus (study 3). As illustrated in Fig. 5, treatment of ovariectomized ewes with P4 for 12 d increased endometrial *IGFBP1* mRNA abundance by about 38-fold ($P < 0.01$, P4+CX *vs.* P4+RU+CX). Intrauterine infusions of IFNT increased endometrial *IGFBP1* mRNA levels by about 2-fold in P4-treated ewes ($P < 0.01$, P4+CX *vs.* P4+IFNT) but had no effect in ewes receiving RU ($P > 0.10$, P4+RU+CX *vs.* P4+RU+IFNT). *In situ* hybridization analysis revealed that effects of P4 to induce and IFNT to stimulate *IGFBP1* expression in the endometrium were confined to the endometrial LE/sGE and GE in the stratum compactum stroma (Fig. 5B).

As illustrated in Fig. 5C, treatment with RU increased endometrial *IGFBP3* mRNA abundance by about 3-fold ($P < 0.01$, P4+CX *vs.* P4+RU+CX). Intrauterine infusion of IFNT did not affect endometrial *IGFBP3* mRNA levels in P4-treated ewes ($P < 0.01$, P4+CX *vs.* P4+IFNT), whereas IFNT decreased *IGFBP3* mRNA abundance by about 2-fold in ewes receiving P4 and RU ($P < 0.05$, P4+RU+CX *vs.* P4+RU+IFNT). *In situ* hybridization analyses were not conducted given that IFNT is not produced in the absence of P4 action and RU is abortifacient in sheep as in other mammals.

IGFBP1 stimulates trophectoderm cell migration and mediates their attachment but does not affect proliferation

The effect of IGFBP1 on trophectoderm migration was determined using oTr1 cells (Fig. 6A). The oTr1 cells are mostly mononuclear and express *IFNT* (43, 47). IGFBP1 stimulated migration of oTr1 cells in serum- and insulin-free medium compared with BSA that was used as a control. Relative to the BSA control, as little as 1 ng/ml IGFBP1 stimulated ($P < 0.01$) oTr1 cell migration, but effects were maximal at 10 and 100 ng/ml and then decreased at 1000 ng/ml (quadratic effect of dose, $P < 0.01$). IGFBP1 and FN stimulated attachment of oTr1 cells in a dose-dependent manner (Fig. 6B) compared with the BSA control wells. An increase in oTr1 cell attachment occurred in wells with as little as 80 ng/ml IGFBP1 and the effect of IGFBP1 was dose dependent (cubic effect of dose, $P < 0.01$). The attachment elicited by IGFBP1 was consistently greater (dose $\times$ treatment, $P < 0.01$) than that elicited by similar concentrations of FN. In contrast, IGFBP1 (0.01–10 μg/ml) had no effect ($P > 0.10$) on proliferation of oTr1 cells in insulin-free medium containing 0, 1, or 10% serum (data not shown).

Discussion

The present studies support previously published results for IGFBP1 in the LE and sGE of sheep (16) and cattle (17) and suggest that IGFBP1 is a common endometrial marker of conceptus elongation and implantation in sheep and cattle that is regulated by ovarian P4 and a conceptus-derived factor. In contrast, expression of *IGFBP3* is different between sheep and cattle. *IGFBP3* is expressed by ovine endometrial LE/sGE (Fig. 3) (17) but predominantly by subepithelial stromal cells in bovine uteri (15). These data agree with emerging

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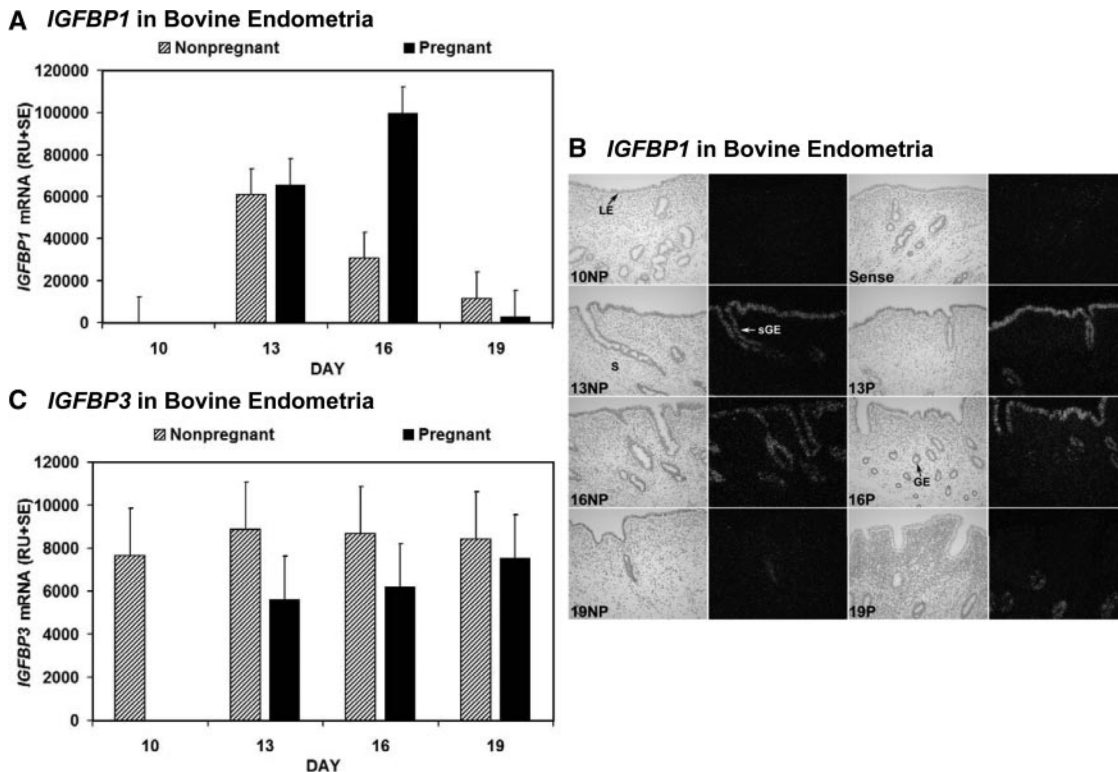

FIG. 4. *IGFBP1* and *IGFBP3* in the bovine uterus. A and C, Steady-state levels of *IGFBP1* and *IGFBP3* mRNA in the endometria of nonpregnant and pregnant heifers. Endometrial mRNA abundance was determined by slot blot hybridization analysis (see *Materials and Methods*). Data are presented as LSM with SE. B, *In situ* hybridization analysis of *IGFBP1* mRNA in uteri of nonpregnant and pregnant heifers. Cross-sections of the uterine wall from nonpregnant (NP) and pregnant (P) heifers were hybridized with radiolabeled antisense or sense ovine *IGFBP1* cRNAs. Note that *IGFBP1* mRNA is most abundant in the endometrial LE. S, Stroma. All photomicrographs are displayed at the same width of field (560 μm).

evidence to indicate that, although both sheep and cattle are ruminants with considerable similarities in conceptus growth, development and implantation during early pregnancy, substantial differences exist in endometrial gene expression between these species. For instance, *LGALS15*, a member of the galectin superfamily, is one of the most abundant mRNAs present in ovine endometria during early pregnancy (48, 49). Although the *LGALS15* gene is present in the bovine genome, *LGALS15* is expressed only in the endometria of sheep and goats (50). These results highlight the importance of caution in translating research findings in sheep directly into cattle when seeking to identify common mediators of endometrial function and conceptus development in ruminants (10).

In sheep, the induction in *IGFBP1* and increase in *IGFBP3* expression in endometrial LE/sGE between d 10 and 12 of the cycle, and pregnancy is temporally associated with loss of *PGR* expression in the same epithelia (51). Similarly, the induction of *IGFBP1* expression in endometrial LE/sGE between d 10 and 13 in nonpregnant and pregnant heifers is also associated with *PGR* loss in those epithelia (52, 53). Likewise, the decrease in *IGFBP1* mRNA in LE/sGE between d 14 and 16 of the estrous cycle in sheep and d 16 and 19 in cattle is coincident with the subsequent reappearance of *PGR* expression in those epithelia (51, 54, 55). Although the cellular and molecular mechanism(s) are not clear, continuous exposure of the sheep uterus to P4 for 8–10 d is required for loss of *PGR* mRNA and PGR protein in endometrial LE and sGE but not stroma or myometrium (56, 57). Indeed, *PGR* expression remains undetectable in the endometrial epithelia throughout pregnancy (8). In the present study 3, *IGFBP1* mRNA was induced by P4 in endometrial LE/sGE, but this effect was blocked by administration of the antiprogestin RU. Treatment of ewes with antiprogestins results in reappearance of PGR in endometrial epithelia (12, 56) because they prevent P4 actions to down-regulate *PGR* expression and production of stromal-derived growth factors (13). In addition to being an antiprogestin, RU is a high-affinity antagonist of the glucocorticoid receptor (58), but little is known of glucocorticoid receptor expression and glucocorticoid effects within the ovine uterus during either the estrous cycle or pregnancy. *IGFBP3* expression in the endometrial LE/sGE did not decline between d 14 and 16 of the estrous cycle in sheep and increased in uteri of ewes treated with RU in study 3, suggesting that *IGFBP3* expression is regulated by a different mechanism than *IGFBP1*.

In addition to induction by ovarian P4 during the cycle and pregnancy, *IGFBP1* expression in the endo-

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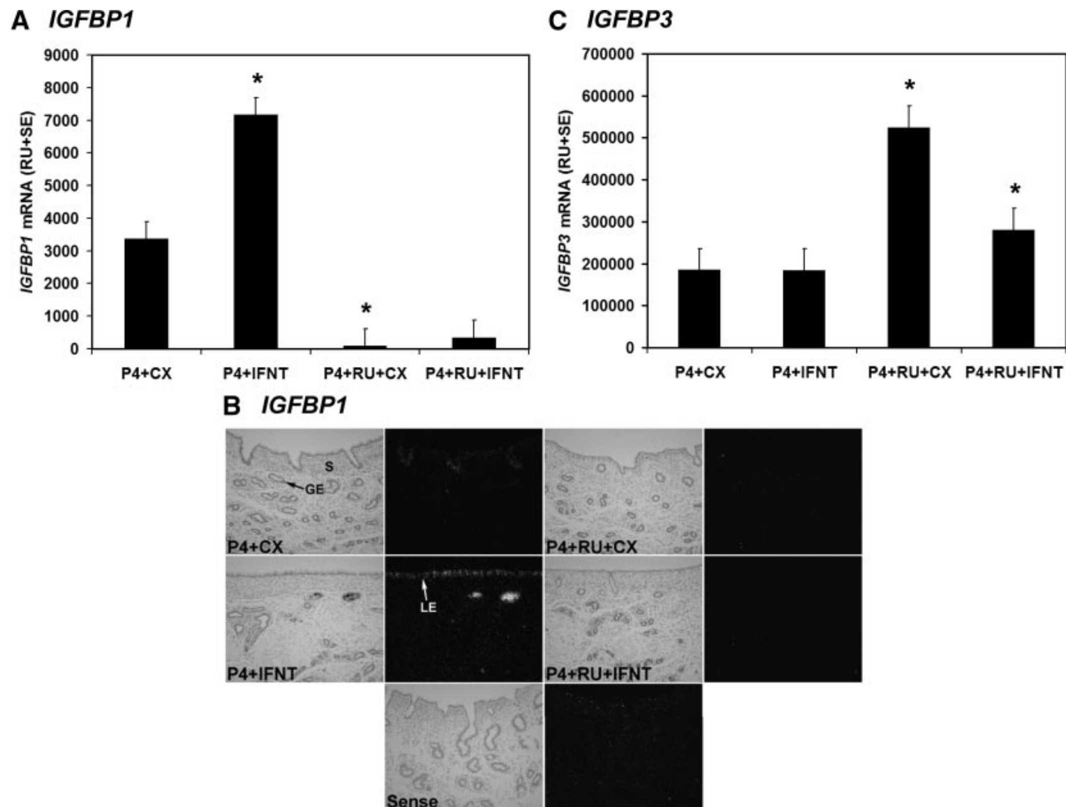

FIG. 5. Effects of progesterone and IFNT on *IGFBP1* and *IGFBP3* mRNA in the ovine uterus. A, Steady-state levels of *IGFBP1* mRNA in endometria were determined by slot blot hybridization analysis. Treatment of ewes with P4 increased (*, $P < 0.01$) endometrial *IGFBP1* mRNA abundance compared with ewes receiving P4 and the antiprogestin RU. Intrauterine IFNT increased *IGFBP1* mRNA ($P < 0.01$) in P4-treated ewes but not P4+RU-treated ewes. B, Steady-state levels of *IGFBP3* mRNA in endometria were determined by slot blot hybridization analysis. Treatment of ewes with P4 and RU increased (*, $P < 0.01$) endometrial *IGFBP3* mRNA abundance compared with ewes receiving P4 alone. In ewes receiving P4+RU, intrauterine IFNT decreased *IGFBP3* mRNA ($P < 0.01$) but not in P4-treated ewes. C, *In situ* hybridization analyses of *IGFBP1* mRNA in the ovine uterus. Cross-sections of the uterine wall from treated-ewes were hybridized with radiolabeled antisense or sense ovine *IGFBP1* cRNA probes. Note the effects of P4 and IFNT on *IGFBP1* expression were manifest on the endometrial LE and upper GE. S, Stroma. All photomicrographs are displayed at the same width of field (560 μm).

metrial LE/sGE was also increased by the presence of a conceptus in both sheep and cattle. During early pregnancy, the ruminant conceptus synthesizes and secretes a number of different factors, but IFNT is the most abundant protein produced by the elongating ruminant conceptus (59). Indeed, infusion of recombinant ovine IFNT into uteri of P4-treated ewes increased *IGFBP1* mRNA abundance by almost 2-fold. However, this stimulation by IFNT was rather modest and did not mimic the approximately 5- and 29-fold increases in endometrial *IGFBP1* expression observed on d 12 and 16, respectively, in pregnant compared with cyclic ewes. In bovine uteri, endometrial *IGFBP1* mRNA abundance was more than 3-fold higher for d 16 pregnant compared with nonpregnant heifers. Indeed, the spherical d 12 conceptus of sheep produces little IFNT compared with large amounts produced by the elongating conceptus that is maximal on d 15–16 (60). These results strongly suggest that another factor produced by the conceptus regulates endometrial *IGFBP1* expression in sheep and perhaps cattle, with prostaglandins being strong candidate factors.

Despite markedly different implantation schemes among primates, rodents, and ruminants, *IGFBP1* is up-regulated in endometria of each of these species during early pregnancy and implicated as a regulator of blastocyst implantation and placental growth and development (61, 62). In humans, *IGFBP1* is a highly up-regulated gene in the human secretory endometrium during the period of receptivity to implantation (63) and localized to endometrial LE, a subpopulation of stromal cells, and the decidua (64, 65). Similarly, IGFBP1 is the primary secretory product of baboon decidua and stimulated by chorionic gonadotropin, the pregnancy recognition signal produced by primate conceptuses (66). In the present studies, IGFBP1 stimulated migration and mediated attachment of oTr1 cells, which are required for elongation and implantation of ruminant conceptuses (1, 3); however, IGFBP1 did not stimulate oTr1 cell proliferation, suggesting that the purified IGFBP1 used in the present studies was not contaminated with IGF1 or another mitogen.

In addition to IGF ligand binding, IGFBP1 contains a conserved RGD sequence that can act as a ligand for the

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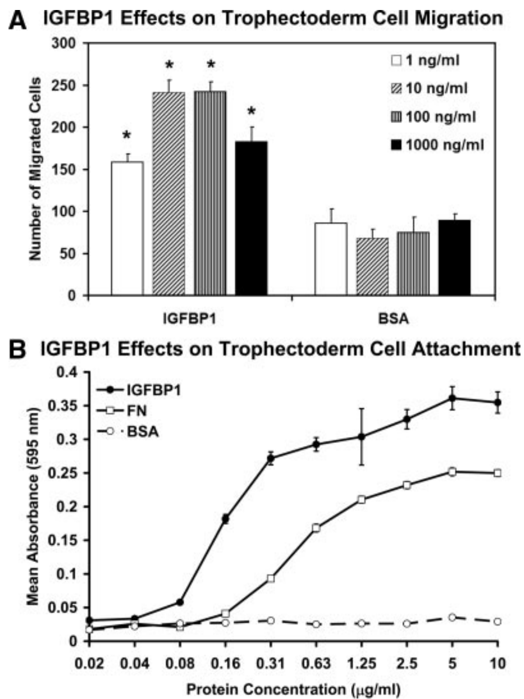

FIG. 6. Effects of IGFBP1 on migration and attachment of oTr1 cells. A, Cell migration. oTr1 cells were cultured in a Transwell plate in serum- and insulin-free medium and treated with IGFBP1 purified from human amniotic fluid or with BSA as a control. The number of cells that migrated was determined after 12 h of treatment. IGFBP1 increased (*, $P < 0.01$) oTr1 cell migration relative to the BSA control. The graph is a compilation of three independent experiments with three replicates per treatment in each experiment. Data are presented as LSM with SE. B, Cell attachment. Wells of suspension culture plates were precoated overnight with increasing amounts of either human IGFBP1, BSA (negative control) or human FN (positive control). Equal numbers of oTr1 cells were added to each well and the number of attached cells determined after 1 h. A dose-dependent increase ($P < 0.01$) in cell attachment was induced by both IGFBP1 and FN but not BSA. The graph is a compilation of three independent experiments with three replicates per treatment in each experiment. Data are presented as LSM with SE.

integrin heterodimer $\alpha5\beta1$ (27, 32). Blocking antibodies against the $\alpha5\beta1$ integrin subunits inhibit trophoblast cell migration (33), and IGFBP1 stimulated migration of trophoblast cells is attenuated by mutation of the RGD integrin binding sequence to Trp-Gly-Asp or pretreatment with an inhibitory peptide (32). In sheep, the $\alpha5$- and $\beta1$-integrin subunits are constitutively expressed on the surface of uterine LE/sGE and conceptus trophectoderm (28), which supports the hypothesis that IGFBP1 from uterine LE/sGE can stimulate migration and adhesion of trophectoderm cells to the uterine LE during the attachment phase of implantation. Indeed, the transient nature of *IGFBP1* expression in uterine LE/sGE is correlated with elongation of conceptuses of both sheep and cattle (1–3, 12). In the present studies, IGFBP1 mediated attachment of oTr1 cells, which is an essential element of blastocyst implantation and trophoblast differentiation in many species (30, 31). Indeed, integrins are proposed to be the dominant glycoproteins that regulate trophectoderm adhesion to endometrial LE during implantation in mammals (31, 67). During the periimplantation period of pregnancy in sheep, integrin subunits-αv, -$\alpha4$, -$\alpha5$, -$\beta1$, -$\beta3$, and -$\beta5$ are constitutively expressed on apical surfaces of the conceptus trophectoderm and endometrial LE (28). Thus, conceptus implantation in sheep does not appear to involve temporal or spatial changes in patterns of integrin expression (28) but may depend primarily on changes in secreted integrin ligands, such as IGFBP1, lectin, galactoside-binding, soluble, 15, and secreted phosphoprotein 1 (or osteopontin) (1, 42, 68, 69). Adhesive LE ligands, normally masked by mucins, become exposed during the receptive period, and various adhesion molecules then function sequentially, or in parallel, to stabilize adhesion of the trophectoderm to the endometrial LE (28, 30, 69).

Although endometrial *IGFBP3* expression differed between sheep and cattle, its expression did increase in endometrial LE/sGE of both cyclic and pregnant ewes after d 10 and was consistently detected in the bovine endometria during early pregnancy. In sheep, the increase and decrease in endometrial *IGFBP3* expression was correlated with the period of rapid elongation of the conceptus. Although distinct differences exist between the cell types expressing IGFBP3 in bovine and ovine uteri, IGFBP3 is the predominant IGFBP in the uterine lumen during early pregnancy in both sheep and cattle (70, 71). Indeed, substances present in the uterus are derived from synthesis and secretions of the endometrium as well as selective transport of serum components (72), and IGFBP3 is the most abundant circulating IGFBP in serum. In serum, IGFBP3 regulates IGF bioavailability by sequestering IGFs in circulating ternary complexes, and it also competitively inhibits IGF action at the cellular level (73). IGFBP3 also has IGF-independent actions that appear to be mediated by a cell surface receptor and/or direct nuclear action (73). Thus, IGFBP3 may have IGF-dependent and -independent activities that modulate conceptus growth and development during early pregnancy in ruminants.

IGF-I and IGF-II possess both mitogenic and differentiative properties and are components of uterine luminal histotroph in sheep and cattle (71, 74, 75). Both ovine and bovine preimplantation embryos (23) as well as d 15 elongated bovine conceptuses express *IGF1R* (76). Indeed, IGF-I stimulates proliferation and inhibits apoptosis in cultured bovine embryos (77), and IGF-II stimulates ovine trophectoderm cell migration (75). Thus, access of the blastocyst to IGF-I and IGF-II in the uterine lumen could be mitigated by the up-regulation of *IGFBP3* and perhaps *IGFBP1* in uterine LE/sGE between d 10 and 12 of pregnancy. IGF-dependent and -independent activities of IGFBP3 are regulated by de-

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activation via several proteases (73). Indeed, treatment of ovariectomized ewes with P4 for 10 d resulted in the proteolysis of IGFBP3 in the uterine lumen, which would theoretically increase bioactive IGF available to the blastocyst (70). Of particular interest, cathepsin L is a P4- and IFNT-stimulated protease expressed by ovine endometrial LE and GE during early pregnancy that may act as an IGFBP protease (78). Furthermore, matrix metalloproteinase-2 and -9 are secretory products of ovine endometria that increase from d 12 to 20 of pregnancy and may regulate IGFBP cleavage and thus IGF bioavailability (79, 80). The IGF-dependent and -independent effects of IGFBP3 on trophectoderm functions need to be investigated to understand the biological role(s) of this IGFBP in the uterine lumen.

In summary, the spatiotemporal alterations in *IGFBP1* mRNA in ovine and bovine uterine LE/sGE during pregnancy, combined with the functional aspects of IGFBP1 discovered in the present studies and in published results, substantially support the hypothesis that IGFBP1 functions as a heterotypic cell adhesion molecule bridging integrins in the endometrial LE and conceptus trophectoderm, which stimulates trophectoderm migration and adhesion that are required for conceptus growth and elongation in ruminants before implantation *in utero*. Future experiments will be directed toward discerning the biological role of conceptus-derived prostaglandins on expression of *IGFBP1* in addition to perhaps other genes in the endometrium that are important for conceptus elongation and development as well as endometrial receptivity to implantation of the conceptus.

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