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Bovine Oviductal and Embryonic Insulin-Like Growth Factor Binding Proteins: Possible Regulators of "Embryotrophic" Insulin-Like Growth Factor Circuits[1]

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ABSTRACT

Bovine oviductal monolayer and vesicle primary cultures express insulin-like growth factor (IGF)-I and -II mRNAs and polypeptides. Early bovine embryos also express IGF-I, IGF-II, IGF-I receptor, IGF-II receptor, and insulin receptor mRNAs. This study reports the expression of IGF binding protein (IGFBP) mRNAs and polypeptides in bovine oviduct primary cultures and IGFBP mRNAs in preattachment embryos. Release of immunoreactive IGF-I and IGF-II by oviduct cultures and bovine blastocysts was also determined. IGFBP-2, -3, -4, and -5 transcripts were observed in oviduct primary cultures throughout an 8-day interval. IGFBP-1 and -6 mRNAs were consistently not detected in the oviduct. Messenger RNAs encoding IGFBPs -2, -3, and -4 were detected throughout bovine preattachment development, while transcripts encoding IGFBP-5 were detected only in blastocysts. IGFBP-1 and -6 transcripts were not detected in early embryos. Ligand blot analysis with ^{125}I-labeled IGF-II revealed the presence of four prominent polypeptide bands of approximate molecular masses 24, 31, and 36 kDa, and a broad band extending from 46 to 53 kDa, in conditioned media samples prepared from oviduct primary cultures. Western immunoblot analysis confirmed the identity of the 24-kDa, 31-kDa, and 36-kDa species as IGFBP-4, -5, and -2, respectively. Levels of the release of IGF-II from oviductal vesicle cultures were significantly greater than levels observed for monolayer cultures ($p < 0.005$). No significant difference in the levels of IGF-I release between monolayer and vesicle cultures was observed. Pools of 10 blastocysts released on average 36.2 ± 3.9 pg of IGF-II per embryo, while the release of embryonic IGF-I was below the levels of detection for our assay. The results suggest that maternally derived IGF may be regulated by IGFBPs to support bovine preattachment development.

INTRODUCTION

The development of specific culture regimes capable of supporting in vitro oocyte maturation (IVM), in vitro fertilization (IVF), and subsequent development of mammalian zygotes to the blastocyst stage has continued unabated [1–12]. Progress has occurred by various routes, including employment of complex media such as Tissue Culture Medium 199 supplemented with serum in combination with embryo coculture [1–7]; employment of simple defined salt solutions (e.g., synthetic oviduct fluid medium) supplemented with a serum source [2, 11–14]; and most recently the construction of chemically defined protein-free media such as hamster embryo culture medium, CZB, or KSOM medium [9, 14]. Variations have included comparing development under varying O_2 levels and supplementing serum-free media with amino acids, synthetic serum substitutes [10–13], BSA, polyvinylalcohol [15], or specific growth factors [16, 17]. Embryo coculture using primary oviduct epithelial cell cultures remains an important method of supporting development of in vivo- and in vitro-matured and -fertilized (IVMF) bovine and ovine zygotes through to the blastocyst stage, particularly under atmospheric conditions of 5% CO_2 in air [1, 2]. Coculture may exert a positive influence on early development by the secretion of "embryotrophic" factors into the culture medium or by reduction of the negative effects of "toxic" components of the culture environment [4]. It seems likely that embryo coculture is effective by both of these routes.

Satokata et al. [18] observed that disruption of Hoxa-10 expression by homologous recombination is associated with early embryonic death just prior to implantation. Hoxa-10 gene products are expressed in several tissues including the distal oviduct and uterus [18]. This result demonstrates that oviductal gene products are necessary for mediating early developmental events. Studies in several animals including the cow, sheep, human, pig, mouse, rabbit, and hamster have reported oviductal-specific glycoproteins associated with the zona pellucida and embryo [19–25]. These glycoproteins may contribute to an exclusive microenvironment for the embryo by entering the perivitelline space. Bovine zygotes cultured on oviductal monolayers are best moved to fresh cultures every 72 h to sustain development [1], whereas microdrop cultures containing oviductal vesicles support development through the first week without transfer to fresh cultures [8, 26]. Our efforts have focused on defining possible embryotrophic and/or "detoxifying" roles of the oviduct by characterizing the expression of mRNAs encoding antioxidant enzymes [26] and growth factor transcripts [27, 28] in primary bovine oviductal cultures.

The precise role(s) of growth factors in supporting early ovine and bovine development remains unclear, but evidence illustrating that a significant number of bovine IVMF zygotes can progress beyond the 16-cell stage, reaching the blastocyst stage [17] in media supplemented with basic fibroblast growth factor (bFGF) and transforming growth factor (TGF)-β, suggests that growth factors could certainly perform roles expected of embryotrophic factors. These molecules are, therefore, good candidates for further experimentation directed at understanding the molecular nature of the beneficial coculture influence on early mammalian development. We have reported that bovine oviductal primary cultures express transcripts encoding bFGF, TGFα, TGFβ1, TGFβ2; platelet-derived growth factor A, and insulin-like growth factors I and II (IGF-I, IGF-II) by

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reverse transcriptase polymerase chain reaction (RT-PCR) [27]. Furthermore, bovine preattachment embryos express the same growth factor transcripts including mRNAs encoding IGF-I, IGF-II, and insulin receptors [27]. We have confined our recent analysis to the IGF family since this family is one of the best-characterized growth factor families in early development. The distribution of both mRNAs and polypeptides encoding IGF-I and IGF-II has been recently mapped out in bovine oviductal monolayer and vesicle cultures [3]. IGF transport and function are modulated by interactions with up to six insulin-like growth factor binding proteins (IGFBPs) [29–32]. The present study was undertaken to further investigate the regulatory interactions of these putative bovine maternal paracrine circuits by characterizing the expression of mRNAs and polypeptides encoding IGFBPs in bovine primary oviduct cultures and IGFBP mRNAs in early preattachment embryos. The levels of IGF-I and -II released by these oviduct cultures and bovine blastocysts were also examined.

MATERIALS AND METHODS

Bovine Embryo Culture

Bovine preattachment embryos were produced by standard in vitro oocyte maturation, fertilization, and embryo culture methods [1, 2, 7, 8] applied to cumulus-oocyte-complexes (COCs) collected from slaughterhouse ovaries. COCs were harvested within 4 h of removal from the animal by a razor blade slashing technique. The contents were pooled and the COCs collected and washed four times with oocyte collection medium (Hepes-buffered Tissue Culture Medium 199 [TCM-199; Gibco, BRL, Burlington, ON, Canada] + 2% steer serum [SS; Cansera, Toronto, ON, Canada]). COCs were placed into maturation medium consisting of TCM-199 (Gibco, BRL) + 10% SS (v:v) supplemented with 35 μg/ml sodium pyruvate (Sigma Chemical Company, St. Louis, MO), 5 μg/ml FSH (Follitropin; Vetrapharm, London, ON, Canada), 5 μg/ml LH (Vetrapharm), and 1 μg/ml estradiol-17β (Sigma) for 22 h at 38.6°C in a humidified atmosphere containing 5% CO_2 in air. Matured oocytes were fertilized in vitro with frozen-thawed bovine semen (Semex Canada Inc., Guelph, ON, Canada) prepared by standard "swim-up" procedures [33]. COCs were removed from the maturation medium and washed four times in Hepes-buffered modified Tyrode's solution [33] just prior to their placement into preequilibrated fertilization drops (50 COCs/300-μl drop) consisting of bicarbonate-buffered modified Tyrode's solution under light paraffin oil (BDH Inc., Toronto, ON, Canada). The sperm/COC droplets (2.25×10^5 motile spermatozoa per drop) were incubated for 18 h at 38.6°C in a humidified atmosphere of 5% CO_2 in air before removal of the remaining cumulus cell investment. Fertilized oocytes were placed into 50-μl culture drops consisting of TCM-199 + 10% SS under oil containing up to 40 oviduct epithelial cell vesicles [2]. To sustain development through to the blastocyst stage, 50 μl of TCM-199 + 10% SS was added to each culture drop after 48 h of culture. No oocyte selection strategy was employed in this study. These conditions routinely support an overall cleavage rate of 70% of inseminated oocytes with up to 25% of the inseminated oocytes (35% of cleaved zygotes) progressing to the blastocyst stage. Pools of 50–100 bovine embryos including 1-cell zygotes, 2- to 5-cell embryos, 6- to 8-cell embryos, morulae, and blastocysts were collected by removing the embryos from culture at the appropriate developmental times.

Primary Oviductal Cultures

Bovine oviductal cultures were established as outlined by Xia et al. [3], Xu et al. [8], and Harvey et al. [26]. For establishment of monolayer cultures, the epithelial cells were isolated by filling the oviduct lumen with 0.05% trypsin in Ca^{2+}-, Mg^{2+}-free Hanks' Balanced Salt Solution (HBSS; Gibco, BRL) and incubating at 38.6°C for 20 min. The oviduct contents were squeezed into a 35-mm petri dish. The cells were dispersed by forcing through an 18-gauge needle attached to a 5-ml syringe three times before transferral of the samples into 15-ml conical tubes containing 10 ml of HBSS for washing by centrifugation. The cells were resuspended in fresh HBSS and washed three more times before final resuspension in the appropriate volume of TCM-199 (Gibco, BRL) + 10% SS (Cansera). The cultures were established by addition of 1×10^6 cells per well of a 24-well plate containing 1 ml of TCM-199 + 10% SS medium per well. By 48 h, approximately 50% of the surface of each well was covered by attached cells. By 72 h the monolayers were confluent. The cultures were maintained for up to 8 days by removal of the old medium and addition of 1 ml of fresh culture medium every 48 h.

For establishment of epithelial vesicle cultures, cell sheets were collected from trimmed oviducts and washed four times with HBSS. Up to 70 μl of cell suspension was placed into individual 35-mm petri dishes containing 3 ml of TCM-199 supplemented with 10% SS. The cell sheets were cultured for 24 h under an atmosphere of 5% CO_2 in air at 38.6°C to allow for the formation of vesicles. From this point on, the vesicle cultures were maintained for up to 8 days by moving vesicles to new culture dishes containing fresh medium every 48 h.

Collection of Conditioned Media

Oviductal monolayers. Conditioned medium was collected from monolayer cultures on Days 2, 5, and 8 by first removing the serum-supplemented medium and then washing the cells three times (1 ml/well) with HBSS followed by two washes in serum-free TCM-199. The final wash medium was collected as a control to ensure that possible transfer of serum proteins into the conditioned medium was avoided. Monolayer-conditioned medium was prepared by adding 200 μl of TCM-199 to each culture well and incubating the cultures at 38.6°C under an atmosphere of 5% CO_2 in air for 24 h. Conditioned medium from 8 wells (1.6 ml) was pooled, filtered through a 0.2-μm filter, and stored for up to 3 wk at −20°C.

Oviductal vesicles. Oviductal vesicle-conditioned medium was collected from Days 2, 5, and 8 cultures by first pooling the oviductal vesicles from 10-ml cultures into 15-ml plastic conical tubes and allowing the vesicles to settle forming a loose 250-μl pellet. The pellet was washed four times with serum-free TCM-199. The final wash was collected and run as control. Approximately half of the vesicles in each sample (i.e., 125 μl of vesicles) were added to culture dishes containing 1 ml of serum-free TCM-199. The vesicles were incubated at 38.6°C under an atmosphere of 5% CO_2 in air for 24 h. Conditioned medium (1 ml) was collected, filtered, and stored for up to 3 wk at −20°C.

Blastocyst-conditioned medium. Blastocyst-stage embryos were removed from culture on Day 7 and washed three times in serum-free medium. Groups of 10 blastocysts were placed in 200 μl of serum-free TCM-199 for a 24-h incubation period. Conditioned medium was collected, lyophilized, and resuspended in 50-μl volumes for RIA.

TABLE I. IGFBP PCR primer sequences.

IGFBP	Primer sequences	Amplicon size (bp)	Identity
BP-1	5′ primer = 5′CGAGCCCTGCCGAATAGAAC 3′ primer = 5′CATCTGGCAGTTGGGGTC	239*	—
BP-2	5′ primer = 5′ACTGTCACAAGCATGGCCTG 3′ primer = 5′CCTCCTGCTGCTCATTGTAGA	186	99.2% (bovine)
BP-3	5′ primer = 5′ACTTCTCCTCTGAGTCCAAGC 3′ primer = 5′CGTACTTATCCACACACCAGC	210	100% (bovine)
BP-4	5′ primer = 5′CTGTGCCCCAGGGTTCCTGC 3′ primer = 5′TCACCCCCGTCTTCCGGTCC	222	100% (bovine)
BP-5	5′ primer = 5′GCTCAAGCCAGCCCACGCAT 3′ primer = 5′GTCGAAGCCGTGGCACTGAA	215	96.1% (human)
BP-6	5′ primer = 5′GACGAGGCGCCTTTGCGGGC 3′ primer = 5′GGAGGAGCGGCACTGCCGCT	345*	—

* Size of predicted human amplicon.

RT-PCR

RNA isolation. Total RNA was extracted as described by Temeles et al. [34]. Bovine zygotes were pooled into the following groups: 1) 1-cell (zygotes), 2) 6- to 8-cell stage, 3) Day 6 morulae, and 4) Day 8 blastocysts. Pools of 50–100 bovine embryos were solubilized at room temperature in 100 μl of 4 M guanidine thiocyanate (Pharmacia, Quebec, PC, Canada), 0.1 M Tris-HCl (pH 7.4), 1 M 2-β mercaptoethanol (Sigma) solution in the presence of 20 μg of *Escherichia coli* rRNA (Gibco, BRL). After vigorous vortex mixing, the samples either were frozen and stored at −70°C or were fully processed by precipitating the RNA by addition of 8 μl of 1 M acetic acid, 5 μl of 2 M potassium acetate, and 250 μl of 100% ethanol. The samples were precipitated overnight at −20°C. Samples then were pelleted by centrifugation at 10 000 × *g* for 20 min at room temperature. The pellets were washed twice with cold 70% ethanol and were air dried before resuspension in 20 μl of resuspension buffer (40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$). Genomic DNA was degraded by incubating the samples with 1 unit of RQI DNase (Promega, Madison, WI) for 30 min at 37°C. The samples were reextracted with phenol and reprecipitated by addition of 5 μl of 3 M potassium acetate, pH 5.2, and three volumes of cold 100% ethanol for overnight at −20°C. The total RNA was collected by centrifugation, the pellets were washed with cold 70% ethanol, and after air drying the samples were dissolved in 10 μl of autoclaved MilliQ (Millipore Corp., Bedford, MA) water. Total RNA was also extracted from bovine oviductal cell cultures by lysis in guanidinium isothiocyanate (Pharmacia) followed by ultracentrifugation through cesium trifluoroacetate [35]. Aliquots of 1 μg of oviduct primary culture total RNA were used for reverse transcription.

Reverse transcription. RNA was reverse transcribed by oligo(dT) priming and reverse transcriptase (Gibco, BRL) [26–28]. The RNA samples were incubated with 1 μg of $oligo(dT)_{12-18}$ primer (Gibco, BRL) for 10 min at 70°C. After cooling on ice, RNA was incubated in 1st Strand Buffer (Gibco, BRL) containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTPs, and 200 U of Superscript Reverse Transcriptase (Gibco, BRL). Reverse transcriptions were accomplished by incubation at 42°C for 1.5 h. The reaction was terminated by heating at 94°C for 4 min and flash cooling on ice. The cDNA was further diluted with sterile distilled water to a concentration of 2 embryo equivalents/μl or the equivalent of 40 ng of oviduct RNA/μl.

Amplification of binding protein cDNAs. Polymerase chain reaction (PCR) was performed as described previously [26–28]. Aliquots of embryo and oviduct cDNA (5 μl) were amplified with 1 U of *Taq* DNA polymerase (Gibco, BRL) in a final volume of 50 μl containing 10-strength *Taq* reaction buffer (200 mM Tris-HCl, pH 8.4, and 500 mM KCl) plus 1.5–2 mM $MgCl_2$, 0.2–0.24 mM dNTPs, and 2 μM of each sequence-specific primer. The mixture was overlaid with mineral oil and then amplified by PCR for up to 40 cycles in a DNA thermal cycler (Perkin Elmer Cetus 480; or Thermolyne, Amplitron; VWR Scientific, Toronto, ON, Canada) with each cycle consisting of denaturation at 94°C for 1 min, reannealing of primers to target sequences at 56–58°C for 30 sec, and primer extension at 72°C for 1 min. PCR products (20 μl) were resolved on 2% agarose gels containing 0.5 μg/ml of ethidium bromide.

PCR primers. Primer pairs were obtained from the Core Molecular Biology Facility, London Regional Cancer Center, University of Western Ontario. The possibility of genomic DNA contamination was assayed for by PCR using β-actin primers that bracket an intron and produce a predicted 243-base pair (bp) fragment for the cDNA and a larger DNA fragment (due to the presence of the intron) if genomic DNA is present [26–28]. The larger genomic DNA product was not detected in any of the cDNA samples employed in this study. Primer pairs were derived from published human and bovine cDNA sequences [36–39], and the sizes of the expected PCR products are shown in Table 1. To confirm identity, each DNA product was cloned into pCRII vector through use of the TA cloning kit (Invitrogen, San Diego, CA) and were sequenced by dideoxy sequencing employing base-specific termination of enzyme-catalyzed primer-extension reactions [40] using a T7 sequencing kit (Pharmacia).

Detection of Oviductal IGFBPs by Western Ligand Blotting

[^{125}I]-IGF-II ligand blot analysis. Oviductal monolayer- and vesicle-conditioned media were concentrated by centrifugal ultrafiltration using a membrane with a molecular size cut-off of 10 kDa (Centricon-10; Amicon, Danvers, MA) [41, 42]. Concentrated samples were subjected to electrophoresis using a nonreducing 10% SDS-polyacrylamide gel. Proteins were then electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Mississauga,

ON, Canada) for 2 h at a constant current of 250 mA. The membrane was washed for 30 min in Tris-NaCl (pH 7.4), 0.5 mg/ml sodium azide (Sigma), and 3% Nonidet P-40 (NP-40; Sigma) before blocking for 2 h in Tris-NaCl (pH 7.4) and 1% BSA (Sigma), all at 4°C. The membrane was washed twice for 20 min in Tris-NaCl (pH 7.4) + 0.1% Tween 20 (Sigma) and was incubated for 20 h with 400 000 cpm [^{125}I]IGF-II (for ligand control, 100 ng/ml unlabeled IGF-II was added to incubating solution) in Tris-NaCl (pH 7.4), 0.1% Tween 20, and 1% BSA at 4°C [41, 42]. Recombinant human IGF-I and IGF-II (Gro-Pep Pty. Ltd., Adelaide, Australia) were iodinated to specific activities of 150–250 μCi/μg of protein using a chloramine-T method [43]. After the incubation, the membrane was taken through a series of 15-min washes, two in Tris-NaCl (pH 7.4) + 0.1% Tween 20 followed by three washes in Tris-NaCl (pH 7.4). The membrane was air dried at room temperature and exposed to x-ray film (XAR; Eastman Kodak, Rochester, NY) with intensifying screens at −70°C for 3–7 days.

Western immunoblots were prepared on the same membranes as described above. Membranes were initially washed for 30 min in 10 mM Tris-HCl containing 0.15 M NaCl, 0.3% NP-40 (v:v), and 0.5 mg/ml sodium azide (pH 7.4). The membrane was then blocked for 1 h in Tris-buffered saline (150 mM NaCl, 50 mM Tris-HCl, pH 7.4) containing 0.05% Tween 20 (v:v) (TTBS) supplemented with 4% BSA (w:v) and was washed three times, 10 min each, in TTBS. Membranes were incubated for 20 h at 4°C in TTBS + 1% BSA (w:v) with one of the following antibodies: anti-IGFBP-2 (rabbit polyclonal antiserum against bovine IGFBP-2 [39], diluted 1:1000; Upstate Biotechnology Inc. [UBI], Lake Placid, NY), anti-IGFBP-4 (rabbit polyclonal antiserum against human IGFBP-4 [44], diluted 1:250; UBI), or anti-IGFBP-5 (rabbit polyclonal antiserum against human IGFBP-5, diluted 1:100; Austral Biologicals, San Ramon, CA). After incubation with primary antiserum, the membranes were washed with TTBS (three times, 10 min each) and then incubated with anti-rabbit IgG biotin conjugates (Sigma) diluted in 1% BSA (1:30, w:v) TTBS for 2 h. Membranes were washed three times for 10 min each in TTBS, in ExtrAvidin (Sigma; 1:30 in PBS) for 1 h, and three times for 10 min each in TTBS. Bands were visualized using a 3,3′-diaminobenzidine tetrahydrochloride (Sigma)/3% hydrogen peroxide reaction. The reaction was then quenched in 50 mM Tris-HCl (pH 7.5), and the membranes were air dried.

RIA of IGF-I and IGF-II

A standard RIA method applied routinely to plasma samples was modified to determine the concentration of the IGF ligands released into oviduct primary culture- and blastocyst-conditioned culture media [45, 46]. Recombinant human IGF-I and IGF-II (GroPep Pty. Ltd.) were iodinated to specific activities of 150–250 μCi/μg of protein using a chloramine-T method [43]. Samples were concentrated by lyophilizing and resuspending in a volume of 100 μl dH_2O. IGFBPs were extracted using an acidic environment (pH < 3.4, formic acid) to release IGF/IGFBP complexes and precipitate the binding proteins. Then 100 μl of media was combined, in a 5-ml polypropylene tube, with 50 μl of 8 M formic acid + 0.5% Tween 20; these were mixed, and 350 μl of acetone was added [47]. The tube was covered to prevent evaporation and was centrifuged at 4°C for 30 min at 3000 × *g*. The supernatant (200 μl) was removed and neutralized in 1 M Tris base (200 μl). The loss of IGF during extraction was determined by the addition of labeled [^{125}I]IGF-I and -II to extraction reactions. The rate of recovery of labeled IGF-I and IGF-II was measured by gamma counting and was 52.2% ± 1.5 cpm/sample. Conditioned media, standard or control samples (100 μl) + primary antibody (100 μl, anti-human IGF-I or IGF-II; GroPep), and RIA buffer (100 μl of 0.01 M phosphate buffer containing 0.1% [w:v] sodium azide, 0.01 M EDTA, and 0.05% [v:v] Tween 20, pH 7.5) were combined in a 5-ml polystyrene test tube. The tubes were mixed briefly and incubated at 4°C overnight. To each tube was added 100 μl of [^{125}I]IGF at 20 000 cpm/tube. Tubes were mixed and incubated for a further 3 days before 250 μl of polyethylene glycol (PEG-2000; Sigma) mix containing bovine gamma globulin (Sigma; 1.5 g/L) was added to each tube. After overnight incubation, the tubes were centrifuged for 30 min at 6500 × *g*, the supernatant was decanted, and the radioactivity of the pellet was determined by λ spectroscopy. A standard curve was generated using 0.15–20 ng/ml recombinant IGF-I or IGF-II (GroPep human recombinant). Minimal detectable dose and half-maximal displacement of the radioligand occurred, respectively, at 0.35 ng/ml and 2.2–2.8 ng/ml for IGF-I and at 0.32 ng/ml and 3.5–7 ng/ml for IGF-II. Results are expressed as nanograms IGF-I or -II released per microgram cell DNA. For DNA quantification, oviductal cells were lysed in 10% trichloroacetic acid at 4°C for 20 min, then solubilized in 0.1 M NaOH overnight at 38.6°C. The solubilized cells were then assayed for DNA content by fluorometric spectroscopy as described by Kissane and Robins [48].

Statistical Analysis

The effects of primary culture (monolayer or vesicle) and culture interval (Days 2, 5, and 8) on IGF-I and IGF-II release from conditioned media was determined by a 2 × 3 factorial analysis of variance. Results were considered significant at $p < 0.05$.

RESULTS

IGFBP Transcripts in Bovine Preattachment Embryos and Primary Oviductal Cell Cultures

RT-PCR assays were repeated a minimum of three times with embryo and oviduct samples derived from replicate cultures. β-Actin amplicons representing the expected size from amplification of cDNA were detected in all embryo and oviduct cell samples (data not shown). At no time was the larger intron spanning genomic β-actin product observed. Figure 1 displays the typical detection pattern for IGFBP mRNAs in cultured bovine preattachment embryos. Figure 2 summarizes the expression pattern for these mRNAs in both oviductal monolayer and vesicle primary cultures.

IGFBP-2, -3, and -4 transcripts were detectable in all stages of bovine preattachment development. Transcripts encoding IGFBP-5 were not detected in early cleavage-stage embryos or morulae, but a weak signal was consistently observed in blastocyst samples. Transcripts for IGFBP-1 were not detected in any preattachment embryo stage (Fig. 1), although a product of expected size was detected in bovine liver and kidney RNA samples (data not shown). The IGFBP-6 amplicon of expected size (345 bp) was not detected in any preattachment embryo stage. Instead a much smaller (166 bp) amplicon was consistently observed in all embryo samples (Fig. 1). This DNA product

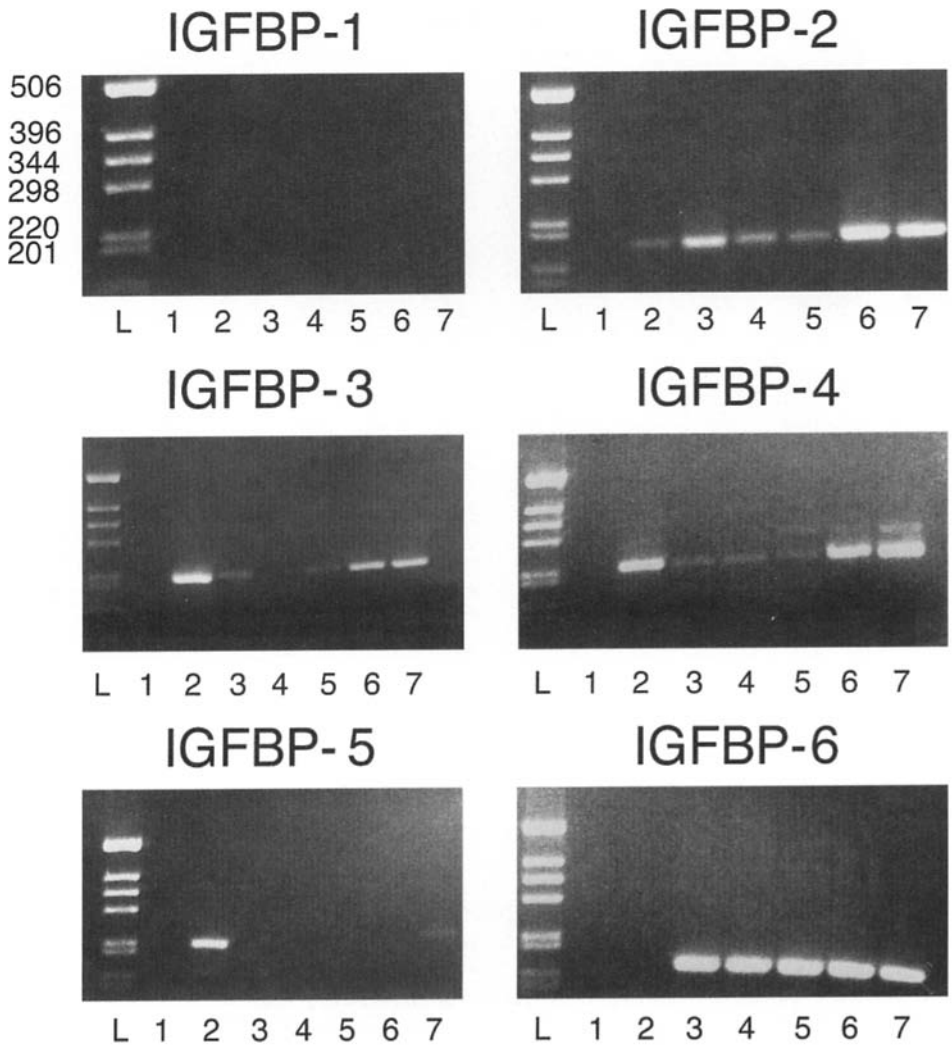

FIG. 1. Detection of mRNAs encoding IGFBPs in preattachment bovine embryos by RT-PCR. Lanes are (L) ladder (size of bands given in base pairs), (1) negative control (no cDNA), (2) oviduct sample, (3) 1-cell zygotes, (4) 2–5-cell embryos, (5) 6–8-cell embryos, (6) morulae, (7) Day 8 blastocysts. Transcripts encoding IGFBP-2, -3, and -4 were detected in oviduct samples and all preattachment embryo stages, while IGFBP-5 mRNAs were detected in oviduct samples and in blastocyst-stage embryos. Messenger RNAs encoding IGFBP-1 and -6 were not detected in any preattachment embryo stage. A PCR amplicon smaller than expected size was detected in all embryo samples employing IGFBP-6-specific primers. The procedures were repeated three times and were applied to pools of embryos obtained from three replicate cultures. The identity of each amplicon was determined by DNA sequence analysis.

was, however, not observed in bovine oviduct samples (Fig. 2). Sequence analysis indicated that, of the first 84 bp of the 166-bp amplicon, 96% were identical to the reported sequence for an *E. coli* aceE gene encoding the E1 component of pyruvate dehydrogenase. This analysis was repeated several times on various proven bovine embryo cDNA samples, and at no time was the expected IGFBP-6 PCR product detected. The identities of the remaining IGFBP amplicons were confirmed by sequence analysis comparing the bovine embryo IGFBP amplicon sequences

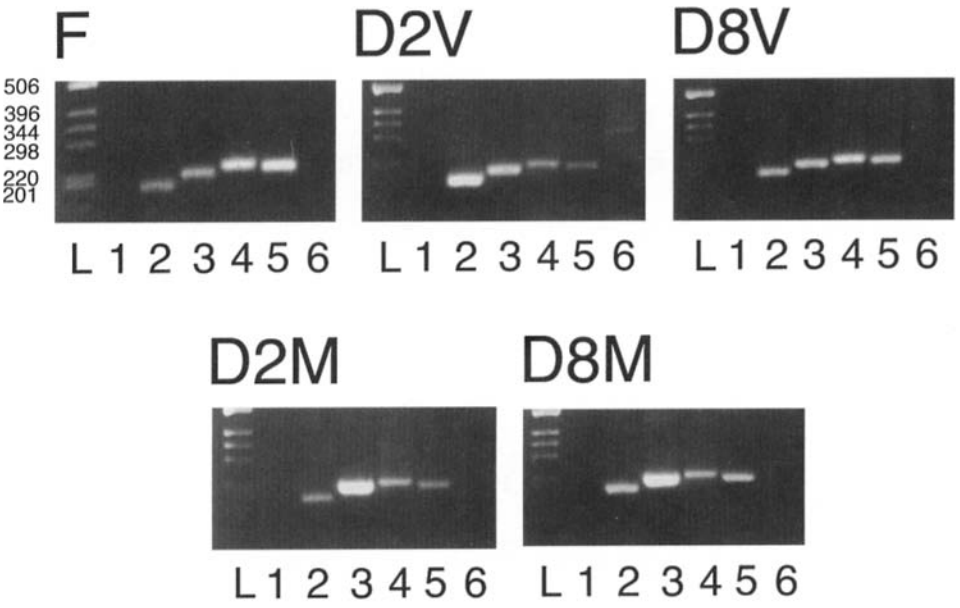

FIG. 2. Detection of IGFBP transcripts in noncultured fresh oviduct samples (F), Day 2 vesicle cultures (D2V), Day 8 vesicle cultures (D8V), Day 2 monolayer cultures (D2M), and Day 8 monolayer cultures (D8M) by RT-PCR. L, ladder (size of bands in base pairs); lanes 1–6 correspond to PCR products encoding IGFBPs 1–6, respectively. Transcripts encoding IGFBPs 2–5 were detected in both vesicle and monolayer primary cultures throughout an 8-day culture interval. IGFBP-6 mRNAs were detected in only a single culture replicate. The procedures were conducted three times and were applied to oviduct RNA samples extracted from three replicate sets of primary oviduct cultures. The identity of each amplicon was determined by DNA sequence analysis.

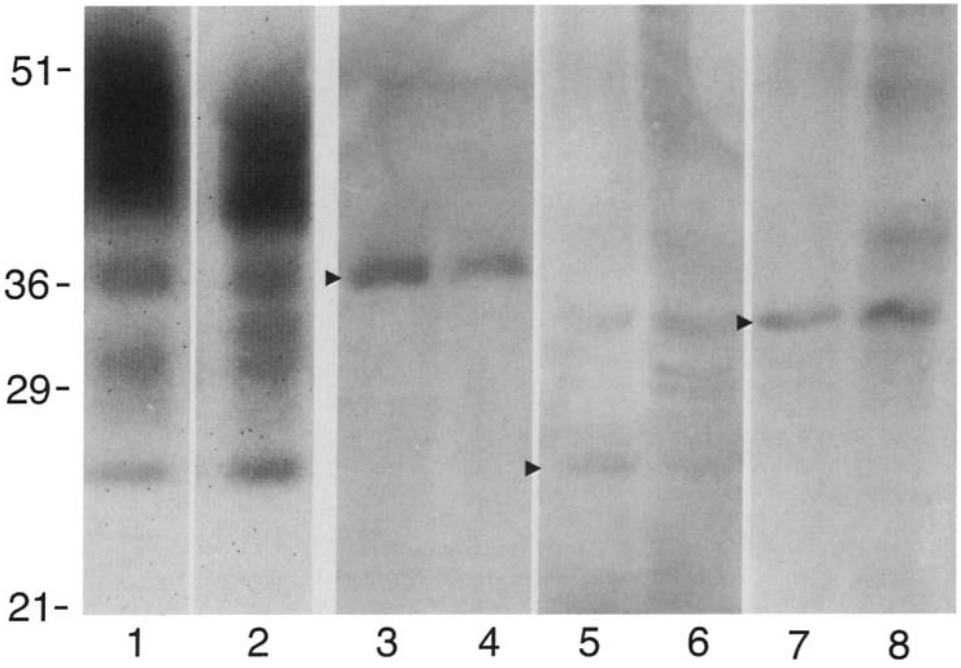

FIG. 3. Western ligand blot and immunoblot analysis of 24-h-conditioned media collected from oviductal cell monolayer (1,3,5,7) and vesicle (2,4,6,8) primary cultures. Concentrated samples were subjected to SDS-PAGE, transferred to nitrocellulose, and incubated with [^{125}I]IGF-II radioligand. A representative autoradiograph reveals 4 prominent bands with IGF-II binding affinity at 24 kDa, 31 kDa, and 36 kDa and a broad band at 46–53 kDa, in both monolayer and vesicle cultures (lanes 1 and 2). Western immunoblot analysis employing polyclonal antisera against IGFBP-2 (lanes 3 and 4), -4 (lanes 5 and 6), and -5 (lanes 7 and 8) identified the 36-kDa, 31-kDa, and 24-kDa bands (arrows), respectively.

to published bovine and human IGFBP cDNA sequences (Table 1) [36–39]. Comparison of the embryonic IGFBP-2 DNA product with those of published sequences revealed a 99.2% sequence identity to the bovine IGFBP-2 sequence. Embryonic IGFBP-3 and -4 products displayed a 100% sequence identity to their respective bovine cDNA sequences, and the IGFBP-5 DNA product displayed a 96.1% sequence identity to that of the human cDNA.

Transcripts encoding IGFBP-2, -3, -4, and -5 were detected in oviduct monolayer and vesicle primary cultures over an 8-day culture interval (Fig. 2). The mRNAs encoding IGFBP-1 and -6 were not consistently detected in oviduct cultures, although a PCR product of expected size (345 bp) encoding IGFBP-6 was observed once in each of the oviduct samples examined (Fig. 2).

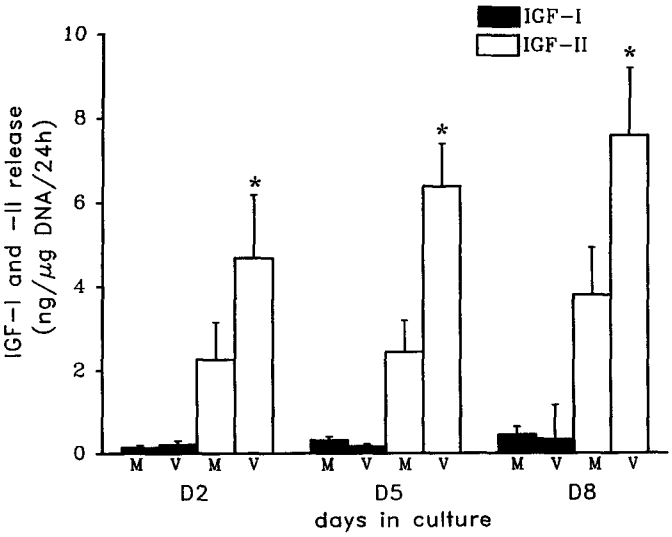

FIG. 4. Release of IGF-I and -II from oviductal cultures over 24 h into serum-free medium. After the extraction of binding proteins, IGF levels were measured by RIA in samples collected at Days 2, 5, and 8 of culture. No significant variation in the levels of IGF-I or -II release was detected for vesicle or monolayer cultures over an 8-day culture interval. IGF-II is released at 25 times higher levels in vesicle cultures and 9 times higher in monolayer cultures than IGF-I. The levels of release of IGF-II from vesicle cultures were significantly greater than those observed for monolayer cultures ($p > 0.005$). No significant difference in the levels of IGF-I release between monolayer and vesicle cultures was observed.

Detection of IGFBPs in Bovine Oviductal Cell-Conditioned Media

Western ligand analysis of conditioned media prepared from monolayer and vesicle cultures over an 8-day culture period revealed four prominent IGFBPs of approximate molecular masses 24 kDa (IGFBP-4), 31 kDa (IGFBP-5), and 36 kDa (IGFBP-2) and a broad band extending from 46 to 53 kDa (IGFBP-3; Fig. 3, lanes 1 and 2). This analysis was repeated four times employing conditioned media collected from replicate monolayer and vesicle cultures established from separate oviduct collections. The specificity for IGF binding displayed by these polypeptides was verified by control analysis consisting of competitive binding assays employing cold IGF-II to displace binding of radiolabeled IGF-II. In all cases this procedure eliminated the detection of any IGFBP signal in these samples (data not shown). Media were also collected from the final washes, and no detectable levels of IGFBPs were observed in these controls. Furthermore, replicate ligand blots were incubated with [^{125}I]IGF-I, and an identical binding pattern was detected (data not shown). Identities of the binding proteins were predicted from the relative molecular sizes, and IGFBP-2, -4, and -5 were confirmed by immunoblotting using IGFBP-specific antisera (Fig. 3, lanes 3–8). A single band of 36 kDa was detected employing a rabbit polyclonal antiserum raised against bovine IGFBP-2 (UBI) in both oviductal monolayers and vesicles (Fig. 3, lanes 3 and 4, arrow). Likewise, bands of appropriate molecular masses for IGFBP-4 (Fig. 3, lanes 5 and 6, arrow) and IGFBP-5 (Fig. 3, lanes 7 and 8, arrow) were detected in oviductal monolayer and vesicle cultures employing rabbit polyclonal antisera specific for human IGFBP-4 (UBI) and human IGFBP-5 (Austral Biologicals), respectively.

Measurement of IGF Levels in Oviduct- and Blastocyst-Conditioned Media

RIAs were conducted to measure IGF-I and -II accumulation in oviductal cell (Fig. 4)- and bovine blastocyst-conditioned media. IGFBP extraction in these samples was confirmed by the absence of an IGFBP signal following Western ligand blot analysis prior to RIA (data not shown). No significant variation in either IGF-I or IGF-II release was detected in either monolayer or vesicle cultures over the 8-day culture interval. However, the release of IGF-II was 25 times that of IGF-I in vesicle cultures (6.25 ± 0.88 ng/μg DNA for IGF-II vs. 0.25 ± 0.05 ng/μg of DNA for IGF-I), with a 9-fold difference in the accumulation of IGF-II over that of IGF-I in monolayer cultures (2.8 ± 0.56 ng/μg DNA for IGF-II vs. 0.31 ± 0.08 ng/μg DNA for IGF-I; Fig. 4). The release of IGF-II by vesicle cultures was significantly greater ($p < 0.005$) than that observed for monolayer cultures. No significant difference in IGF-I release was observed. Pools of 10 blastocysts released on average 36.2 ± 3.9 pg/embryo of IGF-II. Release of IGF-I from blastocysts was below the detectable levels of the assay.

DISCUSSION

We have demonstrated that bovine oviduct monolayer and vesicle cultures both express transcripts encoding IGFBPs 2–5 throughout an 8-day culture interval. Transcripts encoding IGFBP-1 and IGFBP-6 were not consistently detected in oviductal cultures or early embryos. In contrast, IVMF bovine zygotes express mRNAs encoding IGFBPs 2–4 through to the blastocyst stage. Messenger RNAs encoding IGFBP-5 were detected in bovine blastocysts. Each bovine IGFBP DNA product displayed a 96% or greater sequence identity to published cDNAs [36–39]. The 166-bp fragment derived from the IGFBP-6 primers did not display any identity to published IGFBP-6 sequences [38]. This amplicon was also observed by Hahnel and Schultz [36] using identical primers to investigate the expression of IGFBP-6 mRNAs during murine preimplantation development. The IGFBP-6 amplicon of expected size was detected only in murine blastocyst samples; however, the 166-bp product was observed in all preimplantation-stage embryo samples [36]. Our result, indicating a shared homology with an *E. coli* aceE gene encoding the E1 component of pyruvate dehydrogenase, is intriguing and is worthy of further investigation, especially since this DNA product was not detected in bovine oviduct samples. Clearly, differences in the expression of mRNAs encoding the IGFBPs exist between murine and bovine early embryos, as IGFBP-5 transcripts were confined to bovine blastocysts and IGFBP-6 mRNAs were not detected. The significance of these species differences awaits further investigation. No differences in the pools of expressed IGFBP mRNAs were detected in oviduct vesicle or monolayer primary cultures over an 8-day culture interval. The inconsistent detection of IGFBP-1 and -6 mRNAs in both monolayer and vesicle cultures does not rule out the possibility that expression of these gene products may be low, transitory, or linked to ovarian cycles. This possibility has been investigated in the human oviduct [49, 50].

Western ligand blot analysis revealed four prominent IGFBPs of approximate molecular masses 24 kDa, 31 kDa, and 36 kDa and a broad band extending from 46 to 53 kDa in oviductal monolayer- and vesicle-conditioned media samples. Serum was removed prior to collection of conditioned media, and ligand blots of wash media did not result in the detection of any IGFBP signal. Furthermore, all IGFBP signal was eliminated when the blots were coincubated with unlabeled IGF-II. Bands of proteins at 24, 31, 36, and 46–53 kDa correspond in molecular mass to IGFBP-4, -5, -2, and -3, respectively [29, 31, 32, 41, 42, 49, 51]. The 46–53 kDa band likely represents a doublet consisting of the nonglycosylated and glycosylated forms of IGFBP-3, running at the same size as the dominant band present by Western ligand analysis of steer serum (not shown). Absolute identification of this band as IGFBP-3 awaits further determination employing specific antiserum by Western blot analysis. The identities of the 24-, 31-, and 36-kDa proteins were confirmed by immunoblot methods. Bands at the molecular mass range 29–30 kDa that were detected in the ligand blots and in the IGFBP-4 Western immunoblot may represent glycosylated forms of IGFBP-4 [52] or cross-reactivity of antiserum with IGFBP-2 [44]. Since transcripts encoding IGFBP-1 were not detected and IGFBP-6 mRNAs were not consistently detected in these primary cultures, it is unlikely that the 29–30-kDa polypeptides are related to IGFBP-1 or IGFBP-6. Furthermore, no differences in the banding patterns was observed between IGF-I- and IGF-II-radiolabeled ligand blots (data not shown), suggesting that IGFBP-6 is not represented in the banding pattern. IGF-II is reported to have a 10-fold higher affinity for IGFBP-6 than that displayed by IGF-I [53].

IGFBPs are reported to regulate IGF biological activity [31, 32] in at least four ways. These include 1) transportation of IGFs; 2) influences on IGF half-life; 3) contribution to IGF tissue- and cell-specific distribution; and 4) modulation and potentiation of IGF action with receptors [31, 32]. Variations in IGFBP transcript levels have now been associated with several developmental events. For example, IGFBP-2 mRNAs decrease with follicular growth and maturation in the porcine ovary [54], and follicular fluid IGFBP-2 levels increase in porcine atretic follicles over levels observed for follicular fluid derived from healthy follicles [55]. The presence of IGFBPs 2–5 in bovine follicular fluid was reported by de la Sota et al. [56], who also observed that increased levels of IGFBP-2, -4, and -5 were associated with atresia. Porcine corpora lutea express mRNAs encoding IGFBPs 2–5 [57]. Messenger RNAs and polypeptides encoding IGFBPs 1–4 are expressed in human oviduct [49, 50]. Although mRNAs encoding IGFBP-1 were weakly detected by Northern blotting in the human oviduct [49], immunolocalization intensity was the greatest for IGFBP-1 polypeptides in the human oviduct [50]. Cycling IGFBP levels are also correlated with implantation. Endometrial IGFBP-2 mRNA levels increase between Day 18 of the estrous cycle and early pregnancy in cattle [58], and murine uterine stromal IGFBP-4 mRNA levels increase on Day 4 of pregnancy in stromal tissue just underlying the luminal epithelium [59]. These levels of IGFBP expression may be influenced by steroids, as administration of estradiol to ovariectomized rats (1 μg per rat per day for 3 days) dramatically decreases the levels of two IGFBPs with molecular masses of 38–42 and 28 kDa [51]. Furthermore, maternal serum levels of IGFBPs also cycle during pregnancy, as Nason et al. [60] observed that serum levels of all IGFBPs increased between Days 12 and 24 of pregnancy in the rabbit and then declined to term.

Furthermore, IGFBPs may have the capacity to stimulate direct influences on cell processes as exemplified by IGFBP-3 action on human breast cancer cell growth [61]. The nature of IGFBP regulation of IGF activity during early

bovine development is unknown but represents the subject of future experiments.

Bovine oviductal primary cultures express transcripts encoding IGF-I and IGF-II and secrete respective peptides [3, 27]. In addition to these ligand mRNAs, bovine preattachment embryos express mRNAs encoding IGF-I, IGF-II, and insulin receptors [27]. Our present results have confirmed that detectable levels of IGF-I and -II are released by bovine primary oviductal cultures and IGF-II from blastocyst-stage embryos. Both IGF-I and -II have been detected in porcine oviductal fluid, and porcine oviductal primary cultures also secrete IGF-I and -II [62]. The differences in IGF-I and -II levels detected in oviduct-conditioned media are similar to other findings, as Ko et al. [63] observed a 10-fold difference in IGF-II levels over IGF-I in Day 12–14 cyclic and pregnant sheep uterine luminal fluids. The significantly higher levels of IGF-II in vesicle cultures as compared to monolayer cultures is particularly intriguing. It is clear from studies investigating early murine development that insulin and both IGFs are capable of stimulating physiological responses and increases in cell proliferation [30, 64, 65]; similar confirmation of these influences has not been made for early bovine development and awaits further study.

In conclusion, we have demonstrated that bovine preattachment embryos and primary oviductal cultures express transcripts encoding IGFBPs. Bovine primary oviductal cultures release four principal IGFBPs, and oviductal cultures release detectable levels of IGF-I and IGF-II. Bovine blastocysts release detectable levels of IGF-II, and IGF-I release is below the level of detection of the assay. The results support the existence of oviduct paracrine and embryonic autocrine IGF circuits, raising the possibility of the existence of IGFBP modulation in the support of bovine preattachment embryo development.

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