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Characterization and expression of vascular endothelial growth factor (VEGF) in the ovine corpus luteum

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The corpus luteum undergoes tremendous growth, development and regression each oestrous or menstrual cycle. These changes are reflected by equally impressive growth and regression of the luteal vasculature. We have previously shown that angiogenic factors from corpora lutea are primarily heparin binding and that one of these factors is similar to vascular endothelial growth factor (VEGF). In an effort to identify this factor, and to define its role in luteal vascular development, the cDNA for the coding region of ovine VEGF was sequenced and a sensitive RNase protection assay was developed to quantitate mRNA encoding VEGF in luteal tissues from ewes in the early (days 2–4), mid- (day 8) and late (days 14–15) stages of the oestrous cycle. In addition, an N-terminal peptide was synthesized from the translated ovine cDNA sequence for VEGF and an antiserum was raised against this peptide for use in western immunoblotting procedures. Nested reverse transcriptase (RT)-PCR of RNA from ovine corpora lutea resulted in three products that correspond in size to the alternatively spliced variants of VEGF ($VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$) predicted from other species. The RNase protection assay revealed that the proportion of mRNA encoding VEGF was 2- to 3-fold greater on days 2–4 than on day 8 or days 14–15. Densitometric analysis of gels from the RNase protection assay showed that $VEGF_{120}$ represented approximately one third of the total mRNA encoding VEGF in the corpus luteum and that this proportion did not vary with stage of the oestrous cycle. SDS-PAGE and western immunoblot analysis of a homogenate from corpora lutea showed a single 18 kDa protein. These data demonstrate that VEGF is expressed in luteal tissue throughout the ovine oestrous cycle and that expression of mRNA encoding VEGF is upregulated during the period of rapid luteal development, when luteal vascular growth is at its maximum.

Introduction

The corpus luteum is a dynamic endocrine organ that undergoes rapid growth, differentiation and controlled regression every ovarian cycle (Jablonka-Shariff *et al.*, 1993; Reynolds *et al.*, 1993, 1994; Smith *et al.*, 1994; Zheng *et al.*, 1994). Associated with these changes are alterations in luteal vascularity (Reynolds *et al.*, 1992; Zheng *et al.*, 1993; Redmer and Reynolds, 1996). In addition to being one of the most vascular tissues of the body, the corpus luteum also has one of the greatest blood perfusion rates of any tissue (Ellinwood *et al.*, 1978; Redmer and Reynolds, 1996). Therefore, factors that regulate luteal vascular growth are likely to play a major role in regulating luteal function.

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Angiogenesis, which is the growth and development of new blood vessels, has been shown to be regulated by a number of angiogenic factors (Folkman and Klagsbrun, 1987; Klagsbrun and D'Amore, 1991). Indeed, angiogenic factors are produced by the corpus luteum of a variety of mammals (Reynolds *et al.*, 1992; Redmer and Reynolds, 1996). In addition, angiogenic factor(s) produced by the corpora lutea of cows, pigs and sheep are primarily heparin-binding and can be immunoneutralized with antibodies against fibroblast growth factors and vascular endothelial growth factors (VEGF; Gospodarowicz and Thakral, 1978; Zheng *et al.*, 1993; Doraiswamy *et al.*, 1995; Grazul-Bilska *et al.*, 1995; Ricke *et al.*, 1995). Also, a major heparin-binding factor produced by the corpora lutea of these species appears to be a secreted mitogen, specific to endothelial cells; this is not a characteristic of fibroblast growth factors (Grazul-Bilska *et al.*, 1992, 1993, 1995; Redmer and Reynolds, 1996). However, the VEGFs have been shown to be

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endothelial-specific mitogens and chemoattractants (Ferrara *et al.*, 1992), and the presence of mRNA encoding VEGF has been reported in ovarian tissues of several mammalian species (Phillips *et al.*, 1990; Ravindranath *et al.*, 1992; Garrido *et al.*, 1993; Yan *et al.*, 1993).

In an attempt to define further the underlying mechanisms regulating the dynamic vascular changes associated with growth of ovine corpora lutea, we wanted to determine whether VEGF was involved in ovine luteal development. We sequenced the coding region of ovine mRNA encoding VEGF, and herein report the cDNA sequence, concentrations of mRNA encoding VEGF in ovine corpora lutea at several stages of the oestrous cycle, and the presence of immunoreactive VEGF protein in ovine luteal tissue.

Materials and Methods

Tissue collection

Corpora lutea were collected from superovulated ewes at slaughter during the early (days 2–4; $n = 5$), mid- (day 8; $n = 4$), and late (days 14–15; $n = 3$) luteal stage of the oestrous cycle (oestrus = day 0; duration of the oestrous cycle approximately 16.5 days). Superovulation was achieved by using procedures similar to those described by Fricke *et al.* (1991). Starting on day 13 of the oestrous cycle, ewes received two injections (morning and evening) each day of an ovine FSH-pituitary extract (Ovagen; Genus, Newcastle, UK) as follows: day 13, 1.8 mg per injection; day 14, 1.4 mg per injection; day 15, 1 mg per injection (1 mg equivalent to 20 units of NIH-FSH-S1). All ewes received 300 iu of CG (Chorulon, Intervet, Birmingham, UK) at the appearance of the first oestrus after the FSH injections. Corpora lutea were dissected from the ovaries and snap-frozen in liquid nitrogen within 15 min of death. A sample (1.5 g) of the remainder of the ovary (ovary minus corpora lutea) also was snap-frozen in liquid nitrogen.

Preparation of total RNA

Total cellular RNA was isolated by using procedures similar to those described by Chomczynski and Sacchi (1987) and as described in detail by Promega (1991) for the acid guanidinium thiocyanate–phenol–chloroform extraction method. The integrity of the RNA was assessed visually by ethidium bromide staining of the RNA after electrophoresis on a denaturing 1% agarose gel. The quantity and purity of RNA were determined spectrophotometrically.

Cloning of probes

For the VEGF probe, an oligonucleotide primer pair was designed to match consensus sequences near each end of the coding region of human (Leung *et al.*, 1989), rat (Conn *et al.*, 1990) and bovine (Tischer *et al.*, 1989) cDNA and also to contain restriction endonuclease sites (*Bam*H1 and *Eco*R1) for subcloning into Bluescript II SK + (Stratagene, LaJolla, CA). The primer sequences were: 5′-CCGGATCCATGAACTTT CTGCTCT-3′ (near start codon) and 5′-TCGAATTCCC GAAACCCTGAGG-3′ (near stop codon; addition tails as restriction sites are underlined). PCR was then used to amplify cDNA for VEGF from an in-house reverse-transcribed preparation of ovine fetal kidney RNA . The reverse transcription reaction was carried out by using a reverse transcription kit (Promega, Southampton, UK) and 5 μg of total RNA. Taq DNA polymerase (Boehringer Mannheim, Lewes, East Sussex) and the primer pair were used for PCR, which was carried out in a DNA Thermal Cycler (Perkin Elmer/Cetus, Norwalk, CT) as follows: 35 cycles of 94°C for 45 s, 55°C for 40 s, and 72°C for 80 s. The PCR reaction yielded a single faint band between 500 and 600 nucleotides in length following electrophoresis on a 1.5% agarose gel and ethidium bromide staining. A second round of PCR was performed as described above by using the same primer pair and the first PCR reaction solution as a template. Following electrophoresis on a 1.5% agarose gel, the PCR product was isolated by using a QIAEX II gel extraction kit (Qiagen, Dorking, Surrey), then ligated into Bluescript II SK + and cloned as described by Sambrook *et al.* (1989).

To obtain an ovine actin probe, an oligonucleotide primer pair was made using sequences identical to those used by Bacich *et al.* (1994). cDNA for β-actin was then amplified from RNA derived from ovine mid-cycle corpora lutea and also from adult ovine liver by using the RT-PCR similar to procedures described for the VEGF probe above. The PCR products were isolated, ligated into Bluescript II SK + and cloned as described above.

Sequencing

Several clones for each probe were sequenced in both orientations using a Taq DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Warrington, Cheshire) and an automated DNA Sequencer (Applied Biosystems). Sequence analyses were performed by using GCG programs (University of Wisconsin, Madison, WI). Sequences were then compared to other sequences by using FASTA and the EMBL nucleotide sequence database (European Bioinformatics Institute, Cambridge, UK).

Nested PCR

To determine the presence of alternatively spliced variants of mRNA encoding VEGF in ovine luteal tissue, a nested PCR procedure was performed as described by Haining *et al.* (1991). To ensure that the first-round PCR products were indeed cDNA for VEGF, a second round of PCR using an internal (internal to external primers) oligonucleotide primer pair that was synthesized according to the exact cDNA sequence of the ovine cDNA for VEGF was performed. Locations of these specific internal primers with respect to the common external primers (used above for making the VEGF probe) are shown (Fig. 1). The internal primer sequences were: 5′-TTGGATCCTTGCCTTGCTGCTCTACCT-3′ (near start codon) and 5′-GCGAATTCCTCCAGCCCGGCTCACCG CCTC-3′ (near stop codon). For the first round PCR, RT-PCR was conducted by using procedures described above for VEGF, except that several isolates of reverse-transcribed mRNA derived from early and mid-cycle luteal tissue were used as

templates. A second round of PCR was conducted using the specific internal primer pair and 2 μl of the first round PCR solution as a template. For control reactions, cDNA template was omitted from the first round of PCR, and this solution was then used as a 'template' for the second round of PCR. PCR products were visualized on a 1.5% agarose gel containing ethidium bromide.

RNAse protection assay

The RNAse protection assay was based on solution hybridization between the mRNA of interest and a labelled antisense RNA probe, followed by RNAse digestion and subsequent analysis of protected hybrids by electrophoresis. This assay provides not only a very sensitive means to quantitate mRNA, but also discriminates between different alternatively spliced mRNA variants. The *in vitro* transcription reactions for obtaining a labelled probe and the solution hybridization procedures were carried out by using kits (MAXIscript and RPA II, respectively; Ambion, Austin, TX), as described previously (Charnock-Jones *et al.*, 1994). The VEGF probe described above was digested at the start codon with *Bam*H1 and the antisense RNA was transcribed *in vitro* from the T7 promotor. This resulted in a probe of 587 bases which contained bases 1-464 from VEGF (based on the numbering of the cDNA sequence) and another 123 bases transcribed from the vector. For a positive control, an actin probe (described above) also was transcribed from the T7 promoter. This resulted in a probe of 266 bases; 202 bases were transcribed from the actin sequence and another 64 bases were transcribed from the vector. For this assay, excess probe (2–4×10^4 c.p.m.) was coprecipitated with each sample of luteal RNA (50 μg) and resuspended in hybridization buffer. After hybridization and RNAse digestion, the protected hybrid was loaded onto a denaturing 6% polyacrylamide sequencing gel. RNA markers were made during the *in vitro* transcription reactions by using the Century Marker Template Set (Ambion) and were used in each gel. Densitometry was performed on autoradiographs of the gels by using a laser scanning densitometer (Model PDSI; Molecular Dynamics, Sunnyvale, CA). After scanning the autoradiograph, a grid was drawn so that

Fig. 1. The sequence for sheep cDNA for vascular endothelial growth factor (VEGF) and the predicted amino acid sequence for sheep VEGF aligned with the predicted sequences for cow (Tischer *et al.*, 1989), mouse (Claffey *et al.*, 1992), rat (Conn *et al.*, 1990), human (Leung *et al.*, 1989), guinea pig (EMBL database, accession number M84230l), and pig (Sharma *et al.*, 1995) VEGF. Asterisks represent identical amino acids. Numbering of nucleic acids (located at the end of each row) does not include the nucleotides encoding the extra amino acid found in the human sequence (after position 93). The locations of the common primers that were designed from consensus sequences of cDNA for VEGF from several species are indicated. These primers were used for the generation of the sheep VEGF probe and for the first round of a nested PCR reaction. Also indicated are the locations of the specific internal primers that were designed from the sheep VEGF sequence. These primers were used in the second round of the nested PCR reaction. Based on comparisons with other species, locations of exons 1–5 and exon 8 are indicated. The location of a 15 amino acid N-terminal peptide that was synthesized for this study also is indicated.

each sample was contained within boxes of equal size, and the densitometric area contained within each box was given a relative absorbance measurement. A small portion (2 μl) of each protected hybrid sample also was quantified by liquid scintillation counting.

Antibody production

On the basis of the translated sequence of ovine cDNA for VEGF, an N-terminal 15-amino acid peptide was synthesized (Babraham Microchemical Facility, The Babraham Institute, Cambridge, UK) and conjugated to a purified protein derivative of tuberculin. The amino acid sequence of the peptide is NH_4-APMAEGGQKPHEVMK-COOH and is indicated in Fig. 1. The conjugate was used to produce a polyclonal antibody (Red1-b3) in a rabbit.

Western immunoblot analysis

Western analysis was conducted as described by Conley *et al.* (1994). Briefly, corpora lutea from the early stage of the oestrous cycle were homogenized in 10 volumes of buffer (1% cholic acid (w/v), 0.1% SDS (w/v) in PBS) and a pooled sample of protein (180 μg) was added to loading buffer (glycerol (10%, v/v), SDS (2% w/v), EDTA (2 mmol l^{-1}), β-mercaptoethanol (1%, v/v), bromophenol blue (0.05%, w/v), Tris–HCl (63 mmol l^{-1}), pH 6.8), boiled for 2 min, and then applied to an 8% polyacrylamide gel with a 3% stacking gel (Laemmli *et al.*, 1970). A sample of recombinant human VEGF (50 ng; R&D Systems, Minneapolis, MN) was also added to loading buffer, boiled and applied to the gel. Electrophoresis was conducted in electrophoresis buffer (Tris (50 mmol l^{-1}), glycine (383 mmol l^{-1}), SDS (0.1%), EDTA (0.4 mmol l^{-1})) for 18 h at constant current. Separated proteins were electroblotted onto an Immobilon-P membrane (Millipore, Bedford, MA) in electroblot buffer (methanol (20%,v/v), Tris (20 mmol l^{-1}), glycine (150 mmol l^{-1})) for 3 h at constant current, and immunoblotted with the polyclonal antibody (Red1-b3) that was prepared as described above (1:500 in buffer consisting of Tris (10 mmol l^{-1}), NaCl (0.15 mol l^{-1}), fetal calf serum (10%, v/v) and Tween-20 (0.1%, v/v)) for 2 h. Membranes then were incubated with a peroxidase-labelled anti-rabbit antibody (Amersham International plc, Little Chalfont, Bucks; 1:2000 in the same buffer as for primary antibody) for 1 h, followed by detection with ECL reagents (Amersham) and autoradiography.

Statistical analyses

All data were analysed by using the general linear models (GLM) analysis of variance with the main effect of stage of the oestrous cycle (SAS, 1985). When the *F* test was significant ($P < 0.05$), differences between specific means were evaluated by using Bonferroni's multiple comparison procedure (Kirk, 1982).

Results

cDNA sequences for VEGF and actin

The sequence for ovine cDNA for VEGF is shown (Fig. 1) and has been assigned an accession number of X89506 (EMBL database). The coding region contains 441 nucleotides and codes for the 120 amino acid variant of mature VEGF ($VEGF_{120}$), that contains exons 1–5 and exon 8. The cDNA sequence for ovine VEGF exhibits 98.4, 94.8, 93.4, 91.8, 88.9, and 87.3% homology with bovine (Tischer *et al.*, 1989), pig (Sharma *et al.*, 1995), human (Leung *et al.*, 1989), guinea pig (EMBL database, accession number M842301), rat (Conn *et al.*, 1990) and mouse (Claffey *et al.*, 1992) sequences, respectively. Compared with the amino acid sequences reported for other species, ovine $VEGF_{120}$ exhibits greatest homology with bovine VEGF, differing by only two amino acids (Fig. 1). The predicted amino acid sequence for ovine VEGF revealed 98.6, 94.6, 91.8, 91.2, 86.4 and 85.0% homology with those of bovine, pig, guinea pig, human, rat and mouse, respectively (Fig. 1).

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                BamHl_____primer_____
sheep                GCCAACACAGTGCTGTCTGGTGGGACTACCATGTACCCCGGC 42
sheep                IlaAsnThrValLeuSerGlyGlyThrThrMetTyrProGly
Bacich et al.                          *  *  *  *  *  *  *
human                                  *  *  *  *  *  *  *

sheep           ATCGTGGACAGGATGCAGAAAGAGATCACTGCCCTGGCACCCAGCACG 90
sheep           IleValAspArgMetGlnLysGluIleThrAlaLeuAlaProSerThr
Bacich et al.    * Ala *  *  *  *  *  *  *  *  *  *  *  *  *  *
human            * Ala *  *  *  *  *  *  *  *  *  *  *  *  *  *

sheep           ATGAAGATCAAGATCATCGCGTCCCCTGAGCACAAGTACTCCGTGTGG 138
sheep           MetLysIleLysIleIleAlaSerProGluHisLysTyrSerValTrp
Bacich et al.    *  *  *  * Phe *  * Pro *  * Arg *  *  *  *  *
human            *  *  *  *  *  *  * Pro *  * Arg *  *  *  *  *

                                                        primer
sheep           ATTGGCGGCTCCATCCTGGCCTTGCTGTCCAGCTTCCAGCAGATGGAA 186
sheep           IleGlyGlySerIleLeuAlaLeuLeuSerSerPheGlnGlnMetGlu
Bacich et al.    *  *  *  *  *  *  * Ser *  * Thr *  *  *
human            *  *  *  *  *  *  * Ser *  * Thr *  *  *

                 primer con't.  EcoR1
sheep           TCAGCAAGCAGTAGTA 202
sheep           SerAlaSerSerSer
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Fig. 2. The sequence for sheep cDNA for actin and the predicted amino acid sequence for sheep actin (this study) aligned with the predicted amino acid sequences for sheep (Bacich *et al.*, 1994) and human (Erba *et al.*, 1986) actin. Asterisks represent identical amino acids. The locations of the primers used to generate the sheep actin cDNA are indicated. Sequences for these primers were obtained from Bacich *et al.* (1994).

The sequence of the ovine actin cDNA is shown (Fig. 2). In the present study, the cDNA sequences of the actin clones made from RNA from a mid-cycle corpus luteum were identical to those of clones made from liver RNA from a different ewe, and contain 202 nucleotides (including primers but excluding restriction sites). However, the cDNA sequence of our ovine actin clones exhibited only 85.9% homology with an ovine actin sequence from a previous report (Bacich *et al.*, 1994), even though identical primers were used in the PCR reaction. The predicted amino acid sequence for ovine actin in the present study is 93.4, 90.6 and 88.7% homologous to those of frog (Cross *et al.*, 1988), human (Erba *et al.*, 1986) and ovine actin from a previous report (Bacich *et al.*, 1994), respectively.

Nested PCR

Nested PCR consistently revealed bands of three different products (Fig. 3), whereas no detectable products were present in the control reactions. The three products (A, B and C in Fig. 3) were similar in length to three alternatively spliced variants

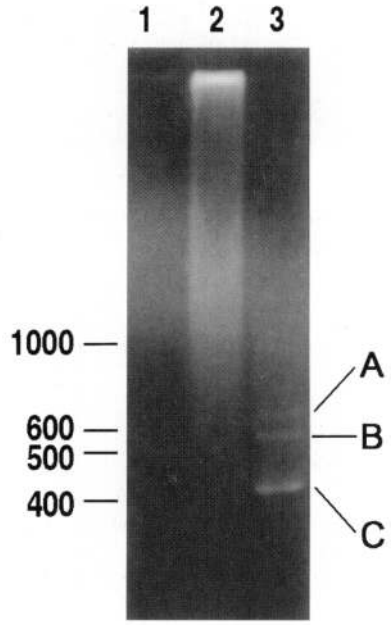

Fig. 3. Agarose gel showing PCR products from the amplification of cDNA from sheep corpora lutea at the early stage of the oestrous cycle. Nested PCR was conducted with the specific internal primers to sheep vascular endothelial growth factor (VEGF) as shown in Fig. 1 and described in Materials and Methods. In lane 3, although not sequenced, bands labelled A, B and C correspond in length to $VEGF_{188}$, $VEGF_{164}$ and $VEGF_{120}$, respectively. Lanes 1 and 2 are control reactions demonstrating the absence of any detectable product. Locations of the standard ladder fragments also are shown.

already described for humans and were likely derived from exons 1–5, 6, 7 and 8 ($VEGF_{189}$); 1–5, 7 and 8 ($VEGF_{165}$); and 1–5 and 8 ($VEGF_{121}$), respectively (Houck *et al.*, 1991). The number of amino acids in the mature protein for each variant is given above for humans; however, there is a single amino acid deletion in other species (see Fig. 1) which gives rise to mature VEGF proteins of 188, 164 and 120 amino acids, respectively.

RNase protection assays

An RNase protection assay was used to detect and quantitate mRNA encoding VEGF. Labelled antisense probes for VEGF and actin (positive control) were fully transcribed from their cDNA clones and appeared as single bands at their predicted lengths of 587 and 266 nucleotides, respectively, following polyacrylamide gel electrophoresis. These probes were completely digested when mixed with yeast RNA and subjected to RNAse treatment. However, when these probes were mixed with RNA isolated from ovine corpora lutea and subsequently subjected to RNAse treatment, the resulting hybrids were protected from RNAse digestion and appeared as specific bands. When the labelled antisense VEGF probe was used for hybridization, a band appeared at the predicted length of 464 nucleotides for mRNA encoding $VEGF_{120}$ (Fig. 4, top arrow) and also at the predicted length of 420 nucleotides for mRNA encoding all other variants of VEGF (Fig. 4, bottom arrow). When the actin probe was used for hybridization, five distinct bands ranging between 100 and 160 nucleotides in length were observed.

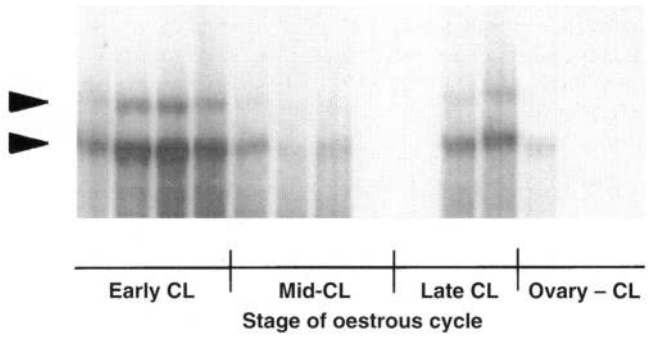

Fig. 4. Autoradiograph of a polyacrylamide gel showing protected mRNA hybrids from hybridization reactions that contained the probe for labelled antisense sheep vascular endothelial growth factor variant $VEGF_{120}$ and total cellular RNA from early (days 2–4), mid- (day 8) and late (days 14–15) cycle ovine corpora lutea (CL), and also from ovaries that had had luteal tissue removed (ovary – CL). The top arrowhead designates protected mRNA hybrids encoding $VEGF_{120}$ at 464 nucleotides in length. The bottom arrowhead designates protected mRNA hybrids encoding other variants of VEGF at 420 nucleotides in length. Each lane represents a hybridization reaction using luteal RNA from an individual ewe.

In all assays, the density of VEGF and actin bands decreased with decreasing amounts of RNA (50–12.5 μg RNA) in the hybridization reactions; this indicates that both labelled probes were used in amounts sufficient to make quantitative comparisons between samples. Scintillation counting of protected hybrids from reactions containing the labelled antisense mRNA encoding VEGF and different doses of RNA from a corpus luteum at the early stage of the oestrous cycle showed that the amount of protected mRNA encoding VEGF decreased proportionately with dose of luteal RNA (870, 476, and 276 c.p.m. for 50, 25 and 12.5 μg RNA, respectively). In addition, when labelled VEGF and actin probes were used in the same hybridization reaction, protected fragments from each probe were resolved, and these were similar to those observed when the probes were used separately.

Protected VEGF hybrids (Fig. 4) and densitometric measurement (Fig. 5) of these protected hybrids are shown for RNA isolated from early, mid- and late cycle ovine corpora lutea. On the basis of the densitometric measurements, mRNA concentrations for all of the forms of VEGF were 2- to 3-fold greater ($P < 0.01$) in early compared with the mid- and late cycle corpora lutea, which in turn were greater ($P < 0.05$) than mRNA concentrations in ovarian tissues without corpora lutea. In another RNase protection assay, when the hybridization reactions were run with only the VEGF probe (that is, actin probe omitted as a control), scintillation counting of the VEGF hybrids confirmed the densitometric measurements in that concentrations of mRNA encoding VEGF were greatest ($P < 0.01$) in early cycle corpora lutea (Fig. 6).

Across all stages of the oestrous cycle, the luteal values of the $VEGF_{120}$ hybrid were less ($P < 0.01$) than those of the hybrid representing the other variants of VEGF (Figs 4, 5), but represented a significant proportion (34.4%) of the total mRNA encoding VEGF. The ratio of mRNA encoding $VEGF_{120}$ to that of the other variants did not differ ($P > 0.05$) across stage of cycle (that is, early versus mid- versus late), or between tissue

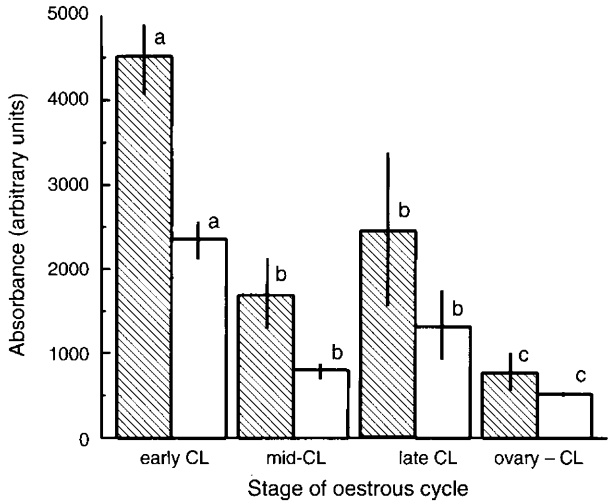

Fig. 5. Absorbance of protected mRNA hybrids encoding vascular endothelial growth factor (VEGF), as shown in Fig. 4, for RNA from early (days, 2–4), mid- (day 8) and late (days 14–15) cycle ovine corpora lutea (CL) and also from ovaries minus luteal tissue (ovary – CL). Absorbances (arbitrary units) were measured by a scanning laser densitometer. For each mRNA variant ((▨) other VEGF or (□) VEGF120), means (± SEM) with different superscripts are significantly different.

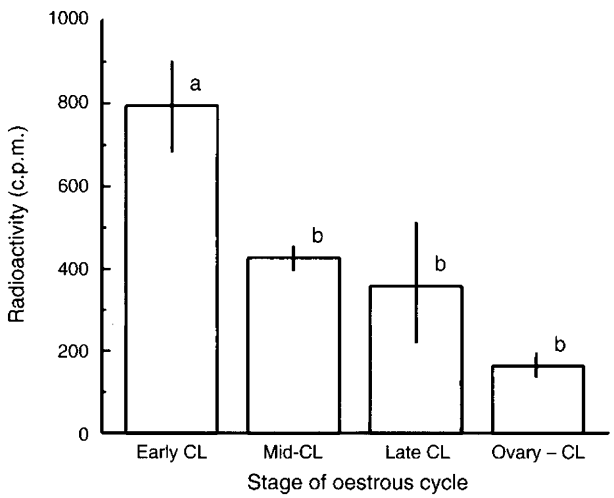

Fig. 6. Quantification by scintillation counting of protected mRNA hybrids for vascular endothelial growth factor (VEGF) (as shown in Fig. 4) for total cellular RNA isolated from early (days 2–4), mid- (day 8) and late (days 14–15) cycle ovine corpora lutea (CL) and also from ovaries minus luteal tissue (ovary – CL). Note that, in contrast with the data shown in Fig. 5; these data are for all of the forms of VEGF, including $VEGF_{120}$. Means (± SEM) with different superscripts are significantly different.

types (that is, corpora lutea versus ovary minus corpora lutea). The density of the actin bands appeared to be relatively constant from sample to sample (data not shown), and for all bands, there was no stage of cycle effect ($P > 0.2$). The consistently low amounts of mRNA encoding VEGF in samples of ovary minus corpora lutea, even though actin values were comparable to corpora lutea samples (data not shown), provides a VEGF-negative control for the protection assay.

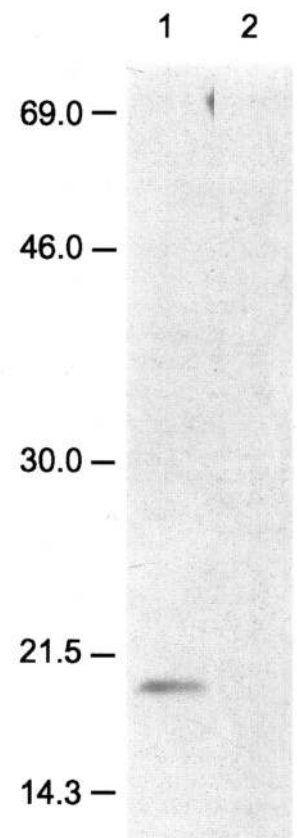

Fig. 7. Western immunoblot after SDS–polyacrylamide gel electrophoresis of a homogenized pool of corpora lutea from sheep during the early part of the oestrous cycle (180 μg per lane). Antiserum against a 15-amino acid peptide from the N-terminal region of ovine vascular endothelial growth factor (VEGF) and peptide neutralized antiserum were used for the immunoblot (lanes 1 and 2, respectively).

Although not shown here, in preliminary experiments, protected hybrids for VEGF were also found in samples of ovine RNA from both kidney and liver of mature ewes.

Western immunoblot analysis

SDS–polyacrylamide gel electrophoresis and western immunoblotting of a pooled, early cycle corpora lutea homogenate showed a single 18 kDa band when probed with an antiserum (Red1-b3) against an N-terminal peptide of ovine VEGF (Fig. 7, lane 1). A lane in which recombinant human VEGF was subjected to electrophoresis failed to show a detectable band (data not shown). When the antibody was presorbed with excess peptide before immunoblotting, the 18 kDa band was no longer present (Fig. 7, lane 2).

Discussion

This study is the first report of the sequence for ovine mRNA encoding VEGF and its use to evaluate expression of VEGF in ovine reproductive tissues. In addition, we report the development and successful use of an antibody against sheep VEGF. This antibody should recognize all of the forms of VEGF since it was made against a peptide that was translated from a region

traversing exons 2 and 3, which are contained in all known forms of VEGF (Tischer *et al.*, 1989, 1991; Conn *et al.*, 1990; Houck *et al.*, 1991; Claffey *et al.*, 1992; Sharma *et al.*, 1995). The reagents developed in this study will be important, not only for elucidating the role of VEGF in vascular development of ovarian, uterine and placental tissues, but also because the sheep is widely used as a model for studies of reproductive function of large animals.

The cDNA sequence for VEGF reported in this study codes for the 120 amino acid variant of VEGF and contains exons 1–5 and 8, as for other species (Tischer *et al.*, 1991). This sequence exhibits a high homology with VEGF from other species, with the greatest variation near the N-terminal region of the mature protein. We have shown, using nested PCR, that there are three splice variants of ovine mRNA encoding VEGF in sheep corpora lutea. Since all splice variants contain exons 1 to 5 and 8, products of a nested PCR reaction using oligonucleotide primers specific for regions near the start and stop codons (exons 1 and 8, respectively) will exhibit high specificity toward the alternatively spliced variants of VEGF and should easily be resolved on agarose gels. Therefore, the positions that PCR products migrated to on gels in this study strongly suggest that they are indeed the three major variants reported for the human VEGF sequences coding for proteins of 189, 165 and 121 amino acids (Houck *et al.*, 1991). For other species, including sheep, a single amino acid deletion in the variable N-terminal region results in a predicted amino acid sequence of 188, 164 and 120 residues.

An RNase protection assay was established and validated in order to determine the relative abundance of mRNA encoding VEGF in ovine corpora lutea throughout the oestrous cycle. Two protected mRNA hybrids were resolved on the gels. If sheep VEGF variants are similar to those from other species, the mRNA for the 120 amino acid variant of VEGF was observed as the longer hybrid because it contains exons 1–5 and 8, which hybridized to the antisense probe containing the same exons (Tischer *et al.*, 1989, 1991; Conn *et al.*, 1990; Claffey *et al.*, 1992; Sharma *et al.*, 1995). Since the antisense probe did not contain exons 6 or 7 (exons which are found in mRNA variants of VEGF other than $VEGF_{120}$), RNase digested the other hybrids (that is, other than $VEGF_{120}$) immediately after exon 5 and presented the variants as a shorter hybrid, which appeared as a single band on the electrophoresis gels. Thus, mRNA for variants of VEGF other than $VEGF_{120}$ was observed as the shorter hybrid because each variant hybridized only to exons 1–5 of the antisense probe, whereas in nested PCR, each splice variant present could be resolved as a separate band.

On the basis of results of the RNase protection assay, $VEGF_{120}$ represented about one-third of the total mRNA encoding VEGF present in the corpora lutea, and this proportion was constant throughout the oestrous cycle. This proportion also was similar to that in ovarian tissue that did not contain luteal tissue. In other species, this 120 amino acid form of VEGF protein does not bind to heparin, is secreted and is freely soluble (Ferrara, 1993). Other forms of VEGF, however, are heparin-binding, can be immobilized in the extracellular matrix and are released from the bound state by a variety of agents (Houck *et al.*, 1992). Taken together, these observations provide evidence for a highly flexible system for the regulation of angiogenesis in the corpus luteum. Determination of proportions of other VEGF variants in the ovine corpus luteum will be the subject of future studies, when cDNAs for these variants have been cloned.

Because the corpus luteum undergoes tremendous neovascularization during its development (Reynolds *et al.*, 1992; Smith *et al.*, 1994; Redmer and Reynolds, 1996), the underlying mechanisms that regulate luteal vascular growth are likely to be intimately associated with the growth and function of this endocrine organ. As discussed earlier, a significant observation from our attempts to characterize factors that stimulate angiogenesis is that all of the activity binds relatively strongly to heparin (Grazul-Bilska *et al.*, 1992, 1993, 1995). In addition, it is apparent from our work and that of others that multiple angiogenic factors are produced by the corpus luteum (Rone and Goodman, 1990; Ricke *et al.*, 1995; Redmer and Reynolds, 1996). An attractive candidate for one of these factors is VEGF, since it binds to heparin with affinity similar to that of one of the luteal angiogenic factors we have reported (Grazul-Bilska *et al.*, 1992, 1993, 1995) and is a relatively specific mitogen and chemoattractant for endothelial cells (Ferrara, 1993). Recently, we have shown that an antibody against VEGF will immunoneutralize the endothelial chemoattractant activity produced by early cycle, but not mid- or late cycle, ovine corpora lutea (Doraiswamy *et al.*, 1995). In addition, a portion (approximately 40%) of the endothelial mitogenic activity produced by pig corpora lutea can be neutralized by VEGF antibody (Ricke *et al.*, 1995).

In this report, we have shown by the RNase protection assay that the ovine corpus luteum contains mRNA encoding VEGF at all stages of luteal development. Moreover, luteal concentrations of mRNA encoding VEGF are greatest during the early stage of the cycle, a time when neovascularization is occurring. Likewise, in monkeys, luteal mRNA encoding VEGF is present in greatest amounts in the early luteal compared with the late luteal phase (Ravindranath *et al.*, 1992). These observations suggest that VEGF plays an important role in vascular development of the corpus luteum.

In addition, Phillips *et al.* (1990) have found VEGF in rat corpora lutea, and others have demonstrated the presence of mRNA encoding VEGF in luteinized human granulosa cells (Yan *et al.*, 1993; Kamat *et al.*, 1995). It has also been shown that treatment with GnRH antagonist reduces concentrations of mRNA encoding VEGF in monkey corpora lutea (Ravindranath *et al.*, 1992). Conversely, hCG or LH induces VEGF transcription in preovulatory rat follicles and in bovine granulosa cells that are undergoing luteinization (Garrido *et al.*, 1993; Koos, 1995). These observations suggest that humoral factors may provide for additional flexibility in regulating angiogenic processes in the corpus luteum by regulating expression of VEGF.

Although it seems likely that the VEGFs are important angiogenic factors during early luteal development, we have shown, in several mammalian species, that angiogenic factors are produced throughout the lifespan of the corpus luteum (Grazul-Bilska *et al.*, 1992, 1993, 1995; Ricke *et al.*, 1995; Redmer and Reynolds, 1996). As mentioned above, it is also apparent that multiple heparin-binding angiogenic factors are produced by these corpora lutea (Redmer and Reynolds, 1996). In this regard, the angiogenic activities produced by the

corpora lutea of cows, pigs and sheep can be partially immunoneutralized with antibody against fibroblast growth factor 2 at all stages of luteal development (Doraiswamy *et al.*, 1995; Grazul-Bilska *et al.*, 1995; Ricke *et al.*, 1995). Fibroblast growth factor 2 has also been detected in bovine and ovine luteal tissues and conditioned media throughout luteal lifespan (Grazul-Bilska *et al.*, 1992; Zheng *et al.*, 1993; Doraiswamy *et al.*, 1995; Ricke *et al.*, 1995), and expression of mRNA encoding fibroblast growth factor 2 in bovine corpora lutea follows a pattern similar to that of angiogenic activity (Redmer *et al.*, 1988; Stirling *et al.*, 1991). Thus, fibroblast growth factor 2 is probably also a major angiogenic factor present in corpora lutea. On the basis of the observation that fibroblast growth factor 2 is produced by corpora lutea at all stages, however, we have suggested that it may be involved not only in luteal vascular development but also in maintaining vascular beds of mature corpora lutea, and might even have other roles in differentiation and function of the corpus luteum (Grazul-Bilska *et al.*, 1992, 1995; Redmer and Reynolds, 1996).

In the present study, SDS-PAGE and western immunoblot analysis demonstrated that at least one VEGF protein is present in corpora lutea at the early stage of the oestrous cycle. This protein is approximately 18 kDa and was not detected when the VEGF antiserum was presorbed with the antigen. As described by Ferrara *et al.* (1992), the human variants $VEGF_{121}$ and $VEGF_{165}$ can exist as glycosylated or unglycosylated species, and each exhibits one form having a molecular mass of 18 kDa. It is not surprising that the Red1 antibody failed to detect recombinant human VEGF because the amino acid sequence for the peptide used to raise the Red1 antibody differed considerably from the human amino acid sequence. Although these data are preliminary, they do show the successful design and production of the 15 amino acid peptide, the successful production of the antipeptide ovine VEGF antibody (Red1), and its successful validation and use for determining VEGF protein via western immunoblot analysis. This antibody, in turn, will be useful for ongoing studies designed to examine more closely the production of VEGF protein in a variety of tissue samples throughout the oestrous cycle as well as to immunolocalize VEGF in corpora lutea at different stages of development.

In summary, in this study we have reported the sequence for one variant of ovine VEGF. In addition, in this study, we have determined the relative abundance of the mRNA transcripts encoding VEGF in ovine corpora lutea throughout the oestrous cycle. Finally, we have identified an 18 kDa VEGF protein in corpora lutea at the early stage of the oestrous cycle. The proportion of other mRNA variants expressed in ovine corpora lutea, and which of these forms of VEGF play physiological roles in the regulation of luteal vascular development, remains to be determined.

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