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Keratinocyte Growth Factor Expression by the Bovine Corpus Luteum

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ABSTRACT

Communication between cells of the corpus luteum (CL) is thought to be necessary for normal luteal function. Keratinocyte growth factor (KGF) is produced by mesenchymally derived cells in numerous tissues and acts on epithelial cells. In bovine follicles, theca cells produce KGF, which can stimulate granulosa cell proliferation. Whether KGF is produced by ovarian cells after luteinization is unknown. Our objective was to determine whether KGF mRNA and protein were present in bovine luteal tissue, and if so, to determine what type(s) of luteal cells contains KGF. CL (n = 3–4/day) were obtained from specific days throughout diestrus. Presence of KGF mRNA in CL was determined using a porcine KGF anti-sense cRNA probe. Northern analyses of luteal tissue poly(A)$^+$ RNA revealed a single transcript (~2.0 kilobases), the quantity of which did not change throughout diestrus. Western analysis revealed an immunoreactive band (28 kDa) in luteal tissues and theca cell homogenates that was absent from granulosa cell homogenates. Immunocytochemistry showed KGF predominantly in theca and small luteal cells. Results indicate that bovine CL produce and contain KGF, which is primarily localized in small luteal cells. Therefore, KGF may participate in paracrine communication within the bovine CL.

INTRODUCTION

The corpus luteum (CL) produces and secretes progesterone, which is required for initiation and maintenance of pregnancy in mammals [1]. In many mammalian species, the CL consists of two different steroidogenic cell types, small and large luteal cells [2–4], formed by differentiation of ovarian follicular cells after ovulation [5]. It has been hypothesized that small and large luteal cells are derived specifically from theca and granulosa cells, respectively [5–7]. Furthermore, some small cells may develop into large cells as the CL ages [5–7].

Just as intercellular communication is thought to be important for function of theca and granulosa cells in the ovarian follicle, it is also thought that intercellular communication is important for CL function. Large and small luteal cells contact each other by developing gap junction-like structures [8]. In response to LH stimulation, cell-to-cell contact between large and small luteal cells increases progesterone production synergistically, reflecting the importance of intercellular contact and communication [9]. Although these cellular interactions are thought to be essential during the life span of a normally functioning CL, little is known about specific autocrine/paracrine mediators of luteal function or their role in this system.

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Expression and production of a variety of growth factors such as insulin-like growth factor I and II (IGF-I, IGF-II), basic fibroblast growth factor (bFGF), and transforming growth factor α (TGFα), and their receptors, have been identified and localized in the ovarian follicle [10–13]. These growth factors appear to mediate interactions between theca and granulosa cells [10]. Messenger RNA expression and production of IGF-I and IGF-II, TGFα, and bFGF have been localized in the CL [14–16] and are proposed to play important roles in mediating progesterone production during the estrous cycle and pregnancy by acting through paracrine mechanisms [17].

Keratinocyte growth factor (KGF), also known as fibroblast growth factor-7 (FGF-7), is a 28-kDa heparin-binding protein that is produced by mesenchymally derived cells and acts in a paracrine manner on epithelial cells to stimulate DNA synthesis and cell differentiation [18]. Parrott et al. [19] identified KGF in the bovine ovarian follicle and showed that KGF is produced exclusively by theca cells. KGF from theca cells can stimulate proliferation of granulosa cells in a paracrine fashion, suggesting that KGF may play an important role in follicular development, steroid production, and/or ovulation. If KGF expression continues in luteal cells after ovulation and luteinization, this growth factor may be involved in intercellular communication within the CL.

The aim of the present study was to determine whether KGF mRNA and KGF protein are present in the bovine CL and, if so, to quantify KGF mRNA during diestrus and to determine the specific cell type(s) that contains immunoreactive KGF.

MATERIALS AND METHODS

Animals

CL were obtained from crossbred beef or Holstein dairy cattle on defined days of the estrous cycle (estrus = Day 0): Days 2–3 (early), 8–10 (mid), 16–18 (late), and 19 (very late). CL from both beef and dairy cattle were included in each day category. Ovaries were collected surgically, via standing flank laparotomy using local anesthesia, or after commercial slaughter, and CL were dissected from ovarian stroma. Tissues used for analysis of RNA and immunohistochemistry were obtained on defined days of the estrous cycle. Tissues used for Western immunoblotting were obtained from a slaughterhouse. All procedures involving animals were approved by the Auburn University Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for Care and Use of Agricultural Animals in Teaching and Research.

Tissue Collection and Preparation

Upon collection, individual CL were isolated from the ovary, washed with sterile Dulbecco's PBS (Sigma, St. Louis, MO), and dissected. One piece of each CL was immediately frozen in liquid nitrogen and stored at −80°C for

later Northern and slot blot analyses of RNA. Another piece of each CL was fixed in 4% paraformaldehyde (in PBS) and then embedded in Paraplast Plus (Fisher Scientific, Denver, CO) for immunocytochemistry.

For total RNA isolation, each CL was weighed and homogenized with a Tissue Tearor (Biospec, Bartlesville, OK) in 1 ml of TRIzol (Life Technologies, Gaithersburg, MD) per 100 μg of tissue. Total RNA was obtained after phenol-chloroform extraction and ethanol wash and stored (100% ethanol:3 M sodium acetate [30:1]) until poly(A)$^+$ RNA isolation using the Promega PolyATtract kit (Madison, WI).

Luteal and follicular tissue samples used for Western immunoblotting were collected from a local slaughterhouse and transported on dry ice after the tissues were snap-frozen in liquid nitrogen. CL obtained from the slaughterhouse were grouped into stages of diestrus (early, mid, and late) on the basis of anatomic characteristics described by Ireland et al. [20]. Thecal and granulosal layers were separated in isolated follicles by freeze/thaw separation [21]. CL were removed from the ovary and decapsulated. Thecal, granulosal, and luteal tissues were homogenized with a glass homogenizer in TE buffer (10 mM Tris, pH 7.4 and 1 mM EDTA). Cytosol was isolated from homogenized samples by centrifugation (10 min at 10 000 × *g*, 4°C) and stored at −80°C until analyzed for the presence of KGF. In order to obtain proteins from granulosa cells, follicular fluid was centrifuged at 4°C for 10 min at 10 000 × *g*. The pellet, consisting of granulosa cells, was resuspended with TE buffer and homogenized with a glass homogenizer in TE buffer. Homogenized samples were centrifuged at 4°C for 10 min at 10 000 × *g* to obtain the cytosol, which was then stored at −80°C until analyzed for the presence of KGF.

Northern Analysis

Individual poly(A)$^+$ RNA samples (~3 μg) from Days 2–3, 8–10, 16–18, and 19 postestrus were electrophoretically separated on a 1% agarose gel containing single-strength 3-(*n*-morpholino)propanesulfonic acid (MOPS; 0.1 M MOPS [pH 7.0], 40 mM sodium acetate, and 5 mM EDTA [pH 8.0]) with 6% formaldehyde (v:v). After the agarose gel was treated with 50 mM NaOH, the RNA was transferred onto Nytran membranes (Schleicher and Schuell Inc., Keene, NH) using the Model 785 Promega Vacuum Blotter (Promega) with 10-strength SSC (single-strength SSC is 150 mM NaCl, 15 mM sodium citrate). Membranes were subjected to UV light at 120 000 kJ (Stratagene, La Jolla, CA) to cross-link RNA onto membranes.

Antisense and sense cRNAs were generated using an in vitro transcription kit (Ambion, Austin, TX) and porcine KGF cDNA template linearized with *Xho* I or *Spe* I endonucleases. The porcine KGF cDNA was from Liu et al. [22]. Probes were labeled with [α-^{32}P]uridine triphosphate (UTP; ICN, Costa Mesa, CA) to a specific activity of approximately 1×10^8 cpm/μg. For analyses, membranes were prehybridized for 5 h at 55°C in buffer containing 50% formamide, 50 mM sodium phosphate, 5-strength SSC, 0.1% SDS, 0.5 M EDTA, 0.5-strength Denhardt's reagent (5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g BSA), and 200 μg/ml of denatured salmon sperm DNA. The labeled probe was denatured at 68°C and added directly to the hybridization buffer. Membranes were hybridized for 48 h and then washed with 0.1-strength SSC containing 0.1% SDS (w:v) three times for 20 min each at 68°C. Signals were detected after exposure of the blots to a beta imaging screen for 14 h and were visualized by a Model GS-525 Molecular Imager (Bio-Rad, Hercules, CA).

Slot Blot Analysis

Two different amounts of poly(A)$^+$ RNA (0.5 and 1.0 μg) from each sample were applied onto the same nitrocellulose membrane using a hybri-slot manifold (Life Technologies, Inc.). Two separate pieces of nitrocellulose membrane were required in order to analyze all samples. Membranes were hybridized with [α-^{32}P]UTP-labeled KGF cRNA probe. Signals were detected by the Model GS-525 Molecular Imager and quantified with Molecular Analyst data analysis software (Bio-Rad). Intensity of each signal was recorded by measuring pixel density in a standard region (18 mm^2). Data obtained from samples of 0.5 μg poly(A)$^+$ RNA were normalized by multiplying by 2 to compare with data obtained from 1.0-μg poly(A)$^+$ RNA samples. Data were corrected for background by subtracting the average background of 6 different randomly chosen regions of the same membrane from each individual sample. After stripping the KGF cRNA probe from the nitrocellulose membrane by washing with 50% formamide and 6-strength SSPE (single-strength SSPE is 0.18 M NaCl, 10 mM sodium phosphate [pH 7.7], 1 mM EDTA) at 65°C for 1 h, blots were rinsed with double-strength SSPE, and rehybridized with an [α-^{32}P]deoxycytidine triphosphate (dCTP)-labeled β-actin cDNA probe generated using the RadPrime DNA labeling system (Life Technologies, Inc.). Signal detection and quantitative evaluation were carried out as described above. Values for steady-state levels of KGF poly(A)$^+$ RNA for each CL were expressed relative to the β-actin signal.

Western Immunoblotting

Sixty-five micrograms of cytosolic protein from early, mid, and late CL and from granulosa and theca cell samples were subjected to SDS-PAGE on a 12% (total monomer) minigel, and separated protein bands were then electroblotted onto nitrocellulose membranes (Bio-Rad). Cytosolic protein obtained from granulosa and theca cells was used as negative and positive control, respectively, for the presence of KGF in Western blot experiments [19]. Membranes were incubated overnight with either a 1:1000 dilution (1.4 μg/ml of protein G-purified immunoglobulin) of polyclonal KGF antibody (raised against a synthetic peptide corresponding to KGF carboxyl-terminal sequence residues 179-194; generously provided by Dr. Jeffery S. Rubin, National Institutes of Health, Bethesda, MD), 1.4 μg/ml of normal rabbit IgG (Jackson Immunological Research Laboratories, West Grove, PA), or secondary antibody alone. Bound primary antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) and Bio-Rad color developing reagents according to the manufacturer's protocol.

Immunohistochemistry

KGF was immunolocalized in tissue sections with the same anti-KGF antibody used for Western immunoblotting. The anti-KGF antibody was prepared in 0.01 M PBS (pH 7.4) containing 1% (w:v) BSA and was used at 5 μg/ml. Staining was accomplished using the peroxidase-based BioStain (anti-rabbit IgG) Super ABC immunoassay detection system (Biomeda, Foster City, CA). Paraplast Plus-(Fisher Scientific) embedded luteal tissues representing dif-

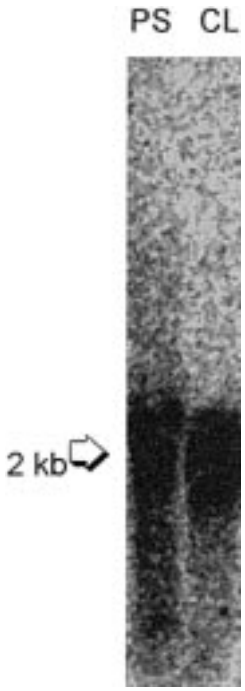

FIG. 1. Northern blot analysis showing a single ~2.0-kb transcript for KGF in pig skin (PS; 15 μg total RNA) and bovine CL (3 μg poly[A]$^+$ RNA) detected using [^{32}P]UTP-labeled KGF antisense cRNA probe.

ferent age groups of CL, as well as antral ovarian follicles (n = 3), were sectioned serially at 6-μm thickness and mounted on poly-L-lysine-coated slides. Sections were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked by incubating sections with 1% H_2O_2 in methanol for 30 min. Antigenic sites were unmasked by pronase (Pronase E; Sigma, type XIV, 0.5 mg/ml in PBS) digestion at 37°C for 5 min. Sections were then rinsed in cold tap water and placed in PBS for 5 min. Subsequent procedures followed were those suggested by the kit manufacturer. Briefly, sections were incubated with the diluted protein blocker reagent for 30 min at room temperature to reduce background staining. Sections were then exposed to anti-KGF. Negative controls consisted of normal rabbit IgG (Jackson Immunological Research Laboratories) in place of anti-KGF, as well as samples incubated with anti-KGF that had been preabsorbed with 300 ng purified KGF (Sigma) to determine nonspecific staining of the first antibody. Additionally, some sections were incubated with PBS and 1% BSA in place of anti-KGF to determine nonspecific staining of the secondary antibody. After application of the first antibody (or negative controls), the slides were incubated overnight at 4°C with KGF antibody at 5 μg/ml in 1% (w:v) BSA in 0.1 M PBS. On the following day, slides were washed in PBS for 5 min, incubated with the biotinylated secondary antibody for 30 min at 37°C in a humidified chamber, and then washed in PBS for 5 min at room temperature. Sections were then incubated with the streptavidin-labeled peroxidase complex for 30 min at 37°C, washed in PBS for 5 min, and placed into a freshly prepared peroxidase substrate solution (0.1% diaminobenzidine tetrahydrochloride in 0.1 M Tris buffer, pH 7.2, and an equal volume of 0.02% H_2O_2 prepared from a 30% stock) until a brown color developed. Sections were counterstained with Harris' hematoxylin (Fisher Scientific), dehydrated, and cleared in HemoDe (Fisher Scientific), and coverslips were attached with Permount.

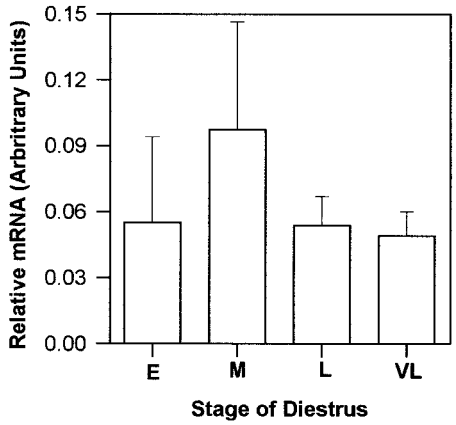

FIG. 2. Expression of KGF mRNA in bovine CL relative to expression of β-actin throughout diestrus. KGF and β-actin expression were detected in two different quantities (0.5 and 1 μg) of poly(A)$^+$ RNA using [^{32}P]UTP-labeled KGF antisense cRNA and β-actin cDNA probes (n = 3–4). E, early CL (Days 2–3 postestrus); M, mid CL (Days 8–10 postestrus); L, late CL (Days 16–18 postestrus); VL, very late CL (Day 19 postestrus).

Luteal and follicular cells were counted and categorized [23, 24] in 20 fields (114 × 114 mm^2/section) chosen randomly on a single section of luteal or follicular tissue from each animal (approximately 1200 cells/section). KGF staining frequency of luteal and follicular cells was evaluated by counting the number of individual stained and nonstained cell types in each randomly chosen field. For luteal tissue sections, categories of cell types were small luteal cells, large luteal cells, fibroblasts, and endothelial cells. For follicular tissue sections, categories of cell types were theca cells, granulosa cells, fibroblasts, and endothelial cells. KGF labeling index (LI), was defined as the proportion (%) of cells of each type that stained positively for KGF and was calculated by dividing the total number of stained cells of one type by the total number of cells of the same type, and multiplying that ratio by 100.

Statistical Analysis

The effect of the stage of diestrus on mean values of KGF poly(A)$^+$RNA and KGF LI was determined by using ANOVA (Sigma Plot; Jandel Scientific, Corte Madera, CA). ANOVA on ranks was performed when heterogeneity of variance was detected. Data for KGF LI are presented as least-square means with standard errors.

RESULTS

Northern blot analysis of 25 μg total cellular RNA from CL did not allow detection of KGF transcripts. Therefore, poly(A)$^+$ RNA was extracted from each CL sample and used for Northern and slot blot analyses. Using the [^{32}P]UTP-labeled porcine KGF antisense cRNA probe, a single transcript was identified at the expected size of approximately 2.0 kilobases (kb) in 3 μg of poly(A)$^+$ RNA samples isolated from CL during diestrus (Fig. 1). An identical transcript was found in total RNA extracted from porcine skin (15 μg). Northern blot analyses of poly(A)$^+$ RNA of luteal samples from early, mid, late, and very late CL all indicated a single KGF transcript at 2 kb (data not shown). Northern blot analyses with [^{32}P]UTP-labeled porcine KGF sense cRNA probe did not result in any specific hybridization.

Because only a single KGF transcript was detected in poly(A)$^+$ RNA from CL, subsequent KGF mRNA expres-

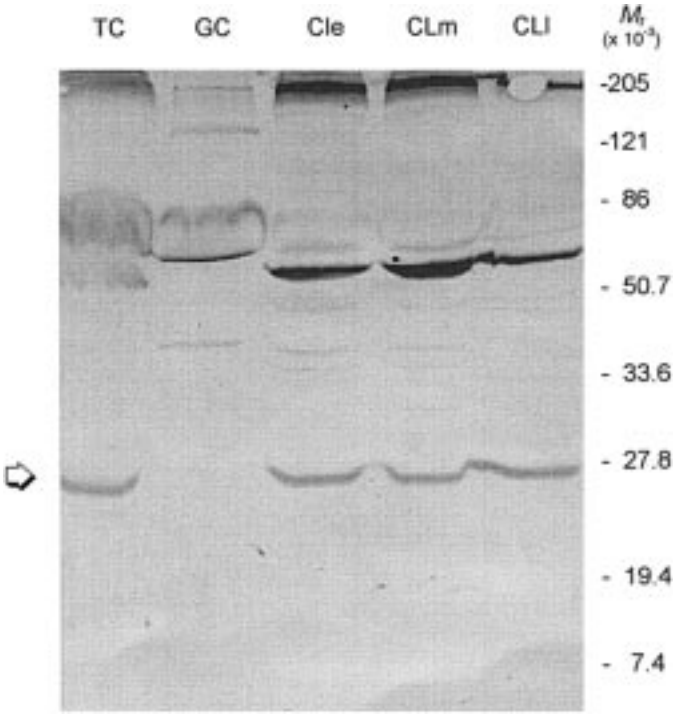

FIG. 3. Presence of KGF immunoreactivity in bovine luteal tissue extracts detected by Western immunoblotting using a polyclonal KGF antibody raised against a synthetic peptide corresponding to KGF carboxyl-terminal sequence. Theca and granulosa cell protein samples were used as positive and negative control groups, respectively. Positions of molecular weight markers are shown on right. The arrow on the left indicates 28 kDa. TC, theca cells; GC, granulosa cells; CLe, early CL; CLm, mid CL; CLl, late CL.

sion was quantified by slot blot analysis. Levels of KGF mRNA expression did not change significantly throughout diestrus (Fig. 2).

Western immunoblotting was employed to evaluate the presence of KGF protein in luteal tissue extracts. Analysis of luteal protein obtained from CL of differing ages indicated the presence of an immunoreactive 28-kDa protein band in all samples (Fig. 3).; This 28-kDa band was co-electrophoretic with a band identified in theca cell protein and was similar in size to KGF as described by Parrott et al. [19]. As expected, there was no band of KGF immunoreactivity at 28 kDa in samples of granulosa cell protein [19]. Some immunoreactive bands of high molecular mass (approximately 80 and over 200 kDa) were also detected. These can be interpreted as nonspecific binding of the polyclonal KGF antibody, since a single faint 28-kDa band was the only signal observed when a monoclonal anti-human FGF-7/KGF neutralizing antibody (R&D; Systems, Minneapolis, MN) was used in Western blot procedures. Moreover, when the polyclonal KGF antibody was preabsorbed with purified KGF, the 28-kDa band was absent, but the bands of larger molecular mass remained (data not shown). Finally, when primary or secondary antibody was omitted, or a normal rabbit IgG was substituted for KGF antibody, no bands appeared on the Western blot.

KGF was immunolocalized in tissue sections using a polyclonal antibody (Fig. 4). Staining was specific as evidenced by the lack of staining in the absence of primary antibody or secondary antibody, or after preabsorption of the primary antibody with purified KGF. The proportion (%) of luteal cells that stained positively for KGF did not change during diestrus. However, KGF LI was higher ($p < 0.05$) in small luteal cells than in other luteal cell types (Fig. 5). Additionally, a higher proportion ($p < 0.05$) of theca cells compared to other ovarian follicular cells stained positively for KGF (Fig. 5). In contrast, granulosa cells did not exhibit any specific staining. Slight, but specific, staining was also observed in ovarian stroma.

DISCUSSION

Results of the present study provide the first demonstration of the expression of KGF mRNA and the presence of KGF protein in bovine luteal tissue. The frequency of KGF immunostained luteal cells was similar to that reported for another growth factor (bFGF) in CL from pregnant ewes [25]. KGF protein was localized predominantly in theca cells and small luteal cells. The use of electron microscopy and cell markers is beneficial for characterizing specific cell types; however, our results using high-resolution light microscopy were very consistent, indicating localization of KGF primarily to theca and small luteal cells. Neither steady-state levels of KGF mRNA nor KGF immunostaining LI in luteal cells was affected by stage of diestrus.

KGF was first identified in a human embryonic lung fibroblast cell line and was found to be a potent mitogenic factor for BALB/MK epidermal keratinocytes. This growth factor is produced exclusively by mesenchymally derived cells and typically has mitogenic effects on epithelial cells [26, 27], indicating that KGF is an important mediator of mesenchymal-epithelial interactions. Since its isolation, KGF has also been shown to participate in a number of physiological processes in addition to mitogenesis, including differentiation, development of intercellular connections, and gene expression, each of which is discussed below. These effects of KGF are typically mediated in a paracrine fashion.

In the bovine ovarian follicle, KGF was found to be produced solely by theca cells [19]. The same study showed that KGF stimulated proliferation of granulosa cells, providing an example of KGF-mediated mesenchymal-epithelial interaction in the ovary. Results from the present study support the idea that a similar relationship may be maintained after luteinization and mediated, at least in part, by KGF. Given the known functions of KGF, numerous processes during luteal development, function, and demise may be influenced by this growth factor.

Formation of the CL (luteinization) involves extensive tissue remodeling [28] and rapid cellular proliferation [29, 30] similar to that which occurs during wound healing and tumor formation [31]. Interestingly, expression of KGF increases dramatically during wound healing in mouse skin [32] and is thought to enhance the rate of re-epithelization and to stimulate proliferation and differentiation of early progenitor cells in rabbit skin [33]. Thus, KGF may act to support luteal development as both a mitogenic factor, stimulating proliferation, and as an effector of luteal cell differentiation. Presently, it is unknown which, if any, cells in the CL are targets for KGF; however, the mitogenic activity of KGF could act to support angiogenesis as well as the proposed differentiation of small luteal cells into large luteal cells.

As in many mammalian CL, bovine CL consist of two distinct steroidogenic cell types: small and large [23]. Both cell types are capable of producing progesterone, but regulation of progesterone production differs between these cells [3, 23, 34]. Basal production of progesterone per cell is higher in large luteal cells than in small cells [23, 35];

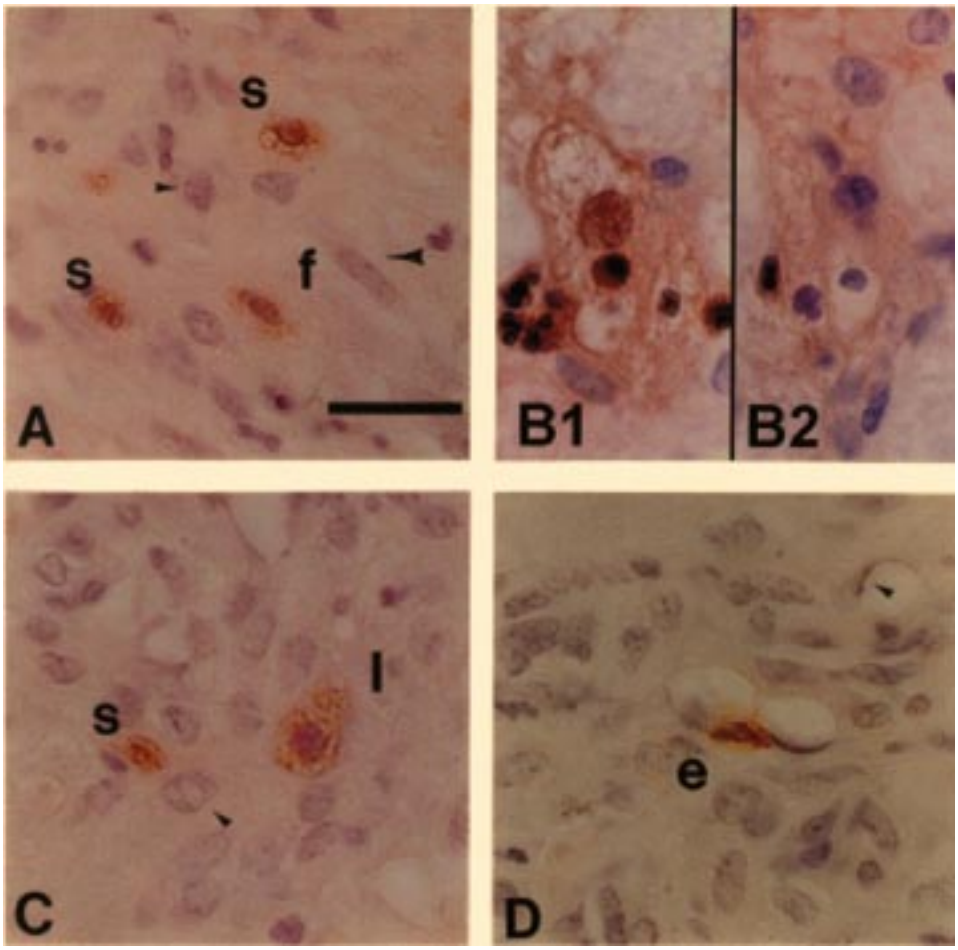

FIG. 4. Photomicrographs depicting KGF immunostaining (identified as a brown precipitate) in sections of a Day 10 CL. **A**) KGF-positive small luteal cell (s) and fibroblast (f). Small arrowhead indicates a nonstained small cell. Large arrowhead indicates a nonstained fibroblast. **B1** and **B2**) Photomicrographs of serial sections of CL after localization of KGF immunostaining with (**B2**) or without (**B1**) preabsorption of the KGF antibody with purified KGF. **C**) KGF-positive small (s) and large (l) luteal cells. Arrowhead indicates a nonstained large luteal cell. **D**) KGF-positive endothelial cell (e). Arrowhead indicates a nonstained endothelial cell. Cell types were distinguished from each other on the basis of their morphology using a light microscope at ×100 magnification. Bar = 25 μm.

however, the regulation of progesterone production by these cells is not yet completely defined. One suggested explanation for regulation of progesterone production by luteal cells [36] involves cellular communication between luteal cell types. Such communication may occur by direct cell-to-cell contact, through gap junction-like structures that allow intercellular exchange of cytoplasmic molecules of less than 1.5 kDa [8, 37]. Communication may also involve paracrine or autocrine effects of substances secreted by luteal cells [9, 38]. KGF promotes formation of compact colonies with extensive intercellular connections in human prostatic cell cultures [39]. These findings raise the possibility that KGF may act to promote cell-to-cell contact in the CL.

During prostaglandin (PG) $F_{2\alpha}$-induced luteolysis in the ewe, the number of small luteal cells decreases before the number of large luteal cells decreases [40], even though the majority of $PGF_{2\alpha}$ receptors are present on large luteal cells [2], indicating that luteolysis involves cellular interactions [38, 41]. The fact that KGF mRNA is expressed and KGF protein is present in luteal tissue even late in diestrus raises the possibility that KGF may have a role in luteolysis. Interleukin-1 (IL-1) has been proposed to be necessary for complete luteolysis [41] and is a potent inducer of both KGF expression and protein in lung [42]. Thus, KGF could mediate some of the luteolytic actions of IL-1, which may explain the presence of KGF during late stages of diestrus.

In contrast, a recent study using keratinocyte cell culture showed that KGF induces a bovine nonselenium glutathione peroxidase-like gene (KRG-1; KGF-regulated gene 1) and its 27-kDa protein [43]. The KRG-1 gene product may

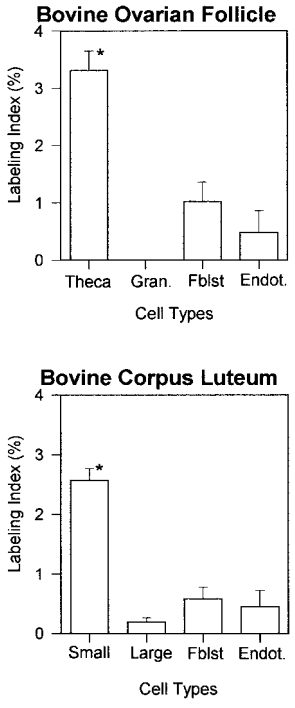

FIG. 5. KGF immunostaining of cells in the bovine CL (n = 17) and ovarian follicle (n = 3). Labeling index = proportion (%) of cells of each cell type that stained positively for KGF. * Mean differs from all other groups ($p < 0.05$). Gran, granulosa; Fblst, fibroblast; Endot, endothelial.

detoxify hydrogen peroxide, a reactive oxygen species [44]. Hydrogen peroxide is produced by bovine luteal cells [45] and is directly luteolytic in isolated rat luteal cells [46]. Although direct evidence in the bovine CL is lacking, KGF may play a role in the production of oxidative stress inhibitors, which, in turn, could protect the CL from the luteolytic effects of reactive oxygen species.

In the current study, presence of KGF mRNA and protein was demonstrated in the bovine CL. KGF was localized primarily to theca and small luteal cells. The levels of KGF mRNA and protein did not change significantly throughout diestrus. KGF may contribute to numerous functions in the CL throughout its life span, including development of the early CL, protection of the CL from accumulation of reactive oxygen species, maintenance of an optimal environment for progesterone production via cell-cell communication, and coordination of luteolysis.

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