Progesterone-dependent Expression of Keratinocyte Growth Factor mRNA in Stromal Cells of the Primate Endometrium: Keratinocyte Growth Factor as a Progestomedin

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Abstract. In vitro studies have shown that keratinocyte growth factor (KGF, also known as FGF-7) is secreted by fibroblasts and is mitogenic specifically for epithelial cells. Therefore, KGF may be an important paracrine mediator of epithelial cell proliferation in vivo. Because stromal cells are thought to influence glandular proliferation in the primate endometrium, we investigated the hormonal regulation and cellular localization of KGF mRNA expression in the rhesus monkey uterus. Tissues were obtained both from naturally cycling monkeys in the follicular and luteal phases of the cycle, and from spayed monkeys that were either untreated or treated with estradiol (E_2) alone, E_2 followed by progesterone (P), E_2 plus P, or E_2 plus P plus an antiprogestin (RU 486). Northern blot analysis of total RNA with ^{32}P-labeled probes revealed that the level of KGF mRNA in the endometrium was 70–100-fold greater in the luteal phase or after P treatment than in untreated, E_2-treated, or follicular phase animals. Northern analysis also showed that KGF mRNA was present in the myometrium but was unaffected by hormonal state. RU 486 treatment prevented the P-induced elevation of endometrial KGF mRNA. P-dependent elevation of endometrial KGF expression was confirmed by measurement of KGF protein in tissue extracts using a two-site enzyme-linked immunosorbent assay. In situ hybridization with nonradioactive digoxigenin-labeled cDNA probes revealed that the KGF mRNA signal, which was present only in stromal and smooth muscle cells, was substantially increased by P primarily in the stromal cells located in the basalis region. Smooth muscle cells in the myometrium and the walls of the spiral arteries also expressed KGF mRNA, but the degree of this expression did not differ with hormonal state. P treatment led to increased proliferation in the glandular epithelium of the basalis region and to extensive growth of the spiral arteries. We conclude that the P-dependent increase in endometrial KGF resulted from a dual action of P: (*a*) a P-dependent induction of KGF expression in stromal cells, especially those in the basalis (zones III and IV), and (*b*) a P-dependent increase in the number of KGF-positive vascular smooth muscle cells caused by the proliferation of the spiral arteries. KGF is one of the first examples in primates of a P-induced, stromally derived growth factor that might function as a progestomedin.

MANY reports suggest that the actions of sex steroids in target tissues are mediated through locally produced growth factors in an autocrine/paracrine fashion. For example, EGF (23, 31), insulin-like growth factor I (IGF-I)[1] (15, 30), and TGF-α (32) are all regarded as possible mediators of the effects of estrogens on uterine growth. Extensive evidence also exists that stromally derived mediators can promote epithelial proliferation and differentiation in steroid hormone responsive tissues (10). Such stromal–epithelial interactions in adult organs may be a special case of the more general phenomenon of mesenchymal-epithelial interactions that occur during embryonic development (9). During the menstrual cycle, the primate

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1. *Abbreviations used in this paper*: Dig, Digoxigenin; E_2, estradiol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; IGF, insulin-like growth factor; KGF, keratinocyte growth factor; KGFR, KGF receptor; oligo-DNA, oligodeoxynucleotides; P, progesterone.

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uterus undergoes dramatic, hormonally driven changes in tissue structure that resemble developmental processes, and we have hypothesized that stromally derived growth factors play important roles in such events (27).

One candidate for such a stromal mediator is keratinocyte growth factor (KGF), which was originally purified from conditioned medium of human embryonic lung fibroblasts (38). KGF is a unique member of the FGF gene family that is mitogenic for a variety of epithelial cell types, but not for fibroblasts, melanocytes, or human saphenous vein endothelial cells (13, 20). KGF mRNA has been detected in a variety of tissues (13). Because KGF has a signal peptide sequence and is produced specifically by cells of mesenchymal origin, it could function as a paracrine mediator of epithelial cell proliferation in vivo during normal growth cycles (13) and in wound healing (40, 43). Moreover, there is evidence that KGF functions as an androgen-dependent epithelial mitogen (an andromedin) in the prostate (reference 45 and Rubin, J., D. Peehl, M. Chedid, W. Taylor, D. Ron, and S. Aaronson, manuscript submitted for publication) and seminal vesicle (1). In the present studies, we examined the hormonal regulation and cellular localization of KGF in the rhesus monkey uterus. Specifically, we examined the effects of estradiol (E_2) and progesterone (P) on KGF mRNA expression by Northern blot analysis, the cellular distribution of KGF mRNA in the uterus by nonradioactive in situ hybridization (25), and the level of KGF protein by various assays.

Materials and Methods

Animals and Tissues

Uteri and oviducts were obtained from rhesus monkeys (*Macaca mulatta*) in the midfollicular ($n = 2$) and midluteal ($n = 4$) phases of natural ovulatory cycles, as determined by prospective assessment of serum E_2 and P levels (5), and from spayed animals that were either untreated ($n = 3$), treated with E_2 alone (14 d of E_2; $n = 5$), E_2 then P alone (14 d E_2, then 14 d P; $n = 2$), E_2 + P (14 d E_2, then 14 d E_2 + P; $n = 6$), or with E_2 + P + RU 486 (14 d E_2, then 14 d of E_2 + P + RU 486; $n = 2$), where RU 486 (1 mg/kg) was injected daily intramuscularly in ethanol (39). Except for RU 486, all hormones were administered in Silastic capsules filled with crystalline steroid, as described previously (44). At the end of each treatment, the animals were laparotomized, the uteri were removed and dissected into endometrial and myometrial segments, and the oviducts were dissected into fimbrial and ampullary regions. For RNA extraction, tissues were frozen in liquid propane and stored in liquid nitrogen. For in situ hybridization, tissues were cut into small pieces, embedded in OCT compound (Tissue Tek, Elkhart, IN), frozen in liquid propane, and then stored in liquid nitrogen (25).

Cells

M426 human embryonic lung fibroblasts were grown in DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin as previously described (38). B5/589 human mammary epithelial cells (41) (a gift of M. Stampfer, University of California at Berkeley) were grown in RPMI 1640 (Gibco Laboratories), to which 10% FBS, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 4 ng/ml human EGF were added.

Probes and Labeling

Human KGF cDNA [0.68 kbp, corresponding to the 5′ untranslated region and first exon (13, 24)], rhesus glial fibrillary acidic protein (GFAP) cDNA (1.2 kbp) (26) human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA [PstI-XbaI fragment of cDNA obtained from American Type Culture Collection (Rockville, MD) (no. 57090) (42)], and linearized pBR 328 DNA (Boehringer Mannheim Corp., Indianapolis, IN) were labeled with [^{32}P-α]dCTP (3,000 Ci/mmol, New England Nuclear, Boston, MA) by nick translation or random priming yielding 2.0–2.4 × 10^9 cpm/μg DNA. Also, the DNAs were labeled with Digoxigenin-dUTP (Dig-dUTP) by random priming with a labeling kit (Boehringer Mannheim Corp.). After ethanol precipitation, the pellet was suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 0.1% SDS. The concentration of Dig-labeled probe was expressed as the amount of template DNA used in the labeling reaction. Immunohistochemical detection (25) of Dig-labeled DNA on nylon membranes (Nytran; Schleicher & Schuell, Inc., Keene, NH) revealed equivalent levels of Dig-labeling among these template DNAs.

Sense and antisense strands corresponding to nucleotides 539–583 of the human KGF cDNA sequence (13) were synthesized on a DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). These 45-mer oligodeoxynucleotides (oligo-DNAs) were labeled at the 3′-end with [^{32}P-α] ATP (3,000 Ci/mmol; New England Nuclear) (21) by terminal deoxynucleotidyl transferase (Boehringer Mannheim Corp.). ^{32}P-labeled oligo-DNAs were separated from unincorporated [^{32}P-α] ATP by Nick Column™ (Pharmacia Fine Chemicals, Piscataway, NJ). The specific activities of these oligo-DNAs were 0.6–1.4 × 10^9 cpm/μg DNA.

RNA Preparation and Northern Blot Analysis

Cell monolayers were grown in 100-mm culture dishes, washed in sterile PBS, and lysed in the presence of RNAzol (Tel-Test Inc., Friendswood, TX). Tissues were pulverized in the presence of liquid nitrogen and homogenized in RNAzol. Total RNA was precipitated with isopropanol (50% vol/vol), washed in 75% ethanol, and resuspended in TE buffer (10 mM Tris-HCl [pH 7.4] 1 mM EDTA). 20-μg samples of RNA were electrophoresed on 1% formaldehyde agarose gels and transferred to Nytran nylon membranes (Schleicher & Schuell, Inc.). To evaluate the integrity of the RNA, gels were stained with ethidium bromide. After cross-linking of the RNA to the membrane, filters were prehybridized for 2 h and hybridized for 20 h at 42°C. When cDNA probes were used, filters were prehybridized and hybridized in Hybrisol (Oncor, Gaithersburg, MD) (40% formamide, 10% dextran sulfate, 1% SDS, 6× SSC, and blocking agents) and washed twice (30 min each time) at room temperature in 2× SSC, 0.1% SDS, and twice at 40°C in 0.5× SSC, 0.1% SDS. When ^{32}P-labeled oligonucleotides were used as probes, filters were prehybridized at 42°C for 2 h and hybridized for 17-72 h at 42–45°C in a solution containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.6 M NaCl, 1× Denhardt's solution, 250 μg/ml yeast transfer RNA, 125 μg/ml salmon testis DNA, 10% dextran sulfate, and 40% formamide. Membranes were washed in 0.5× SSC, 0.1% SDS at 45°C. Filters were exposed to x-ray film (Eastman Kodak, Rochester, NY) or phosphor intensifying screens. Densitometric analysis was performed with a scanner densitometer (Bio-Rad Laboratories, Richmond, CA) or a phosphorimager (ImageQuant; Molecular Dynamics, Inc., Sunnyvale, CA).

Measurement of KGF Protein

Tissue samples were thawed and homogenized with a Polytron tissue disrupter (Brinkmann Instruments, Inc., Westbury, NY) in a solution (2 ml/g wet wt) consisting of 1.0 M NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. After sonication for 30 s × 3 (power setting = 10; Heat Systems-Ultrasonics Inc., Plainview, NY) and centrifugation at 40,000 *g* for 30 min at 4°C, supernatants were analyzed for KGF using either a radioimmunoassay (4) or a two-site ELISA. The total protein concentration of the extracts was measured (Bio-Rad Laboratories), and all samples were adjusted to a uniform concentration before assay (typically concentrations varied ≤10% before adjustment). For the ELISA, all steps were performed at room temperature. In brief, 96-well polyvinyl microtiter plates (no. 3912; Falcon Labware, Oxnard, CA) were precoated with 50 μl/well of a KGF monoclonal antibody (1G4, 8 μg/ml, (4) overnight and subsequently blocked with 4% bovine serum albumin. Serial dilutions of tissue extracts (protein concentrations ≤11 mg/ml) were incubated at 50 μl/well for 5 h; then wells were washed extensively with 0.05% Tween, 0.02% sodium azide in PBS, and further incubated overnight with a rabbit polyclonal antibody (designated no. 9492) raised against recombinant human KGF. After extensive washing as above, alkaline phosphatase–conjugated goat anti-rabbit IgG (Tago Inc., Burlingame, CA) (1:15,000) was added to the wells. After 2 h, the wells were again washed and *p*-nitrophenyl phosphate (concentration 2 mg/ml) was introduced. Optical density was measured at 405 nm with an ELISA scanner (Bio-Rad Laboratories). The concentration of the recombinant human KGF standard (37) was based on amino acid analysis and extinction coefficient.

In Situ Hybridization

The procedures for preparing frozen sections (5 μm), attaching them to gelatin-coated slides, fixation, pretreatments, and hybridization of the sections have been described previously (25). Briefly, the slides were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 20 min and treated with 0.2 *N* HCl, 0.2% Triton X-100 in PBS, and proteinase K (1 μg/ml, 37°C, 15 min), successively. Hybridization was carried out at 37°C overnight in medium containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.6 M NaCl, 1× Denhardt's solution, 250 μg/ml yeast transfer RNA, 125 μg/ml salmon testis DNA, 10% dextran sulfate, 20 pmol/ml random oligo-DNA (NEP-505; Du Pont Pharmaceuticals, Wilmington, DE), 0.01% SDS, 400 U/ml heparin, 40% deionized formamide, and 0.45–0.5 μg/ml Dig-cDNA.

After hybridization, the slides were washed three times with 2× SSC/0.075% Brij 35 (Sigma Chemical Co., St. Louis, MO) at 37°C, twice with 0.5 × SSC/0.075% Brij 35 at 37°C for 1 h each, and finally washed with 0.2 × SSC at 45°C for 30 min. After successive treatments with ethanol and acetone to delipidate the sections, the signals were detected enzyme-immunohistochemically with horseradish peroxidase–labeled anti-Dig antibody, as described previously (25). We found in preliminary work that delipidation removed nonspecifically bound Dig-cDNA without affecting specific hybrids. Most of the above protocol was as previously described (25), except for the use of an oligonucleotide mixture (Randomer-36) in the hybridization medium and for the delipidation step after hybridization, both of which helped to eliminate nonspecific binding of probe to cell nuclei. The staining was observed by either bright field or phase microscopy. Because stromal cells have very scanty cytoplasm, phase microscopy was used to enhance the hybridization signal. Photomicrographs of the nonradioactive in situ hybridization preparations were prepared with 35-mm film (Technical Pan; Eastman Kodak).

Controls for In Situ Hybridization

To evaluate the specificity of KGF mRNA signals, various types of control experiments were conducted on adjacent sections. As a control probe, GFAP cDNA was used, and some sections were treated with RNase A (100 μg/ml; 37°C, 1 h) before hybridization. Some sections were hybridized with labeled KGF cDNA probes in the presence of an excess amount of unlabeled KGF cDNA (17-fold) to validate the sequence specificity of the signal. On some sections, after hybridization with KGF cDNA, excessively stringent washing conditions were used. The calculated melting temperature of the KGF cDNA-mRNA hybrids in 50% formamide/0.2× SSC is 22–29°C (24) assuming 90–95% homology in KGF base sequences between monkey and human. For an excessively stringent wash, we used the same conditions at 37°C.

Identification of Proliferating Cells

A nuclear antigen associated with proliferation was immunohistochemically localized in frozen sections of uteri with antibody Ki-67 (DAKO PATTS Inc., Santa Barbara, CA). We have described our immunocytochemical technique for use of this antibody in a previous paper on the monkey endometrium (39). Several studies have shown that quantitation of the number of cells positively stained for this antigen in frozen sections provides a reliable index of proliferation (14).

Histology of Monkey Uterus

Glycolmethacrylate sections of monkey uterus were prepared and stained with hematoxylin as described previously (39).

Results

Hormonal Regulation of KGF mRNA in Primate Uterus

To investigate possible hormonal influences on KGF expression in the primate uterus, endometrial and myometrial RNAs were prepared under various treatment conditions. As shown in Fig. 1, the 2.4-kb KGF transcript was barely detectable in endometrium of spayed (lane *1*) or E_2-treated (lane *3*) monkeys. However, the KGF mRNA was abundant in the endometrium of animals treated with E_2 and P (lane *5*). While the transcript was detectable at low level in myometrium of spayed animals (lane 2), its level of expression was essentially unchanged by E_2 or E_2 + P treatment (lanes *4* and *6*). The slight increase in signal from the E_2 + P–treated myometrial sample is probably caused by contamination with endometrial tissue. Under the same experimental conditions, a typical 2.4-kb band of KGF mRNA was detected in total RNA from M426 human fibroblasts, a KGF-positive line (lane *7*), but not from B5/589 cells, a KGF-negative cell line (lane *8*). The lower portion of the figure illustrates the signal on these blots after reprobing with a cDNA probe against GAPDH, a housekeeping gene, confirming that equivalent amounts of RNA were loaded and transferred in all lanes.

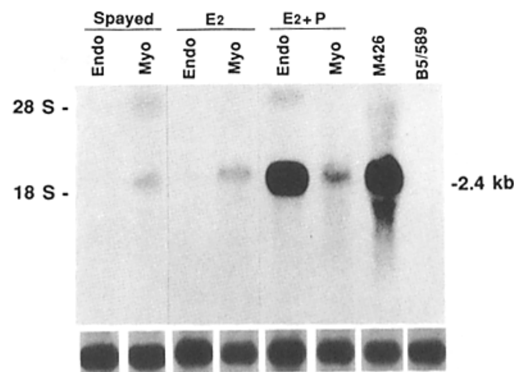


Figure 1. Northern blot of total uterine RNA from hormonally treated animals hybridized with ^{32}P-labeled KGF cDNA. (Lanes *1* and 2) Spayed animals; endometrium in lane *1* and myometrium in lane *2*. (Lanes *3* and *4*) E_2-treated animals; endometrium in lane *3* and myometrium in lane *4*. (Lanes *5* and *6*) E_2 + P-treated animals; endometrium in lane *5* and myometrium in lane *6*. (Lane *7*) KGF-positive M426 cell line. (Lane *8*) KGF-negative B5/589 cell line. 2.4 kb marks KGF mRNA, which was markedly enhanced by E_2 + P treatment in endometrium, and was present but invariant in myometrium under all hormonal conditions. The lower portion of the figure illustrates the signal on these blots after reprobing with a cDNA probe against GAPDH, confirming that equivalent amounts of RNA were loaded and transferred in all lanes.

Fig. 2 (lanes *1* and *3*) shows that in animals treated sequentially with E_2 followed by P, the degree of endometrial KGF mRNA expression was very high, whether the E_2 was continued along with the P treatment (*E_2 + P*; lane *1*) or the E_2 was stopped and P was administered alone (*E_2, P*; lane *3*). In striking contrast, administration of RU 486 (lane *2*) blocked the effect of P in upregulating endometrial KGF mRNA. Fig. 2 also shows that during the natural menstrual cycle, endometrial KGF mRNA expression was minimal during the follicular phase (lane *4*) and highly abundant in the luteal phase (lane *5*). The level of KGF mRNA was equivalent in P-dominated tissues, whether sampled during the natural cycle or after hormone administration. The lower portion of the figure shows the signal obtained with the GAPDH probe to control for RNA loading and transfer.

Fig. 3 *A* shows that upregulation of KGF mRNA by P was tissue specific. When Northern blots of total RNAs from endometrium and oviduct of E_2- or E_2 + P–treated monkeys were probed with ^{32}P-labeled KGF antisense oligo-DNA,

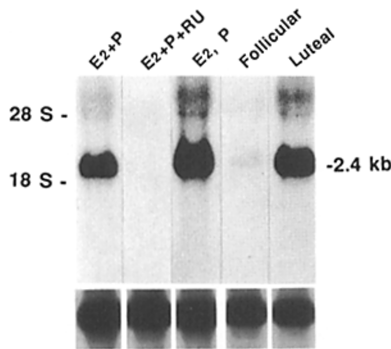


Figure 2. Northern blot of total endometrial RNA from hormonally treated and naturally cycling macaques hybridized with ^{32}P-labeled KGF cDNA. Hormonal conditions for each lane were as follows: Lane *1*, E_2 + P combined; lane *2*, E_2 + P + RU 486; lane *3*, E_2 followed by P alone (*E_2, P*); lane *4*, follicular phase of the menstrual cycle; and lane *5*, luteal phase of the menstrual cycle. 2.4 kb marks KGF mRNA, which was elevated in P-dominated tissues, whether E_2 was present or absent. This effect of P was suppressed by RU 486. The lower portion of the figure illustrates the signal on these blots after reprobing with a cDNA probe against GAPDH, confirming that equivalent amounts of RNA were loaded and transferred in all lanes as above.

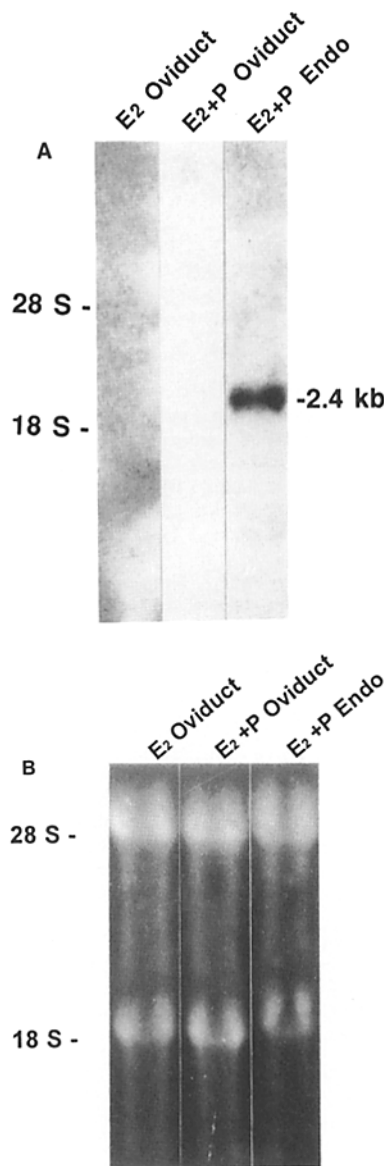


Figure 3. (*A*) Northern blot of oviductal and endometrial total RNA hybridized with ^{32}P-labeled antisense oligo-DNA. Tissues and hormonal conditions for each lane were as follows: Lane *1*, oviduct, E_2 treated; lane 2, oviduct, E_2 + P treated; and lane 3, endometrium, E_2 + P treated. 2.4 kb marks KGF transcript that was increased by P only in the endometrium, not in the oviduct. (*B*) Ethidium bromide-stained gel used to produce the blot shown in Fig. 3. Essentially equivalent amounts of RNA were loaded into each lane.

the 2.4-kb band of KGF mRNA was detected in the endometrial (lane *3*), but not the oviductal RNA preparation (lanes *1* and *2*). Ethidum bromide staining of the agarose gel used to prepare the blot in Fig. 3 *A* indicated that similar amounts of RNA were loaded in each lane (Fig. 3 *B*). All of these results indicated that KGF mRNA was markedly upregulated in the endometrium but not the myometrium or oviduct in response to P.

Elevated KGF Protein Levels in Endometrium after P Treatment

In an effort to determine whether KGF mRNA expression was correlated with the detection of KGF protein, we developed a sensitive two-site ELISA as described in Materials and Methods. In this assay, recombinant KGF was detectable at subnanogram amounts (Fig. 4). In this same assay, endometrial tissue extracts showed readily detectable KGF-immunoreactive material. As illustrated in Fig. 4, the titration patterns exhibited by these extracts in the ELISA closely matched that of the recombinant human KGF standard, strongly suggesting that the cross-reactivity resulted from endogenous KGF. Moreover, the results summarized in Fig. 4 and Table I demonstrated that endometrial tissue from animals in the luteal phase or treated with E_2 + P showed more KGF immunoreactivity than tissue from animals in the follicular phase or exposed only to E_2. Similar findings were obtained when samples were surveyed in a KGF radioimmunoassay (data not shown). These data indicate that KGF protein was present in the endometrium and was elevated in P-dominated tissue.

Cellular Localization of KGF mRNA in the Uterus

To examine the cellular localization of KGF transcript in the uterus, we performed in situ hybridization. During all hormonal conditions, there was a distinct but essentially invariant, cytoplasmic signal for KGF mRNA in the smooth muscle cells within the myometrium (Fig. 5, *A–C*) and the walls of the spiral arteries (Fig. 5, *D–F*). Endometrial glandular epithelial cells (Fig. 5, *G–K*) and vascular endothelial cells (Fig. 5, *D–F*) were negative for KGF mRNA under all hormonal conditions. Myometrial fibroblasts were also generally negative for KGF mRNA under all hormonal conditions (Fig. 5, *A–C*).

In animals that were spayed or E_2 treated (or in the follicular phase), stromal cells were negative in all endometrial zones (Fig. 5, *G* and *H*) except for a few stromal cells closely associated with spiral arteries (Fig. 5, *D* and *E*). In P-treated

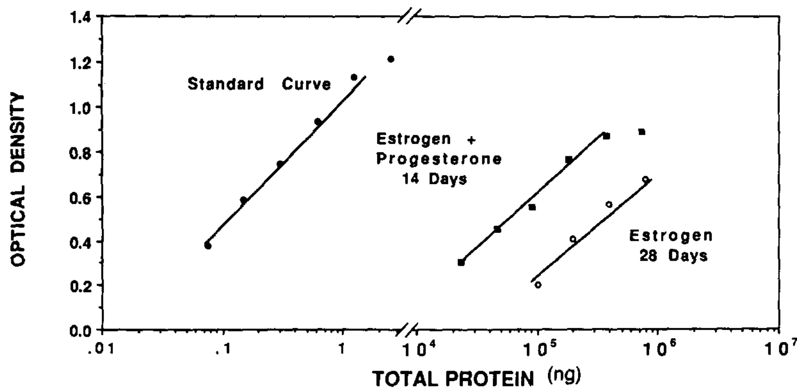


Figure 4. ELISA analysis of KGF protein in homogenates of rhesus monkey endometrium. Protein was extracted from the endometrium of animals subjected to different hormonal treatments, and serial dilutions of each sample were assayed along with dilutions of a recombinant human KGF standard as described in Materials and Methods. Each data point was the mean value of duplicate measurements.

(or luteal phase) animals, the stromal cells in the functionalis zone appeared negative (Fig. 5 *I*); however, the stromal cells of the basalis zone showed a definite cytoplasmic signal for KGF mRNA (Fig. 5 *J*). The cellular specificity of the positive KGF mRNA signal was even more obvious when the signal was enhanced by phase microscopy (Fig. 5 *K*). During P domination, the stromal cells in the perivascular regions around the spiral arteries also showed a small increase in the level of staining (Fig. 5 *F*). Sequence specificity was shown by the great reduction of signal intensity in competition controls (Fig. 5 *L*), excess stringency controls (Fig. 5 *M*), and GFAP probe controls (Fig. 5 *N*). Positive signals were also absent in RNase-pretreated controls (Fig. 5 *O*). Signals were also absent from the smooth muscle cells of the myometrium and the walls of the spiral arteries in all of the above control preparations (data not shown).

The in situ hybridization signal was stronger in the smooth muscle cells of the myometrium and the spiral arteries than in the endometrial stromal cells, even though the signal in Northern blots was greater in the total RNA extracted from endometrium than myometrium. The reason for this discrepancy is not clear, but may be related to differences in probe penetrability between different cell types.

P-dependent Glandular and Vascular Proliferation

In E_2-treated and follicular phase animals, there was a substantial number of Ki-67–positive cells in the glands of the functionalis (Fig. 6 *A*) and only a minimal number in the glands of the basalis (Fig. 6 *C*). In contrast, in E_2 + P-treated and luteal phase animals, glandular proliferation was not observed in the functionalis (Fig. 6 *B*), but was stimulated in the basalis (Fig. 6 *D*). In addition, the spiral arteries proliferated under P influence. During the follicular phase (Fig. 6 *A*), there were few Ki-67 cells in the walls of the spiral arteries, while in P-dominated animals (Fig. 6 *B*), there were numerous Ki-67–positive vascular smooth muscle cells, perivascular stromal cells, and endothelial cells in the spiral arteries. The P-dependent growth of the spiral arteries was also evident in GMA sections (Fig. 7, *A* and *B*).

Table I. KGF Concentration in Rhesus Monkey Endometrium

Animal number	Hormonal state	KGF concentration (ng/mg tissue protein)
11762	E_2 + P 14 d	4.0
14958	E_2 + P 14 d	3.5
11989	E_2 + P 14 d	2.1
14581	Luteal phase	1.1
9360	Follicular phase	0.26
11671*	Follicular phase + E_2	0.12

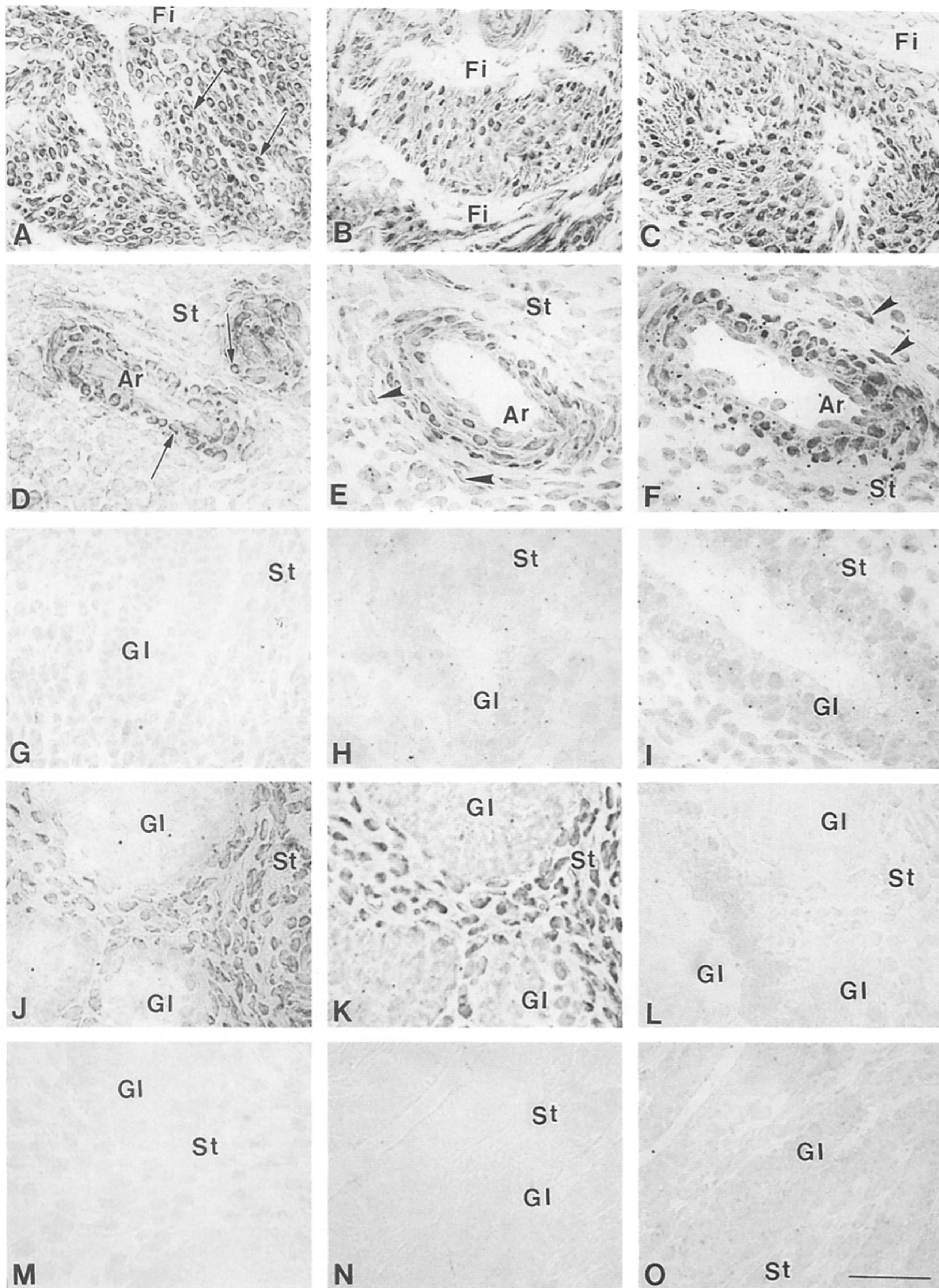
"Hormonal state" indicates phase of menstrual cycle or hormone treatment. 14 d E_2 + P, treated for 14 d with an E_2 implant and then for 14 d with implants of E_2 and progesterone. * Rhesus 11671 (follicular phase + E_2) was an animal injected with 42 μg/kg E_2 28 h before surgery during late follicular phase of the menstrual cycle to produce highly elevated levels of E_2 to mimic the natural preovulatory E_2 surge.

Discussion

Three different phases of epithelial proliferation occur in the macaque endometrium during the menstrual cycle. The first is the repair phase, immediately after menstruation, when the ragged surface of the endometrium heals. This surface healing, highly analogous to reepithelialization during wound healing, is hormone independent (6). The second occurs during the follicular phase, in which the glandular epithelium in the functionalis, but not the basalis, proliferates. This proliferation is driven by E_2 (27). Finally, during the luteal phase, the basalis glands and the spiral artery system proliferate in a P-dependent manner, while the glands of the functionalis cease proliferating. The P dependence of this burst of proliferation in the basalis of the macaque endometrium during the luteal phase has been well documented (2, 3, 33, 35).

Our present findings revealed that KGF mRNA levels were dramatically elevated in the endometrium, specifically during the luteal phase in naturally cycling animals or after progesterone administration to estrogen-primed spayed animals. This rise in KGF expression also was demonstrated by measurement of KGF-immunoreactive protein in tissue extracts with a two-site ELISA. In situ hybridization showed that the P-dependent increase in KGF mRNA expression was strongest in the stromal cells located around and between the glands of the basalis. This temporal and spatial correlation between KGF mRNA expression and epithelial proliferation in the basalis implies a possible role for KGF on these specific cells as a "progestomedin," a paracrine factor that mediates the actions of progesterone.

Other potential targets of KGF action in the rhesus monkey are the epithelial cells that form the surface plaque characteristic of this species (11) during either normal implantation or trauma-induced decidualization (17). Ghosh et al.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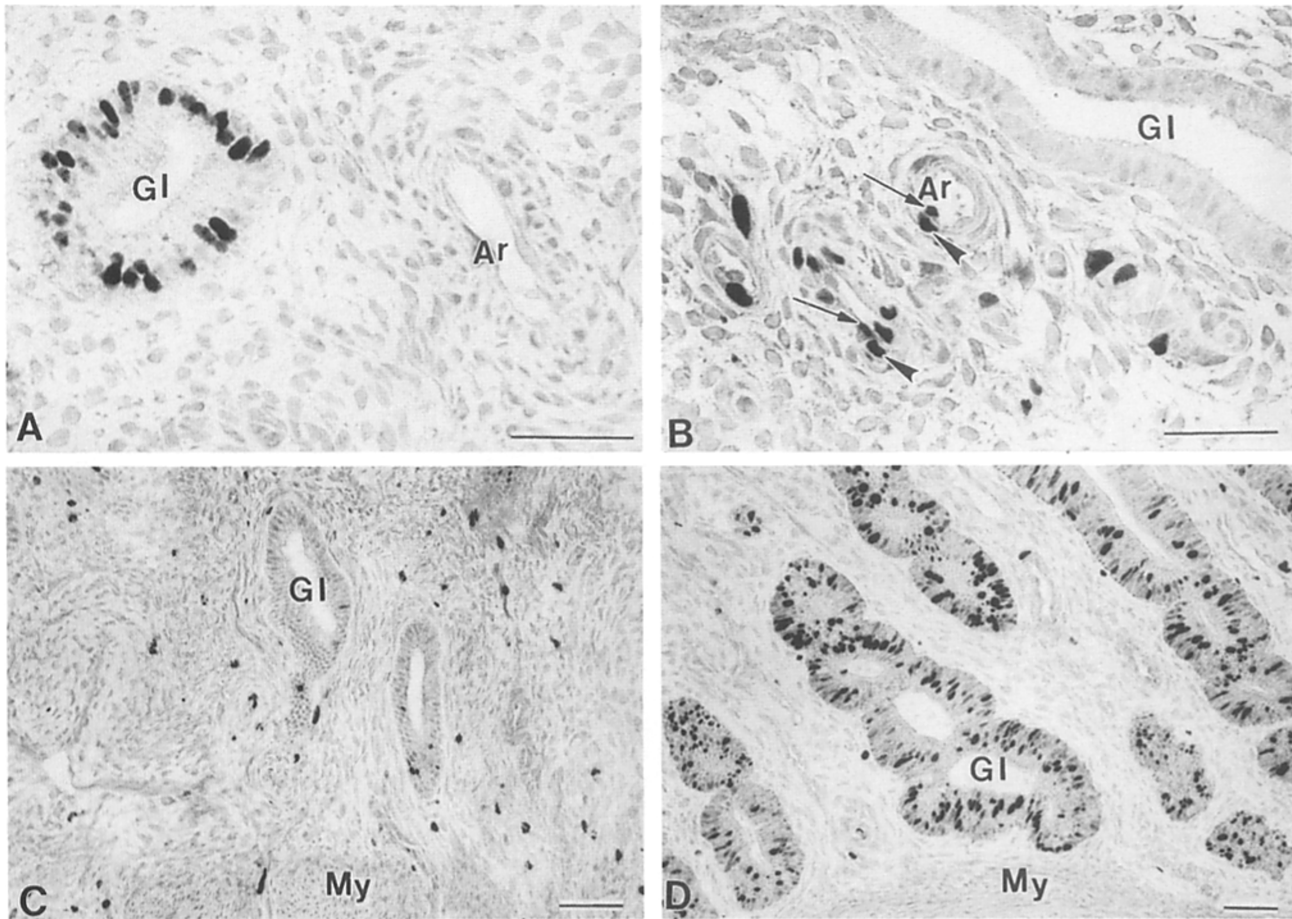

Figure 6. Proliferating cells as indicated by Ki-67 staining. Micrographs on the left (*A* and *C*) are from an E_2-treated animal, and on the right (*B* and *D*) are from an animal sampled on day 21 of the natural luteal phase (P dominated). Original magnification in *A* and *B* is 400; bar, 10 μm. Original magnification in *C* and *D* is 160; bar, 12.5 μm. (*A*) Functionalis, E_2 treated. Ki-67 staining is evident in many glandular epithelial cells, but is absent from the walls of the adjacent artery. (*B*) Functionalis, P dominated. Ki-67 staining is absent from the glandular epithelial cells, but is evident in many of the endothelial (*arrows*) and smooth muscle cells (*arrowheads*) of the spiral arteries. (*C*) Basalis, E_2 treated. A region of the basalis near the myometrial (*My*) border. Ki-67 staining is evident in some stromal cells but is minimal in the glandular epithelium. (*D*) Basalis, P dominated. A region of the basalis near the myometrial border. Ki-67 staining is now greatly increased in the glandular epithelium and minimal in the stroma.

Figure 5. In situ hybridization of KGF mRNA with dig-labeled cDNA. In the upper three rows of this figure (includes *A–I*), the micrographs in the left column are from spayed (untreated) animals, those in the center column are from E_2-treated animals, and those in the right column are from P-treated animals. In the last two rows (includes *J–O*), all the micrographs are from P-treated animals. All original magnifications were ×400. The bar in *O* represents 10 μm. (*A*) Myometrium, spayed. A distinct signal for KGF mRNA is evident in the cytoplasm of the smooth muscle cells of the muscle bundles. The fibroblasts (*Fi*) in the connective tissue separating the muscle bundles are negative. (*B*) Myometrium, E_2 treated. The KGF mRNA signal is essentially identical to that in the spayed animals. (*C*) Myometrium, P treated. The KGF mRNA signal is essentially identical to that in the E_2-treated and spayed animals. (*D*) Artery, spayed. A distinct signal is evident in the cytoplasm of the smooth muscle cells that constitute the tunica media of the artery (*Ar*). Stromal (*St*) cells lack any significant signal. (*E*) Artery, E_2 treated. The signal evident in the cytoplasm of the smooth muscle cells of the artery wall is about the same strength as in the spayed animals. A few perivascular stromal cells (*arrowheads*) show a distinct signal. (*F*) Artery, P treated. The signal in the cytoplasm of the smooth muscle cells of the artery wall is about the same as in the E_2-treated animals. Some perivascular stromal cells show an increased signal. (*G*) Functionalis, spayed. The glands (*Gl*) and the periglandular stromal regions are negative for KGF mRNA. (*H*) Functionalis, E_2 treated. The glands and the periglandular stroma are negative as in the spayed animals. (*I*) Functionalis, P treated. The glands and the periglandular stroma are negative as in the E_2-treated animals. (*J*) Basalis, P treated. The glands are negative, but the stroma shows a substantially increased, distinct signal for KGF mRNA. (*K*) Basalis, P treated. This is a phase micrograph of the identical section as in *J*. The signal in the stroma is enhanced, while the glands remain negative. (*L*) Basalis, P treated, competition control. Competition with excess unlabeled probe greatly suppresses the stromal signal. (*M*) Basalis, P treated. Excess stringency control. Washing at excessively high stringency removes the signal from the stromal cells. (*N*) Basalis, P treated. GFAP probe control. Hybridization with GFAP cDNA probe produces no signal in stromal cells. (*O*) Basalis, P treated. RNase pretreated control. Treatment with RNase before hybridization with the KGF cDNA probe eliminates the signal in the stromal cells.

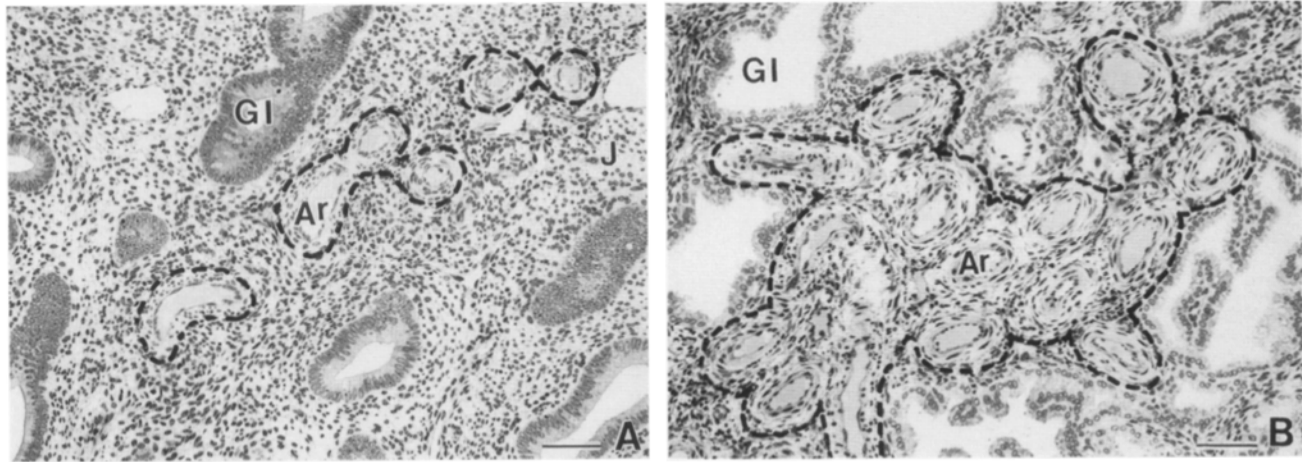

Figure 7. GMA sections to illustrate histology of spiral arteries. *A*, E_2-treated; *B*, P-treated. ×100; bar, 20 μm. (*A*) Functionalis, E_2 treated. The perimeter of a spiral artery in a GMA section is indicated with a dashed line. (*B*) Functionalis, P treated. The dramatic increase in the spiral arteries under P influence is indicated by the increase in the perimeter outlined by the dashed line.

(16) recently showed that this response was P dependent, since RU 486 could significantly inhibit plaque formation during experimental decidualization. Because the fetal trophoblast and the epithelial cells in zones I and II that form the plaque lack progesterone receptors (7, 22, 34), it is likely that only those cells that retain their progesterone receptors during the luteal phase, such as stromal cells, spiral artery wall cells, and glandular basalis cells (7, 22, 34), could play a role in plaque formation. Of note, in situ hybridization also localized KGF transcripts to the smooth muscle cells of the spiral arteries. These vessels terminate in a rich vascular bed near the surface of the endometrium where the epithelial plaque forms. Consequently, this region might contain KGF released from the spiral vasculature that could promote epithelial proliferation. In addition, KGF produced by cells in and around the spiral arteries could directly enhance the proliferation and/or migration of the embryonic trophoblast, which invades and canalizes the spiral arteries during the early stages of implantation (12).

Endothelial cells and smooth muscle cells in spiral arteries, as well as some perivascular stromal cells, proliferate during P domination, as indicated by positive staining with the Ki-67 antibody. KGF does not act as a mitogen in vitro for endothelial cells, fibroblasts, or vascular smooth muscle cells isolated from other tissues (reference 38 and unpublished observations). Thus, KGF expression in the spiral arteries probably does not have a direct impact on the growth of these vessels, unless the components of these unique arteries possess a distinctive responsiveness to KGF.

In primates and many other mammals, oviductal proliferation is E_2-dependent and occurs only during the follicular phase or during E_2 treatment of spayed animals (6). P antagonizes this effect of E_2 (6). Consequently, the lack of P-dependent KGF mRNA in this organ is physiologically consistent with the absence of P effects on growth in this tissue. P may only upregulate KGF expression in tissues where P stimulates the proliferation and perhaps differentiation of epithelial cells (8).

Members of the IGF system may interact with and complement the actions of KGF during P domination in the primate endometrium. Together, KGF and IGF-I (or insulin at pharmacologic doses) stimulated proliferation of BALB/MK mouse epidermal keratinocytes in a chemically defined medium more effectively than KGF alone (38). P has been shown to increase the levels of several IGF-binding proteins (19), and IGF-II mRNA was detected in secretory phase human endometrium (18). Thus, during P domination, increased levels of IGF-binding proteins, IGF-II, and KGF may act in concert, together with other growth factors, to mediate P action in the primate endometrium.

A high affinity KGF receptor (KGFR) has been identified as an alternatively spliced variant of FGFR-2 (bek) with exon IIIb in place of IIIa (4, 28, 29). A recent report indicates that KGF and KGFR mRNA are both present in the human endometrium (36). We also have evidence that the KGFR is expressed in the rhesus monkey endometrium (unpublished observations). Studies on the hormonal control, regulation, and cellular localization of the KGFR are currently underway to directly identify those cells in the uterus that are capable of responding to this newly identified progestomedin.

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