0021-972X/00/$03.00/0
The Journal of Clinical Endocrinology & Metabolism

Vol. 85, No. 9
Printed in U.S.A.

Progesterone Withdrawal Up-Regulates Vascular Endothelial Growth Factor Receptor Type 2 in the Superficial Zone Stroma of the Human and Macaque Endometrium: Potential Relevance to Menstruation*

NIHAR R. NAYAK, HILARY O. D. CRITCHLEY, OV D. SLAYDEN, ANDREAS MENRAD, KRISTOF CHWALISZ, DAVID T. BAIRD, AND ROBERT M. BRENNER

Oregon Regional Primate Research Center (N.R.N., O.D.S., R.M.B.), Beaverton, Oregon 97006; Center for Reproductive Biology (H.O.D.C., D.T.B.), EH3 9ET Edinburgh, United Kingdom; Schering AG (A.M.), D13342 Berlin, Germany; and Jenapharm (K.C.), 07745 Jena, Germany

ABSTRACT

Several reports indicate that vascular endothelial growth factor (VEGF) expression is increased in endometrial glands and stroma during the menstrual phase in the human endometrium. Here we report that VEGF receptor type 2 (KDR), normally expressed only in the vascular endothelium, was dramatically up-regulated in the stromal cells of the superficial endometrial zones during the premenstrual phase in both human and macaque endometrium. This increase was detectable by Northern analysis, *in situ* hybridization, and immunocytochemistry and was cell specific, zone specific, cycle phase specific, and VEGF receptor type specific. That is, it only occurred during the premenstrual/menstrual phase, did not occur in glandular epithelium, endothelium, or stromal cells of the deepest endometrial zones, and was not observed for VEGF receptor type 1. The up-regulation of stromal KDR was induced by progesterone (P) withdrawal in both women and macaques, and adding back P 24 h after P withdrawal in macaques blocked stromal, but not vascular, endothelial KDR expression. Promatrix metalloproteinase-1 (MMP-1) was coordinately up-regulated in the same stromal cell population by P withdrawal. Because of reports that VEGF can enhance MMP expression, we hypothesize that VEGF-KDR interactions may influence MMP expression in the superficial zones of the primate endometrium during the premenstrual phase, and that these interactions play a role in the induction of menstruation. (*J Clin Endocrinol Metab* **85:** 3442–3452, 2000)

IN PREPARATION for implantation, the primate endometrium undergoes sequential phases of proliferation and secretory differentiation under the influence of ovarian steroid hormones. In the absence of implantation, the corpus luteum declines, and the subsequent fall in progesterone (P) leads to the characteristic dissolution, sloughing, and menstrual bleeding of the endometrium. The classic study of menstruation conducted about 60 yr ago in rhesus monkeys by Markee (1) demonstrated that the demise of the corpus luteum was followed by a rapid shrinkage of the endometrium and an intense vasoconstriction of the spiral arteries that preceded the bleeding and sloughing of the upper endometrial zones. Clinically, extensive bleeding from the endometrium results in a range of disorders, which significantly reduce the quality of life for women and accounts for about 70% of the hysterectomies performed annually (2). Despite the vital physiological role of menstruation and the varied pathological implications of abnormal bleeding, a full understanding of the local factors and regulatory mechanisms involved in menstruation is not yet at hand.

Recent data indicate that a close association exists between menstruation and the expression of matrix metalloproteinase (MMP) enzymes (3–5). MMPs are a multigene family of zinc-requiring enzymes that degrade components of the extracellular matrix and various types of collagen, including those in basement membranes. Most of the MMPs are synthesized by endometrial stromal cells, except for matrilysin (MMP-7), which is localized in glandular epithelial cells (6, 7). In the human and the macaque endometrium, expression of most MMPs is greatest during the immediate premenstrual and menstrual phases and is confined to the upper functionalis zone, which sheds off during menstruation. Expression of most MMPs declines substantially during the remainder of the follicular phase and is negligible during the luteal phase after P levels rise (5). We and others have hypothesized that constriction of the spiral arteries after P withdrawal induces a local endometrial hypoxic injury, that this hypoxia is greatest in the upper endometrial zones supplied by the spiral arteries, and that this hypoxic insult is the initiating factor that begins a molecular cascade culminating in MMP expression and tissue dissolution (4, 5). The precise mechanisms and local mediators regulating the endometrial zone-specific expression of MMPs *in vivo* are not known.

Several reports indicate that vascular endothelial growth

Received March 13, 2000. Revision received May 10, 2000. Accepted May 22, 2000.

Address all correspondence and requests for reprints to: Dr. Robert M. Brenner, Reproductive Sciences, Oregon Regional Primate Research Center, 505 N.W. 185th Avenue, Beaverton, Oregon 97006. E-mail: brennerr@ohsu.edu.

* This work was supported by the Lalor Foundation (to N.R.N.). In Oregon the research was also supported by the NICHHD, NIH through Cooperative Agreement U54 18185 as part of the Specialized Cooperative Centers Program in Reproductive Research and by NIH Grants RR-00163 and HD-19182.

factor (VEGF), which is present in the endometrium throughout the menstrual cycle (8, 9), is up-regulated during menstruation (10, 11). A hypoxic injury might be the stimulus for this increase *in vivo*; there are several reports indicating that hypoxia can up-regulate VEGF expression in endometrial stromal cells (10, 12) and gland cells (10) *in vitro*. Here we report that VEGF receptor type 2 (KDR) was dramatically up-regulated in the stromal cells (not the endothelial cells) of the upper zones of the human and macaque endometrium during the premenstrual phase of the cycle. At other times in the cycle, KDR expression was limited to the vascular endothelium. Because this enhanced stromal KDR expression was coordinate in time and space with the expression of MMPs, and because VEGF can stimulate the expression of MMPS (13–15) we suggest that VEGF, acting through KDR, could play an important role in the up-regulation of MMPs in the premenstrual endometrium. In the present report we provide definitive evidence for the regulation and expression of KDR in the stromal cell population of the superficial zones of both the human and macaque endometrium during the premenstrual phase, data that for the first time implicate a potential VEGF-KDR link in the menstrual induction cascade.

Experimental Subjects

Human subjects were fertile women with regular cycles (25–35 days) undergoing hysterectomy for benign indications during the proliferative phase (n = 4), secretory phase (n = 5), and premenstrual phase (n = 3). Subjects with fibroids or benign polyps and women who had been administered hormonal preparations in the preceding 3 months were excluded. All subjects provided written informed consent, and ethical approval was granted by the local research ethics committee. Full thickness endometrial samples were obtained that included superficial and basal endometrium plus the endometrial-myometrial junction.

Endometrial tissue was also collected from 15 fertile women with regular cycles in whom the onset of menstruation was experimentally simulated *in vivo* as previously reported (16). Briefly, P, in the form of 200 mg Cyclogest (Hoechst UK Ltd., Hounslow, UK), was administered vaginally twice daily starting 8–10 days after the surge (LH peak + 8–10 days). Treatment was stopped after 4 days to mimic luteal regression. Endometrial biopsies were collected with an endometrial suction curette (Pipelle, Laboratoire CCD, Paris, France) at three time points: midluteal phase (n = 5; LH peak + 8–10 days) and 24 h (n = 5) and 48 h (n = 5) after stopping P. All subjects provided a venous blood sample at the time of biopsy collection for measurement of circulating 17β-estradiol (E_2) and P concentrations by RIA as previously described (17).

Biopsies for studies requiring frozen tissue sections were placed in Hanks' Balanced Salt Solution (Life Technologies, Inc., Gaithersburg, MD) supplemented with 0.7% HEPES buffer (Sigma, St. Louis, MO) and microwaved at low power for 12 s as previously described (18). Tissue was then placed in cold phosphate-buffered saline (PBS) containing 10% sucrose for 15–30 min before immersion in OCT freezing compound (Tissue-Tek, Miles, Inc., Elkhart, IN) in liquid nitrogen and stored at −70 C. Some blocks were cryosectioned and processed for immunocytochemistry (ICC) of KDR as described below. Other blocks were shipped on dry ice to the Oregon Regional Primate Research Center for *in situ* hybridization (ISH) studies. Remaining tissues were fixed in 10% neutral buffered formalin at 4 C overnight, rinsed, stored in 70% ethanol, and thereafter routinely wax embedded for subsequent immunolocalization of leukocyte subpopulations.

Experimental Animals

Animal care during these studies was provided by the veterinary staff of the Division of Animal Resources of the Oregon Regional Primate Research Center, in accordance with the NIH policy for the care and use of laboratory animals. Fourteen adult female rhesus macaques (*Macaca mulatta*) and 16 pigtailed macaques (*Macaca nemestrina*) were ovariectomized and treated sequentially with E_2 and P to create artificial menstrual cycles as described previously (5, 19). All macaques received sc implants of 3-cm SILASTIC brand capsules (Dow Corning Corp., Midland, MI) packed with crystalline E_2 (Sigma) to stimulate development of an artificial proliferative phase endometrium. After 14 days, a 6-cm SILASTIC brand capsule packed with crystalline P (Sigma) was implanted sc, and both implants remained in place for 14 days to stimulate an artificial secretory phase endometrium. To induce menstruation, the P implant was removed, and usually the E_2 implant was left in place. In some cases, as noted below, the P and E_2 implants were both withdrawn to induce menstruation, and these animals were referred to as aspays, because they lacked both E_2 and P. No substantial differences in endometrial responsiveness were noted between the pigtailed and rhesus macaques.

Seven different hormonal states of the endometrium were achieved by sequential treatment and withdrawal of E_2 and P as follows: 1) early premenstrual phase, endometrium collected after 1 day of P withdrawal (E-1P; n = 2); 2) premenstrual phase, endometrium collected after 2 days of P withdrawal (E-2P; n = 4); 3) early proliferative phase, endometrium collected after 4–5 days of P withdrawal (E-4/5P; n = 4); 4) late proliferative phase, endometrium collected after 14 days of P withdrawal (E-14P; n = 4); 5) secretory phase, endometrium collected at the end of the secretory phase (E+14P; n = 4); 6) 2 day spay, endometrium collected after 2 days of withdrawal of both E_2 and P (2d sp; n = 2); and 7) 4–5 day spay, endometrium collected after 4–5 days of E_2 and P withdrawal (4/5d sp; n = 2). Endometrial tissue samples were collected by laparotomy and hysterectomy as described previously (5, 19). In each case, serum was harvested at the time of tissue collection, and concentrations of serum E_2 and P were determined by RIA as previously validated (20).

We further examined the effects of adding back P after P withdrawal on the expression of KDR. In this study six pigtail macaques were artificially cycled as described above. At the end of one cycle, P implants were removed from all of the macaques, whereas E_2 implants remained in place. Endometrium was collected from three animals 48 h after P withdrawal. In the remaining three animals, P implants were added back 24 h after P withdrawal, and at the same time a single im dose of P (1 mg/kg in ethanol) was administered to rapidly elevate serum P levels. The endometrium from these two animals were collected 24 h later (E-24hP+24hP). The goal of this design was to compare regulation of KDR expression in the vascular endothelium *vs.* the stroma of the superficial zones during both P withdrawal and P add-back and to validate that KDR up-regulation was due to P withdrawal.

Materials and Methods

ICC

ICC detection of KDR, VEGFR-1 (the Fms-like tyrosine kinase-1 receptor, or Flt-1), and pro-MMP-1 (interstitial collagenase) were performed in frozen sections of human and macaque endometrium following the same procedural details described previously (5, 18). Briefly, fresh tissues were microwaved for 7 s before being embedded in OCT, frozen in liquid propane, and cryosectioned at 5–7 μm. Cryosections were mounted on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA), freeze-substituted with absolute acetone at −80 C for 2–4 days (21), fixed in 2% paraformaldehyde in phosphate buffer at pH 7.3 for 10 min at room temperature, immersed twice for 2 min each time in 85% ethanol and 1.5% polyvinylpyrolidone (PVP) at 4 C, rinsed in PBS, immersed twice for 7 min each time in 0.37% glycine in PBS and PVP, and then immersed in 0.1% gelatin in PBS and PVP at 4 C. To inhibit endogenous peroxidase activity, the sections were incubated with a solution containing glucose oxidase (1 U/mL), sodium azide (1 mmol/L), and glucose (10 mmol/L) in PBS for 45 min. Sections were then incubated with blocking serum for 20 min and with the primary antibody (KDR at 20 μg/mL; Flt-1 at 0.1 μg/mL; pro-MMP-1 at 2 μg/mL) overnight at 4 C. Antihuman mouse monoclonal antibodies for KDR (22) and Flt-1 were provided by A. Menrad, and pro-MMP-1 was purchased (R&D Systems, Minneapolis, MN). Omission of the first antibody, incubation of tissue sections with irrelevant, isotype-matched mouse IgG, and comparison with a standard endothelial marker [von Willebrand factor (vWF), DAKO Corp., Carpinteria, CA] were used as method controls. After rinsing and immersion in blocking serum again, sections were incubated with second antibody for 30 min at room temperature. Final visualiza-

tion was achieved using the ABC kit (Vector Laboratories, Inc. Burlingame, CA), 0.025% 3,3′-diaminobenzidine/4HCl (Dojindos DAB, Wako Chemicals, Richmond, VA) in Tris buffer, 0.03% H_2O_2 (Fisher Scientific), and 0.026% osmium tetroxide, as described previously (5, 18). Tissue sections were then postfixed with 2% paraformaldehyde and lightly counterstained with hematoxylin to facilitate identification of cellular elements.

To ascertain whether any of the endometrial leukocytes scattered throughout the endometrium contributed to the increased KDR expression observed in the superficial zones, we prepared near-serial cryosections (4 μm) of macaque premenstrual stage endometrium and performed ICC for KDR and various leukocyte-specific markers. Specifically, we used commercial antihuman mouse monoclonal antibodies from DAKO Corp. for vWF at 1:500 for endothelial cells, CD14 at 1:30 for monocytes/macrophages, CD45 at 1:30 for all leukocytes, and CD68 at 1:50 for a variety of macrophages (DAKO Corp.). Human endometrial premenstrual samples were also stained for these leukocytic cell types.

RT-PCR

We used RT-PCR for preparation of monkey-specific KDR and Flt-1 complementary DNA (cDNA) amplified from monkey endometrial ribonucleic acid (RNA) in the Molecular Core Facility, Oregon Regional Primate Research Center. Five micrograms of total RNA prepared from menstrual cycle day 5 endometrium were reverse transcribed in 20 μL reaction using an oligo(deoxythymidine) primer and SuperScript II Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). Five microliters of the RT were then amplified in a 50-μL reaction using 50 pmol of the 5′- and 3′-primers in a standard PCR reaction for 35 cycles of 92 C for 30 s, 50 C for 30 s, and 72 C for 1 min. Amplified bands of the right size were gel isolated and subcloned into pGEM-T (Promega Corp., Madison, WI). Two to four clones with the correct size inserts were miniprepped (Perfect Preps, 5′-3′, Boulder, CO) and were subjected to automated sequencing on an ABI 373 XL sequencer (Applied Biosystems, Inc., Foster City, CA). A sequence was obtained on at least two independent subclones to rule out any PCR errors. Primers for the 437-bp KDR and 411-bp Flt-1 cDNAs were selected based on the homologous human KDR (1124–1560 bp; accession no. AF035212) and human Flt-1 (3431–3841 bp; accession no. AF063657) sequences. The forward and reverse primers used to amplify KDR were ACC GAG ACC TAA AAA CCC AG and GAC ATA CAC AAC CAG AGA GAC C, respectively, and those for Flt-1 were GGC CAA GAT TTG CAG AAC TTG and CCT TAC TTT TAC TGG TTA CTC, respectively. The partial cDNA sequences for macaque KDR and Flt-1 have been submitted to GenBank with accession numbers AF235161 and AF235162, respectively.

Northern blot analysis

Total RNA was isolated from macaque endometria using RNA STAT-60 (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. Ten micrograms of total RNA were electrophoresed on 1% formaldehyde agarose gels and transferred to nylon membranes (Nytran Plus, Schleicher & Schuell, Inc., Keene, NH). The RNA was UV cross-linked to the membranes using the UV Stratalinker 1800 (Stratagene, La Jolla, CA). The membranes were then hybridized with ^{32}P-labeled random primed cDNA probes (SA, 10^6 cpm/mL). Probes were prepared using the random primed DNA labeling kit from Roche Molecular Biochemicals (Indianapolis, IN), and hybridization was performed according to the manufacturer's instructions using ExpressHyb hybridization solution (CLONTECH Laboratories, Inc., Palo Alto, CA). The same blot was probed separately for KDR and a rat 18S cDNA (23) to control equal loading after stripping in boiling 0.5% SDS solution. The blots were exposed to Kodak BioMax MS film with TranScreen-LE intensifying screen at −70 C (Eastman Kodak Co., Rochester, NY). Densitometric analysis of the autoradiographs was performed using a Hewlett-Packard Co. ScanJet 4C/T scanner, and NIH Image Analysis software was used to measure KDR expression, to equalize with 18S expression, and to normalize to the percentage of most intense signal as previously described (5).

In situ hybridization

[^{35}S]UTP-labeled (NEN Life Science Products, Boston, MA) sense and antisense riboprobes from KDR and Flt-1 cDNAs were prepared for ISH with the MAXIscript *in vitro* transcription kit from Ambion, Inc. (Austin, TX), following the manufacturer's instructions. Processing was generally as previously described (24) with the following modifications: 10-μm frozen sections of endometrium mounted on SuperFrost Plus slides (Fisher Scientific) were fixed in 4% paraformaldehyde in PBS for 20 min at 4 C. The tissue sections were rinsed in 2 × SSC (standard saline citrate), then acetylated with 0.25% acetic anhydride in 0.1 mol/L triethanolamine (pH 8.0) for 10 min. Slides were then rinsed in 2 × SSC, dehydrated through an ascending series of alcohols, and air-dried. At this point at least one slide per tissue group was treated with ribonuclease A [RNase A; 20 mg/mL, 0.5 mol/L NaCl, 0.01 mol/L Tris, and 1 mmol/L ethylenediamine tetraacetate (EDTA), pH 8.0] as a negative control. The slides were prehybridized for 1 h at 42 C in 10 mmol/L dithiothreitol, 0.3 mol/L NaCl, 20 mmol/L Tris (pH 8.0), 5 mmol/L EDTA, 1 × Denhardt's solution, 10% dextran sulfate, and 50% formamide. Sections were then incubated at 55 C overnight in the same solution containing the appropriate concentrations of the sense and antisense probes (5 million cpm/mL). The most appropriate concentrations of labeled probes were empirically determined by serial dilution trials. RNase-treated sections were incubated with the antisense probe. After treatment with RNase A-containing buffer (20 mg/mL; 0.5 mol/L NaCl, 0.01 mol/L Tris, and 1 mmol/L EDTA, pH 8.0) at 37 C for 30 min to inactivate nonhybridized probe, the slides were rinsed in a descending series of SSC (2, 1, and 0.5 ×) and then incubated in 0.1 × SSC at 65 C (high stringency) for 30 min. Sections were dehydrated in an ascending series of alcohol dilutions, vacuum-dried, coated with NTB2 autoradiographic emulsion (Eastman Kodak Co., Rochester, NY), stored at 4 C for 2 weeks, developed in D-19 (Eastman Kodak Co.), lightly counterstained with hematoxylin, dehydrated in an ascending series of alcohol dilutions, cleared with xylene, and coverslipped with Permount (Fisher Scientific). Sense- and RNase-treated controls had no specific signals.

Statistical tests

Data were tested by ANOVA, and significance between groups was assessed with Fisher's protected least significant difference test (25).

Results

Steroid hormone levels in macaques

Serum concentrations of E_2 and P in macaques, produced by the sc E_2 and P implants, and also serum concentrations after removal of E_2 and/or P implants are presented in Table 1. E_2 levels were not different among monkeys receiving E_2 alone compared with those receiving both E_2 and P during artificial cycles. When P was withdrawn, and the E_2 implant remained, there was no change in serum E_2 levels. When both

TABLE 1. Serum concentrations of E_2 and P achieved during different hormonal states in artificially cycled rhesus and pigtail macaques

	E-14P (n = 4)	E+14P (n = 4)	E-2P (n = 4)	E-4/5P (n = 4)	2d sp (n = 4)	4/5d spE-14P (n = 4)
E_2 (pmol/L)	311.03 ± 20.30	335.80 ± 3.40	297.27 ± 3.13	294.51 ± 13.48	18.35 ± 0.00[a]	18.35 ± 0.00[a]
P (nmol/L)	0.60 ± 0.09	21.68 ± 1.58[b]	0.47 ± 0.03	0.98 ± 0.11	0.34 ± 0.01	0.31 ± 0.00

Values are the mean ± SE.
[a] Significantly ($P < 0.05$) different from other E_2 concentrations.
[b] Significantly ($P < 0.05$) different from other P concentrations.

the E_2 and P were withdrawn, E_2 levels dropped to less than 18.35 pmol/L. In the P add-back experiment, the mean serum level of P at the end of an artificial cycle was 18.44 nmol/L, and removal of P implants resulted in a decrease in serum P to less than 0.31 nmol/L by 24 h. P add back 24 h after P removal resulted in a rapid increase in serum levels to 143.41 nmol/L after 12 h, followed by a decline to 52.78 nmol/L by 24 h.

Steroid hormone levels in women

In the naturally cycling group, serum levels of P were as follows: proliferative phase, 6.77 ± 2.2 nmol/L; secretory phase, 33.92 ± 9.17 nmol/L; and premenstrual phase, 4.86 ± 1.59 nmol/L. Serum P concentrations in the premenstrual samples were significantly lower than those in the secretory samples ($P < 0.05$). In the group of women in whom the onset of menses was simulated, serum P levels in the 48-h withdrawn group (3.5 ± 0.7 nmol/L) were significantly lower ($P < 0.001$) than those in the LH+8–10 day group (50 ± 11 nmol/L), as previously reported (16).

Antibody specificity

KDR antibody 2–10−1 is a mouse monoclonal antihuman antibody specific for domains 6 and 7 of the extracellular region of human KDR (22). The antibody works in ICC on cells in culture and in frozen sections, but not in paraffin sections. In various tissues, including spleen, lymph node, and ovary, it stained only the vascular endothelium. Background staining with this antibody is extremely low. To exemplify both the specificity and clean-working properties of this antibody, we prepared very thick (~50–100 μm) cryosections and immunostained them to reveal the three-dimensional vascular pattern of the endometrium. Only the blood vessels were strongly stained in this proliferative phase specimen (Fig. 1).

The Flt-1 antibody detected vascular endothelium in cryosections in an equivalent manner as standard markers such as vWF. The pro-MMP-1 antibody and the CD antibodies are well characterized, highly specific mouse monoclonal antibodies. Irrelevant or isotype control mouse monoclonals were all negative or produced very faint background staining in human and macaque endometrial cryosections.

FIG. 1. A thick (100-μm) cryosection of a proliferative stage rhesus macaque endometrium immunostained with the KDR antibody. The high specificity and clean-working properties of this antibody permit delineation of the three-dimensional pattern of the vascular endothelium in this thick section of endometrium. (original magnification, ×50).

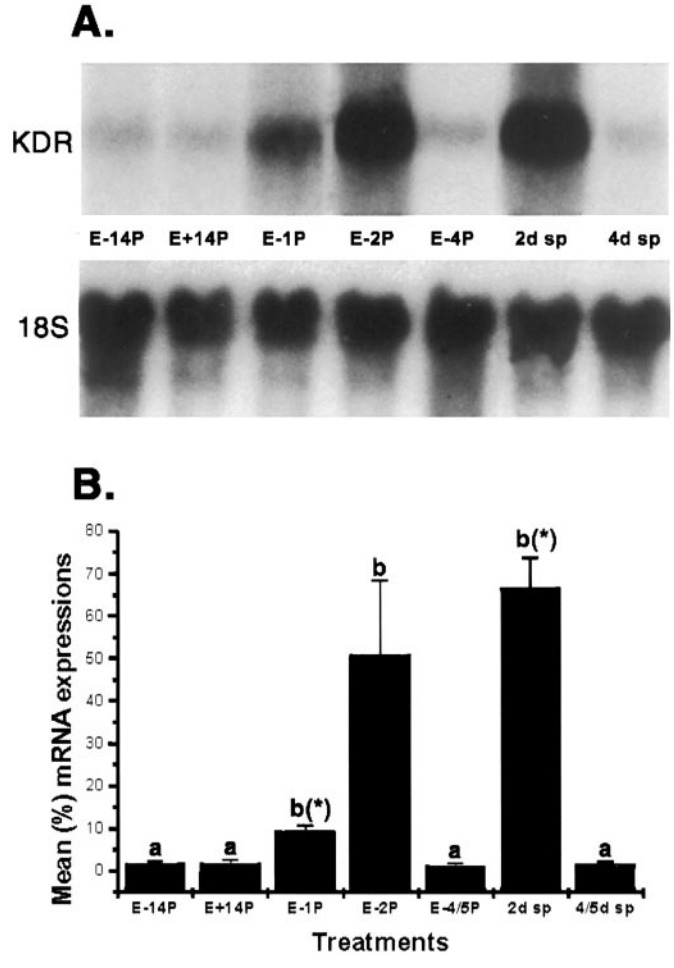

FIG. 2. Hormonal effects on KDR mRNA expression. A, Representative Northern blot showing KDR mRNA expression during different hormonal states and the respective 18S mRNA levels to demonstrate uniform loading. B, Bar graphs (mean ± SE) representing densitometric analysis of Northern blots after correction for loading differences and normalization to the percentage of the most intense signal. *Bars with different letters* and *bars with asterisks* are significantly different ($P < 0.05$). KDR mRNA is low under all hormonal conditions, except for the period from 1–2 days after P withdrawal regardless of whether E_2 is maintained.

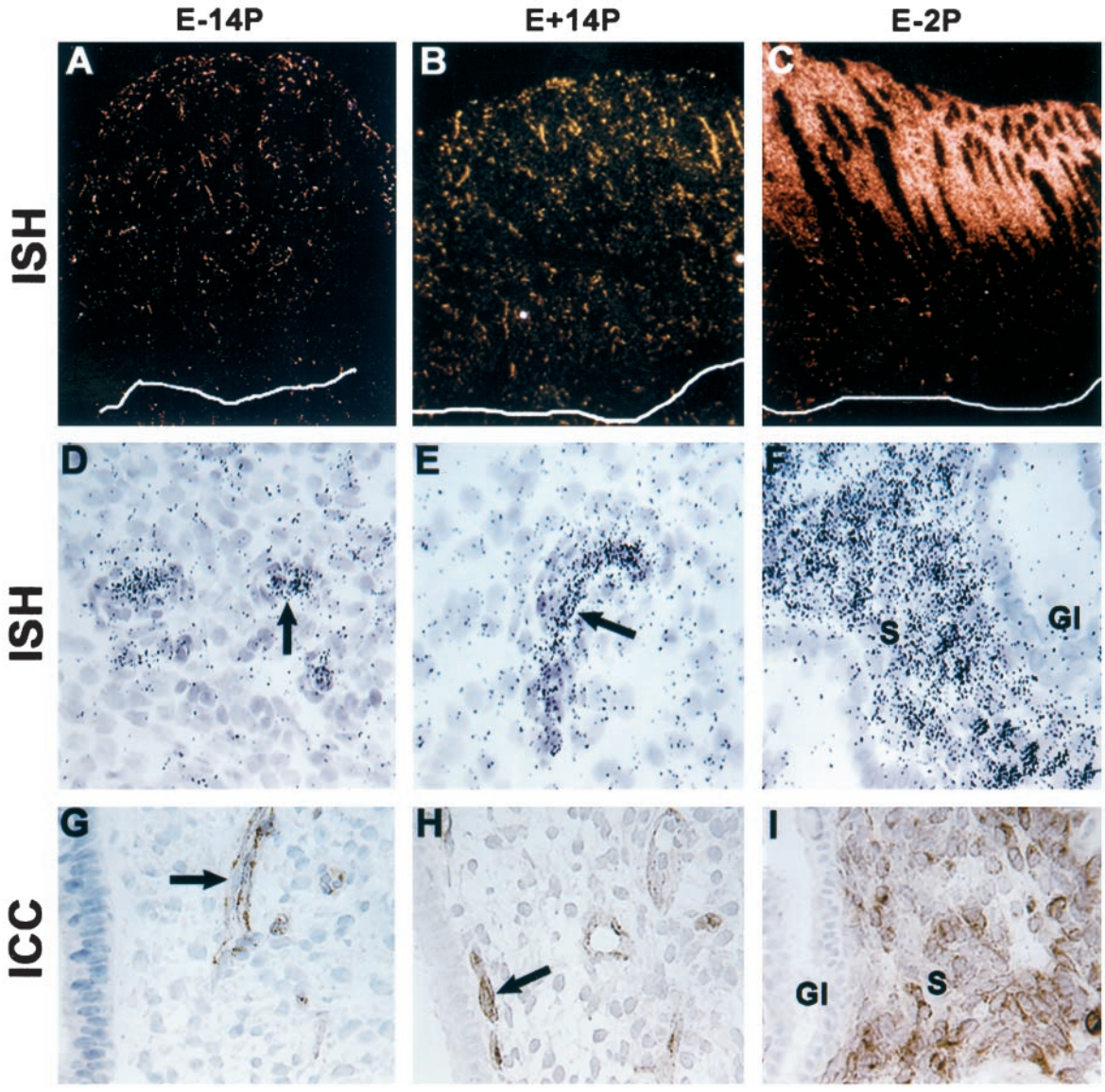

FIG. 3. Cellular localization of KDR mRNA and protein in the rhesus macaque endometrium. The first two rows are ISH preparations, and the third row shows ICC preparations. The three columns represent, *left* to *right*, E-14P, E+14P, and E-2P treatments. In this and succeeding plates: Gl, gland; S, stroma; *arrows*, blood vessels. A–C, ISH shown as darkfield images (magnification, ×25). D—F, ISH shown as brightfield images (magnification, ×312). G—I, ICC shown as brightfield images (magnification, ×312). KDR expression is restricted to the vasculature during the E-14P and E+14P treatments and is strongly up-regulated in the stroma of the upper endometrial zones only after E-2P treatment.

Expression of KDR messenger RNA (mRNA) in the macaque endometrium

The relative abundance of KDR transcripts in both pigtail and rhesus macaque endometrium was analyzed by Northern hybridization. Figure 2A shows a representative Northern blot probed with KDR-specific, random primed probes. Densitometric analyses (Fig. 2B) showed a low level signal at the late proliferative (E-14P) and late secretory (E+14P) stages. After P withdrawal, there were approximately 5- and 29-fold increases in the levels of KDR mRNA 1 day (E-1P) and 2 days (E-2P) later, respectively, and these increases were significantly higher than KDR mRNA levels in the E+14P (secretory phase) endometrium. By day E-4P (early proliferative phase), KDR transcripts had significantly declined to match those evident during the late proliferative and secretory phases. When E_2 and P implants were both removed, menstruation ensued normally, as previously reported (5). KDR mRNA levels were significantly increased (~38-fold) 2 days after both E_2 and P were withdrawn compared to those in the E+14P group, and then declined to low levels by day 4/5 spay. There was no significant difference in KDR mRNA expression between the E-2P and 2d spay groups.

Localization of KDR in endometrial stromal cells: dramatic increase during the premenstrual phase and after experimental P withdrawal

During the proliferative and secretory stages, the expression of KDR protein and mRNA was confined to the vascular

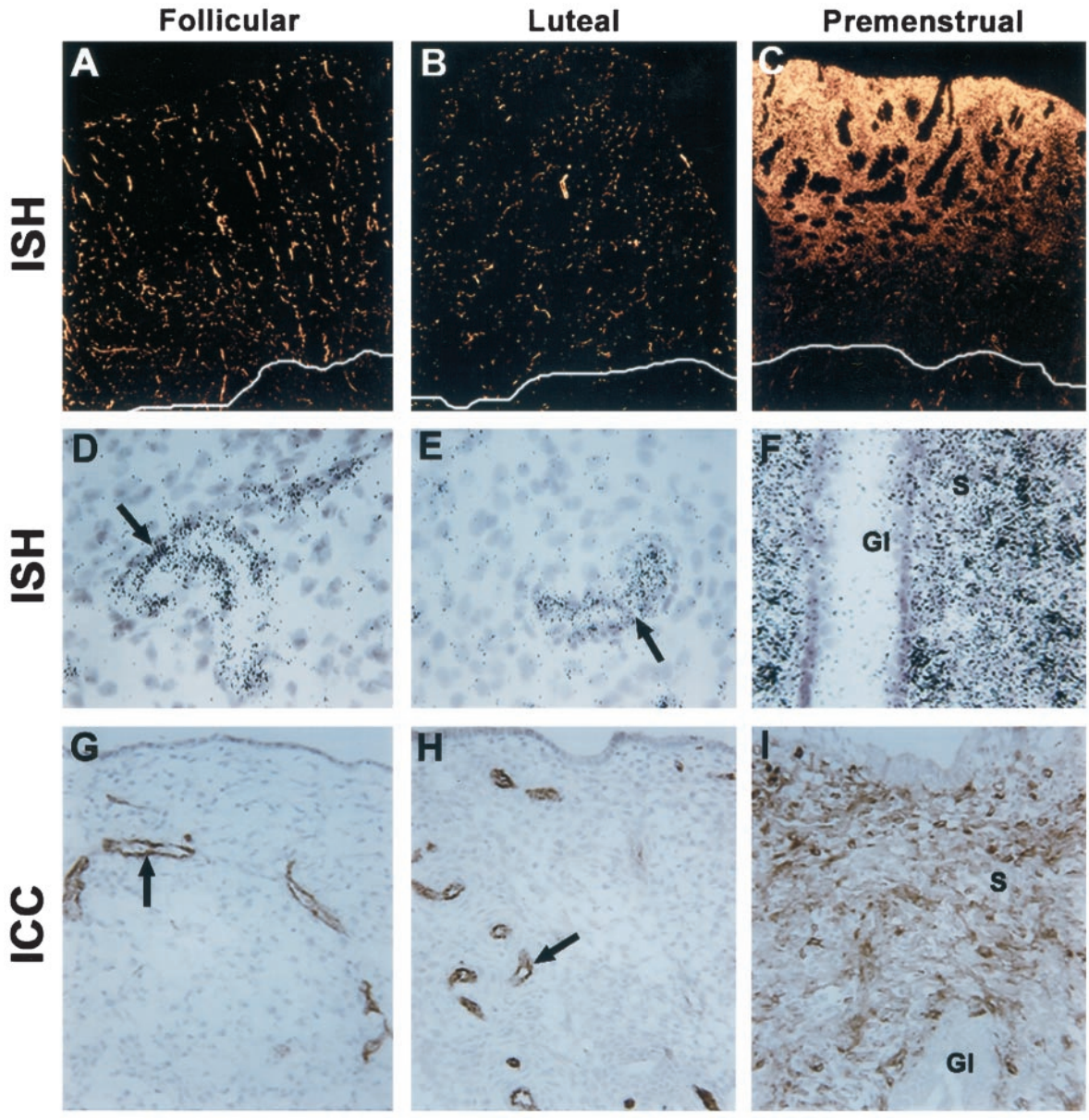

FIG. 4. Cellular localization of KDR mRNA and protein in the human endometrium. As in Fig. 3, the first two rows are ISH preparations, and the third row shows ICC preparations. The three columns represent, *left* to *right*, the proliferative, secretory, and premenstrual phases of the natural menstrual cycle. A—C, ISH shown as darkfield images (magnification, ×25). D—F, ISH shown as brightfield images (magnification, ×312). G—I, ICC shown as brightfield images (magnification, ×312). KDR expression is restricted to the vasculature during the proliferative and secretory phases and is strongly up-regulated in the stroma of the upper endometrial zones during the premenstrual phase.

endothelium in the rhesus macaque (Fig. 3, A, B, D, E, G, and H) and human (Fig. 4, A, B, D, E, G, and H) endometrium. After experimental P withdrawal in the rhesus macaque (Fig. 3, C, F, and I) and during the premenstrual stage in women (Fig. 4, C, F, and I), expression of KDR protein and mRNA was very strongly up-regulated in the endometrial stromal cells of the superficial zone. A marked gradient of KDR mRNA expression, from surface to midfunctionalis, was evident in both the human and macaque premenstrual endometrium (Figs. 3C and 4C). Exactly the same up-regulation and localization of KDR mRNA and protein were found on d2 spay, after both P and E_2 were withdrawn (data not shown). In the samples obtained from women before and after experimental P withdrawal, KDR immunoreactivity in the superficial zone stromal cells was undetectable in the LH peak + 8–10 day samples (similar to Fig. 4H) and greatly increased 24–48 h after P treatment ceased (similar to Fig. 4I). In pigtail macaques, the pattern of expression and cellular localization of KDR protein and mRNA was essentially similar to that seen in the rhesus and human endometrium.

P add back suppresses KDR expression in endometrial stromal cells, but not in vascular endothelium

In the experiments performed in pigtail macaques, Northern analysis revealed that in this species there was also a huge

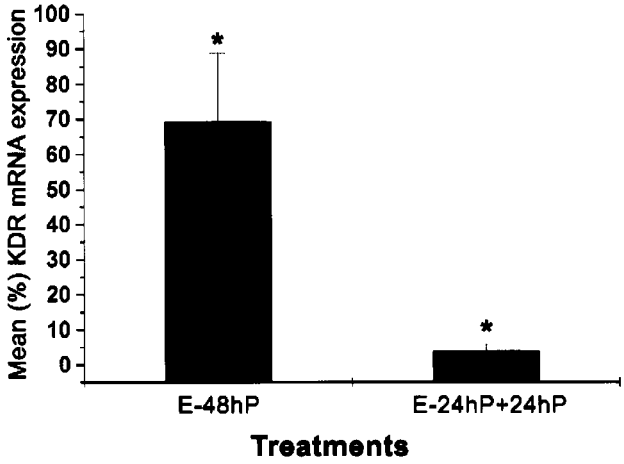

FIG. 5. Effects of P add back on KDR mRNA expression in the pigtail macaque endometrium. The figure shows a densitometric analysis of Northern blots after correction for loading and normalization as described in Fig. 2. Adding back P at 24 h after P withdrawal for 24 h (E-24hP+24hP) significantly suppresses the level of KDR mRNA expression induced by 48-h P withdrawal (E-48hP). *Asterisks* indicate significant differences at $P < 0.05$.

up-regulation of KDR mRNA in the endometrium 48 h after P withdrawal, and that this expression was dramatically reduced when P was added back 24 h after P withdrawal (Fig. 5). In addition, ICC (Fig. 6, A and B) and ISH (Fig. 6, C and D) revealed that KDR protein and mRNA were dramatically suppressed in the stromal, but not the vascular, endothelium after P add back. The regulation of vascular endothelial KDR and that of endometrial stromal KDR are clearly independent.

In pigtail macaques, pro-MMP-1 is expressed by the same population of cells that expresses KDR

We reported that in the rhesus macaque, several MMPs are up-regulated in the upper endometrial zones by P withdrawal (5). Here we note that in pigtail macaques, pro-MMP-1 protein was strongly expressed 48 h after P withdrawal in the same population of stromal cells expressing KDR (Fig. 6E). Furthermore, P add back after 24 h of P withdrawal completely suppressed the expression of pro-MMP-1 by these cells, as it had for KDR (Fig. 6F). E_2 and P levels in pigtailed macaques during artificial cycles were not distinguishable from those in rhesus macaques. As noted above (see *Steroid hormone levels in macaques)* P add back rapidly and effectively elevated P levels.

Infiltrating leukocytes in the premenstrual stage endometrial stroma do not contribute to the KDR signal; Flt-1 and vWF are not up-regulated in stromal cells by P withdrawal

Because leukocytes infiltrate the endometrium in the premenstrual period, we performed ICC for KDR, different markers of leukocytes, and vWF in consecutive sections of premenstrual stage macaque endometrium to determine whether any cell types other than resident endometrial stromal cells expressed KDR. Figure 7 shows an approximately consecutive series of sections of rhesus macaque endometrium immunostained for KDR, CD45, CD68, CD14, and

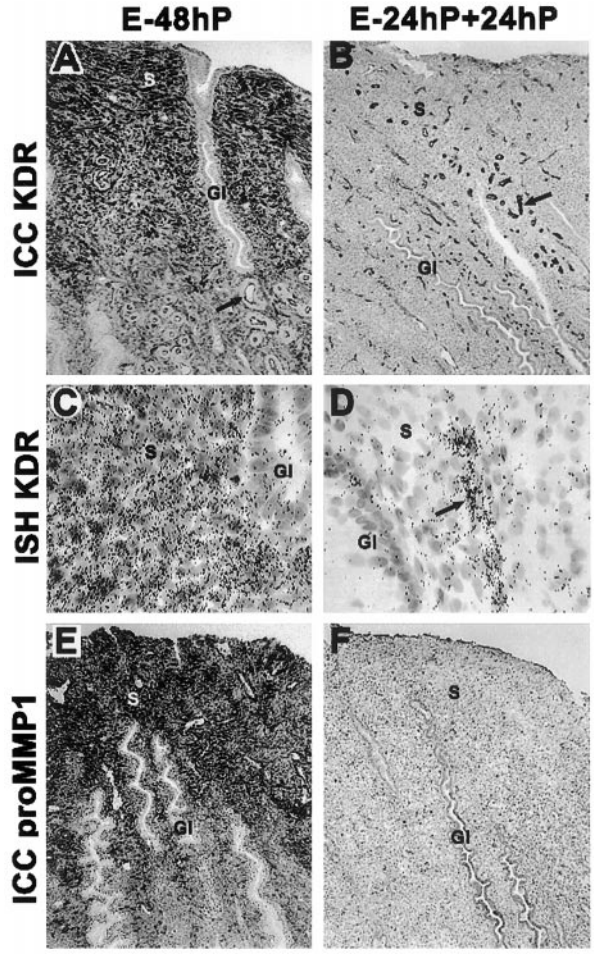

FIG. 6. Effects of P add back on KDR and pro-MMP1 cellular localization in the pigtail macaque endometrium. KDR protein (A; magnification, ×50) and transcript (C; magnification, ×312) were highly expressed in the upper zones of endometrial stroma after 48 h of P withdrawal. This up-regulation was completely suppressed (B and D) in the stroma by P add back for 24 h (E-24hP+24hP), but the vascular endothelium expression of KDR protein (B; magnification, ×50) and mRNA (D; magnification, ×312) was unaffected. Pro-MMP-1 protein, which was strongly expressed after 48 h of P withdrawal in the same population of stromal cells that expressed KDR (E; magnification, ×50), was also completely suppressed by P add back (F; magnification, ×50).

vWF. In macaque endometrium, CD45, CD68, and CD14 cells were widely scattered throughout the endometrium. Although these cells overlapped the zone of KDR-positive stromal cells, it was clear from their sparse distribution that they could not be the source of the uniform, extensive KDR expression evident in the upper zones. Moreover, there were large numbers of scattered cells positive for CD45, CD68, and CD14 in the lower zones of the endometrium (Fig. 7, G–I) regions that, except for the vasculature (Fig. 7F), were always negative for KDR. Similarly, in the human endometrium, most leukocytes were scattered in a loosely distributed pattern that could not account for the extensive, uniform KDR signal in the superficial zones (data not shown).

vWF staining of the upper zones of premenstrual (E-2P-treated) rhesus macaque endometrium (Fig. 7E) demonstrated that endothelial cells were only present within ves-

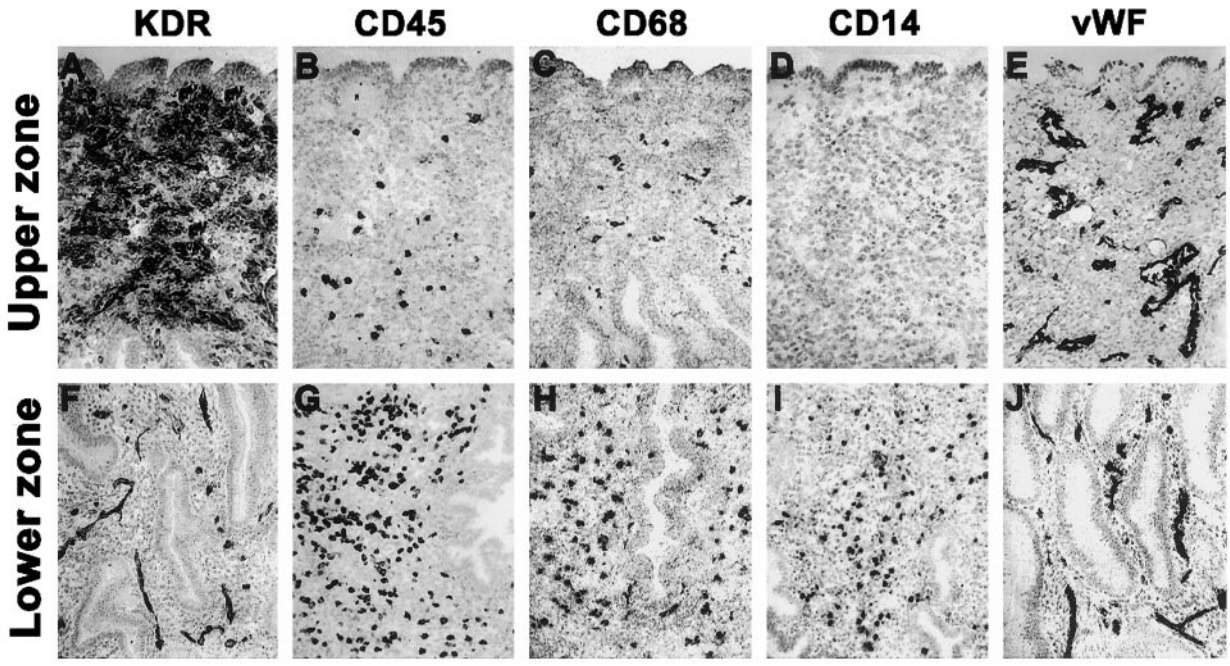

FIG. 7. ICC of KDR, vWF, and various leukocyte-specific markers (CD45, CD68, and CD14) in the premenstrual stage rhesus macaque endometrium. The *upper* (A–E) and *lower* (F–J) *halves* of this plate show the upper and lower endometrial zones respectively. In the upper zones, the extensive KDR signal (A) is not matched by the scattered distribution of CD45 (B)-, CD68 (C)-, or CD14 (D)-positive cells. In the lower zones, KDR is only expressed in blood vessels (F), whereas CD45 (G)-, CD68 (H)-, and CD14 (I)-positive cells are more densely distributed than in the upper zones. VWF immunostaining was evident only in blood vessels in both the upper (E) and lower (J) zones. L, Lumen. Magnification, ×125.

sels, not scattered throughout the stroma, a further indication that the resident endometrial stromal cells were the only source of the extensive expression of KDR during the premenstrual phase (E-2P). Flt-1 mRNA and protein, which were both clearly expressed in the vascular endothelium, were not expressed by endometrial stromal cells in the superficial zone after P withdrawal (Fig. 8, A–D).

Discussion

Our findings indicate that in both the human and macaque endometrium, there is a remarkable up-regulation of KDR expression that is cycle stage specific, cell type specific, zone specific, and VEGF receptor type specific.

Cycle stage specificity

The premenstrual stage was the only time in the natural cycle when a major increase in KDR expression by the human endometrium was evident. This is the stage when P levels precipitously decline due to luteolysis. In women whose cycle was experimentally controlled, KDR expression was maximal 48 h after P withdrawal. Similarly, in the macaque endometrium, stromal KDR expression was only increased when a premenstrual stage was experimentally induced at the end of an artificial cycle by P withdrawal. Northern analysis of macaque endometrial RNA at other cycle stages revealed that KDR transcript levels were minimal, reflecting KDR expression confined to the vascular endothelium. In macaques, the degree of KDR up-regulation was similar when either P alone was withdrawn and E_2 levels were maintained, or both P and E_2 were withdrawn. Therefore, P withdrawal is the key signal that results in up-regulation of KDR mRNA expression. A concurrent decline in E_2 does not appear to contribute to this increase.

Cell type specificity

The only cell type that responded dramatically to the withdrawal of P was the resident endometrial stromal cell in the upper functionalis. ISH and ICC revealed that glandular epithelial cells, myometrial cells, and stromal cells in the deeper zones were KDR negative at all times. The vascular endothelium was KDR positive at all time points we examined, and there was no evidence from Northern blots, ISH, or ICC for any substantial changes in endothelial KDR expression. A recent report (26) states that changes in the degree of vascular endothelial KDR expression were quantifiable by ICC in the human endometrium during the cycle. We have not yet performed quantitative studies of endothelial KDR expression throughout the cycle to assess this matter.

Scattered leukocytes, macrophages, and lymphocytes were present throughout the upper endometrial zones, but their sparse distribution made it evident that they could not contribute substantially to the uniform KDR signal evident in this zone. Moreover, the same leukocytes were scattered throughout the deep endometrial zones, regions that never showed any KDR signal except for that in the vasculature. It has been reported that CD68-positive macrophages can express KDR during the menstrual phase in human endometrium (26), but we saw no evidence for macrophage KDR with the antibody we used, nor did the pattern of ISH for KDR match the distribution of CD68-positive macrophages in the deeper zones.

It has also been reported that human peritoneal fluid

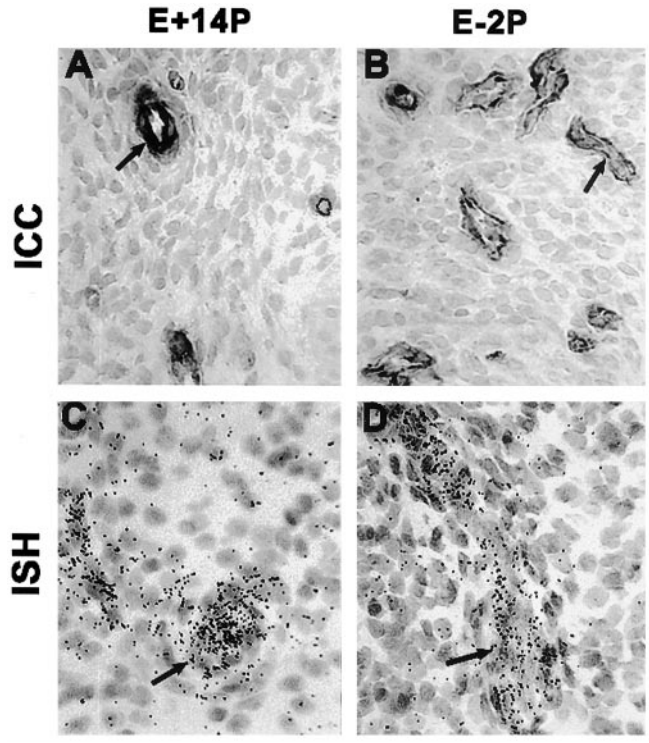

FIG. 8. Cellular localization of Flt-1 protein and mRNA in rhesus macaque endometrium at the secretory (E+14P) and premenstrual stages (E-2P). Flt-1 protein (A and B) and mRNA (C and D) remained localized within the vascular endothelium both before (A and C) and after (B and D) P withdrawal. No stromal expression of Flt-1 was evident at any time. Magnification, ×312.

macrophages, especially those positive for CD14, could express both Flt-1 and KDR as well as VEGF (27), but we found no evidence for such production by CD14-positive endometrial macrophages. The differences in the microenvironment between the peritoneal fluid and the endometrium may be responsible for these observed differences in macrophage expression of KDR. Finally, the possibility that the KDR signal we observed was due to endothelial cells that had emigrated from blood vessels was eliminated by vWF staining. This staining revealed that in the upper endometrial zones, in which the stroma strongly expressed KDR, the vasculature was intact (compare Fig. 7A with Fig. 7E).

VEGF receptor type specificity

KDR and Flt-1 were both present in the vascular endothelium of the endometrium, but only KDR was up-regulated by P withdrawal in the endometrial stromal cells. ISH and ICC for Flt-1 showed that this receptor was confined to the vascular endothelium throughout the entire cycle, including the premenstrual phase.

Zonal specificity

The restriction of the KDR signal to the upper endometrial zones after P withdrawal indicates that changes specific to this zone play key roles in elevating KDR expression. Adding back P for 24 h completely suppressed KDR expression in this zone, which confirmed that the effects on this zone are due to P withdrawal. Because our human samples were full thickness wedges rather than curettage samples, they included all endometrial zones down to the myometrium, and these wedges revealed that a zonal gradient of KDR expression occurred in the human as well as the macaque endometrium. The most likely explanation for this zonal restriction lies in the unique vascular pattern of the primate endometrium. The upper zones are primarily vascularized by the spiral artery system, and the lower zones by the basilar arteries (28). Vasoconstriction of the spiral arteries after P withdrawal, as originally described by Markee (1), would lead to localized ischemic hypoxia that could initiate a molecular cascade of signals leading to up-regulation of KDR.

Effects of hypoxia

VEGF itself is directly up-regulated by hypoxia in analogous fashion to other hypoxia-sensitive genes such as erythropoietin, through hypoxia-inducible factor (29). Pronounced up-regulation of Flt-1 and KDR genes have been reported in the lung vasculature in rats exposed to acute or chronic hypoxia (30) and throughout the heart in rats subjected to myocardial infarction (31). Hypoxia has been shown to induce KDR in endothelial cells in culture (32). Although we did not observe any dramatic increase in KDR mRNA expression in upper zone vascular endothelial cells by ISH on days 1–2 after P withdrawal, any increase in such expression may have been masked by the very strong expression of KDR mRNA in upper zone stromal cells.

However, the KDR gene is probably not directly up-regulated by hypoxia, as Gerber *et al.* (33) reported that the KDR gene is lacking the hypoxia-inducible enhancer element in its promoter. However, evidence is accumulating that VEGF itself can stimulate KDR expression. For example, in an avian embryonic limb bud model, overexpression of VEGF resulted in up-regulation of KDR (34), and addition of VEGF to mouse brain cerebral slices enhanced KDR expression in the vascular endothelium (35). Therefore, the hypoxia-induced enhancement of VEGF expression in the glands and stroma of the upper endometrial zones could be responsible, through an autocrine or paracrine mechanism, for the up-regulation of stromal KDR.

Functional correlates of premenstrual stromal KDR expression

The entire upper third to one half of the endometrium is destined to be sloughed off during menstruation, and the MMPs, which are presumed to be responsible for this tissue destruction, are expressed specifically by the stromal cells in this zone just before and during menstruation (5). Therefore, our current working hypothesis is that VEGF-KDR interactions in premenstrual stromal cells play some role in the initiation of MMP expression. There are several reports that VEGF receptors are expressed in several nonendothelial cell types (14, 36, 37). In these cells as well as in endothelial cells (15), one of the defined actions of VEGF is the receptor-mediated enhancement of several proteolytic enzymes, including the MMPs. For example, VEGF can stimulate ex-

pression of MMP-1, -3, and -9 in vascular smooth muscle cells (14) and MMP-1 in endothelial cells (15). A role of VEGF in cervical ripening has also been suggested based on the observation that VEGF stimulates the production of MMP-2 and MMP-9 from cervical fibroblast explants acting through Flt-1 and KDR (13). Additional experimental analysis is required to add strength to this hypothesis, including ligand binding studies to assess the binding capacity of the stromal KDR and the use of VEGF-specific and/or KDR-specific antibodies as blocking agents to disrupt MMP expression *in vivo* or *in vitro*.

In summary, our data indicate that in women and two nonhuman primate species, P withdrawal *in vivo* induces KDR up-regulation in the stromal cells of the upper endometrial zones. These zones are destined to slough and bleed, and there is a tight temporal and spatial relationship among VEGF, KDR, and MMPs in this tissue. We conclude that a VEGF-KDR-MMP link may be a component of the premenstrual/menstrual process. Of course, many other factors are operative during the premenstrual phase (38–40), and several reports note that P withdrawal itself can induce MMP expression in endometrial stromal cells cultured *in vitro* under presumably normoxic conditions (41–43). Consequently, additional experimental studies are needed to ascertain the details of the various interactions involved, both *in vivo* and *in vitro*. Nevertheless, our data suggest that VEGF could play a previously unappreciated role during the premenstrual phase that is different from its usual angiogenic role; however, the two roles may be linked by the production of MMPs. Research into these interactions should increase our knowledge of the control of natural menstrual bleeding, and ultimately benefit the large number of women who suffer from menorrhagia and breakthrough bleeding.

Acknowledgments

We thank Teresa A. Drudy (Edinburgh, Scotland) and Kunie Mah (Beaverton, OR) for superb immunocytochemical technical support. Dr. Brenner expressly thanks the Burroughs-Wellcome Foundation for support during his sabbatical in 1997 at the Center for Reproductive Biology (Edinburgh, Scotland), where this work was initiated in the laboratory of Profs. David T. Baird and Hilary O. D. Critchley.

References

1. **Markee JE.** 1940 Menstruation in intraocular endometrial transplants in the rhesus monkey. Contrib Embryol. 28:219–308.
2. **Stirrat GM.** 1999 Choice of treatment for menorrhagia. Lancet. 353:2175–2176.
3. **Hulboy DL, Rudolph LA, Matrisian LM.** 1997 Matrix metalloproteinases as mediators of reproductive function. Mol Hum Reprod. 3:27–45.
4. **Salamonsen LA, Woolley DE.** 1996 Matrix metalloproteinases in normal menstruation. Hum Reprod. 11(Suppl 2):124–133.
5. **Rudolph-Owen LA, Slayden OD, Matrisian LM, Brenner RM.** 1998 Matrix metalloproteinase expression in *Macaca mulatta* endometrium: evidence for zone-specific regulatory tissue gradients. Biol Reprod. 59:1349–1359.
6. **Rodgers WH, Matrisian LM, Giudice LC, et al.** 1994 Patterns of matrix metalloproteinase expression in cycling endometrium imply differential functions and regulation by steroid hormones. J Clin Invest. 94:946–953.
7. **Rodgers WH, Osteen KG, Matrisian LM, Navre M, Giudice LC, Gorstein F.** 1993 Expression and localization of matrilysin, a matrix metalloproteinase, in human endometrium during the reproductive cycle. Am J Obstet Gynecol. 168:253–260.
8. **Bausero P, Cavaillé F, Méduri G, Freitas S, Perrot-Applanat M.** 1998 Paracrine action of vascular endothelial growth factor in the human endometrium: production and target sites, and hormonal regulation. Angiogenesis. 2:167–182.
9. **Shifren JL, Tseng JF, Zaloudek CJ, et al.** 1996 Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. J Clin Endocrinol Metab. 81:3112–3118.
10. **Sharkey AM, Day K, McPherson A, et al.** 2000 Vascular endothelial growth factor expression in human endometrium is regulated by hypoxia. J Clin Endocrinol Metab. 85:402–409.
11. **Charnock-Jones DS, Sharkey AM, Rajput-Williams J, et al.** 1993 Identification and localization of alternately spliced mRNAs for vascular endothelial growth factor in human uterus and estrogen regulation in endometrial carcinoma cell lines. Biol Reprod. 48:1120–1128.
12. **Popovici RM, Irwin JC, Giaccia AJ, Giudice LC.** 1999 Hypoxia and cAMP stimulate vascular endothelial growth factor (VEGF) in human endometrial stromal cells: potential relevance to menstruation and endometrial regeneration. J Clin Endocrinol Metab. 84:2245–2248.
13. **Ahmed A, Sisi P, Gee H, Li X-F, Khaliq A.** Role of vascular endothelial growth factor (VEGF) in human cervical ripening: activation of matrix metalloproteinases (MMPs) via VEGF receptors. Proc of the 44th Annual Meet of the Soc for Gynecol Invest, 1997. Abstract 736.
14. **Wang H, Keiser JA.** 1998 Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells. Role of flt-1. Circ Res. 83:832–840.
15. **Unemori EN, Ferrara N, Bauer EA, Amento EP.** 1992 Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. J Cell Physiol. 153:557–562.
16. **Critchley HOD, Jones RL, Lea RG, et al.** 1999 Role of inflammatory mediators in human endometrium during progesterone withdrawal and early pregnancy. J Clin Endocrinol Metab. 84:240–248.
17. **Yong EL, Glasier A, Hillier H, et al.** 1992 Effect of cyclofenil on hormonal dynamics, follicular development and cervical mucus in normal and oligomenorrhoeic women. Hum Reprod. 7:39–43.
18. **Slayden OD, Koji T, Brenner RM.** 1995 Microwave stabilization enhances immunocytochemical detection of estrogen receptor in frozen sections of macaque oviduct. Endocrinology. 136:4012–4021.
19. **Brenner RM, Rudolph L, Matrisian L, Slayden OD.** 1996 Non-human primate models: artificial menstrual cycles, endometrial matrix metalloproteinases and s.c. endometrial grafts. Hum Reprod. 11(Suppl 2):150–164.
20. **West NB, Hess DL, Brenner RM.** 1986 Differential suppression of progesterone receptors by progesterone in the reproductive tract of primate macaques. J Steroid Biochem. 25:497–503.
21. **McClellan MC, Rankin S, West NB, Brenner RM.** 1990 Estrogen receptors, progestin receptors and DNA synthesis in the macaque endometrium during the luteal-follicular transition. J Steroid Biochem Mol Biol. 37:631–641.
22. **Menrad A, Thierauch K-H, Martiny-Baron G, Siemeister G, Schirner M, Schneider MR.** 1997 Novel antibodies directed against the extracellular domain of the human VEGF-receptor type II. Hybridoma. 16:465–471.
23. **Low KG, Nielsen CP, West NB, et al.** 1989 Proenkephalin gene expression in the primate uterus: regulation by estradiol in the endometrium. Mol Endocrinol. 3:852–857.
24. **Koji T, Chedid M, Rubin JS, et al.** 1994 Progesterone-dependent expression of keratinocyte growth factor mRNA in stromal cells of the primate endometrium: keratinocyte growth factor as a progestomedin. J Cell Biol. 125:393–401.
25. **Petersen RG.** 1985 Design and analysis of experiments, 66th Ed. New York: Marcel Dekker.
26. **Meduri G, Bausero P, Perrot-Applanat M.** 2000 Expression of vascular endothelial growth factor receptors in the human endometrium: modulation during the menstrual cycle. Biol Reprod. 62:439–447.
27. **McLaren J, Prentice A, Charnock-Jones DS, et al.** 1996 Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. J Clin Invest. 98:482–489.
28. **Daron GH.** 1936 The arterial pattern of the tunica mucosa of the uterus in macacus rhesus. Am J Anat. 58:349–419.
29. **Goldberg MA, Schneider TJ.** 1994 Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. J Biol Chem. 269:4355–4359.
30. **Tuder RM, Flook BE, Voelkel NF.** 1995 Increased gene expression for VEGF and the VEGF receptors KDR/flk and Flt in lungs exposed to acute or to chronic hypoxia. Modulation of gene expression by nitric oxide. J Clin Invest. 95:1798–1807.
31. **Li J, Brown LF, Hibberd MG, Grossman JD, Morgan JP, Simons M.** 1996 VEGF, flk-1, and flt-1 expression in a rat myocardial infarction model of angiogenesis. Am J Physiol. 270:H1803–H1811.
32. **Brogi E, Schatteman G, Wu T, et al.** 1996 Hypoxia-induced paracrine regulation of vascular endothelial growth factor receptor expression. J Clin Invest. 97:469–476.
33. **Gerber H-P, Condorelli F, Park J, Ferrara N.** 1997 Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. *Flt*-1, but not *Flk*-1/KDR, is up-regulated by hypoxia. J Biol Chem. 272:23659–23667.
34. **Flamme I, Reutern MV, Drexler HCA, Syed-Ali S, Risau W.** 1995 Overexpression of Vascular endothelial growth factor in the avian embryo induces hypervascularization and increased vascular permeability without alterations of embryonic pattern formation. Dev Biol. 171:399–414.
35. **Kremer C, Breier G, Risau W, Plate KH.** 1997 Up-regulation of flk-1/vascular endothelial growth factor receptor 2 by its ligand in a cerebral slice culture system. Cancer Res. 57:3852–3859.

36. **Brown LF, Tognazzi K, Dvorak HF, Harrist TJ.** 1996 Strong expression of kinase insert domain-containing receptor, a vascular permeability factor/vascular endothelial growth factor receptor in AIDS-associated Kaposi's sarcoma and cutaneous angiosarcoma. Am J Pathol. 148:1065–1074.
37. **Boocock CA, Charnock-Jones DS, Sharkey A, et al.** 1995 Expression of vascular endothelial growth factor and its receptors flt and KDR in ovarian carcinoma. J Soc Gynecol Invest. 87:506–516.
38. **Tabibzadeh S.** 1996 The signals and molecular pathways involved in human menstruation, a unique process of tissue destruction and remodelling. Mol Hum Reprod. 2:77–92.
39. **Schatz F, Aigner S, Papp C, Toth-Pal E, Hausknecht V, Lockwood CJ.** 1995 Plasminogen activator activity during decidualization of human endometrial stromal cells is regulated by plasminogen activator inhibitor 1. J Clin Endocrinol Metab. 80:2504–2510.
40. **Baird DT, Cameron ST, Critchley HOD, et al.** 1996 Prostaglandins and menstruation. Eur J Obstet Gynecol. 70:15–17.
41. **Lockwood CJ, Krikun G, Hausknecht VA, Papp C, Schatz F.** 1998 Matrix matalloproteinase and matrix metalloproteinase inhibitor expression in endometrial stromal cells during progestin-initiated decidualization and menstruation-related progestin withdrawal. Endocrinology. 139:4607–4613.
42. **Irwin JC, Kirk D, Gwatkin RBL, Navre M, Cannon P, Giudice LC.** 1996 Human endometrial matrix metalloproteinase-2, a putative menstrual proteinase. Hormonal regulation in cultured stromal cells and messenger RNA expression during the menstrual cycle. J Clin Invest. 97:438–447.
43. **Marbaix E, Kokorine I, Moulin P, Donnez J, Eeckhout Y, Courtoy PJ.** 1996 Menstrual breakdown of human endometrium can be mimicked *in vitro* and is selectively and reversibly blocked by inhibitors of matrix metalloproteinases. Proc Natl Acad Sci USA. 93:9120–9125.