Expression of Vascular Endothelial Growth Factor and Its Receptors in the Rhesus Monkey (*Macaca mulatta*) Endometrium and Placenta During Early Pregnancy

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ABSTRACT Vascular endothelial growth factor (VEGF) is fundamental for development and maintenance of endometrial and placental vascular function during pregnancy. While there are a number of studies on VEGF in the human placenta, they are mostly restricted to late pregnancy. To further understand the role of VEGF in mediating angiogenesis during human early pregnancy, we employed a rhesus monkey early pregnancy model to study the temporal and spatial expression of VEGF and its receptors, fms-like tyrosine kinase (Flt)-1, and kinase-insert domain-containing receptor (KDR) mRNAs and proteins in the uteri on day 12, 18, and 26 of pregnancy using in situ hybridization, RT-PCR, and immunohistochemistry. VEGF mRNA had been identified in the luminal epithelium on day 12, in the glandular epithelium on day 12 and 18, and the highest expression was detected in the walls of some spiral arterioles adjacent to the implantation site on day 18, in the placental villi and in the fetal-maternal border on day 18 and 26. Besides, immunostaining of VEGF was detected in the placental villi and endometrial compartments including spiral arteries walls and the glandular epithelium. The localization of VEGF in the endothelium correlates with the presence of Flt-1 and KDR receptors on vascular structure. All the results above suggest that VEGF-VEGFR pairs were involved in the process of trophoblast invasion, maternal vascular transformation, and fetoplacental vascular differentiation and development during the rhesus monkey early pregnancy. Expression of VEGF, Flt-1, and KDR in the epithelial cells also hints some additionally functional roles of VEGF during early pregnancy. *Mol. Reprod. Dev. 65: 123–131, 2003.* © 2003 Wiley-Liss, Inc.

Key Words: endometrium; implantation; placenta; VEGF; VEGFR

INTRODUCTION

Successful pregnancy requires the development of a vascular network that facilitates the maternal-fetal communication (Gordon et al., 1995). Angiogenesis is a fundamental component in the blastocyst implantation and the development of endometrium in early pregnancy. Changes in the vascular compartments occur within the placental villi, as well as in the maternal deciduas, to establish the vascular structures involved in placental exchange during early pregnancy (Reynolds et al., 1992; Athanassiades et al., 1998). The formation of new vessels depends on interactions between various hormones, growth factors, inhibitors, and insoluble extracellular matrix molecules (Ingber and Folkman, 1989). The vascular endothelial growth factor (VEGF), as one of the most predominant regulator of angiogenesis and vascular permeability, may play an active role during early pregnancy (Chakraborty et al., 1995; Athanassiades et al., 1998; Gerwins et al., 2000; Mueller et al., 2000).

VEGF is a mitogen for endothelial cells and a potent inducer of angiogenesis. It is a homodimeric glycoprotein of two 23 kDa subunits exhibiting sequence homology with platelet-derived growth factor (PDGF) (Keck et al., 1989; Conn et al., 1990; Ghosh et al., 2000). At present, five isoforms of human VEGF containing 121, 145, 165, 189, and 206 amino acids have been identified by analyses of genomic and cDNA clones (Houck et al., 1991; Charnock-Jones et al., 1993), and their actions are mediated via two tyrosine kinase family receptors, fms-like tyrosine kinase (Flt)-1 (VEGFR-1) and the kinase-insert domain-containing receptor (KDR, VEGFR-2) (de Vries et al., 1992; Kendall and Thomas, 1993; Boocock et al., 1995). For VEGF, it is

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known that binding to Flt-1 mediates endothelial cell interaction and tubule formation (Fong et al., 1995), whereas binding to KDR causes endothelial cell differentiation and proliferation (Bernatchez et al., 1999). Studies involving gene knockout mice have implicated that *Flt-1*-deficient mice develop endothelial cells but with greatly disturbed vascular lumen formation (Fong et al., 1995), whereas mice deficient in *Flk-1* (the mouse homolog of KDR) did not develop any endothelial cells or blood islands (Shalaby et al., 1995), indicating that *vegf* and its two receptors genes *flk-1* and *flt-1* were necessary for angiogenesis and vascular development, and the loss of these genes led to embryonic lethality (Ferrara et al., 1996).

The expression of VEGF and its receptors has been examined in the cyclic human (Charnock-Jones et al., 1993) and mouse uteri (Shweiki et al., 1993), as well as in the human, rat and mouse embryos and uteri during the post-implantation and mid-gestational periods (Matthews et al., 1991; Shweiki et al., 1993; Charnock-Jones et al., 1994). Until recently, very little is known about the functional role of VEGF in rhesus monkey placental development, and observations on VEGF receptors in the uterus appear not to be available in rhesus monkey. Besides, using primate monkey as a model system, which is highly relevant to the human reproductive biology, will contribute to the study on the functional mechanisms of VEGF in human.

To determine the spatiotemporal expression relationship between VEGF and its receptors in the macaque endometrium and placenta during early pregnancy, experiments were designed to detect mRNAs for VEGF and its receptors—Flt-1 and KDR, and to localize their proteins in the macaque endometrium and placenta on day 12, 18, and 26 of pregnancy.

MATERIALS AND METHODS

Animals, Tissue Collection, and Processing

The uteri of 12 pregnant rhesus monkeys (*Macaca mulatta*) were collected at the Center for Medical Primate, Institute of Medical Biology, Chinese Academy of Medical Sciences. Sexually matured female rhesus monkeys, with a history of regular menstrual cycles and prior pregnancies, were housed individually and maintained in compliance with the standards of the Ethical Committee, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. Female macaques were placed with the males for 2 days from the anticipated time of ovulation, and the anticipated day of ovulation was designated as day 0 of pregnancy. On estimated day 12, 18, and 26 of pregnancy, monkeys were laparotomized under ketamine hydrochloride anesthesia. Uteri were removed and trimmed laterally to expose the primary implantation site. The implantation site was cut into two parts, with one part embedded in embedding medium (Triangle Biomedical Sciences, Durham, NC) for frozen tissue specimens and the other frozen in liquid nitrogen and stored at −80°C until analyzed.

In Situ Hybridization

The plasmid containing VEGF cDNA was kindly provided by Dr. S.K. Dey (Department of Molecular and Integrative Physiology, Kansas University Medical Center, KS). Probe labeling was performed according to the instructions of digoxigenin RNA labeling mix (Boehringer-Mannheim, Indianapolis, IN). In situ hybridization was detailed previously (Braissant and Wahli, 1998). In brief, adjacent cryostat sections (10 μm) were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.5) for 15 min, then incubated in PBS containing 0.1% active diethylpyrocarbonate (DEPC) for 30 min at room temperature (RT). Prehybridization was carried out at 50°C for 2 hr in a buffer containing 50% deionized formamide, 5 × SSC [1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate], and 120 μg/ml salmon sperm DNA. Slides were then hybridized with 400 ng/ml of the labeled probe for 18 hr at 50°C. This was followed by serial washings as follows: 2 × SSC at RT for 30 min, 2 × SSC at 65°C for 1 hr, 0.1 × SSC at 65°C for 1 hr. The slides were then incubated for 2 hr at RT with anti-digoxigenin-alkaline phosphatase (dilution 1:3,000; Roche Diagnostics Ltd., Hong Kong, China), and rinsed in buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5) for 30 min to remove excessive antibody. Color development was carried out using nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer-Mannheim). Sense probe hybridizations were used as a control for the background level.

RT-PCR

The presence of mRNAs for Flt-1 and KDR in the macaque endometrium was examined by RT-PCR. Total RNA of approximately 100 mg of macaque endometrium was isolated using Trizol reagent (Gibco-BRL, Grand Island, NY). The integrity of the purified RNA was confirmed by visualization of the 28 S and 18 S rRNA bands after the electrophoresis of RNA through a 1% agarose-formaldehyde gel. The quantity of total RNA was determined by spectrophotometric measurement at 260 nm. cDNAs were obtained from 2 μg RNA using superscript II reverse transcriptase (Gibco-BRL). The resulting cDNA samples were amplified by PCR. Specific primer pairs used in this study are as follows: (1) Flt-1, anti-sense 5′-CCA CCA CTC AAG ATT ACT CCA-3′ and sense 5′-ATC ATA GGG CAG CCG TTC-3′, giving a PCR product of 620 base pairs (bp); (2) KDR, anti-sense 5′-CGT CCT CCT TCC TCA CTC T-3′ and sense 5′-CCA CAG CCT CTG CCA ATC-3′ to amplify a PCR product of 433 bp. Amplification of the human β-actin gene transcripts was used to control the efficiency of RT-PCR among the samples. Sequences of anti-sense and sense primers for β-actin were 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′and 5′-GTG GGG CGC CCC AGG CAC CA-3′, respectively, and the expected size of the PCR product was 548 bp. PCR was conducted in the exponential range of amplification for each set of primers. The ranges in which the exponential amplification

were observed were 25 cycles for Flt-1, KDR, and β-actin, therefore PCR was performed by 25 cycles for Flt-1, KDR, and β-actin. The parameters used for PCR amplification were as follows: denaturation, 45 sec at 94°C; annealing, 45 sec at 56°C for Flt-1 and 55°C for KDR; extension, 45 sec at 72°C. The products of the amplification were separated on 1 % agarose containing ethidium bromide and photographed under UV transilluminator. As internal controls for the RT, samples without RNA or without reverse transcriptase were prepared in parallel, and these yield no amplification products (data not shown). As negative controls for the PCR, samples without reverse-transcribed cDNA or without Taq enzyme were used (data not shown). As positive controls, samples containing Flt-1 or KDR cDNA were performed (data not shown).

Immunohistochemistry

A standard immunohistochemical technique, avidin-biotin-peroxidase complex method (Vectastain ABC kit, Vector Labs., Burlingame, CA) was adopted to visualize VEGF, Flt-1, and KDR immunostaining and to identify the detailed endometrial and placental tissue morphology in the cryosections of rhesus monkey uterine samples. Briefly, cryosections (10 μm) were fixed in 4% paraformaldehyde for 10 min and washed with PBS. They were then sequentially incubated at RT with normal horse serum (dilution 3:200, Vector Labs.) for 20 min, the primary antibody for 30 min, the biotinylated secondary antibody (dilution 1:200, Vector Labs.) for 30 min, methanol containing 0.3% H_2O_2 for 30 min, and the AB reagent (dilution 1:100, Vector Labs.) for 40 min. Intervening PBS washes were necessary after each incubation. Sites of the bound enzyme were visualized by the application of 3,3′-diaminobenzidine (DAB) in H_2O_2 solution. Slides were washed, mounted, and examined under light microscopy. In this study, polyclonal rabbit anti-VEGF, anti-Flt-1, and anti-Flk-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used to immunohistochemically localize VEGF, Flt-1, and KDR, respectively. A monoclonal mouse anti-vimentin was used to identify the detailed morphology of the cryosections. A monoclonal mouse anti-actin and a polyclonal rabbit anti-Factor VIII were employed to respectively recognize smooth muscle cells and endothelial cells of the arterioles. Immunohistochemical controls consisted of substituting the primary antibody or the biotinylated anti-rabbit serum by pre-immune serum.

Statistical Analysis

The quantity of the PCR products was determined by densitometric analysis of the intensities of the bands (MetaView image analyzing system, version 4.50, Universal Imaging Corp., Downington, PA). The relative levels of Flt-1 or KDR mRNAs normalized to β-actin mRNA were calculated. Signal intensities of VEGF mRNA and VEGF, Flt-1, and KDR protein messages detected by in situ hybridization and immunohistochemistry were determined by computer-aided laser scanning densitometry (Personal Densitometer SI; Molecular Dynamics Inc., Sunnyvale, CA). In order to make the statistical significance of the quantitative difference credible, at least three slides from four animals of each group were examined. In each compartment on a slide, at least 80 spots were randomly selected. Statistical analysis was performed using Statistical Package for Social Science (SPSS for Windows package release 10.0; SPSS, Inc., Chicago, IL). The nonparametric Mann–Whitney match pair test was used in the evaluation of the data. $P < 0.05$ was considered to be statistically significant.

RESULTS

Localization of VEGF mRNA in the Macaque Endometrial and Placental Compartments During Early Pregnancy

In order to study the gene expression of VEGF in the placental villi, pregnant endometrium, as well as in the fetal-maternal border during rhesus monkey early pregnancy, in situ hybridization using VEGF RNA probes was carried out. As shown in Figure 1, VEGF mRNA messages were expressed in the luminal epithelial cells and glandular epithelial cells, but were absent from the decidual cells (Fig. 1a) on day 12 of pregnancy. On day 18 of pregnancy, the syncytiotrophoblast and cytotrophoblast cells lining the placental villi (Fig. 1b), as well as the glandular epithelial cells (data not shown) both exhibited numerous VEGF transcripts. VEGF mRNA was also well defined and strong in the walls of some spiral arteries situated adjacent to the implantation site on day 18 of pregnancy (Fig. 1b). A high level of VEGF transcripts were detected in the fetal-maternal border on day 26 (Fig. 1c), while the syncytiotrophoblast and cytotrophoblast cells lining the placental villi and the endometrial compartments expressed relatively faint VEGF mRNA (Fig. 1c). Figure 1g–i show respectively the detailed tissue morphology of the uterus and the placenta on day 12, 18, and 26 of pregnancy by vimentin immunostaining. Control sections corresponding to Figure 1a–c hybridized to the sense probes are shown in Figure 1d–f, respectively, in which no specific signal was detectable above a low background.

Expression of Flt-1 and KDR mRNAs in the Macaque Endometrium by Using RT-PCR

Using RT-PCR, mRNAs for Flt-1 and KDR were detected in total RNA samples from the endometrium of rhesus monkeys on day 12, 18, and 26 of pregnancy. Representative pictures of PCR-amplified products are shown in Figure 2a. Flt-1 mRNA was detected at all the three time points examined, and the Flt-1 mRNA expression in the endometrium was the most intense on day 12 of pregnancy (Fig. 2a,b). There was an overall expression of KDR mRNA in the endometrium on day 12, 18, and 26 of pregnancy (Fig. 2a,c), and the expression levels did not change substantially at these three time points examined ($P > 0.05$).

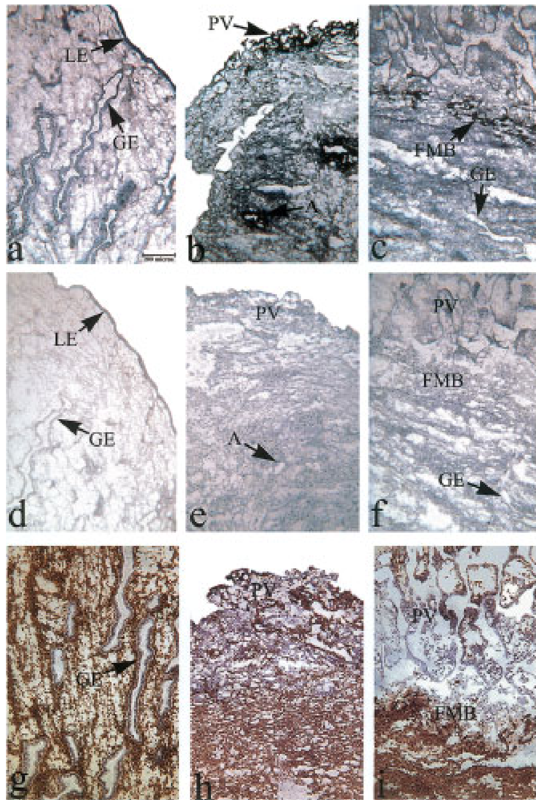

Fig. 1.

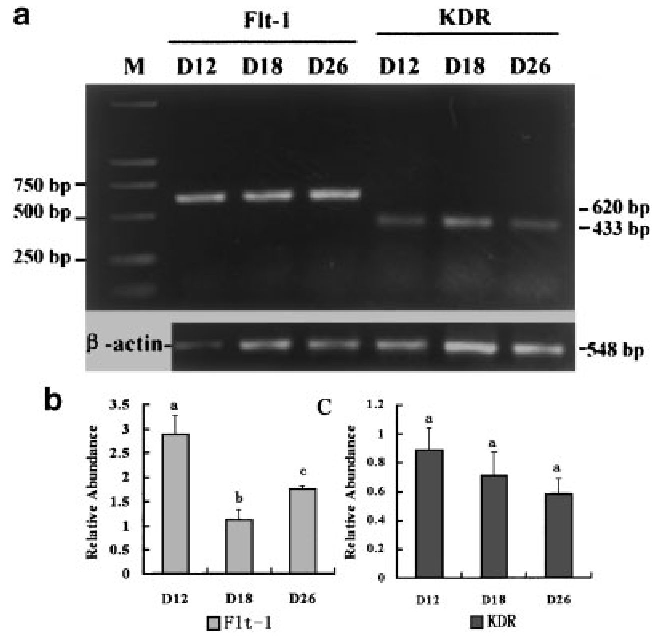

Fig. 2.

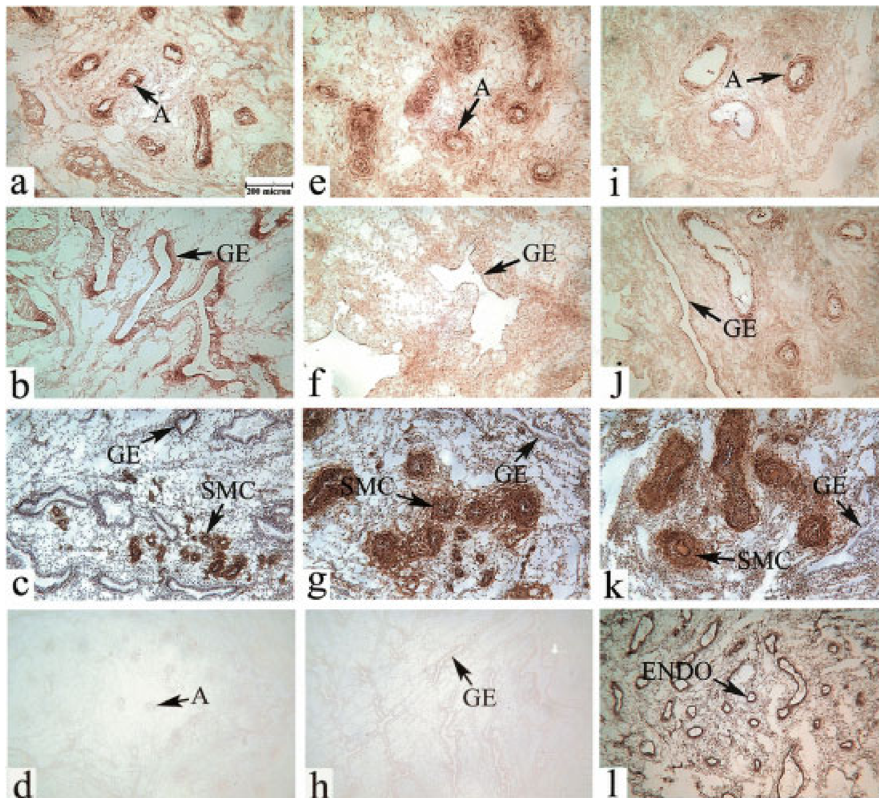

Fig. 3.

TABLE 1. Quantification of VEGF Proteins at Different Localizations of Macaque Uteri on Day 12 (D12), 18 (D18), and 26 (D26) of Pregnancy

	D12	D18	D26
Decidualized stromal cells (background)	1.01 ± 0.02	1.06 ± 0.03	1.13 ± 0.02
Smooth muscle cells of the arterioles	$1.49 \pm 0.05^{**,a}$	$1.42 \pm 0.05^{**,a}$	$1.29 \pm 0.05^{**,b}$
Endothelial cells of the arterioles	$1.67 \pm 0.08^{**,a}$	$1.87 \pm 0.08^{**,a}$	$1.52 \pm 0.11^{**,a}$
Glandular epithelium	$1.34 \pm 0.04^{**,a}$	0.96 ± 0.02^{b}	$1.32 \pm 0.34^{**,a}$
Placental villi	—	1.00 ± 0.03^{a}	$1.80 \pm 0.07^{**,b}$

Numbers are means ± SEM. Compared with its corresponding decidualized stromal cells: $^*P < 0.05$; $^{**}P < 0.01$. Values within rows with different letters (a, b, or c) are significantly different. — represents nonexistent tissues.

Immunohistochemical Localization of VEGF, Flt-1, and KDR in the Pregnant Uteri

Figure 3 and Table 1 show the distribution of VEGF protein in the endometrial compartments of rhesus monkeys on day 12, 18, and 26 of pregnancy. Cells immunoreactive with antibodies to VEGF can be found in the spiral arterial walls on day 12 (Fig. 3a). Anti-VEGF immunoactivities in the arterial endothelial cells on day 18 and 26 did not change substantially compared with that on day 12 ($P = 0.156$, $= 0.095$, respectively) (Fig. 3e,i), but its expression in the arterial smooth muscle cells decreased significantly ($P < 0.05$) on day 26 (Fig. 3i). Besides, VEGF immunostaining was found in the glandular epithelial cells on day 12 and 26 of pregnancy (Fig. 3b,j), but it was hardly detectable on day 18 of pregnancy (Fig. 3f). Decidualized stromal cells did not express VEGF proteins on day 12, 18, and 26 of pregnancy. Figure 3c,g,k show respectively the immunostaining of actin in the macaque endometrial cryosections on day 12, 18, and 26 of pregnancy, with hematoxylin counterstaining. Positive actin staining is present in the smooth muscle cells circling the arterioles. Figure 3l shows the specific immunostaining of Factor VIII in the arterial endothelial cells on a representative section of day 12 pregnant endometrium. Control sections treated similarly but substituting the primary antibody by normal rabbit IgG were representatively shown in Figure 3d,h corresponding with Figure 3a,b.

As to the placental compartments (Fig. 4, Table 1), there was no detectable staining of VEGF antigen in the placental villi on day 18 of pregnancy (Fig. 4a), but significant amounts of VEGF immunostaining were detected in the syncytiotrophoblast and cytotrophoblast cells lining the placental villi on day 26 (Fig. 4b). The trophoblast shell also exhibited significant VEGF immunostaining (Fig. 4b). Control sections substituting the primary antibody by normal rabbit IgG were representatively shown in Figure 3d,h.

Immunoreactive Flt-1 was detected throughout the macaque early gestation in all the sections examined (Fig. 5, Table 2). On day 12 of pregnancy, Flt-1 was mainly present in the arterial endothelial cells and the glandular epithelial cells (Fig. 5a,b). Immunoreactive Flt-1 in the arterial smooth muscle cells and in the endothelial cells was significantly ($P < 0.01$) increased on day 18 and 26 of pregnancy (Fig 5c,e), compared with that on day 12. Besides, immunostaining of Flt-1 in the glandular epithelial cells on day 26 was significantly ($P < 0.01$) decreased (Fig. 5f). Decidualized stromal cells at these three time points did not show any positive

Fig. 1. Localization of VEGF transcripts in the uterus and placenta of rhesus monkeys on day 12 (**a**), 18 (**b**), and 26 (**c**) of pregnancy by in situ hybridization. All the pictures are of identical magnification as shown in (a). Detailed tissue morphology was shown by vimentin immunostaining (**g**,**h**,**i**). Note that villous mesenchyme and general decidual cells in the endometrium were vimentin-positive, while cytotrophoblast and syncytiotrophoblast lining placental villi, extravillous trophoblast cells, and glandular epithelium of the endometirum were vimentin-negative. Control sections (**d–f**) corresponding to (a–c), respectively, hybridized to the sense probes show absence of the specific signals. (LE, luminal epithelium; GE, glandular epithelium; PV, placental villi; A, arteriole; FMB, fetal-maternal border.)

Fig. 2. RT-PCR analysis of Flt-1 and KDR transcripts in the macaque endometrium during early pregnancy. **a**: Agarose-gel electrophoresis of Flt-1, KDR, and β-actin (used as an internal control) during day 12 (D12), 18 (D18), and 26 (D26) of pregnancy (indicated above the gel). The leftmost lane is the DNA marker lane followed by Flt-1 products on D12, D18, and D26; and KDR products on D12, D18, and D26. The PCR product sizes in base pair (bp) are shown on the right. **b**,**c**: Graphical illustrations of Flt-1 and KDR mRNA relative levels in the macaque endometrium at different stages of pregnancy. The relative levels for Flt-1 or KDR in each tissue were determined as the ratio of Flt-1 or KDR mRNA/β-actin mRNA measured by densitometry. Time points with no characters in common are different at $P < 0.05$.

Fig. 3. Immunohistochemical signals for VEGF in the macaque endometrial compartments on day 12 (**a**,**b**), 18 (**e**,**f**), and 26 (**i**,**j**) of pregnancy. All the pictures are of identical magnification as shown in (a). Actin immunostaining of the macaque endometrial cryosections on day 12, 18, and 26 are respectively shown in (**c**,**g**,**k**). Note that positive actin staining is present in the endometrial smooth muscle cells circling the arterioles. **l**: It shows the Factor VIII staining in the endothelial cells of the arterioles. Control sections of day 12 pregnant endometrium treated similarly but with omission of the primary antibodies were shown in (**d**) and (**h**). (A, spiral arteries; GE, glandular epithelium; SMC, smooth muscle cells of the arterioles; ENDO, endothelial cells of the arterioles.)

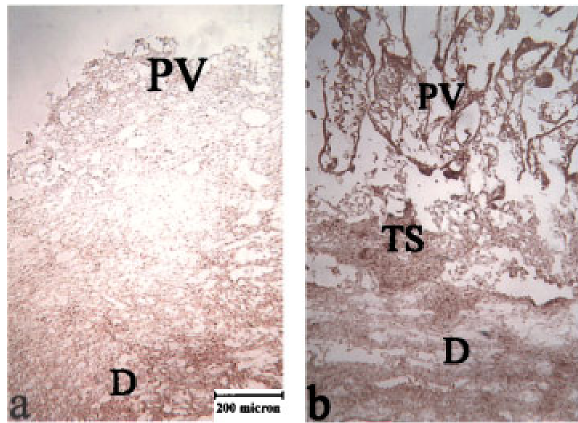

Fig. 4. Immunohistochemical localization of VEGF in the macaque placental compartments on day 18 (**a**) and 26 (**b**) of pregnancy. Both pictures are of identical magnification as shown in (a). Control sections treated similarly but substituting the primary antibody by normal rabbit IgG were representatively shown in Figure 3d,h. (PV, placental villi; TS, trophoblast shell; D, deciduas.)

immunostaining of Flt-1. Control sections incubated with normal rabbit IgG, but not the primary antibody were representatively shown in Figure 3d,h.

As shown in Figure 6 and Table 3, KDR was strongly expressed in the smooth muscle cells and the endothelial cells around the spiral arteries, as well as in the glandular epithelial cells on day 12 of pregnancy (Fig. 6a,b). With the progress of gestation, immunostaining of KDR in these endometrial compartments was significantly ($P < 0.01$) decreased (Fig 6c–f). Decidualized stromal cells only showed a background level of KDR immunostaing on day 12, 18, and 26 of pregnancy. Control sections substituting the primary antibody by normal rabbit IgG were representatively shown in Figure 3d,h.

DISCUSSION

While there are a number of investigations of VEGF in the human and nonhuman primate placenta, they are mostly restricted to late pregnancy (Wulff et al., 2002). So, very little is known about the functional role of VEGF in human placental development during peri-implantation for ethical and practical reasons. Especially, possible relationship between VEGF and its receptors during early pregnancy remains to be further investigated. A rhesus monkey early pregnancy system was used to study the above problems. We collected rhesus monkey uteri at very early stages of pregnancy (day 12, 18, and 26 of pregnancy), and observed that transcripts and proteins for VEGF and its receptors were expressed in a spatiotemporal manner during rhesus monkey early pregnancy, and importantly, we demonstrate that the two receptors, Flt-1 and KDR, apart from their endothelial localization, are also localized in nonendothelial cells.

During blastocyst implantation and early placentation of rhesus monkey, cytotrophoblast and syncytiotrophoblast cells lining the placental villi will invade uterine epithelium and subepithelial maternal arterioles. Extensive angiogenesis occurs in the endometrium, in the fetal-maternal border, and in the placenta; and various angiogenic factors may play important roles during these processes. In this study, VEGF mRNA was detected in the macaque placental compartments on day 18 and 26 of pregnancy. Expression of VEGF proteins in the placental compartments was hardly detectable on day 18, but they were highly expressed in the placental villi and the trophoblast shell on day 26. Our results are in agreement with earlier studies by Jackson, Ahmed and Clark (Jackson et al., 1994; Ahmed et al., 1995; Clark et al., 1996) in human placenta throughout gestation, and those of Shiraishi in the human placenta from 6 to 41 weeks of gestation (Shiraishi et al., 1996).

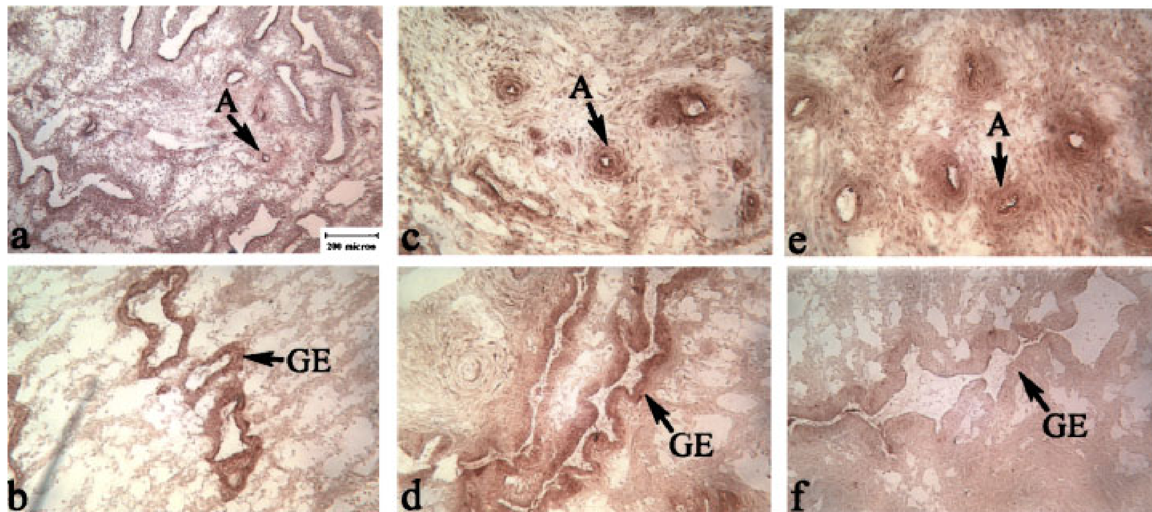

Fig. 5. Immunohistochemical localization of Flt-1 in the macaque endometrial compartments on day 12 (**a**,**b**), 18 (**c**,**d**), and 26 (**e**,**f**) of pregnancy. All pictures are of identical magnification as shown in (a). Control sections treated similarly but substituting the primary antibody by normal rabbit IgG were representatively shown in Figure 3d,h. (A, spiral arteries; GE, glandular epithelium.)

TABLE 2. Quantification of Flt-1 at Different Localizations of Macaque Endometrium on Day 12 (D12), 18 (D18), and 26 (D26) of Pregnancy

	D12	D18	D26
Decidualized stromal cells (background)	1.01 ± 0.02	0.99 ± 0.02	1.13 ± 0.02
Smooth muscle cells of the arterioles	1.07 ± 0.05^{a}	$1.38 \pm 0.03^{**,b}$	$1.67 \pm 0.03^{**,c}$
Endothelial cells of the arterioles	$1.72 \pm 0.05^{**,a}$	$2.15 \pm 0.05^{**,b}$	$2.46 \pm 0.04^{**,c}$
Glandular epithelium	$1.54 \pm 0.04^{**,a}$	$1.42 \pm 0.05^{**,a}$	1.15 ± 0.03^{b}

Numbers are means ± SEM. Compared with its corresponding decidualized stromal cells: *$P < 0.05$; **$P < 0.01$. Values within rows with different letters (a, b, or c) are significantly different.

All the results above support a role of VEGF in placental angiogenesis during human and nonhuman primate early pregnancy.

As to the endometrial VEGF expression, VEGF mRNA was highly expressed in the walls of the spiral arteries adjacent to the implantation site on day 18. Besides, VEGF proteins were localized mainly in the arterial walls on day 12, 18, and 26 of pregnancy. The existence of VEGF mRNA and its proteins in the endometrial arteries adjacent to the implantation site hints that angiogenic growth factor—VEGF—may be involved in promoting endometrial vascular growth and vascular reconstruction required for adequate receptivity during macaque early pregnancy. Studies on VEGF in human and nonhuman primate early pregnant endometrium are lacking, but interestingly, our results are compatible with previous findings of Li (Li et al., 1994) and Moller (Moller et al., 2001) in human menstrual cycle. Considering the great similarity between rhesus monkey and human pregnancy, we hypothesize that VEGF may be primarily involved in the angiogenesis during human early pregnancy.

Besides, VEGF mRNA expression in the endometrium from day 18 pregnant rhesus monkey had a similar overall distribution with matrix metalloproteinase (MMP)-2 and -14 in the walls of the spiral arterioles adjacent to the implantation site reported in our previous studies (Wang et al., 2001). Although the correlation between VEGF and various MMPs has been reported in other cells and tissues such as cultured human smooth muscle cells (Wang and Keiser, 1998), human umbilical vein endothelial cells (HUVECs) (Zucker et al., 1998), or the hepatic metastasis of human colon cancer (Arii et al., 1998), very little is known about their possible relationship in the endometrium during early pregnancy. So the co-expression of MMP-2, -14 and VEGF in the walls of the arterioles adjacent to the implantation site supports roles of MMP-2, -14 and VEGF in the angiogenesis during macaque blastocyst implantation, and also hints that VEGF may be crucial in the regulation of MMP-2 and -14 expression during rhesus monkey early pregnancy.

VEGF mRNA had been identified in the glandular epithelial cells on day 12 and 18 of pregnancy, and strong immunostaining of VEGF protein was also localized in the glandular epithelium on day 12. The existence of VEGF transcripts and proteins in the glandular epithelium, together with previous studies of Greb (Greb et al., 1995) who demonstrated that VEGF was secreted by polarized human epithelial cells both in vivo and in vitro,

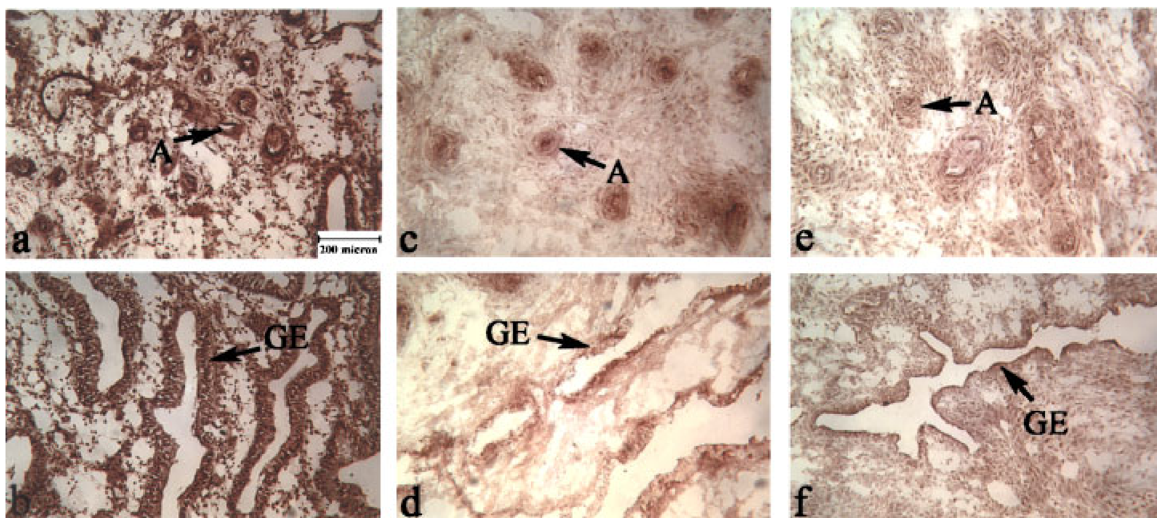

Fig. 6. Immunohistochemical localization of KDR in the macaque endometrial compartments on day 12 (**a**,**b**), 18 (**c**,**d**), and 26 (**e**,**f**) of pregnancy. All pictures are of identical magnification as shown in (a). Control sections treated similarly but substituting the primary antibody by normal rabbit IgG were representatively shown in Figure 3d,h. (A, spiral arteries; GE, glandular epithelium.)

TABLE 3. Quantification of KDR at Different Localizations of Macaque Endometrium on Day 12 (D12), 18 (D18), and 26 (D26) of Pregnancy

	D12	D18	D26
Decidualized stromal cells (background)	1.05 ± 0.03	0.92 ± 0.02	0.99 ± 0.02
Smooth muscle cells of the arterioles	$1.79 \pm 0.04^{**,a}$	$1.30 \pm 0.06^{**,b}$	$1.09 \pm 0.03^{*,c}$
Endothelial cells of the arterioles	$2.41 \pm 0.02^{**,a}$	$1.79 \pm 0.11^{**,b}$	$1.16 \pm 0.03^{**,c}$
Glandular epithelium	$1.82 \pm 0.07^{**,a}$	$1.47 \pm 0.03^{**,b}$	$1.27 \pm 0.03^{**,c}$

Numbers are means ± SEM. Compared with its corresponding decidualized stromal cells: $^{*}P < 0.05$; $^{**}P < 0.01$. Values within rows with different letters (a, b, or c) are significantly different.

hints that the epithelium may be a primary site of VEGF production in the pregnant uterus, and VEGF may serve as a mediator of cellular growth and differentiation in addition to its function as an endothelial mitogen, and they may be also involved in the effects of ovarian steroids on endometrial vascular development during early gestation (Torry et al., 1996).

We also examined the expression and modulation of the receptors Flt-1 and KDR, key modulators of VEGF effects. Flt-1 and KDR mRNAs had been, for the first time, detected in the endometrial compartments of rhesus monkey during early pregnancy. Expression of KDR mRNA was at a stable level, but Flt-1 mRNA was expressed in a dynamic manner, and the highest expression level was appeared on day 12 of pregnancy. Flt-1 protein was primarily in association with the endothelial cells on day 12 and 18 of pregnancy, which is similar to the distribution of KDR. Combined with the immunohistochemical results of VEGF, which showed intense signals for VEGF protein in the arterial walls on day 12 and 18, the co-localization of VEGF and its receptors in the endometrial spiral arteries strongly suggests that VEGF action is mediated through the regulation of KDR and Flt-1. On day 26 of gestation, immunostaining of Flt-1 in the vascular walls was intense, but KDR immunoactivity was significantly decreased in the spiral arteries. Immunoactivity of VEGF on day 26 tended to co-localize with the staining for KDR, hinting that KDR, but not Flt-1, is the primary regulator of VEGF function on day 26 of pregnancy. The expression relationship between VEGF receptors and VEGF in human or nonhuman primate early gestation is not well understood, and similar findings were only reported by Meduri in human normal menstrual cycle (Meduri et al., 2000).

Apart from being expressed in the endothelial cells, the two receptors Flt-1 and KDR have also been reported in other cell types. Charnock-Jones et al. (1994) found that human trophoblast cells expressed a high level of Flt-1 transcripts and protein. Besides, Flt-1 and KDR were localized in human uterine smooth muscle cells (Brown et al., 1997), and KDR is also expressed in human breast cancer cells (Kranz et al., 1999). Winther et al. localized Flt-1 and KDR in porcine and mink luminal epithelium, glandular epithelium, and trophoblast. In this study, we found that Flt-1 and KDR were highly expressed in the glandular epithelial cells on day 12 of pregnancy, and with the progress of gestation, immunostaining of Flt-1 and KDR in glandular epithelium was significantly decreased. The intense immunoreactivity of the Flt-1 and KDR in the macaque uterine glandular epithelia suggests a role of these receptors on the uterine glandular cell differentiation and secretory activity during early pregnancy.

Taken together, we localized VEGF, Flt-1, and KDR in the endothelial cells and nonendothelial cells including smooth muscle cells, glandular epithelial cells, and trophoblast cells in the macaque endometrium and placenta. Our investigations on the expression of VEGF and its receptors in the endothelial cells implicate participation of VEGF and its receptors in the endometrial and placental angiogenesis during rhesus monkey early pregnancy; and the observation of VEGF, Flt-1, and KDR being localized in nonendothelial cells reflects that VEGF-receptor pair must have additional possible functional roles including influencing macaque placentation during early pregnancy, and cellular differentiation of the glandular epithelium and its secretory capacities. These observations will permit further investigation of the precise mechanism of angiogenesis during human early gestation.

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