Expression of platelet-derived endothelial cell growth factor and its mRNA in uterine endometrium during the menstrual cycle

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Steroid hormones, e.g. progesterone and oestradiol, may be responsible for the production and expression of a variety of angiogenic growth factors present in endometrial tissue. The expression of platelet-derived endothelial cell growth factor (PD-ECGF) in neovascularization after regression of the microvessels in the endometrium was examined. PD-ECGF protein expression in the endometrium during the menstrual cycle was determined by a sandwich enzyme immunoassay. Transcription levels of PD-ECGF were measured by a quantitative reverse transcription–polymerase chain reaction (RT–PCR) Southern blot technique. The data show that levels of PD-ECGF protein and mRNA in uterine endometrium did not alter during the proliferative phase prior to ovulation. During the midcycle phase a sharp transient fall in mRNA levels accompanied by a gradual drop in protein levels was observed. After ovulation transcription of PD-ECGF recovered with a sharp increase in mRNA levels which persisted during the ovulatory phase. PD-ECGF protein levels were temporarily low after ovulation, but increased remarkably through the late secretory phase. PD-ECGF expression in the endometrium seems to be inversely correlated with oestradiol concentrations during the menstrual cycle.

Key words: PD-ECGF/neovascularization/uterine endometrium

Introduction

Generally, the turnover of capillary endothelial cells is extremely slow (months or years) in physiological neovascularization, while the turnover of uterine endometrium is rapidly altered along with the ovarian steroidal cycle (Denekamp, 1984). The endometrial vasculature during the menstrual cycle consists of four different stages or conditions: vascular growth, implantation, vascular regression and menstruation (Rogers, 1996), and the endothelial cells that are renewed from degenerated coiled arteries migrate and form microvessel tubes at the final stage of menstruation (Markee, 1978). The steps of neovascularization are regulated by angiogenic factors, and involve the following events: dissolution of basement membrane of endothelial cells by proteases released from host cells, migration and proliferation of endothelial cells, and microvessel tube formation (Folkman and Haudenschild, 1980).

The angiogenic factors, basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), placenta growth factor (PIGF), epidermal growth factor (EGF), transforming growth factor (TGF-α, TGF-β), platelet-derived growth factor (PDGF), platelet-derived endothelial cell growth factor (PD-ECGF), hepatocyte growth factor (HGF), tumour necrosis factor (TNF-α, pleiotrophin, proliferin, angiogenin, oestadiol, interleukin (IL)-1, IL-6, IL-8, have been identified. Among them, basic FGF, VEGF, PDGF, TGF-α, TGF-β, IL-1, IL-6, IL-8 (Rogers, 1996; Stewart and Nowak, 1996), and PD-ECGF (Osga et al., 1995) play a role in controlling angiogenesis for the endometrial vasculature. Angiogenic factors expressed in the endometrium, which is sex steroid-dependent during growth and differentiation, might be considered to be regulated by sex steroids.

In a previous study, we showed that basic FGF mRNA expression in the endometrium did not alter during the proliferative phase, but decreased in the secretory phase, and that oestrogen treatment increased its expression in secretory phase endometria (Fujimoto et al., 1996). Basic FGF levels in the endometrium were increased up to the late proliferative phase, and decreased during the secretory phase, but were increased by oestrogen treatment in the endometrium of the secretory phase (Fujimoto et al., 1996). Additionally, the expression and secretion of basic FGF was more dominant in glandular epithelial cells than in stromal cells (Fujimoto et al., 1997a,b). Therefore, it is suggested that sex steroid regulation in basic FGF is oestrogen dependent to support endometrial angiogenesis mainly via glandular cells.

There are some immunohistochemical reports of endometrial expression of VEGF altering during the menstrual cycle (Li et al., 1994; Shifren et al., 1996). During the early proliferative phase, strong staining was seen in the glandular epithelial cells, while staining of the stroma was confined to a subpopulation of stromal cells, and staining in endometrial blood vessels appeared to be negative. In contrast, the greatest degree of immunostaining of stromal cells was observed in the mid to late proliferative endometrium. Throughout the secretory phase no staining was seen around the endometrial blood vessels, and obvious staining of endometrial stromal cells was confined to the early secretory endometrium. In the late secretory endometrium, the glands alone were positive to VEGF antibody (Li et al., 1994). Ribonuclease protection analysis demonstrated...
that the expression of VEGF mRNA increased by 1.6-, 2.0-, and 3.6-fold in the mid-proliferative, late proliferative, and secretory endometria respectively, compared with that of the early proliferative endometrium (Shifren et al., 1996). In the monkey, the staining intensities for VEGF in endometrial glandular epithelial cells and stromal cells were higher in the secretory phase than in the proliferative phase (Greb et al., 1995). Furthermore, gene expression of VEGF189, but not other isoforms, was higher in progesterone- and progesterin-exposed endometria than in antiprogestin-exposed endometria from anovulatory cycles. Antiprogestin abolished VEGF staining in the glandular cell almost completely (Greb et al., 1997). The intensities are more dominant in the endometrial glandular epithelial cells than in the stromal cells. Taken together, it is suggested that sex steroidal regulation in VEGF might be primarily oestrogen-dependent with the adjunctive effect of progesterone to support endometrial angiogenesis, mainly via glandular cells.

Neovascularization occurs after menstrual regression of the microvessels in the uterine endometrium, which might be related to the expression of basic FGF and VEGF and to that of PD-ECGF as the third sex steroid regulated-angiogenic factor. In this study, the expression of PD-ECGF in the uterine endometrium was determined during the menstrual cycle by a sandwich enzyme immunoassay and reverse transcription–polymerase chain reaction (RT–PCR)/Southern blotting in order to understand the sex steroidal regulation of endometrial neovascularization.

Figure 1. Signal intensity curve for platelet-derived endothelial cell growth factor (PD-ECGF) mRNA level in a series of reverse-transcribed total RNA of uterine endometrium by reverse transcription–polymerase chain reaction (RT–PCR)/Southern blot analysis. The levels of mRNA expression in normal uterine endometrium were assigned as arbitrary units/GAPDH mRNA (AU/GAPDH mRNA). Data are the mean ± SD of nine determinations.

Materials and methods

Endometria

Agreement for the studies was obtained from all patients and the Research Committee for Human Subjects, Gifu University School of Medicine, Japan. Women volunteers (n = 46) aged 33–45 years, with a regular menstrual cycle, as confirmed by body basal temperature, and without any disease of the reproductive organs underwent endometrial biopsy at the Department of Obstetrics and Gynecology, Gifu University School of Medicine, Japan, between August 1996 and May 1997. None of the patients had received any therapy. Part of each endometrial sample was snap-frozen in liquid nitrogen for protein and mRNA studies, and a neighbouring part of the endometrium was submitted for histological endometrial dating (Noyes et al., 1950). To confirm the histological endometrial dating, the levels of oestradiol and progesterone in peripheral blood were measured with Coat-A-Count Estradiol (Diagnostic Products Corporation, Los Angeles, CA, USA) and Coat-A-Count Progesterone (Diagnostic Products Corporation) respectively, in triplicate.

Sandwich enzyme immunoassay for determination of human PD-ECGF antigen

All steps were carried out at 4°C. Tissues (wet weight 10–20 mg) were homogenized in HG buffer [5 mM Tris HCl, pH 7.4, 5 mM NaCl, 1 mM CaCl₂, 2 mM ethyleneglycol-bis-(β-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid, 1 mM MgCl₂, 2 mM dithiothreitol (DTT), 25 µg/ml aprotinin, and 25 µg/ml leupeptin] with a Polytron homogenizer (Kinematics, Luzern, Switzerland). This suspension was centrifuged in a microfuge at 12 000 r.p.m. for 3 min to remove the nuclear pellet. The protein concentration of samples was measured using the
PD-ECGF in uterine endometrium

Figure 2. Levels of platelet-derived endothelial cell growth factor (PD-ECGF) and its mRNA in the endometrium. The levels of PD-ECGF and its mRNA were determined by a sandwich enzyme immunoassay and reverse transcription–polymerase chain reaction (RT–PCR)/Southern blot analysis respectively. Histological endometrial dating was done according to Noyes’ criteria (Noyes, 1950). To confirm histological endometrial dating, the levels of oestradiol and progesterone in peripheral blood are shown. The same numbers in O for the expression of PD-ECGF and its mRNA indicate an identified case. Data are the mean ± SD of nine determinations.

Bradford assay (Bradford, 1976) to normalize PD-ECGF antigen levels.

PD-ECGF antigen levels in the sample were determined by the acid guanidium thiocyanate–phenol–chloroform extraction method (Chomozynski et al., 1997). Total RNA (3 µg) was reverse-transcribed with Moloney murine leukemia virus–reverse transcriptase (MMLV–RTase, 200 IU; Gibco BRL, Gaithersburg, MD, USA) in a buffer of 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 2.5 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 10 mM DTT, and 0.5 mM deoxyribonucleotides to generate cDNA using random hexamer (50 ng, Gibco BRL) at 37°C for 60 min. The RT reaction mixture was heated at 94°C for 5 min to inactivate MMLV–RTase.

The signal intensity curve for mRNA expression is necessary for accurate measurement of the mRNA by RT–PCR. The PCR template was prepared from reverse-transcribed total RNA (100 µg) of the normal uterine endometrium as follows: 1/4×, 0.75 µg total RNA reverse-transcribed (RNA–RT); 1/2×, 1.5 µg RNA–RT; 1×, 3 µg RNA–RT; 2×, 6 µg RNA–RT; 4×, 12 µg RNA–RT and 8×, 24 µg RNA–RT.

Five cycles of PCR for PD-ECGF mRNA, consisting of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C was carried out with reverse-transcribed cDNA and 0.1 µM specific primers using the Iwaki thermal sequencer TSR-300 (Iwaki Glass, Tokyo, Japan), with Vent DNA polymerase (New England Biolabs, Beverly, MA, USA) in a buffer of 10 mM KCl, 20 mM Tris–HCl, pH 8.8, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, and 0.15 mM deoxynucleotide phosphates. Additionally, 23 cycles of PCR for PD-ECGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were run simultaneously in the same manner, as an internal standard. Thus a similar intensity of bands amplified from PD-ECGF and GAPDH mRNAs could be compared.

The oligodeoxynucleotides of specific primers in PCR were synthesized according to the published information on cDNA for PD-ECGF (Hagiwara et al., 1991) and GAPDH (Arcari et al., 1984) as follows: sense primer for PD-ECGF mRNA 5'-AGTCGGATGCGGCAATAG-3' (in exon 2); antisense primer for PD-ECGF mRNA 5'-TGGATGCTTGCCCAAGCA-3' (in exon 2); sense primer for GAPDH mRNA: 5'-TGAAGGTCGGAGTCAACGGAATTTGG-3' (in exon 2); antisense primer for GAPDH mRNA, 5'-CATGTGGGCCC-3' (in exon 8).

Southern blot analysis for quantities of PD-ECGF mRNA expression

PCR products were applied to 1.2% agarose gel, and electrophoresis was performed at 50–100 V. PCR products were capillary-transferred to an Immobilon transfer membrane (Millipore Corporation, Bedford, MA, USA) for 16 h. The membrane was dried at 80°C for 30 min, and was UV-irradiated to fix the PCR products. PCR products on the membrane were prehybridized in a buffer of 1 M NaCl, 50 mM Tris–HCl, pH 7.6, and 1% sodium dodecyl sulphate at 42°C for 1 h, and hybridized in the same solution with the biotinylated oligodeoxynucleotide probes synthesized from the sequences of PD-ECGF and GAPDH cDNAs between the specific primers at 65°C overnight. Specific bands hybridized with the biotinylated probes were detected with Plex Luminescent Kits (Millipore Corporation), and X-ray film was exposed on the membrane at room temperature for 10 min.
quantification of Southern blot was carried out with Bio Image (Millipore, Ann Arbor, MI, USA). The intensity of specific bands was standardized with that of GAPDH mRNA.

**Statistical analysis**

The levels of PD-ECGF and its mRNA were measured from three parts of the same tissue in triplicate, and the results were the mean ± SD of nine determinations.

**Results**

The signal intensity curve for PD-ECGF mRNA levels ranging from 1/4× to 4× of reverse transcribed-total RNA of the uterine endometrium by RT–PCR/Southern blot was linear (Figure 1). Therefore, semiquantitative determination of mRNA levels is thought to be reliable.

The expression of PD-ECGF mRNA in uterine endometrium did not significantly alter during the proliferative phase before ovulation. PD-ECGF mRNA expression was transiently reduced towards the date of ovulation, and increased sharply immediately after ovulation, in contrast to the concentration of oestradiol in peripheral blood. PD-ECGF mRNA expression persisted during the secretory phase (see Figure 2). The expression of PD-ECGF in the uterine endometrium did not change remarkably during the proliferative phase. However, its expression tended to decline transiently toward the date of ovulation, and to increase afterward through the late secretory phase following its mRNA expression and progesterone level in peripheral blood, as shown in Figure 2.

**Discussion**

Menstrual bleeding from arterioles occurs after the constriction of coiled arteries in the uterine endometrium. Thereafter, the endothelial cells are renewed from degenerated coiled arteries, and migrate to form microvessel tubes, a process known as neovascularization (Markee, 1978). An endometrial capillary network is accomplished up to the late proliferative phase, and neovascularization (Markee, 1978). An endometrial capillary and migrate to form microvessel tubes, a process known as endothelial cells are renewed from degenerated coiled arteries, of coiled arteries in the uterine endometrium. Thereafter, the

In the present study, PD-ECGF mRNA and its protein was simultaneously demonstrated in human endometrium during the menstrual cycle. PD-ECGF mRNA expression dropped at least 2 days before ovulation, and recovered 1 day after ovulation. Therefore, the transient drop of PD-ECGF mRNA expression might occur through an oestrogen-induced negative feed back. The PD-ECGF expression was slightly decreased a few days following the transient drop of its mRNA expression, and was induced several days after the ovulation following the alteration of its mRNA expression and progesterone level in peripheral blood. Therefore, PD-ECGF expression might be up-regulated by progesterone under oestrogen-primed conditions in the secretory phase. The expression of PD-ECGF in human uterine endometrium was demonstrated by Western blot and immunohistochemical staining (Osuga et al., 1995). There are no appreciable changes in the intensity of immuno-reactivity for PD-ECGF in the epithelial cells. On the other hand, the intensity was relatively sparse and weak in the stromal cells at the proliferative and early secretory phases. Furthermore, a marked increase is found in deciduized endometrial stromal cells (Osuga et al., 1995). In the present study, the PD-ECGF expression in the uterine endometrium was remarkably increased by the appearance of stromal mitoses and the static proliferation of the epithelial cells through the late secretory phase. These observations suggest that PD-ECGF might be predominantly provided by endometrial stromal cells. Alternatively, progesterone with oestrogen might induce the angiogenic potential from a unique aspect via the PD-ECGF expression in uterine endometrium, which might be related to a contribution to support implantation of the fertilized oocyte. Further experiments are needed to test this hypothesis.

**References**


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