Estrogen and selective estrogen receptor modulators regulate vascular endothelial growth factor and soluble vascular endothelial growth factor receptor 1 in human endometrial stromal cells

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Objective: To determine whether 17β-estradiol (E_2) and selective estrogen receptor modulators can regulate vascular endothelial growth factor (VEGF) and soluble VEGF receptor 1 (sVEGFR-1) as a VEGF antagonist in human endometrial stromal cells (ESCs).
Design: In vitro experiment.
Setting: Research laboratory at Kansai Medical University.
Patient(s): Sixteen patients undergoing hysterectomy for benign reasons.
Intervention(s): The ESCs were cultured with E_2, 4-hydroxytamoxifen (OHT), and raloxifene.
Main Outcome Measure(s): The VEGF and sVEGFR-1 messenger RNA (mRNA) levels in ESCs were determined using quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR). Free (unbound) VEGF and sVEGFR-1 protein levels from ESCs were measured using ELISA kits.
Result(s): The E_2 significantly induced VEGF mRNA levels, whereas E2 caused a significant decrease in sVEGFR-1 messenger RNA (mRNA) levels. The E_2 or OHT significantly increased the VEGF production levels and attenuated the sVEGFR-1 production compared with control, but raloxifene had no significant effect. The decrease in levels of free VEGF was proportional to the increase in sVEGFR-1 levels in the culture media of ESCs.
Conclusion(s): The E_2 or OHT stimulates VEGF production and concurrently attenuates sVEGFR-1 production in ESCs. This consequential increase in VEGF:sVEGFR-1 ratio might enhance the biological effects of VEGF on the angiogenic environment in human endometrium. (Fertil Steril® 2010;93:2680–6. ©2010 by American Society for Reproductive Medicine.)

Key Words: Estrogen, endometrial stromal cells, selective estrogen receptor modulator, sVEGFR-1, VEGF

Human endometrium is a dynamic active tissue that undergoes a series of physiological changes during the menstrual cycle of women of reproductive age. Endometrial growth and repair after menstruation are associated with profound angiogenesis (1–3). Vascular endothelial growth factor (VEGF) is the most prominent and well-characterized angiogenic factor. Seven VEGF subtypes (VEGF-A to VEGF-F and placental growth factor) and three VEGF receptor (VEGFR) subtypes (VEGFR-1 to VEGFR-3) have been identified (4, 5). The VEGF-A, usually referred to as VEGF, seems to be one of the most important angiogenic factors in the regulation of angiogenesis in human endometrium (6, 7). Alternative splicing of VEGF messenger RNA (mRNA) results in at least four polypeptide isoforms of 121, 165, 189, and 206 amino acids (8). The most widely expressed forms, $VEGF_{121}$ and $VEGF_{165}$, are freely soluble, whereas $VEGF_{189}$ and $VEGF_{206}$ remain in the extracellular matrix (9).

The VEGF actions are mediated through binding to two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR -2 (KDR). Although VEGFR-1 binds to VEGF with high affinity, most of the biological effects of VEGF are mediated by VEGFR-2 (5). The VEGFR-1 is also produced as a soluble form (sVEGFR-1, also referred to as sflt-1) through the alternative splicing of the precursor mRNA. This sVEGFR-1 is known to act as a specific high-affinity antagonist of VEGF function by competitively binding VEGF and by preventing their interaction with its cell surface receptors VEGFR-1 and VEGFR -2 (10).

Estrogen (E) has a pivotal role in establishing the new vascular bed and promoting growth and cellular differentiation within the endometrium during each menstrual cycle. The E receptor (ER) ligands are clinically used for a variety of indications including assisted reproductive technology (ART), hormone replacement therapy (HT), and the prevention of breast cancer and osteoporosis. Several of the ER ligands used in the clinic are selective ER modulators (SERMs) such as tamoxifen and raloxifene (11, 12). The 17β-estradiol (E_2) has a cyclophenanthrene structure, whereas tamoxifen has a triphenylethylene structure and raloxifene has a benzothiophene structure. Although the primary structure of these SERMs differs strikingly from that of E, they have a conformation that allows them to bind to the ligand-binding domain of ER (13). Unlike E, the SERMs exert selective agonist or antagonist effects on various E target tissues (12, 14). However, the distinct regulatory effects on molecules in human endometrium among E_2 and SERMs are largely unknown.

The E_2 has effects on the growth and angiogenesis in human endometrium, which may be direct or mediated by VEGF systems. The

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VEGF and sVEGFR-1 expressions have been previously shown to be detected in human endometrium (6–8, 15, 16). The VEGF expression can be caused by hypoxia, inflammatory cytokines, growth factors, and sex hormones in various tissues including the human endometrium (17–19). However, the regulation of sVEGFR-1 secretion, and the balance between VEGF and sVEGFR-1 in human endometrium remain largely unknown. In the present study we investigated the direct effects of E_2 or SERMs on VEGF and sVEGFR-1 production using isolated endometrial stromal cells (ESCs) in vitro.

MATERIALS AND METHODS

Tissue Collection and Culture of ESCs

All human tissues were obtained using a protocol for the protection of human subjects approved by the ethical committee of Kansai Medical University, together with informed consent from all patients and in accordance with the Declaration of Helsinki. Human endometrial tissues were obtained from 16 patients in the proliferative phase, aged 32–44 years, with regular menstrual cycles, who underwent hysterectomies for the treatment of myoma uteri (n = 14) and pelvic relaxation (n = 2) without hormonal therapy and submucosal fibroids.

The ESCs were purified by the standard enzyme digestion method as described previously (20, 21). The purity of ESCs was determined by morphology and by immunohistochemical staining as described previously (22, 23) with markers specific to ESCs (vimentin). The percentage of vimentin-positive cells in confluent ESCs was more than 99% by immunohistochemical staining.

Steroid Hormone Treatment

When ESCs were nearly confluent, cells were trypsinized and replated in 6-well plates (1×10^6 cells/well) for real-time polymerase chain reaction (PCR) analyses, and in 24-well plates (2×10^5 cells/ well) for ELISA. After removing the effect of endogenous steroid hormones (24), ESCs were cultured in 10% dextran-coated charcoal-stripped fetal calf serum (FCS)-supplemented medium containing various amounts of E_2 (Wako, Osaka, Japan), 4-hydroxytamoxifen (OHT; active metabolite of tamoxifen) (Sigma, St. Louis, MO), raloxifene (Sigma), or dimethyl sulfoxide (DMSO) as vehicle control. The number of ESCs was counted by the citric acid–crystal violet method, as described previously (23).

RNA Extraction and Real-Time PCR Analysis

Total RNA was isolated from cultured ESCs using an RNeasy Minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the SYBR green I nucleic acid Gel Stain (Roche, Mannheim, Germany) as described previously (25). The elongation factor-1α (EF-1α), as an internal control, is valid as reference housekeeping gene for transcription profiling, which is also used for real-time PCR experiments (26). The sequences of the primers are VEGF forward 5′- CGAAACCATGAACTTTCTGC -3′, VEGF reverse 5′- CCTCAGTGGG CACACACTCC -3′; sVEGFR-1 forward 5′-GCACCTTGGTTGTGGCT GACT-3′, sVEGFR-1 reverse 5′-GAGCCCGGGGGTCTCATTATT-3′; EF-1α forward 5′- TCTGGTTGGAATGGTGACAACATGC -3′, and EF-1α reverse 5′- AGAGCTTCACTCAAAGCTTCATGG -3′.

The PCR of all standards and samples was performed using duplicate reactions, after which a melting curve analysis was performed to monitor PCR product purity. The concentration of each sample was calculated from the threshold cycle (Ct) and the final result was expressed as $2^{-\triangle Ct\ sample}$ (25).

VEGF and sVEGFR-1 Assay by ELISA

The VEGF and sVEGFR-1 in cell culture supernatants were assessed using commercially available sandwich ELISA kits (Quantikine ELISA kit; R&D systems, Minneapolis, MN). The kit has a lower detection limit of 5 pg/mL for VEGF and 3.5 pg/mL for sVEGFR-1. To examine whether coexisting sVEGFR-1 interfered with VEGF protein levels in culture media of ESCs, free VEGF was measured using the VEGF ELISA kit with various amounts of recombinant human sVEGFR1-Fc (R&D systems) or control.

Statistical Analysis

Data are expressed as mean±SEM. Results were analyzed with a statistical software package, StatView II version 4.0 (Abacus Concepts, Berkeley, CA). Differences in the measured parameters across the different groups were statistically assessed using analysis of variance (ANOVA) with repeated measurements, followed by Fisher's protected least significant difference, multiple range test. A level of $P<.05$ was considered statistically significant.

RESULTS

Expression of VEGF and sVEGFR-1 mRNA in ESCs

We evaluated whether E_2 regulates the expression VEGF and sVEGFR-1 mRNA using the cultured ESCs. A single specific band corresponding to the expected size was obtained for amplifications (Fig. 1A). Real-time PCR analysis demonstrated that the addition of E2 to the culture medium had a significant increase on the levels of VEGF mRNA expression after 12 days of culture (Fig. 1B). In contrast, E2 caused a significant decrease in sVEGFR-1 mRNA levels of ESCs ($P<.01$) (Fig. 1C).

Effects of E2 on VEGF Secretion From ESCs

To study the secretion of VEGF we measured the free (unbound) VEGF concentration in the cell culture supernatants using ELISA kits. The temporal release of VEGF from ESCs exposed to E_2 is shown in Figure 1D. The E2 caused a significant increase in VEGF production after 3 days of culture compared with control (vehicle) ($P<.05$), and this continued to increase until the end of these studies at 12 days ($P<.01$). To analyze the dose-dependent effects of E_2 on VEGF production ESCs were incubated with various doses of E_2 for 12 days. The E_2 enhanced the VEGF production levels in a dose-dependent manner, with E_2 (10^{-9}, 10^{-8}, and 10^{-7} mol/L) resulting in 2.1-, 3.3-, and 3.7-fold increases, respectively, compared with the level without E_2 (Fig. 1E). These findings suggest that E_2 participates in the induction of VEGF production by cultured ESCs.

Decreased Free VEGF in Culture Media with sVEGFR-1

The E_2 significantly increased VEGF mRNA levels and attenuated sVEGFR-1 mRNA levels ($P<.01$) (Fig. 1). The decrease in sVEGFR-1 production by E_2 may have a synergistic effect for the increase in free VEGF by E_2, because sVEGFR1 is known as an antagonist of VEGF and binds to VEGF. We examined whether coexisting sVEGFR-1 in culture media might decrease the VEGF level. Human VEGF protein in culture media was measured in the absence (control) or in the presence of various doses of recombinant human sVEGFR-1 using the ELISA kit for VEGF (Fig. 2A). Free VEGF (not bound to sVEGFR-1) levels are decreased in the culture media with exogenous sVEGFR-1. The decrease in levels of free VEGF was proportional to the increase in sVEGFR-1 levels in the culture media (Fig. 2B). We examined whether coexisting VEGF in culture media might interfere with the measured sVEGFR-1 value. The sVEGFR-1 value did not interfere with coexisting recombinant human VEGF (Fig. 2C).

Effects of E2 on sVEGFR-1 Secretion From ESCs

To study the secretion of sVEGFR-1 we measured the concentration in the cell culture supernatants using the ELISA kit. The sVEGFR-1 production was significantly attenuated in ESCs after 12 days of treatment with E2 in comparison with controls ($P<.01$) (Fig. 3).