Endocrine gland–derived vascular endothelial growth factor stimulates proliferation and tube formation in human uterine microvascular endothelial cell through the mitogen-activated protein kinase but not through the Akt pathway

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Objective: To study the angiogenic functions of endocrine gland–derived vascular endothelial growth factor (EG-VEGF) on a normal myometrial uterine microvascular endothelial cell line (UtMVEC-Myo) and the signaling pathways elicited by EG-VEGF in UtMVEC-Myo.

Design: Experimental laboratory study.

Setting: University gynecology unit.

Patient(s): Infertile women undergoing diagnostic laparoscopy for assessment of tubal patency.

Intervention(s): Real-time polymerase chain reaction (PCR) analysis of mRNA of EG-VEGF and its receptors, PKR1 and PKR2, in UtMVEC-Myo and endometrial samples. The effects of EG-VEGF on the cell proliferation, tube formation, and cell signaling pathways of UtMVEC-Myo were studied.

Main Outcome Measure(s): Cell proliferation, tube formation, and molecules of cell-signaling pathways in the treated UtMVEC-Myo.

Result(s): UtMVEC-Myo cells had PKR1 and PKR2 but not EG-VEGF mRNA. EG-VEGF significantly stimulated cell proliferation and tube formation in UtMVEC-Myo cells. EG-VEGF activated p44/42 mitogen-activated protein kinase (MAPK) but not Akt signaling pathway. The effects of EG-VEGF on p44/42 MAPK phosphorylation and cell proliferation were nullified by the specific MAPK inhibitor, PD98059.

Conclusion(s): EG-VEGF has a direct angiogenic effect on UtMVEC-Myo that expresses EG-VEGF receptors (PKR1 and PKR2) and modulates cell proliferation and sprouting of the endothelial cells. It is suggested that EG-VEGF enhanced cell proliferation through the activation of MAPK pathway but not through the Akt pathway. (Fertil Steril 2009;91:2163–71. ©2009 by American Society for Reproductive Medicine.)

Key Words: EG-VEGF, PKR1, PKR2, angiogenesis, cell-signaling pathways, UtMVEC-Myo

Angiogenesis refers to the generation of new blood capillaries from the preexisting vessels. It is critical during normal embryonic development (1) and during the reproductive cycle and pregnancy (2). Successful implantation and placentation requires increased vascular permeability and angiogenesis. Some angiogenic factors, such as hepatocyte growth factor, acidic and basic fibroblast growth factors (FGF-1 and FGF-2), and interleukin-8, have pleiotropic activities (2), while vascular endothelial growth factor (VEGF) and angiopoietins are endothelial cell specific (3–5).

Endocrine gland–derived VEGF (EG-VEGF) belongs to the prokineticin family. It is also known as prokineticin 1 (PK1) and shares about 44% amino acid homology with another family member, PK2 (also known as Bv8) (6). EG-VEGF was previously thought to be selectively expressed in steroidogenic glands stimulating the growth of endocrine gland endothelium (7). However, it has recently been found to be present also in nonendocrine tissue, for example, endometrium (8). EG-VEGF promotes proliferation, survival, and chemotaxis of endothelial cells from steroidogenic tissue, such as adrenal cortex capillary endothelial cells, but not those from other tissues, such as human umbilical vein endothelial cells and other nonendothelial cell types (9). It acts via G-protein coupled receptors, PKR1 and PKR2 (10), and induces mitogen-activated protein kinase (MAPK) and Akt serine/threonine kinase phosphorylation (11). Although EG-VEGF is structurally distinct from VEGF, they induce a similar angiogenic response in the ovary (12).

Prokinetics and their receptors are expressed in male tissues including the testis and prostate (13, 14) and in female tissues such as the ovary, uterus, and various other tissues during pregnancy (8, 15, 16). During early pregnancy, EG-VEGF and PKR1 in placenta may be under hypoxic regulation, as the hypoxia-inducible factor binding site is present
were cultured in a 5% CO2 humidified incubator at 37°C in RPMI 1640 medium supplemented with 10% FBS, hydrocortisone, human fibroblast growth factor, epidermal growth factor, insulin-like growth factor-1 (IGF-1), and PKR2 mRNA in the endometrial tissues, endometrial cells, and UtMVEC-Myo was performed using an ABI 7500 Sequence Detector (PE Applied Biosystems, Foster City, CA). Real-time PCR analysis of human EG-VEGF, PKR1, and PKR2 mRNA in the endometrial tissues, endometrial cells, and UtMVEC-Myo was performed using an ABI 7500 Sequence Detector (PE Applied Biosystems). Water was used as no template control. Real-time PCR analysis was done by the sequence detection software supplied with the ABI 7500 Sequence Detector. The threshold cycles (Ct) for each reaction were calculated and used for quantifying the amount of starting template in the reaction. A difference of Ct values (∆Ct) was obtained by subtracting the Ct value of 18S from the Ct value of the genes of interest. The relative gene expression values were calculated by the 2^−ΔΔCt method (18).

Cell Viability Test
UtMVEC-Myo were seeded at 10,000 cells/well in a 24-well plate with 1 mL of endothelial basal medium-2 (EBM-2) culture media supplemented with Bulletkit (sEBM-2, Cambrex) and cultured in an atmosphere containing 5% CO2 in air at 37°C. Before experimentation, the cells were starved in 1 mL EBM-2 containing 5% charcoal-stripped FBS without supplementation of VEGF and FGF-2 (sEBM-2) for 24 hours. Different concentrations (0, 1, 10, 100 ng/mL) of recombinant EG-VEGF, VEGF, and FGF-2 (PeproTech, London, UK) were then added into each well and incubated for 48 hours. All the treatments were duplicated in each experiment. After trypsinization, the total cell numbers in the wells were determined with a hemocytometer. The experiment was repeated 5 times.

Fibrin Gel In Vitro Angiogenesis Assay
The Fibrin Gel In Vitro Angiogenesis Assay (Chemicon International, Temecula, CA) was used for in vitro biological activity study of EG-VEGF, VEGF, and FGF on tube formation of UtMVEC-Myo. The manufacturer’s protocol was followed. Briefly, 30 μL of fibrinogen solution was added into the wells in a 96-well plate and mixed with 20 μL of thrombin solution with shaking. The gel was left to polymerize at 37°C for 1 hour. UtMVEC-Myo at a density of 5 × 10^3 cells in 100 μL of culture medium alone or in 100 μL of medium containing either 100 ng/mL of EG-VEGF, VEGF, FGF-2, or combined EG-VEGF/VEGF/FGF-2 were added into each well and incubated at 37°C overnight. Another layer of fibrin gel was then added for capillary network formation. The cells
were further cultured for 24 hours before being examined under a phase-contrast microscope (Axioskop, Zeiss, Oberkochen, Germany). Five random fields of each well were captured and examined by an investigator who was blinded to the treatment of the cells. The vascular tube formation was numerically scored as described in the manufacturer’s protocol: 0, cells isolated or in a sheet-like monolayer; 1, cells begin to migrate and align themselves; 2, capillary tubes visible, no sprouting; 3, sprouting of new capillary tubes visible; 4, closed polygons begin to form; 5, complex mesh-like structure develop (Chemicon). The values for duplicated wells were averaged, and the experiment was repeated 4 times.

**Signaling Pathway(s) Induced by EG-VEGF in UtMVEC-Myo**

To study whether p44/42 MAPK and Akt signaling pathways were involved in EG-VEGF signaling in UtMVEC-Myo, subconfluent culture of UtMVEC-Myo in a 24-well plate were incubated in csEBM-2 for 24 hours and treated with recombinant VEGF at a concentration of 10 ng/mL or EG-VEGF at concentrations of 25, 50, and 100 ng/mL for 15 minutes. The time-course effect of EG-VEGF was studied by treating the cells with 100 ng/mL of EG-VEGF for 15, 30, and 60 minutes. In the control experiment, MCF-7 cells were treated the cells with 100 ng/mL of EG-VEGF at a concentration of 10 ng/mL or EG-VEGF at concentrations of 25, 50, and 100 ng/mL for 15 minutes. The specificity of the p44/42 MAPK pathway was further confirmed by preincubating UtMVEC-Myo with PD98059 at concentrations of 1, 2, 5, 10, and 20 μM for 30 minutes before addition of 100 ng/mL of EG-VEGF. To study whether the EG-VEGF activated pathway(s) was (were) mediated indirectly through release of VEGF, antibodies (4 μg/mL against EG-VEGF (R&D Systems, Minneapolis, MN) and VEGF (Zymed, San Francisco, CA) were used to pretreat UtMVEC-Myo for 2 hours before EG-VEGF (100 ng/mL) and VEGF (10 ng/mL) treatment for 15 minutes. The treated UtMVEC-Myo were then lysed in cold RadioImmuno Precipitation Assay Buffer (RIPA) (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 0.1 mg/mL phenylmethylsulfonyl fluoride (PMSF) in isopropanol, 10 μL/mL aprotinin, and 1 mM sodium orthovanadate for 20 minutes. The lysates were subjected to Western blot analysis using monoclonal antibody against a synthetic phosphopeptide corresponding to residues around Thr202/ Tyr204 of human p44 MAPK and polyclonal antibody against Ser473 and Thr308 of phosphorylated Akt (Cell Signaling Technology, Danvers, MA). The signals were compared with the total amount of p44/42 MAPK and Akt proteins, respectively.

**Cell Proliferation Assay**

Cell proliferation was quantified by the XTT assay (Roche Diagnostic, Basel, Switzerland). UtMVEC-Myo was grown at 2500 cells/well in a 96-well plate in 100 μL of sEBM-2 (Cambrex) and then cultured in csEBM-2 for 24 hours. The cells were treated with 100 ng/mL of EG-VEGF with or without the addition of 10 μM of PD98059 for 48 hours. XTT labeling mixture (50 μL) was then added, and the cells were then incubated for a further 4 hours. The absorbance of the medium at 450–500 nm with a reference wavelength at 650 nm was measured using a spectrophotometer (Dynatech MR5000, Embrach, Switzerland).

**Statistical Analysis**

Data were analyzed using the SigmaPlot software package (Jandel Scientific, San Rafael, CA). Statistical analysis was performed by the Kruskal-Wallis test and Mann-Whitney U-test (followed by multiple pair-wise comparisons using the Student-Newman-Keuls method). P < .05 was considered statistically significant.

**RESULTS**

**Relative Expression of EG-VEGF and Its Receptors in UtMVEC-Myo and Endometrial Cells**

Multiplex real-time quantitative polymerase chain reaction (qPCR) using 18S as internal control was used to study the presence of EG-VEGF, PKR1, and PKR2 mRNA in UtMVEC-Myo at passages 5 (Fig. 1, column 1), 9 (column 2), and 12 (column 3), endometrial stromal (column 4) and epithelial (column 5) cells, and normal secretory endometrial biopsy (column 6). The results showed that no EG-VEGF mRNA was detected in UtMVEC-Myo at the three passages. Endometrial biopsy at the secretory phase contained the most abundant EG-VEGF mRNA, and both the endometrial glandular epithelial and stromal cells possessed the EG-VEGF transcript. PKR1 mRNA was detected in all the cells and tissues tested, while that of PKR2 was only detected in UtMVEC-Myo and endometrial stromal cells (Fig. 1B, 1C). The expression levels of PKR2 were much lower than those of PKR1, with the difference ranging from 89- to 556-fold in different cells and tissues tested.

**EG-VEGF Promotes UtMVEC-Myo Cell Viability and Sprouting**

The mitogenic properties of EG-VEGF, VEGF, and FGF-2 on UtMVEC-Myo were compared. The proliferative effect of EG-VEGF, VEGF, and FGF-2 at concentrations from 1 to 100 ng/mL were significantly increased when compared with the controls (P < .05; Fig. 2). There was a dose-dependent increasing trend of EG-VEGF and FGF-2 effect on the proliferation of UtMVEC-Myo, although the trend had not yet reached statistical significance.

The effect of EG-VEGF on UtMVEC-Myo tube formation was assessed and compared with that after VEGF, FGF-2, and combined EG-VEGF/VEGF/FGF-2 treatment. The vascular tube formation was numerically scored as described above. Morphologically, UtMVEC-Myo formed a monolayer on noncoated culture flask (Fig. 3A, inset a). Culture on fibrin gel without angiogenic factors resulted in the elongation of cells (Fig. 3A, inset b). Treatment of UtMVEC-Myo with VEGF (Fig. 3A, inset c), FGF-2 (Fig. 3A, inset d), EG-VEGF (Fig. 3A inset e), and combined VEGF/FGF-2/EG-
VEGF (Fig. 3A, inset f) induced formation of angiogenic sprouting patterns when compared with the controls. Higher angiogenic scores were obtained after VEGF (Fig. 3B, column c), FGF-2 (Fig. 3B, column d), EG-VEGF (Fig. 3B, column e), and combined treatment (Fig. 3B, column f) when compared with the medium control (Fig. 3B, column b).

**EG-VEGF Induced p44/42 MAPK but Not Akt Phosphorylation in UtMVEC-Myo**

Since the MAPK and Akt signaling pathways are involved in EG-VEGF signaling in other endothelial cell types (11), the phosphorylation of MAPK and Akt after treating the UtMVEC-Myo with EG-VEGF was studied. Treatment with 10 ng/mL of VEGF induced a strong signal of p44/42 MAPK phosphorylation in UtMVEC-Myo (Fig. 4A). Although to a much lesser extent, EG-VEGF at concentrations of 25, 50, and 100 ng/mL also increased the phosphorylation of p44/42 MAPK (upper panel, Fig. 4A). Using an inhibitor specific to the MAPK pathway, PD98059, the EG-VEGF-induced p44/42 MAPK phosphorylation was suppressed in a dose-dependent manner (upper panel, Fig. 4A). The action of EG-VEGF on p44/42 MAPK phosphorylation in UtMVEC-Myo was rapid; the response peaked at 15 minutes and decreased thereafter (Fig. 4B).

In the present study, UtMVEC-Myo expressed VEGF mRNA (data not shown), and VEGF activated the MAPK signaling pathway. Therefore, neutralizing antibodies against VEGF and EG-VEGF were used to determine whether the action of EG-VEGF on p44/42 phosphorylation was mediated indirectly through the release of VEGF from the UtMVEC-Myo. As expected, when compared with the untreated
FIGURE 3

Effects of EG-VEGF, VEGF, and FGF-2 on UtMVEC-Myo tube formation using Fibrin Gel In Vitro Angiogenesis Assay under phase-contrast microscopy. (A) UtMVEC-Myo was cultured to confluence on plastic culture flask (inset a) and subcultured to a fibrin gel–coated 96-well plate (inset b). The cells were treated with 100 ng/mL of VEGF (inset c), 100 ng/mL of FGF-2 (inset d), 100 ng/mL of EG-VEGF (e), and combined EG-VEGF/VEGF/FGF-2 treatment (f). (B) The angiogenic score was evaluated as described in the Materials and Methods section using a 5-step scoring system. *Significant increase in the angiogenic score when compared with controls ($P<.05$).

FIGURE 4

Effects of EG-VEGF on MAPK and Akt cell signaling pathways in UtMVEC-Myo cells. (A) Exposures of UtMVEC-Myo for 15 minutes to 10 ng/mL of VEGF induced a prominent phosphorylation of p44/42 MAPK (P-p44/42 MAPK). EG-VEGF at 25, 50, and 100 ng/mL also activated the p44/42 MAPK pathway, although to a lesser extent. The specificity of EG-VEGF-induced phosphorylation of p44/42 MAPK was confirmed by the dose-dependent inhibitory effects of PD98059. (B) Time-course effects of EG-VEGF-induced phosphorylation of p44/42 MAPK. The response of UtMVEC-Myo to EG-VEGF treatment peaked after 15 minutes but declined thereafter. (C) Dependency of EG-VEGF induced p44/42 MAPK phosphorylation on VEGF. UtMVEC-Myo was preincubated with 4 μg/mL of antibodies (Ab) against VEGF or EG-VEGF for 2 hours, followed by EG-VEGF (100 ng/mL) and VEGF (10 ng/mL) treatment for 15 minutes. EG-VEGF-induced p44/42 MAPK phosphorylation was independent of VEGF. (D) Exposures of UtMVEC-Myo to 10 ng/mL VEGF or 25, 50, and 100 ng/mL of EG-VEGF at different time intervals did not significantly induce Akt phosphorylation (both Thr308 and Ser473). (E) Phosphorylation of Akt (P-Akt) was found in a human breast cancer cell (MCF-7) line treated with 10 ng/mL IGF-1 for 15 and 60 minutes. (F) Effect of PD98059 on EG-VEGF induced cell proliferation. UtMVEC-Myo was treated with 100 ng/mL of EG-VEGF with or without preincubation of 10 μM PD98059 for 30 minutes. VEGF was used as a control. The cell proliferation assay XTT was performed, and the absorbances were recorded. Data represent mean ± SEM of three replicates of three individual experiments. *Significant increase in the cell proliferation when compared with controls (P<.05).

control, both antibodies alone have no effect on phospho-p44/42 expression but suppressed the response of the cells to their corresponding exogenous ligands. Although anti-VEGF antibody could not completely block the effect of VEGF, p44/42 MAPK phosphorylation was greatly reduced. On the other hand, anti-VEGF antibody had no effect on EG-VEGF-induced activation of p44/42 MAPK, and anti-EG-VEGF did not affect VEGF-induced p44/42 phosphorylation (Fig. 4C), indicating that EG-VEGF and VEGF activated the p44/42 MAPK pathway independently. The expression levels of total Akt and activated Akt (P-Akt) were low in the UtMVEC-Myo cells. There was no significant change in the expression of P-Akt in cells treated with VEGF or EG-VEGF for 15–60 minutes (Fig. 4D). Treatment of MCF-7 cells with IGF-1 for 15–60 minutes was used as a positive control to confirm the detection of P-Akt in our assay (Fig. 4E). The EG-VEGF-induced activation of p44/42 MAPK in UtMVEC-Myo was related to EG-VEGF-produced cell proliferation as 10 μM of PD98059 nullified the effect of EG-VEGF on UtMVEC-Myo cell proliferation (Fig. 4F).

DISCUSSION

The coexpression of EG-VEGF and its receptors, PKR1 and PKR2, in human endometrial biopsies suggests that the growth factor may act as a paracrine factor regulating angiogenesis in the uterus (17). Estrogen stimulates the production of EG-VEGF by endometrial glandular epithelial cells (17). In a coculture model with endometrial glandular epithelial cells, estrogen induces tube formation of the human myometrial microvascular endothelial cells (19). These studies suggest that EG-VEGF from the endometrial glandular epithelial cells may mediate the action of estrogen on tube formation of the myometrial microvascular endothelial cells and that the UtMVEC-Myo cells can be used as a model for studying the biological activity of EG-VEGF.

In the endometrium, angiogenesis is involved in repairing the vascular bed after menstruation, rapid endometrial growth in the proliferative phase, and growth and coiling of the spiral arteries in the secretory phase of the menstrual cycle (20). Radial arteries arising from the arcuate arteries within the myometrium are responsible for the blood supply to the endometrium. They split into small basal arteries in the basal layer and spiral arteries in the functional layer of the endometrium after passing through the myometrial-endometrial junction (21). The study of myometrial endothelial cells could therefore be relevant to angiogenesis in the endometrium. Moreover, an endothelial cell line of endometrial origin is not available.

The effect of the EG-VEGF on endothelial cells varies with the origin of the cells; EG-VEGF promotes the proliferation of adrenal cortex capillary endothelial cells but not that of the human umbilical vein endothelial cells (9). This study demonstrated for the first time that EG-VEGF has angiogenic properties in endothelial cells of uterine origin. The UtMVEC-Myo cells were effector cells of EG-VEGF as the cells contained PKR1 and PKR2 but did not synthesize EG-VEGF. This possibility is supported by the observation that EG-VEGF induced proliferation and tube formation of UtMVEC-Myo.

The relative amount of PKR1 in UtMVEC-Myo is about 100-fold higher than that of PKR2 (data not shown). The two receptor molecules share 87% homology in amino acid sequences and have an almost identical transmembrane domain sequence (22), suggesting that they may have a similar activation mechanism (6). The much higher expression of PKR1 than PKR2 in the normal endometrium and UtMVEC-Myo indicates that EG-VEGF may mainly bind to PKR1 to produce its angiogenic effects. This possibility is in line with the previous observation that EG-VEGF induced phosphorylation of p44/42 MAPK in the PKR1-transfected cells but not in the PKR2-transfected cells (10) and the observation in the present study that EG-VEGF activated the p44/42 MAPK pathway. The possibility can be confirmed in the future by studying the angiogenic effects of EG-VEGF after knocking down PKR1 and/or PKR2 in UtMVEC-Myo. In mice, PKR2 is essential for maturation of the reproductive system as its deficiency is associated with atrophy of the reproductive tract including gonads and endometrium, which has been linked to the lack of gonadotropin-releasing hormone neurones in the hypothalamus (23). On the other hand, no abnormality in the reproductive tract of PKR1 knockout mice has been found (23, 24).

Endothelial cell sprouting is one of the mechanisms of angiogenesis in which new outgrowths of capillaries are formed that ultimately connect with each other to form a vessel network (25). In vitro tube formation assay showed that capillary sprouting of UtMVEC-Myo occurred more rapidly and vigorously with the addition of EG-VEGF, VEGF, or FGF-2 or in combination when compared with the control without supplementation of growth factors. VEGF is generally accepted as a critical angiogenic factor associated with implantation and early pregnancy (26), although this view is not universally held (27, 28). FGF-2 is a potent pleiotropic heparin-binding mitogen acting synergistically with VEGF in stimulating new vessel formation (29). The observation that EG-VEGF supported the proliferation and sprouting of UtMVEC-Myo to an extent similar to that of VEGF and FGF-2 suggested that EG-VEGF was important in the development of endothelial cells in the uterus. In the ovary, the angiogenic response induced by EG-VEGF is indistinguishable from that induced by VEGF (12), which indicates that EG-VEGF may play a complementary or overlapping role in vascular functions as VEGF. Like VEGF, the expression of EG-VEGF in steroid-producing cells and endometrium is controlled by low oxygen tension and hormones (8, 12, 17).

The present results demonstrated that phosphorylation of the p44/42 MAPK signaling pathway was induced in UtMVEC-Myo in response to EG-VEGF, although to an extent much smaller than that of VEGF treatment. The effects of VEGF on UtMVEC-Myo are in line with previous results showing that VEGF stimulated the MAPK cascade in endothelial cells (30, 31). The activation of the MAPK signaling...
pathway by EG-VEGF was specific because it could be inhibited by the specific MAPK kinase inhibitor, PD98059. The effect of EG-VEGF on the cell proliferation of UT-MVEC-Myo was also inhibited by PD98059, suggesting that the activation of the p44/42 MAPK signaling pathway contributes directly to EG-VEGF-induced proliferation of the cells, consistent with the critical role of MAPK activation in angiogenesis (32).

Apart from the MAPK signaling pathway, EG-VEGF activates phosphatidylinositol 3-kinase (PI3K) and its downstream target serine/threonine kinase, Akt in neuroblastoma (11, 33), and adrenal cortex capillary endothelial cells (11). VEGF also activates the PI3K pathway in a number of endothelial cells (34, 35). Although no study has yet demonstrated a direct effect of VEGF on Akt activation of uterine endothelial cells, Kazi and Koos recently showed that estrogen-induced VEGF expression in the rat uterus was mediated mainly through PI3K pathways (36). However, neither VEGF nor EG-VEGF activates Akt in UTMVEC-Myo, or, if they do, the level of activation is too low to be detected by Western blotting. In bovine adrenal cortex capillary endothelial cells, Akt phosphorylation also contributes less to EG-VEGF-induced cell growth than the phosphorylation of p44/42 MAPK does (11). On the other hand, the lack of p44/42 MAPK activation in a neuroblastoma cell line after EG-VEGF treatment (33) revealed a context-dependent functionality of EG-VEGF.

Human myometrial microvascular endothelial cells contain estrogen receptor-α and respond to estrogen treatment, resulting in increased expression of VEGF receptor and enhanced cell proliferation (37). The present data showed that UTMVEC-Myo expressed VEGF mRNA. Thus the possibility that the action of EG-VEGF on tube formation of UTMVEC-Myo was mediated through UTMVEC-Myo-derived VEGF was investigated. Using antibodies against VEGF and EG-VEGF, it was demonstrated that the two endothelial growth factors activated the p44/42 MAPK pathway independently.

No additive effect of EG-VEGF, VEGF, and FGF-2 was detected on UTMVEC-Myo proliferation and sprouting in this study, despite previous demonstrations of potent synergistic effects of VEGF and FGF-2 in different endothelial cells (38–41) and additive induction of fenestrate by VEGF and EG-VEGF in adrenal cortex capillary endothelial cell culture (12). There are two possible explanations for the observations. First, the three growth factors activate the same signaling pathway, probably the MAPK pathway, in UTMVEC-Myo, and the amount of individual growth factor used had already maximally stimulated the pathway. Second, the pathways in UTMVEC-Myo induced by the three growth factors are different from those in other endothelial cells. Experiments are being performed to distinguish these possibilities. The cell proliferation and sprouting induced by EG-VEGF is modest in UTMVEC-Myo as compared with its effects on adrenal cortex capillary endothelial cells (11). However, a similar proliferation rate was also obtained after treating enteric neural crest cells with EG-VEGF in vitro (42). As angiogenesis in vivo is a process involving the interaction of the mural cells and endothelial cells, the modest response detected in the present study could be due to the suboptimal culture condition in vitro or lack of interaction with appropriate cells in culture.

In conclusion, this study showed that EG-VEGF has a direct angiogenic effect on EG-VEGF receptors (PKR1 and PKR2) expressing uterine myometrial endothelial cells in terms of higher cell proliferation and increased sprouting of the endothelial cells. EG-VEGF enhanced cell proliferation through the activation of MAPK pathway but not through the Akt pathway.

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