Stimulatory Interaction Between Vascular Endothelial Growth Factor and Endothelin-1 on Each Gene Expression

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Abstract—The precise regulation of cell growth in the vascular wall maintains vascular integrity, and its disruption leads to cardiovascular disorders including atherosclerosis and restenosis. Vascular endothelial growth factor (VEGF) is a specific mitogen for endothelial cells, and endothelin-1 (ET-1) is known to stimulate the proliferation of smooth muscle cells. The aim of this study was to explore a potential interaction between VEGF and ET-1 on each expression in vascular cells. VEGF enhanced preproET-1 mRNA expression and ET-1 secretion in bovine aortic endothelial cells (BAECs). Similarly, in rat vascular smooth muscle cells (VSMCs), ET-1 enhanced VEGF mRNA expression and stimulated VEGF secretion. ET-1–induced VEGF mRNA expression was abolished by a selective ETα receptor antagonist, BQ-485, but not by an ETβ-selective blocker, BQ-788. It was also inhibited by pretreatment with actinomycin D but not by pretreatment with cycloheximide. Furthermore, the actinomycin D chase experiment revealed that ET-1 did not alter VEGF mRNA stability. Coculture of BAECs and VSMCs enhanced both ET-1 and VEGF gene expression in these cells, and the conditioned media from BAECs and VSMCs reproduced the augmentation of each gene expression, which was partially inhibited by BQ-485 or an antibody specific to VEGF. Our results indicate that VEGF and ET-1 have stimulatory interactions on each expression, which may play an important role in concomitant proliferation of endothelial and smooth muscle cells in the vascular wall. (Hypertension. 1998;32:89-95.)

Key Words: endothelial growth factors  ■  endothelin-1  ■  gene expression  ■  coculture  ■  atherosclerosis

Vascular endothelial and smooth muscle cells are major components of vascular wall; they interact with each other in regulating vascular tone and in vascular growth or remodeling through various vasoactive substances, growth factors, and cytokines, which they produce under physiological or pathological conditions such as atherosclerosis. Recently, VEGF has been identified and characterized as a homodimeric protein of approximately 34- to 46-kDa that binds to the receptors Flt and Flk (expressed mainly in endothelial cells) and promotes proliferation, migration, and permeability of endothelial cells and angiogenesis.2,3 VEGF is produced in several cell types, such as smooth muscle cells and macrophages, as well as transformed cells, and its production is stimulated by a variety of factors including growth factors, cytokines, and hypoxia.3 Recently, immunohistochemical studies have demonstrated VEGF expression localized predominantly to smooth muscle cells in normal human vessels, as well as in atherosclerotic or restenotic human coronary arteries. These findings suggest that VEGF may play a role in the maintenance and repair of vascular endothelium other than promoting angiogenesis. However, the mechanisms of the action of VEGF on endothelial cells are poorly understood, and it remains unknown whether VEGF induces other growth factors or cytokines in endothelial cells.

ET-1 is a vasoactive peptide purified from the endothelial supernatant as a potent vasoconstrictor,5 and it has potent effects on cell growth and stimulates DNA synthesis of various cells, including VSMCs6 and mesangial cells.7 ET-1 is produced primarily in endothelial cells and also is expressed in VSMCs and macrophages in atherosclerotic lesions.8,9 In addition to its vasoconstricting and mitogenic properties, ET-1 can stimulate the production of vasoactive substances such as adrenomedullin in VSMCs10 and atrial natriuretic peptide in cardiomyocytes.11 Therefore, there is a possibility that ET-1 regulates vascular tone or cell growth indirectly by inducing other cytokines or growth factors under physiological or pathological circumstances.

The disruption of regulation of cell growth in the vascular wall leads to cardiovascular disorders such as atherosclerosis and restenosis, in which injury to endothelium induces migration and proliferation of smooth muscle cells, leading to the formation of plaque or neointima. There are several reports demonstrating the association of VEGF or ET-1 with intimal hyperplasia after balloon denudation. VEGF is shown to modify the extent of neointimal thickening of injured vessels.12,13 Similarly, it was reported that neointimal formation after balloon injury was augmented by exogenous ET-1 and decreased by an ET receptor antagonist.14 These findings suggest that VEGF and ET-1 may serve as key regulators of cell proliferation and interact with each other in atherosclerotic or restenotic lesions. In the present study, we examined a potential interaction between VEGF and ET-1 on each expression in vascular endothelial and smooth muscle cells.

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VEGF and ET-1 Interaction on Gene Expression

Methods

Materials
ET-1 was obtained from Peptide Institute. Human recombinant VEGF, cycloheximide, and actinomycin D were obtained from Sigma Chemical Co. BQ-485 and BQ-788 were purchased from Calbiochem-Novabiochem. The polyclonal antibody against VEGF was from Santa Cruz Biotechnology. Enzyme immunoassay kits for ET-1 peptide and VEGF protein were from Wako Pure Chemical Industries and American Research Products, respectively. Human VEGF cDNA was a gift from Dr. Hikaru Ueno (Kyushu University School of Medicine, Fukuoka, Japan). Bovine preproET-1 cDNA was generously provided by Dr. Masashi Yanagisawa (University of Texas Southwestern Medical Center at Dallas). Other materials and chemicals were obtained from commercial sources.

Cell Culture
VSMCs were isolated from rat thoracic aorta by enzymatic dissociation as described previously.16 Cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum at 37°C in 5% CO2/95% air. For experiments, cells between passage levels 5 and 15 were seeded into dishes and grown in DMEM/10% fetal calf serum. The media were changed to DMEM/15% fetal calf serum. Confluent cells were fed with serum-free DMEM for 12 to 24 hours before experiments.

Coculture of BAECs and VSMCs was performed using the Transwell culture system (Corning Costar). BAECs were seeded into Transwell inserts (24-mm diameter) with permeable membrane supports (0.4-μm pore size) and grown in DMEM/15% fetal calf serum. VSMCs were spread into 6-well cluster plates and grown in DMEM/10% fetal calf serum. The media were changed to serum-free DMEM at confluence. After 24 hours, serum-free DMEM was replaced, and Transwell inserts were transferred into the 6-well plates where VSMCs were cultured so that BAECs and VSMCs could share the media. The cells were used for experiments 24 hours after coculture.

RNA Isolation and Northern Blot Analysis
Total RNA was extracted from VSMCs and BAECs by guanidium isothiocyanate and phenol extraction as described previously.17 Total RNA (15 to 30 μg) was subjected to electrophoresis on 1% agarose gels containing formaldehyde and transferred to nitrocellulose filters. The filters were hybridized with a random-primed, [32P]labeled full length of human VEGF cDNA or bovine preproET-1 cDNA17 as a probe at 42°C for 16 hours in 4× SSC, 1× Denhardt’s solution, 40% (vol/vol) formamide, 10% SDS, and 40 μg/mL herring sperm DNA. The hybridized filters were then washed in 0.1× SSC/0.1% SDS at 55°C for 30 minutes twice and analyzed using Bio-Imaging analyzer BAS2000.

Assay of mRNA Stability
VSMCs were incubated with or without ET-1 (10 nmol/L) for 2 hours, and then actinomycin D (5 μmol/L) was added to the media. During the following 6 hours, cells were harvested at various times as indicated; total RNA was extracted and Northern blot analysis was performed as described above.

Assay of ET-1 Peptide
The concentrations of ET-1 peptide were measured by sensitive sandwich-type enzyme immunoassay as described previously.19 Briefly, BAECs in 100-mm culture dishes were incubated with or without VEGF (1 nmol/L) for 24 hours in 5 mL of serum-free DMEM. The conditioned media were directly subjected to the assay for ET-1 peptide. The cells were washed with PBS, trypsinized for 2 minutes, and then neutralized with fetal calf serum. After centrifugation, the supernatants were removed, and the cells were resuspended in PBS. In a 100-μL aliquot of the cell suspension, the number of cells was determined using a hemocytometer. The remaining cells were solubilized with sonication, and protein concentration was determined by the method of Bradford with bovine serum albumin as a standard protein.

Assay of VEGF Protein
The concentrations of VEGF protein were measured using a competitive enzyme immunoassay kit (Cytokine Duplex VEGF) as recommended by the manufacturer. The conditioned media of VSMCs treated with or without ET-1 (100 nmol/L) for 24 hours were directly subjected to the assay for VEGF protein. The number of cells was determined as described in “Assay of ET-1 Peptide.”

Statistical Analysis
Results were expressed as mean±SEM. The F test was first performed on the data, and significant differences were determined by unpaired t test. A value of P<0.05 was considered significant.

Results

Effect of VEGF on preproET-1 mRNA Expression in BAECs
First, we examined the effect of VEGF on ET-1 gene expression in BAECs. BAECs grown to confluence were treated with VEGF (1 nmol/L) for various periods as indicated (Figure 2A). Although the baseline level of preproET-1 mRNA was low, it increased in response to VEGF treatment within 2 hours and reached a maximum (5.4-fold increase) at 3 hours. Thereafter, the level of preproET-1 mRNA decreased gradually but still was higher than control (2.4-fold increase) at 24 hours. The treatment with VEGF also increased the preproET-1 mRNA level in a dose-dependent manner (Figure 1B). VEGF caused the maximal enhancement at 1 nmol/L.

Effect of VEGF on ET-1 Secretion From BAECs
To confirm that the induction of ET-1 gene expression resulted in increased synthesis and release of ET-1 peptide, we measured the level of ET-1 peptide in the conditioned media of BAECs. As shown in Figure 2C, the treatment of BAECs with VEGF (1 nmol/L) for 24 hours increased the level of ET-1 peptide in conditioned media by 1.5-fold (582±27 pg/mL per 107 cells for VEGF-stimulated versus 408±6 pg/mL per 106 cells for unstimulated, P<0.05). As we examined the effect of VEGF on the confluent culture of BAECs, there were no significant differences in the number of cells and total protein content between VEGF-treated and untreated BAECs (Figure 2A and 2B).
Effect of ET-1 on VEGF mRNA Expression in VSMCs

Next, we tested whether ET-1 induced VEGF mRNA expression in VSMCs. As shown in Figure 3A, treatment of VSMCs with ET-1 (10 nmol/L) resulted in a rapid increase in the level of VEGF mRNA that peaked at 2 hours and declined to the basal level within 6 hours. As shown in Figure 3B, 0.1 to 10 nmol/L of ET-1 showed dose-dependent effects on the increase in VEGF mRNA expression.

Effect of ET-1 on VEGF Secretion From VSMCs

To confirm that the increase of VEGF mRNA expression resulted in increased synthesis and release of VEGF protein, we measured the level of VEGF protein in the conditioned media of VSMCs. As shown in Figure 4, treatment of VSMCs with ET-1 (10 nmol/L) for 24 hours increased the level of VEGF protein in conditioned media (4.31 ± 0.23 ng/mL per 10^6 cells for ET-1–stimulated versus 3.31 ± 0.28 ng/mL per 10^6 cells for unstimulated, P<0.05).

Effects of ET Receptor Antagonists on ET-1–Induced VEGF mRNA Expression in VSMCs

There are two subtypes of ET receptors, i.e., the ET<sub>A</sub> receptor, which is expressed abundantly on VSMCs and cardiac myocytes, and the ET<sub>B</sub> receptor, which is expressed predominantly on endothelial cells. To determine which receptor mediated the induction of VEGF mRNA expression by ET-1, we examined the effect of an ET<sub>A</sub>-selective receptor antagonist, BQ-485, and a highly potent ET<sub>B</sub> receptor antagonist, BQ-788, on VEGF mRNA expression. As shown in Figure 5A, 100 nmol/L of BQ-485 completely blocked the effect of ET-1 on VEGF mRNA expression, whereas BQ-788 had no effect. These results indicated that ET-1 induced VEGF mRNA expression through the ET<sub>A</sub> receptor in VSMCs.

Effects of Actinomycin D and Cycloheximide on ET-1–Induced VEGF mRNA Expression in VSMCs

To determine whether ET-1 induced VEGF mRNA expression by increasing transcription or stability of mRNA, VSMCs were treated with actinomycin D. As shown in Figure 5B, pretreatment of VSMCs with actinomycin D completely abolished the effect of ET-1 on VEGF mRNA expression. The actinomycin D chase experiment revealed that ET-1 did not alter the stability of VEGF mRNA (Figure 5C), suggesting that ET-1 regulated VEGF mRNA expression by the transcriptional mechanism. To determine whether new protein synthesis was necessary for ET-1–induced VEGF mRNA expression, VSMCs were treated with ET-1 in the presence or absence of cycloheximide. As shown in Figure 5B, cycloheximide did not inhibit ET-1–induced VEGF mRNA expression. Therefore, new protein synthesis was not required for ET-1–induced VEGF mRNA expression.
ET-1 and VEGF Gene Expression on Coculture of BAECs and VSMCs

To demonstrate the physiological significance of the mutual induction of ET-1 and VEGF in vascular cells, we investigated whether the coculture of BAECs and VSMCs yielded effects on the expression of ET-1 and VEGF in these cells. PreproET-1 mRNA was upregulated in BAECs cocultured with VSMCs for 24 hours, approximately 4.5 times as much as that in BAECs cultured separately (Figure 6A and 6C). Similarly, the expression of VEGF mRNA was also enhanced in VSMCs cocultured with BAECs for 24 hours compared with that in VSMCs cultured separately (Figure 6B and 6C).

Effects of Conditioned Media on ET-1 and VEGF Gene Expression

To confirm that the enhancement of the expression of ET-1 and VEGF mRNA by coculture was mediated by soluble and secreted factors, we examined effects of the conditioned media of BAECs and VSMCs on the expression of ET-1 and VEGF mRNA. Confluent BAECs and VSMCs in 100-mm dishes were cultured separately in 5 mL of serum-free media for 24 hours, and the conditioned media were collected from culture dishes of BAECs and VSMCs, respectively. As shown in Figure 7, the conditioned media of VSMCs enhanced preproET-1 mRNA expression in BAECs, which was partially inhibited by pretreatment of the conditioned media with the antibody against VEGF. Similarly, the conditioned media of BAECs induced VEGF mRNA expression in VSMCs, which was blocked partially and dose-dependently by BQ-485.

Discussion

In this study, we demonstrated the stimulatory interaction between VEGF and ET-1 on each other’s gene expression in vascular endothelial cells and smooth muscle cells. Coculture of BAECs and VSMCs resulted in enhanced gene expression in these cells of ET-1 and VEGF, respectively. Furthermore, the augmentation of their expression was reproduced by addition of the conditioned media to each of these cells. This augmentation was partially inhibited by the specific antibody against VEGF or the ETA receptor antagonist, indicating that a part of this augmentation was mediated through VEGF and ET-1, respectively. However, other factors in the conditioned media that are involved in the enhancement of each gene expression remain to be determined. ET-1 is synthesized abundantly in endothelial cells and acts locally as a mitogenic factor as well as a constrictor on VSMCs. The production of ET-1 is regulated at the level of mRNA transcription and stimulated by many factors such as TGF-β, IL-1β, and hypoxia in cultured vascular endothelial cells. On the other hand, VEGF has been reported to be produced by VSMCs and exerts the proliferative or permeabilizing effect on endothelial cells. VEGF is induced by various factors, including platelet-derived growth factor, TGF-β, angiotensin II, and IL-1β and hypoxia in cultured vascular endothelial cells. On the other hand, VEGF has been reported to be produced by VSMCs and exerts the proliferative or permeabilizing effect on endothelial cells. VEGF is induced by various factors, including platelet-derived growth factor, TGF-β, angiotensin II, and hypoxia in a variety of cultured cells including VSMCs and cardiac myocytes.

Endothelial cells express two high-affinity receptors for VEGF, termed Flk-1 and Flt-1, but several reports have shown that biological activities of VEGF on endothelial cells, such as chemotaxis and proliferation, appear to be mediated exclusively by Flk-1. With reverse transcription–polymerase chain reaction, we detected only Flk-1 in the BAECs
we used in our experiments (data not shown), which was consistent with a previous report demonstrating that BAECs expressed only Flk-1.30 In addition, we found that placenta growth factor, which is a member of the family of VEGF and binds with high affinity to Flt-1 but not to Flk-1,31 did not enhance the expression of preproET-1 mRNA in BAECs (data not shown). These findings indicate that the action of VEGF in ET-1 induction in BAECs was mediated by Flk-1. Activated by VEGF, VEGF receptors phosphorylate several cytoplasmic proteins containing SH2 domains and can participate in downstream signal transduction.32 Thus, it is possible that receptor-mediated activation of protein tyrosine kinase contributes to ET-1 induction by VEGF, but the distinct signal transduction pathway from VEGF receptors to ET-1 gene expression remains to be elucidated. For this experiment, we used postconfluent quiescent BAECs, which did not proliferate in response to VEGF. A previous report showed that DNA synthesis and ET-1 gene expression did not increase in the presence of serum in the postconfluent BAECs.33 Therefore, it is suggested that ET-1 induction elicited by VEGF was not cell-cycle dependent in the present study.

In this report, we showed that ET-1 induced VEGF mRNA expression through the ET2 receptor in VSMCs, which is consistent with the previous report that the VSMCs of relatively early passage (up to the 15th), which we used for this experiment, express predominantly ET2 receptors.34 Each ET receptor subtype is a seven-transmembrane receptor coupled with G protein35,36 and elicits phosphoinositide breakdown to produce inositol triphosphate and diacylglycerol, causing intracellular calcium mobilization and protein kinase C activation, respectively.37 The upstream promoter region of VEGF gene has several AP-1 sites,38 and phorbol ester and calcium ionophore are reported to induce transcription of VEGF mRNA.39 Therefore, it is presumed that VEGF induction by ET-1 is coupled with the phosphoinositide turnover signaling pathway, but further investigations are required for its elucidation.

We found that the mechanism of the VEGF induction by ET-1 was activation of mRNA transcription, since actinomycin D inhibited the ET-1 effect on VEGF induction and ET-1 had no effect on VEGF mRNA stability. There are several studies clarifying the mechanism of VEGF gene induction by cytokines, growth factors, or hypoxia in various cultured cells. Ryuto et al39 demonstrated that VEGF gene expression was induced transiently within 3 hours by basic fibroblast growth factor or tumor necrosis factor-α in human glioma cells due to transcriptional activation without changing sta-
bility of mRNA. In VSMCs, IL-1β was reported to upregulate VEGF mRNA gradually and persistently up to 48 hours by increasing the rate of transcription and the half-life of mRNA. Finally, Ikeda et al. showed that hypoxic induction of VEGF in C6 glioma cells was due to both early transcriptional activation and later increased stability of mRNA during the time course under hypoxia. In our experiment, ET-1 enhanced VEGF gene expression transiently within 3 hours. The time course of induction is similar to that by another vasoactive peptide, angiotensin II. This rapid and transient induction of VEGF elicited by ET-1 is likely related to the fact that ET-1 affected the transcription but not the stability of VEGF mRNA.

In pathological conditions such as the progression of atherosclerosis and the formation of neointima after angioplasty, there is a possibility that both VEGF and ET-1 are upregulated by various stimuli including inflammatory cytokines, growth factors, or a hypoxic condition. In atherosclerotic lesions, neovascularization is frequently observed and possibly contributes to progression of atherosclerosis by providing nutrient influx and cell recruitment. In this regard, the interaction of VEGF and ET-1 may increase neovascularization and promote the progression of atherosclerosis, constituting a vicious circle. In addition, recent studies have demonstrated the vasorelaxing activity of VEGF. Because ET-1 is a potent vasoconstrictor, the opposite effect of VEGF on vascular tone may play some role in the regulation of vascular tone under normal or pathological conditions.

In summary, we have shown the stimulatory interaction between VEGF and ET-1 on each gene expression and secretion in vascular cells. The interaction between them may augment vascular endothelial and smooth muscle cell proliferation and play an important role in the development of cardiovascular disorders generated by disruption of the regulation of vascular cell proliferation.

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