In Vivo Evidence of Angiogenesis Induced by Transcription Factor Ets-1

Ets-1 Is Located Upstream of Angiogenesis Cascade

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Background—A transcription factor, ets-1, regulates the transcription of metalloproteinase genes, the activity of which is necessary for matrix degradation and the migration of endothelial cells. However, no study has demonstrated that ets-1 itself has an angiogenic action in vivo. Thus, we examined (1) the effects of overexpression of the ets-1 gene on angiogenesis in a rat hindlimb ischemia model, and (2) how ets-1 induced angiogenesis.

Methods and Results—In this study, we used the HVJ-liposome method, which is highly effective for transfection, to transfet the human ets-1 gene. At 4 weeks after transfection, the capillary density and blood flow were significantly increased in a hindlimb transfected with the human ets-1 gene compared with control. These data clearly demonstrated that ets-1 has the ability to stimulate angiogenesis in vivo. To elucidate the molecular mechanisms by which ets-1 induced angiogenesis, we focused especially on the expression of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF), potent angiogenic growth factors, because the promoter regions of both genes contain ets binding sites. Interestingly, overexpression of ets-1 upregulated both tissue HGF and VEGF concentrations in rat hindlimb. More importantly, administration of neutralizing antibody against HGF and VEGF attenuated the increase in blood flow and BrdU-positive cells induced by ets-1. Upregulation of HGF and VEGF by ets-1 was also confirmed by in vitro experiments using human vascular smooth muscle cells.

Conclusions—The present study demonstrated that ets-1 regulated angiogenesis through the induction of angiogenic growth factors (VEGF and HGF). Overexpression of ets may provide a new therapeutic strategy to treat peripheral arterial disease. (Circulation. 2004;109:3035-3041.)

Key Words: angiogenesis ■ gene therapy ■ growth substances ■ viruses

Development of new blood vessels begins with activation of parent vessel endothelial cells by angiogenic growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF).1-4 Because endothelial cells express various genes during angiogenesis, an understanding of the transcriptional control of these genes in endothelial cells is important. From this viewpoint, ets-1 might be important, because it is well known to be expressed in endothelial cells during angiogenesis.5,6 The ets family may activate the transcription of genes encoding collagenase 1, stromelysin 1, and urokinase plasminogen activator, which are proteases involved in extracellular matrix degradation.7-9 Recent reports also documented that integrin β3 and VE-cadherin are downstream targets of ets-1 in endothelial cells.10,11 Moreover, endothelium-specific, receptor-type tyrosine kinases such as Flt-1, KDR, TIE-1, and TIE-2 contain the ets binding motif in their promoter/enhancer regions, and the ets family transcription factors are suggested to stimulate their promoter activities.12-17 It is believed that the ets family takes part in regulating angiogenesis by controlling the transcription of those genes whose activity is necessary for the migration of endothelial cells from preexisting capillaries. However, no report has documented in vivo evidence of angiogenesis induction by ets-1 itself, although ets-1 stimulated invasive activity but not the growth of endothelial cells using endothelial cells stably expressing a high level of ets-1.18 In this study, we examined (1) the effects of overexpression of ets-1 gene on angiogenesis in a rat hindlimb ischemia model and (2) how ets-1 induced angiogenesis. The present study...
demonstrated that ets-1 regulated angiogenesis through the induction of angiogenic growth factors (VEGF and HGF).

Methods

Construction of Plasmids
To produce an HGF expression vector or an ets expression vector, human HGF cDNA (2.2 kb) or human ets cDNA was inserted into a simple eukaryotic expression plasmid that utilizes the cytomegalovirus promoter/enhancer.18–20 This promoter/enhancer has been used to express reporter genes in a variety of cell types and can be considered to be constitutive. The vector used as a control was a cytomegalovirus expression vector plasmid, which contained neither HGF cDNA nor ets DNA.

In Vivo Gene Transfer Using Direct Intramuscular Injection Approach
A hindlimb ischemia model of Sprague-Dawley rats (400 to 500 g; Charles River Breeding Laboratories, Wilmington, Mass) was created as previously described.18,19 Because we have previously reported a high efficacy of transfection with hemagglutinating virus of Japan (HVJ)–coated liposomes,19,21 we used the HVJ-liposome method to transfected plasmid DNA. The HVJ-liposome complex was carefully injected directly into the ischemic limb of rats with a 27-gauge needle (Terumo) at 1 week after surgery. The injection volume of plasmid was 100 μL.

The measurement of blood flow with a laser Doppler imager (LDI) has been described previously.18–20 Because laser Doppler flow velocity correlates well with the capillary density,18–20 we measured the blood flow by means of a laser Doppler flowmeter (Laser Doppler Imager, Moor Instruments). Consecutive measurements were obtained over the same regions of interest (leg and foot). LDI uses a 12-nm helium-neon laser beam that sequentially scans a 5×5-cm surface area at extremely high speed to be able to measure the blood flow in the ischemic hindlimb. The blood flow at 1 mm under the surface can be measured. During scanning, blood cells that are in motion shift with the frequency of projected light according to the Doppler principle. Low or no perfusion is displayed as dark blue, whereas the highest perfusion interval is displayed as white. The stored perfusion values behind the color-coded pixels remain available for data analysis. These laser images were quantitatively converted into histograms that represented the amount of blood flow on the x-axis and the number of pixels on the y-axis in the traced area. The average blood flow in each histogram was calculated.

Alkaline phosphatase staining was used as a specific marker for endothelial cells in paraffin-embedded sections.18–20 Three individual sections from the middle of the transsected muscle were analyzed. The number of vessels was counted under a light microscope (magnification, ×100) in a blinded manner. The total number of vessels in each section was summed and expressed as the number per section. At least 10 individual sections were evaluated from each muscle. The areas in which the number of vessels was quantified were randomly selected in the injected site and around the injected site. The animals were coded so that analysis was performed without any knowledge of which treatment each had received. The reproducibility of the results was assessed.

Measurement of HGF and VEGF
Tissue samples were rapidly frozen in liquid nitrogen and homogenized in lysis buffer (Pierce Chemical Co) containing protease inhibitors. Protein concentration was measured by the method of bicinchoninic acid assay (Pierce Chemical Co). One week after transfection, endogenous rat HGF concentration in tissue was measured by electroimmunoassay using anti-rat HGF antibody, because the antibody against rat HGF reacts only with rat HGF and not with human HGF.25 In contrast, the concentration of VEGF protein in muscles was determined by Western blotting using anti-VEGF antibody (1:1000; Santa Cruz Biotechnology, Inc). To quantify and compare levels of proteins, the density of each band was measured by densitometry. Finally, the amounts of loaded proteins were confirmed to be equal by Western blotting of tubulin using anti-tubulin antibody (1:1000; Calbiochem).

In Vitro Transfection Using Cationic HVJ-Liposomes
Human aortic vascular smooth muscle cells (VSMCs) (passage 5) were obtained from Clonetics Corp and cultured in modified MCDB131 medium supplemented with 5% FCS, 100 U/mL penicillin, 100 mg/mL streptomycin, 10 ng/mL epidermal growth factor, 2 ng/mL bFGF, and 1 mmol/L dexamethasone in the standard manner.23 All the cells were used within passage 5 to 6.

The procedure for preparation of HVJ-cationic liposomes was the same as that for the HVJ-liposome method except for the lipid compounds used. The lipid mixture was 6 μg phosphatidylcholine, 3 mg cholesterol, and 0.75 mg 3-[N-(N,N-dimethylaminoethane)carbamoyl] cholesterol. VSMCs were grown to confluence in 5% calf serum. After confluence, the medium was changed to fresh defined serum-free medium. HVJ-cationic liposome complex (100 μL) containing the human HGF gene or human ets gene was added to the dishes. Two days after transfection, HGF and VEGF concentrations were measured by electroimmunoassay.

Administration of anti-HGF and anti-VEGF Antibodies in Mouse Hindlimb Ischemia Model
A mouse ischemia model was created according to previous reports.18–20 Consequently, blood flow to the ischemic limb was dependent on collateral vessels developing from the internal iliac artery. HVJ-liposome vector containing plasmid DNA encoding ets-1 or control vector (10 μg/body) was carefully injected directly into the ischemic limb with a 27-gauge needle at 10 days after surgery (day 10). Four separate injections of vector locally (intra-muscular into the ischemic limb near both the proximal and distal arterial stump) were performed. The Osaka University Institutional Animal Care and Use Committee approved all protocols. In all experiments, investigators performing the follow-up examinations were blinded to the identity of the treatment administered. The measurement of blood flow with an LDI and BrdU-positive cells was performed at 2 weeks after transfection. In vivo suppression of endogenous VEGF activity was performed by use of VEGF-specific neutralizing rabbit polyclonal IgG (cross-reactive to human and murine VEGF; NeoMarkers Co).26 Suppression of endogenous HGF activity was also performed using HGF-specific neutralizing rabbit polyclonal IgG (cross-reactive to human and murine VEGF).

Figure 1. Effect of injection of naked human HGF plasmid and/or human ets plasmid in rat ischemic limb model at 4 weeks after transfection. Quantitative analysis of blood flow in hindlimb expressed as perfusion ratio of ischemic hindlimbs to untreated opposite limbs. Control indicates control vector; HGF, human HGF plasmid; Ets, human ets plasmid; HGF+Ets, co-injected with human HGF plasmid and human ets plasmid. *P<0.05 vs control. Each group contains 7 or 8 animals.
osmotic pump (Alza Co) with either nonimmunized rabbit IgG or anti-VEGF IgG was implanted into the peritoneal cavity. Soon after the ischemia-related surgery, additional bolus administration of these antibodies (100 μg) was given via the pineal vein.

**Statistical Analysis**

All values are expressed as mean±SEM. ANOVA with subsequent Duncan’s test was used to determine the significance of differences in multiple comparisons. Differences with a value of $P<0.05$ were considered significant.

**Results**

**Angiogenesis Induced by Transfection of Human ets Gene**

Initially, we examined transfection of the human ets-1 using naked plasmid DNA, because overexpression of human HGF or VEGF was able to stimulate angiogenesis using naked plasmid DNA. However, we failed to demonstrate induction of angiogenesis by ets-1 using naked plasmid DNA. As shown in Figure 1, 500 μg naked ets-1 plasmid (without HVJ vector) did not have any effect on blood flow in the ischemic hindlimb, although injection of naked HGF plasmid induced angiogenesis. Because ets-1 is not a secretable protein, unlike HGF and VEGF, we thought that the transfection efficiency using naked plasmid DNA ($\approx$1% to 5%) would not be enough to express a transcription factor such as ets-1. Thus, we used the HVJ-liposome method to test the feasibility of induction of angiogenesis by ets-1 gene transfer, because the transfection efficiency with the HVJ-liposome method was much higher than that with naked plasmid injection. Then, HVJ-liposome complex containing human ets-1 plasmid was injected intramuscularly into the rat ischemic hindlimb. Intramuscular injection of ets-1 vector into the ischemic limb produced significant augmentation of collateral vessel development as assessed by LDI after 4 weeks in the ischemia model (Figure 2). Interestingly, the increase in blood flow by ets-1 was equivalent to that by HGF (Figure 2). Moreover, angiogenesis induced by ets-1 was also confirmed by a significant increase in the capillary density in the ischemic hindlimb around the injection site (Figure 3, $P<0.01$). As shown in Figure 3, transfection of ets-1 gene induced newly
formed microvessels and transfection of HGF. These results clearly demonstrated that transfection of the human ets-1 gene into the ischemic hindlimb induced angiogenesis in vivo. Edema formation was not observed in rats transfected with ets-1. Interestingly, cotransfection of the HGF gene with the ets-1 gene revealed an additive effect on angiogenesis (Figure 3, \( \text{**P}<0.01 \)).

Ets-Induced Upregulation of HGF and VEGF

To clarify the mechanisms by which ets-1 induced angiogenesis, we examined the effects of ets-1 overexpression on HGF and VEGF. As shown in Figure 4a, transfection of the ets-1 gene significantly increased rat endogenous HGF concentration in the rat hindlimb compared with the control vector at 1 week after transfection (\( \text{P}<0.01 \)). Because the promoter regions of HGF contain ets binding sites,\textsuperscript{26} the endogenous HGF level would be directly enhanced by ets-1. Interestingly, cotransfection of the HGF gene with the ets-1 gene further increased the endogenous rat HGF level (Figure 4a, \( \text{P}<0.01 \)). More importantly, VEGF was also significantly induced by transfection of the ets-1 gene (Figure 4, b and c). Upregulation of HGF and VEGF by overexpression of ets-1 was also confirmed by in vitro experiments using cultured human VSMCs. At 1 week after transfection, a significant increase in human HGF protein was observed in the conditioned medium from VSMCs transfected with the ets-1 gene (Figure 5a, \( \text{P}<0.01 \)). Moreover, cotransfection of the ets-1 gene with the HGF gene further enhanced the upregulation of HGF compared with single gene transfection (Figure 5a). Similarly, a significant increase in VEGF protein was observed in the conditioned medium from VSMCs transfected with the ets-1 gene (Figure 5b, \( \text{P}<0.01 \)). Cotransfection of the ets-1 gene with the VEGF gene also further enhanced the upregulation of VEGF compared with single gene transfection (Figure 5b).

Finally, we investigated whether the angiogenic property of ets-1 was mediated by the induction of VEGF and HGF using neutralizing antibodies. Because of the limitation of the available species of antibody, we used the mouse hindlimb ischemia model in this study. As shown in Figure 6a, administration of neutralizing anti-VEGF antibody partially, but significantly, abolished the increase in blood flow induced by ets-1 gene, whereas the complete inhibition of induction of blood flow by ets-1 gene was achieved by coadministration of anti-HGF and anti-VEGF antibodies (\( \text{P}<0.01 \)). In addition, the increase in BrdU-positive endothelial cells was observed in mice transfected with the ets-1 gene, as shown in Figure 6b (\( \text{P}<0.01 \)). Administration of neutralizing anti-VEGF antibody partially, but significantly, abolished the increase in BrdU-positive cells induced by ets-1 gene. In contrast, the complete inhibition of increase in BrdU-positive cells by the ets-1 gene was achieved by coadministration of anti-HGF and anti-VEGF antibodies (\( \text{P}<0.01 \)).
Discussion

Critical limb ischemia is estimated to develop in 500 to 1000 individuals per million per year. Recently, the efficacy of therapeutic angiogenesis using VEGF gene transfer has been reported in human patients with critical limb ischemia.\textsuperscript{27–30} The strategy for therapeutic angiogenesis using angiogenic growth factors should be considered for the treatment of patients with critical limb ischemia. In addition to VEGF, FGF and other angiogenic factors such as hypoxia inducible factor-1 genes have been tested in the clinic.\textsuperscript{30} We have also started a human clinical trial using the HGF gene, because HGF is a potent angiogenic growth factor.\textsuperscript{31} In the present study, we identified that ets-1 is a potent stimulator of angiogenesis in vivo. Here, we demonstrated that ets-1

Figure 4. a, Effect of transfection of human HGF plasmid and/or human ets plasmid on expression of endogenous rat HGF at 1 week after transfection. Control indicates control vector; HGF, HGF vector; Ets, ets-1 vector; HGF+Ets, rats cotransfected with HGF vector and ets-1 vector. * \( P \leq 0.05 \), ** \( P \leq 0.01 \) vs control; # \( P \leq 0.05 \) vs HGF. Each group contains 7 or 8 animals. b, Typical example of VEGF Western blot after ets-1 gene transfer. c, Effect of transfection of human ets plasmid on expression of rat VEGF as assessed by Western blot. * \( P \leq 0.05 \) vs control. Each group contains 5 animals.

Figure 5. Induction of secreted HGF (a) and VEGF (b) from cultured human VSMCs transfected with human HGF plasmid and/or human ets plasmid at 1 week after transfection. Control indicates control vector; HGF, HGF vector; Ets, ets-1 vector; HGF+Ets, rats cotransfected with HGF vector and ets-1 vector. * \( P \leq 0.05 \), ** \( P \leq 0.01 \) vs control; # \( P \leq 0.05 \) vs HGF. n=7 or 8.
activated the transcription of 2 important angiogenic growth factor genes (HGF and VEGF). In addition, the coordinated induction of protease activities and cell migration is a principal feature of endothelial cells invading the interstitial space in the initial step of angiogenesis. The ets family of transcription factors has a common DNA-binding domain that binds to a core GGA(A/T) DNA sequence.5,6 Because previous reports documented the induction of genes encoding collagenase 1, stromelysin 1, and urokinase plasminogen activator, which are proteases involved in extracellular matrix degradation,7-9 the invasiveness was enhanced in high ets-1-expressing cells.7 Although the present study clarified the upregulation of VEGF and HGF by ets-1, previous reports demonstrated contrary findings that 4 typical angiogenic growth factors, acidic FGF, bFGF, VEGF, and epidermal growth factor, induced the expression of ets-1 mRNA in several endothelial cells.32,33 Interestingly, tube formation by endothelial cells in type 1 collagen gel stimulated with epidermal growth factor was abrogated by ets-1 antisense ODN.34 We have also reported that HGF also activated ets-1 mRNA and protein in human VSMCs and endothelial cells and myocardium.21,35 The present study revealed that induction of ets-1 upregulated HGF and VEGF expression. Thus, upregulation of ets-1 by HGF or VEGF activated its own gene expression. This auto-loop of upregulation of angiogenesis might have a role in the regulation of natural neovascularization (see Figure 7). The angiogenic property of ets-1 might be regulated primarily by induction of VEGF and HGF, because administration of both anti-HGF and anti-VEGF completely abolished the angiogenesis induced by the ets-1 gene. Interestingly, co-transfection of the ets-1 gene with the HGF gene enhanced angiogenesis in ischemic tissues. The complexity of angiogenesis related to ets-1 appears to be supported by this phenomenon. Consistent changes were observed in the morphology of cells stably transfected with the ets-1 gene, with increased spreading and modifications in the organization of the cytoskeleton and increased cell adhesion.36 On Matrigel, all endothelial cell lines formed a cord-like network, with an increased ability to spread on this substrate by ets-1.36 Thus, ets-1 might contribute to all aspects of angiogenesis. In the present study, we did not study the angiogenic activity of other ets family members, which may also modulate angiogenesis in vivo in various physiological conditions. One of the important findings is no edema formation by ets-1 overexpression, whereas VEGF gene therapy reported a high prevalence of edema in human patients.27-29 Although further studies are necessary to explore the mechanisms, in clinical application, no induction of edema might be a favorable feature.

The present study demonstrated that ets-1 regulated angiogenesis through the induction of angiogenic growth factors (VEGF and HGF), in addition to endothelial cell migration and matrix degradation. Overexpression of ets may provide a new therapeutic strategy to treat peripheral arterial disease, although high expression of the transgene by use of a viral vector is necessary.

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