Angiogenic Property of Hepatocyte Growth Factor Is Dependent on Upregulation of Essential Transcription Factor for Angiogenesis, ets-1

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Background—Although hepatocyte growth factor (HGF) is an angiogenic growth factor, it is still unclear how it exerts its angiogenic effects. Thus, we focused on the role of an essential transcription factor for angiogenesis, ets-1. In this study, we addressed the following specific questions: (1) what genes responsible for angiogenesis can be regulated by HGF and (2) whether upregulation of gene expression for angiogenesis is dependent on ets-1.

Methods and Results—In human endothelial cells, HGF significantly stimulated the matrix-degrading pathway, such as the production of matrix metalloprotease-1 (MMP-1) through its specific receptor, c-met. In addition, HGF also significantly increased HGF itself and its specific receptor, c-met. Moreover, HGF significantly increased the transcription activity and mRNA expression of ets-1 in a time-dependent manner. Importantly, transfection of antisense ets-1 oligodeoxynucleotides (ODN) resulted in a significant reduction in MMP-1, HGF and c-met. Interestingly, HGF also stimulated ets-1 mRNA in vascular smooth muscle cells, similar to endothelial cells. Of importance, transfection of antisense ets-1 ODN resulted in a significant decrease in vascular endothelial growth factor (VEGF) and HGF expression, whereas HGF stimulated both HGF and VEGF expression. Moreover, in vivo transfection of ets-1 antisense ODN resulted in an inhibition of angiogenesis induced by the HGF gene in a rat ischemic hindlimb model.

Conclusions—Here, we demonstrated that HGF stimulated the expression of MMP-1, VEGF, HGF itself, and c-met in human endothelial cells and vascular smooth muscle cells. Upregulation of angiogenesis-related genes was largely dependent on the induction of ets, especially ets-1. These data provide new information about the mechanisms of angiogenesis. (Circulation. 2003;107:1411-1417.)

Key Words: angiogenesis ■ metalloproteinases ■ cells ■ growth substances
we addressed 2 specific issues: (1) the molecular mechanisms involved in angiogenesis induced by HGF and (2) the role of ets-1 in angiogenesis induced by HGF.

**Methods**

**Transfection of Antisense ets-1 ODN**

Human aortic endothelial cells and VSMCs (passage 3) were obtained from Clonetics Corp and cultured in modified MCDB131 medium supplemented with 5% FCS, 10 ng/mL epidermal growth factor, 2 ng/mL basic fibroblast growth factor, and 1 mmol/L dexamethasone. VSMCs were maintained in Waymouth media with 5% fetal calf serum. Endothelial cells and VSMCs were seeded in 6-well plates and transfected with antisense ets-1 ODN using LipofectAMINE PLUS (Gibco-BRL). Briefly, 2 μg ODN was mixed with 10 μg liposomes for 30 minutes at room temperature. Then, liposome complex was added to the medium, which was maintained at 37°C for 4 hours. To demonstrate successful transfection, we used FITC-labeled antisense ODN. The 3' and 5' ends of the ODN were labeled with FITC by Nihon Seifun. Cells were harvested 2 and 24 hours after transfection and fixed with paraformaldehyde. Cells were examined by fluorescence microscopy. Fluorescence was readily distinguishable from the specific FITC-labeled ODN. The sequences of antisense and sense ets-1 ODN were as follows: antisense, 5'-AGATCGAGGCGGCGCTTCACT-3'; sense, 5'-ATGAAAGGCG-GCGCTGATCT-3'.

**Measurement of MMP-1, HGF, and VEGF in Conditioned Medium**

Endothelial cells and VSMCs were seeded at a density of 5×10^4 cells/cm^2 and cultured for 24 hours. After the medium was replaced with fresh defined serum free (DSF) medium and after culture for 24 hours, the concentrations of matrix metalloproteinase-1 (MMP-1) and HGF in the medium were determined by enzyme immunoassays (EIAs) (MMP-1 Biotrack, Amersham; HGF, Tokushu-Meneki Co Ltd). The concentration of HGF in the medium was determined by ELISA using anti-human HGF antibody. This ELA specifically detects only human HGF, because of lack of cross-reactivity of antibodies. To study the effects of HGF on endogenous HGF production, we used rat recombinant HGF (rHGF) to stimulate endogenous human HGF. The concentration of VEGF in the medium was determined by EIAs (VEGF; R&D systems). The binding mixtures (10 μL), including 3P-labeled primers (0.5 to 1 ng, 10 000 to 15 000 cpm) and 1 μg polydeoxyinosinic-deoxyctylic acid (Sigma Co), were incubated with 10 μg nuclear extract for 30 minutes at room temperature and then loaded onto 5% polyacrylamide gel. As a control, samples were incubated with an excess (50×) of nonlabeled ets-1 ODN, which completely abolished binding. Gels were analyzed by autoradiography.

**In Vivo Gene Transfer by Intramuscular Injection**

A rat ischemic hindlimb model was created. Consequently, blood flow to the ischemic limb was dependent on collateral vessels developing from the internal iliac artery. Naked human HGF vector and control vector (500 μg per body) were carefully injected directly into the ischemic limb with a 27-gauge needle (Terumo) at 10 days after surgery. Four separate injections of plasmid vectors (intramuscular into the ischemic limb near both the proximal and distal arterial stump) with or without sense or antisense ets-1 ODN (10 μmol/L) were performed. The injection volume of plasmid DNA was 100 μL. All protocols were approved by the Osaka University Institutional Animal Care and Use Committee.

**Measurement of Blood Flow and Capillary Density**

Measurement of blood flow with a laser Doppler imager has been performed by means of a laser Doppler flowmeter (Laser Doppler Imager, Moor Instruments), because laser Doppler flow velocity correlates well with capillary density. Consecutive measurements were obtained over the same regions of interest (leg and foot). 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 for evaluation. Alkaline phosphatase staining was used as a specific marker of endothelial cells in paraffin-embedded sections. Three individual sections from the middle of the transfected muscle were analyzed. The number of vessels was counted under a light microscope (magnification, ×100) in a blinded manner.

**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 probability value of P<0.05 were considered significant.

**Results**

**Uprogulation of ets-1 in Endothelial Cells**

To investigate the molecular mechanisms of the angiogenic actions of HGF, we initially focused on the transcription factor ets-1. Expression of ets-1 mRNA was readily detected in human aortic endothelial cells without any stimulation (Figure 1A). Interestingly, rHGF significantly increased ets-1 mRNA expression from 1 hour, as assessed by Northern blotting (P<0.01) (Figure 1A). This increase in ets-1 mRNA by rHGF continued for at least 6 hours after treatment. Consistent with the significant increase in mRNA level, ets-1 activity was also markedly increased in endothelial cells treated with rHGF compared with vehicle, as assessed by gel mobility shift assay at 24 hours after stimulation (Figure 1B). To elucidate the role of ets-1 in the angiogenesis induced by rHGF, we used an antisense strategy. For transfection into human aortic endothelial cells, we used cationic liposomes.
As shown in Figure 2, our present study using FITC-labeled ODN demonstrated the feasibility of transfecting antisense ODN into endothelial cells, because marked fluorescence could be detected in cells transfected with FITC-labeled antisense ets-1 ODN but not in untransfected cells. The average transfection rate was 80% to 90% in human endothelial cells. The fluorescence was localized primarily in cell nuclei, and some could be detected in the cytoplasm. Consistent with a previous report, we confirmed the specificity of antisense ets-1 ODN. Transfection of antisense ets-1 ODN attenuated the increase in ets-1 mRNA induced by rHGF at 24 hours, whereas sense ODN or liposomes alone did not alter ets-1 mRNA expression (P<0.01) (Figure 3). Using this specific antisense ets-1 ODN, we studied further the role of ets-1 on HGF-mediated angiogenesis-related proteins.

As shown in Figure 4A, human rHGF (100 ng/ml) significantly increased the production of MMP-1 (P<0.01) and MMP-1 activity. In contrast, no detectable amount of MMP-3 and MMP-9 was observed in the conditioned medium of human endothelial cells. Of importance, transfection of antisense ets-1 ODN resulted in a significant decrease in MMP-1 production induced by rHGF (P<0.01) (Figure 4A), whereas sense ets-1 ODN or liposomes alone did not affect MMP-1 production. These findings are consistent with previous reports that the MMP-1 gene has ets binding sites in its promoter region. Because the promoter regions of HGF and c-met both contain ets binding sites, we studied further the role of ets-1 in upregulation of HGF and c-met. As expected, transfection of antisense ets-1 ODN resulted in significant attenuation of endogenous human HGF expression induced by exogenously added rat rHGF (P<0.01) (Figure 5).
ODN. Antisense, cells treated with rHGF (100 ng/mL) with antisense ODN; and lane 4, with rHGF (100 ng/mL) without ODN; lane 3, Sense, cells treated with vehicle; lane 2, Vehicle, cells treated with rHGF (100 ng/mL) with sense ODN; and lane 1, Control, cells treated with vehicle. n = 5 to 8 per group calculated from 5 independent experiments. Lane 1, human endothelial cells at 24 hours after transfection. n = 5 to 8 independent experiments. Control indicates cells treated with vehicle and sense ODN (P < 0.01) (Figure 5). The inhibition was also attenuated by antisense ets-1 ODN compared with vehicle (P < 0.01) (Figure 5). The inhibition of HGF-induced c-met expression by antisense ets-1 ODN was also confirmed at the mRNA level, as assessed by Northern blotting (data not shown). These data clearly revealed that HGF activated angiogenesis through the autocrine system of HGF and c-met mediated by ets-1.

4B). Similarly, the increase in c-met protein by human rHGF was also attenuated by antisense ets-1 ODN compared with vehicle and sense ODN (P < 0.01) (Figure 5). The inhibition of HGF-induced c-met expression by antisense ets-1 ODN was also confirmed at the mRNA level, as assessed by Northern blotting (data not shown). These data clearly revealed that HGF activated angiogenesis through the autocrine system of HGF and c-met mediated by ets-1.

Upregulation of ets-1 in VSMCs
We have previously reported that HGF stimulated endothelial cell growth exclusively without replication of VSMC growth. However, we were aware that the expression of the specific receptor of HGF, c-met, could be detected in VSMCs. Indeed, the presence of c-met was clearly demonstrated by Western blotting, although the amount of c-met protein in human VSMCs was obviously lower than that in endothelial cells (VSMCs, 100%; endothelial cells, 492 ± 42%; P < 0.01). Moreover, it has been reported that HGF induced migration of VSMCs. Therefore, we elucidated the role of c-met in VSMCs. As in endothelial cells, rHGF significantly stimulated the production of MMP-1 (vehicle, 31.5 ± 2.5 ng/mL; HGF, 47.2 ± 2.2 ng/mL; P < 0.01). Thus, HGF may stimulate the expression of angiogenesis-related genes in VSMCs. Interestingly, the expression of ets-1 mRNA was significantly increased by rHGF in a time-dependent manner, as assessed by Northern blotting (P < 0.01) (Figure 6A), whereas transfection of antisense ets-1 ODN inhibited the upregulation of ets-1 mRNA induced by rHGF (data not shown). Accordingly, transfection of antisense ets-1 ODN also resulted in significant inhibition of endogenous human HGF production induced by rat HGF (P < 0.01) (Figure 6B). Finally, we examined the effects of HGF on other angiogenic growth factors such as VEGF, because a previous report documented that HGF stimulated VEGF expression. In this study, we confirmed the previous observation that addition of rHGF resulted in a significant, but weak, increase in VEGF protein in human VSMCs (P < 0.01) (Figure 6C). Interestingly, upregulation of VEGF by rHGF was also diminished by treatment with antisense ets-1 ODN (P < 0.01) (Figure 6C). In contrast, rHGF did not affect basic fibroblast growth factor expression (data not shown). These results demonstrated that HGF upregulated the degradation pathway of extracellular matrix, such as production of MMP-1, via the induction of HGF and VEGF through the upregulation of ets-1, without affecting the growth of VSMCs.

Role of ets-1 in Angiogenesis Induced by HGF In Vivo
To clarify the role of ets-1 in angiogenesis induced by HGF, we also used a rat hindlimb ischemia model in vivo. Injection of human HGF vector into the ischemic hindlimb resulted in a significant increase in blood flow at 4 weeks after transfection, as assessed by laser Doppler imager, compared with ischemic hindlimb transfected with control vector (P < 0.01), as shown in Figure 7. Moreover, transfection of human HGF vector significantly increased the number of capillary arteries in the ischemic hindlimb of rat around the injection site compared with control vector (P < 0.01) (Figure 8A). Importantly, cotransfection of antisense ets-1 ODN resulted in a significant decrease in blood flow induced by the HGF gene compared with sense ets-1 ODN transfection (P < 0.01).Capillary density was also significantly decreased in rats transfected with antisense ets-1 ODN compared with sense ODN (P < 0.01) (Figure 8). There was no significant difference in the blood flow or the capillary density between rats transfected with sense ODN and untransfected rats.
Discussion

Members of the ets family play important roles in regulating angiogenesis for the migration of endothelial cells from preexisting capillaries. In this study, we demonstrated that HGF upregulated ets-1 mRNA and activity in human endothelial cells. With an antisense strategy, inhibition of ets-1 attenuated the increase in MMP-1 induced by HGF. These results were consistent with the previous report that endothelin-1 and phorbol 12-myristate 13-acetate induced MMP-1 through ets-1 in endothelial cells. Interestingly, similar results were observed in another cell type, the human hepatic stellate cell line. In addition, we also demonstrated upregulation of endogenous HGF and c-met expression by exogenously added HGF, consistent with our previous in vivo findings.

Interestingly, induction of ets activity by HGF regulated this autoloop upregulation of the HGF system. This phenomenon is consistent with the previous report that the promoter region of the HGF gene contains putative regulatory elements, such as a B-cell- and macrophage-specific transcription factor binding site (PU.1/ETS). The upregulation of c-met has been reported in the acute phase of a myocardial infarction model. Considering the clinical usefulness of therapeutic angiogenesis by HGF, it is important to continue stimulation of the angiogenesis pathway by autoinduction of the HGF system after a single stimulation. Because our previous study clearly demonstrated induction of ets binding activity in infarcted myocardium by HGF gene transfer in vivo, an increase in ets may play a pivotal role in the regulation of angiogenesis by HGF in physiological situations.
release of blood-derived cells into the extracellular space, al-
though further studies are needed. The functionality of c-met in
VSMCs was confirmed by the present study, which shows that
HGF stimulated ets-1 mRNA and MMP-1 production.

Importantly, HGF also stimulated the production of VEGF
in VSMCs, consistent with a previous report.8 Thus, it is
believed that HGF may exert a potent combination of direct
and indirect effects, including direct effects on endothelial
cells and indirect effects mediated via an increase in the
production of VEGF.8 However, no report has documented
how HGF induced VEGF expression. Here, using an anti-
sense strategy, we clearly demonstrated that the induction of
VEGF by HGF was also mediated by ets-1. This finding is
supported by the observation that the promoter region of
VEGF contains ets binding sites.28 Furthermore, HGF is
known to increase the expression of the VEGF receptor
(VEGFR) flk-1 in human endothelial cells.29 Interestingly,
both the VEGFRs Flt-1/VEGFR-1 and KDR/VEGFR-2 carry
a putative ets-responsive element in their promoters.30,31 It
has also been proposed that ets-1 stimulates its own expres-
sion in vitro.32,33 These results extend our findings, suggest-
ing that the ability of HGF to induce angiogenesis by direct
effects on the proliferation and migration of endothelial cells
may be potentiated by its ability to induce angiogenesis
indirectly by upregulating 1 or more cytokines, such as
VEGF, through ets-1. Although the angiogenesis pheno-
menon is multicomplex, including migration, proliferation,
and tubular morphogenesis of endothelial cells, in this study we
did not perform experiments regarding proliferation and
migration so as to avoid duplication of previous works.
Transfection of transdominant mutant ets-1 cDNA into endo-
thelial cells resulted in a decrease of bromodeoxyuridine
incorporation and DNA synthesis, a lesser migration of
endothelial cells stimulated with basic fibroblast growth
factor in vitro.34 In addition, transfection of the mutant ets-1
gene also resulted in a significant decrease in cell number and DNA synthesis
endothelial cells stimulated with basic fibroblast growth
factor in vitro. The length of tube-like formation stimulated
by basic fibroblast growth factor was also smaller in cells
transfected with the mutant ets-1 gene.35 Most importantly,
the present study proved that the in vivo angiogenesis
induced by HGF was inhibited by antisense, but not sense,
ets-1 ODN in the ischemic hindlimb model. The present
studies revealed that HGF is located upstream of the angio-
genesis cascade and acts through induction of the coordinated
trans-activating genes necessary for the processes for angiogenesis
modulated by ets-1. The widespread activation of
these genes by HGF is critical in treating ischemic disease.

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