Potential Role of Hepatocyte Growth Factor, a Novel Angiogenic Growth Factor, in Peripheral Arterial Disease

Downregulation of HGF in Response to Hypoxia in Vascular Cells

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Background—Although hepatocyte growth factor (HGF), a novel angiogenic growth factor, plays an important role in angiogenesis, regulation of local HGF production under hypoxia has not yet been clarified in vascular smooth muscle cells (VSMC) and endothelial cells (EC). Thus, we have studied the role of HGF in hypoxia-induced endothelial injury and the regulation of local vascular HGF expression in response to hypoxia.

Methods and Results—HGF attenuated hypoxia-induced endothelial cell death. Importantly, hypoxic treatment of EC resulted in a significant decrease in local HGF production according to the severity of hypoxia and increased VEGF. Similarly, hypoxia significantly decreased in mRNA and protein of HGF and increased VEGF production in VSMC. In organ culture system, local HGF production was also significantly decreased by hypoxia (P<0.01). Downregulation of HGF by hypoxia is due to a significant decrease in cAMP, as hypoxic treatment decreased cAMP, a stimulator of HGF. Although active TGF-β, a suppressor of HGF, was increased at 72 hours after hypoxic treatment, treatment of anti-TGF-β antibody did not attenuate decreased HGF production. Finally, rHGF was intra-arterially administered into unilateral hind limb ischemia rabbits, to evaluate in vivo angiogenic activity. Of importance, a single intra-arterial administration of rHGF reduced severe necrosis due to ischemia in rabbit muscle, accompanied by a significant increase in angiographic score (P<0.01).

Conclusions—Overall, these data demonstrated that hypoxic treatment of vascular cells significantly downregulated HGF production due to decreased cAMP, suggesting their potential roles in the pathophysiology of ischemic diseases. Moreover, administration of rHGF induced therapeutic angiogenesis, accompanied by improvement of necrotic changes in the ischemic hind limb model, as cytokine supplement therapy for peripheral arterial disease. (Circulation. 1999;100[suppl II]:II-301–II-308.)

Key Words: endothelium ■ peripheral vascular disease ■ angiogenesis ■ myocardial infarction ■ remodeling

The development of new blood vessels, or angiogenesis, begins with activation of parent vessel endothelial cells. Growth factors shown to be mitogenic for endothelial cells in vitro, as well as stimulating angiogenesis in vivo, have been referred to as angiogenic growth factors. These include vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). In addition, recent studies identified that hepatocyte growth factor (HGF) is also a member of angiogenic growth factors. HGF is a mesenchyme-derived pleiotropic factor which regulates cell growth, cell motility, and morphogenesis of various types of cells and is thus considered a humoral mediator of epithelial-mesenchymal interactions responsible for morphogenic tissue interactions during embryonic development and organogene-

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neovascularization in order to satisfy the needs of the tissues, knowing the triggers of natural neovascularization is important. Hypoxia is generally considered to represent a fundamental stimulus for angiogenesis, although the mechanisms responsible for its angiogenic activity remain enigmatic. Numerous studies have indicated that VEGF is upregulated by hypoxic treatment in various cells, thereby indicating its pathophysiological role in the pathogenesis of ischemic diseases. In contrast, no report has described the regulation of HGF expression in response to hypoxia, although HGF is a potent angiogenic growth factor. Therefore, in this study we hypothesized that HGF may also modulate the ischemic condition in cardiovascular disease. To understand the molecular mechanisms of neovascularization, we have addressed the following issues: (1) the role of HGF in hypoxia-induced endothelial injury, and (2) the regulation of local HGF expression in response to hypoxia. In addition, we tested whether it is possible to promote therapeutic angiogenesis by means of HGF in the rabbit ischemia model, to examine the feasibility of therapy for critical limb ischemia.

Methods

Experiment 1

Cell Culture
Human aortic endothelial cells (passage 3) and human aortic VSMC (passage 3) were obtained from Clonetics Corp, and cultured in modified MCD131 medium supplemented with 5% fetal calf serum, 10 ng/mL epidermal growth factor, 2 ng/mL bFGF, and 1 mmol/L dexamethasone. Cells were cultured at 37°C in a humidified atmosphere of 95% air/5% CO₂ with medium changes every 2 days. Immunohistochemical examination and morphological observation showed these cells exhibiting specific characteristics of endothelial cells and VSMC. Briefly, human aortic endothelial cells tested positive for factor VIII antigen and for uptake of diacetylated LDL. In contrast, human aortic VSMC tested negative for expression of factor VIII antigen. All the cells were used within passage 3-5.

Hypoxic Treatment
Hypoxia was induced with an anaerobic device. Briefly, a hypoxic condition was achieved using BBL GasPak (Becton Dickson, Cockeysville, Md), which catalytically reduces O₂ to undetectable levels in the severe condition by GasPak plus or to 10% in the mild condition by Campy Pak within 90 minutes, as assessed by an oxygen electrode. In the preparation of experiments for determination of cell death, endothelial cells were grown to confluence. After reaching confluence, the medium was changed to fresh DSF (defined serum free) medium containing HGF, bFGF, VEGF, or vehicle. DSF medium was supplemented with insulin (10⁻⁷ M), transferrin (5 mg/mL), and ascorbate (0.2 mmol/L), as previously described. The cells were then incubated. On days 1, 2, and 3, an index of cell proliferation was determined, as described below.

Cell Counting Assay
An index of cell proliferation was determined using WST (sulfonated tetrazolium salt, 4-[-3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate)-cell counting kit, because this compound produces a highly water-soluble formazan dye which makes the assay procedure easier to perform. Tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate-cell counting kit, because this compound produces a highly water-soluble formazan dye which makes the assay procedure easier to perform. An increase in absorbance of 0.2 reflects an increase in cell number to 20,000 cells/well.

Transfection of Human HGF Vector into Endothelial Cells Using HVJ-Liposomes
To produce an HGF expression vector, human HGF cDNA (2.2 kb) was inserted into the Not I sites of pUC-SRα expression vector plasmid. In this plasmid, transcription of the HGF cDNA was under the control of the SRα promoter. As the control, we constructed a control expression vector without HGF gene. We have previously reported the high efficiency of transfection of cells in culture using Hemagglutinating Virus of Japan (HVJ)-coated liposomes, in this study, we used the HVJ-liposome method. Endothelial cells (1×10⁵) were seeded onto 6-well plates (Corning) and grown to 80% confluence. Cells were washed 3 times with BSS containing 2 mmol/L CaCl₂, and then incubated with 1 mL HVJ-liposomes-DNA complex (2.5 mg lipid and 10 μg encapsulated DNA) at 4°C for 5 minutes followed by 37°C for 30 minutes. To study the release of HGF, transfected cells (48 hours posttransfection) were washed and fed with 1 mL DSF medium. Twenty-four hours later, conditioned medium was collected, centrifuged at 600g for 10 minutes and stored at −20°C. The concentration of HGF in the medium was determined by enzyme immunoassay using anti-human HGF antibody, as described below. On day 4, an index of cell proliferation was also determined using WST-cell counting kit (Wako).

Organ Culture System
The carotid arteries of Sprague-Dawley rats (weighing 400 to 450 g) were dissected as previously reported. A 20-mm segment of the carotid artery was immediately transferred to DMEM medium with 30% fetal calf serum, penicillin (100 U/mL), and streptomycin (1000 μg/ml). Previous studies in our laboratory and others have documented viability of the intact vessel maintained under these conditions. After 48 hours of normoxic or hypoxic treatment, vessels were homogenized, as previously reported. HGF concentration was measured by enzyme immunoassay using rat anti-HGF antibody, as described below.

Measurement of HGF and VEGF in Conditioned Medium
Human endothelial cells and VSMC were seeded on 6-well plates (Corning) at a density of 5×10⁵ cells/cm² and cultured for 24 hours. After replacing the medium with fresh DSF (for VSMC or DSF) with 0.5% fetal calf serum (for endothelial cells) and following culture under hypoxic or normoxic condition, the concentration of HGF in the medium was determined by enzyme immunoassay using anti-human HGF antibody, as described previously. This ELISA specifically detects only human HGF because of lack of cross-reactivity of antibodies. For the organ culture experiment, rat HGF concentration was measured using anti-rat HGF antibody. Measurement of VEGF was also performed using ELISA kit (R & D systems), as recommended by the manufacturer. DSF medium did not contain immunoreactive HGF and VEGF assessed by enzyme immunoassay (data not shown).

Northern Blot Analysis
RNA was extracted by RNAzol (Tel-Test Inc), from cells after 2, 6, and 12 hours of hypoxic or normoxic condition for Northern blot analysis. For Northern blot analysis, 20 μg total RNA was subjected to electrophoresis on 1.5% agarose-formaldehyde denaturing gel and transferred to a nitrocellulose membrane (Amersham International plc, Amersham, UK). The filter was baked, prehybridized, and hybridized. Full-length cDNA for HGF, labeled by random-primer kit (Amersham), were used as probes for Northern blotting. The filter was then washed and exposed to x-ray film.

Measurement of Intracellular CAMP Level
VSMC were grown to confluence in DSF with 0.5% FCS; cells were then exposed to hypoxia. Intracellular cAMP was measured using an
enzyme immunoassay kit from Amersham. In brief, culture medium was removed at the indicated times, and cells were washed twice in phosphate-buffered saline and a third time in the same buffer containing 3-isobutyl-1-methylxanthine. Cells were then lysed by the addition of ice-cold trichloroacetic acid (5%). The trichloroacetic acid–soluble supernatant was removed from the well, extracted 3 times with 10 mL ether, dried (SpeedVac), and resuspended in 0.4 mL/sample of sodium acetate buffer (pH 6.2). The enzyme immunoassay was then performed.

**Effect of Neutralizing Anti-HGF Antibody**

The effect of endogenously produced HGF was examined by a neutralization procedure, using rabbit anti-human HGF antibody. For the antibody, the IgG fraction (purified with protein A–agarose) was able to neutralize a biological activity of 10 ng/mL HGF, at a concentration of 10 μg/mL. Normal rabbit serum IgG fraction (10 μg/mL) was used as a control.

**ELISA of TGF-β**

Conditioned medium was collected from VSMC, maintained in DSF 24, 48, and 72 hours later, centrifuged at 600g for 10 minutes, and stored at −20°C. ELISA for immunoreactive TGF-β1 in the supernatant was performed using ELISA kit (Amersham). The antibody against TGF-β1, cross-reacts with rat active TGF-β1, but not with rat latent TGF-β1, TGF-β2, or TGF-β3.

**Effect of Neutralizing Anti-TGF-β Antibody**

Augmentation of decreased local HGF production in human endothelial cells and VSMC was characterized as TGF-β specific by a neutralization procedure, using rabbit anti-human TGF-β (R&D Research). The IgG fraction (purified with protein A–agarose) was able to neutralize a biological activity of 10 ng/mL TGF-β1, at a concentration of 10 μg/mL. Normal rabbit serum IgG fraction (10 μg/mL) was used as a control.

**Experiment 2**

**Rabbit Ischemic Hind Limb Model**

The physiological response to administration of rhHGF was investigated in the rabbit ischemic hind limb model, described in previous reports. Male New Zealand White rabbits (3.5 to 4.0 kg) (Kitayama Rabes, Nagano, Japan) were anesthetized with a mixture of xylazine (5 mg/kg) and ketamine (50 mg/kg). A longitudinal incision was then performed, extending inferiorly from the inguinal ligament to a point just proximal to the patella. Through this incision, using surgical loupes, the operator dissected free the left femoral artery along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex, and superficial epigastric arteries, were also dissected free. After dissection of the popliteal and saphenous arteries distally, the external iliac artery and all of the mentioned arteries were ligated with 4-0 silk (Ethicon). Finally, the left femoral artery was completely excised for the ischemia limb model, from its proximal origin as a branch of the external iliac artery to the point distally where it bifurcates to form the saphenous and popliteal arteries. Excision of the femoral artery results in retrograde propagation of thrombus and occlusion of the external iliac artery. Consequently, blood flow to the ischemic limb is dependent on collateral vessels developing from the internal iliac artery. Using this ischemia model, we administered a single injection of 500 μg rhHGF locally (intra-arterially into the ischemic limb) on day 10 after the operation. Animals received the first intra-arterial bolus of rhHGF (500 μg/body) or vehicle (3 mL saline with 0.1% rabbit serum albumin; Sigma) administered as a bolus over 1 minute through a 3F end-hole infusion catheter (Terumo) positioned in the internal iliac artery of the ischemic limb.

The angiographic luminal diameter of the internal iliac artery in the ischemic limb at baseline and after drug infusion was determined on days 0, 10, and 30 by previously described techniques. Briefly, morphometric analysis of collateral vessel development in the ischemic limb was performed from 4-second angiograms recorded after injection of contrast medium into the internal iliac artery. A grid overlay composed of 2.5-mm (diameter) circles arranged in rows spaced 5 mm apart was placed over the angiogram in the region of the medial thigh. The number of contrast-opacified arteries crossing over circles and the total number of circles encompassing the medial thigh area were counted in a blinded fashion. The angiographic score was calculated as the ratio of overlying opacified arteries divided by the total number of circles in the ischemic thigh. This angiographic score reflects vascular density in the medial thigh.

**Materials**

Human recombinant HGF was purified from the culture medium of Chinese hamster ovary cells or C-127 cells transfected with expression plasmid containing human HGF cDNA. bFGF and VEGF were obtained from Pepro Tec EC Ltd.

**Statistical Analysis**

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

**Results**

**Effect of HGF on Hypoxia-Induced Endothelial Cell Death**

Initially, we tested the effect of hypoxic treatment on endothelial cell growth. After 12 hours after hypoxia, some cells started to become round and eventually detached from the plate and floated in the medium, leaving many holes in the sheet of confluent cells (data not shown). The floating cells could be recovered with the medium and neither attached onto a new plate nor proliferated. Consistent with this morphological observation, cell death rate after hypoxia was significantly increased in a time-dependent and hypoxia-dependent manner, as shown in Figure 1. Therefore, we examined the effects of HGF, VEGF, and bFGF, because these growth factors have been reported to act as survival factors against endothelial cell death. As shown in Figure 2, addition of HGF (10 ng/mL) resulted in partial attenuation of cell death mediated by hypoxia in a dose-dependent manner. Similarly, addition of bFGF or VEGF attenuated endothelial cell death induced by hypoxia in a dose-dependent manner.

There was no significant difference in the attenuation of cell death among HGF, bFGF, and VEGF. These results suggest...
that endothelial cell death induced by hypoxia may be mediated by these growth factors.

Differential Regulation of HGF and VEGF in Vascular Cells in Response to Hypoxia

We focused on HGF and VEGF as candidates, because they are endothelium-specific growth factors.5,6 In particular, VEGF has been reported to be upregulated by hypoxia.10–12 Consistent with previous reports,10–12 VEGF concentration in the medium of endothelial cells was significantly higher under hypoxic treatment than that under normoxic treatment after 24 and 72 hours of treatment (P<0.01), as shown in Figure 3. Our previous studies demonstrated that local HGF production by endothelial cells and VSMC regulates growth of endothelial cells in an autocrine-paracrine manner.8 Consistent with our previous finding,8 secretion of HGF in human endothelial cells was also readily detected by ELISA using specific human anti-HGF antibody. Surprisingly, HGF concentration in the conditioned medium of endothelial cells was 4- to 5-fold higher than VEGF concentration (Figure 3). In addition, our previous studies demonstrated that addition of neutralizing anti-HGF antibody to human aortic endothelial cells resulted in a significant decrease in number of endothelial cells22 suggesting that HGF secreted from endothelial cells maintain the endothelial cell growth in the autocrine manner. Therefore, we studied the effect of hypoxic treatment on endogenously produced HGF from vascular cells. Importantly, hypoxic treatment of human aortic endothelial cells resulted in a significant decrease in local HGF production according to the severity of hypoxia (P<0.01), as shown in Figure 3. From these results, we hypothesized that endothelial cell death induced by hypoxia might be mediated by decreased vascular HGF production. This hypothesis is supported by the results of transfection of human HGF gene. As shown in Figure 4, transfection of human HGF gene into endothelial cells significantly attenuated hypoxia-induced endothelial cell death (P<0.01), accompanied by increased immunoreactive HGF (control vector, 85±12 pg/10^6 cells per 24 hours; HGF vector, 203±14 pg/10^6 cells per 24 hours, P<0.01).

Figure 2. Effect of human recombinant HGF on human aortic endothelial cell death induced by hypoxia: comparison of inhibitory effects of VEGF, HGF, and bFGF at 48 hours after stimulation with growth factors. n=8 per group. *P<0.05, **P<0.01 vs Untreat; #P<0.05, ##P<0.01 vs Vehicle. Untreat indicates untreated cells under normoxic condition; vehicle, untransfected cells under severe hypoxic conditions.

Figure 3. Effect of hypoxia on concentrations of HGF and VEGF released from human aortic endothelial cells at 24 (left) and 72 hours (right). Values are expressed as HGF or VEGF concentration adjusted for cell number. n=8 per group. *P<0.05, **P<0.01 vs normoxia; #P<0.05, ##P<0.01 vs mild hypoxia.

Figure 4. Effect of gene transfer of human HGF into human aortic endothelial cells induced by hypoxia. n=8 per group. HGF Gene indicates cells transfected with human HGF vector under severe hypoxic conditions; Control Vector, cells transfected with control vector under severe hypoxic conditions. Other abbreviations as in Figure 2. *P<0.01 vs Untreat, ##P<0.01 vs Control Vector.

Figure 5. Effect of hypoxia on concentrations of HGF and VEGF released from human aortic VSMC at 24 hours (left), 48 hours (middle), and 72 hours (right). Values are expressed as HGF or VEGF concentration adjusted for cell number. n=8 per group. *P<0.05, **P<0.01 vs normoxia; ##P<0.01 vs mild hypoxia.
Because VSMC also secrete HGF in addition to endothelial cells, we also examined the effect of hypoxia on HGF production in human aortic VSMC. As shown in Figure 5, hypoxic treatment also resulted in a significant decrease in local HGF production in VSMC after 24, 48, and 72 hours of hypoxic treatment, compared with normoxic control assessed by enzyme immunoassay (P<0.01), whereas VEGF was significantly increased by severe hypoxic treatment (P<0.01). Increased severity of hypoxic condition further inhibited local HGF production (Figure 5). Consistent with the decreased local HGF production assessed by enzyme immunoassay, HGF mRNA was also decreased by hypoxic treatment in VSMC (P<0.01; Figure 6).

Finally, we examined the effect of hypoxia on local HGF production under more physiological conditions: in an organ culture system. Rat carotid arteries were exposed to normoxic and hypoxic conditions in culture medium. Similar to cultured vascular cells, mild as well as severe hypoxic treatment significantly decreased vascular HGF concentration according to the severity of hypoxia (P<0.01. Figure 7). There was no significant difference in protein contents among normoxia- and hypoxia-treated vessels during the experimental period (48 hours; data not shown).

Molecular Mechanisms of Downregulation of Vascular HGF in Response to Hypoxia

Finally, we studied the molecular mechanisms of downregulation of local HGF production in vascular cells in response to hypoxia. Although regulation of HGF is not yet fully understood, one of the upregulators of local HGF production is cAMP,26,27 and TGF-β negatively regulates local HGF production.28,29 Thus, cAMP level and TGF-β concentration were measured under normoxic and hypoxic conditions. Importantly, hypoxic treatment significantly decreased cAMP content in VSMC and endothelial cells in a time-dependent manner, as shown in Figure 8. During the time period of the experiments, cAMP levels in normoxic condition were not significantly changed in either VSMC or endothelial cells. Moreover, the decrease in local HGF production by hypoxic treatment was significantly attenuated by treatment with a cAMP analogue, 8-bromo cAMP, in VSMC (Figure 9). Of importance, addition of the cAMP analogue significantly attenuated endothelial cell death induced by hypoxia, whereas addition of neutralizing anti-HGF antibody attenuated the prevention of endothelial cell death by the cAMP analogue.
Figure 10. Effect of hypoxia on active TGF-β concentration in human aortic VSMC. Values are expressed as TGF-β concentration adjusted for cell number. n=8 per group. **P<0.01 vs normoxia, ##P<0.01 vs mild hypoxia.

Angiogenesis Induced by Intra-Arterially Injected rhHGF

Given the significant decrease in endogenous HGF production in response to hypoxia, we hypothesized that administration of rhHGF into the ischemic limb might result in a beneficial effect in hypoxia. Therefore, rhHGF was intra-arterially administered via the internal iliac artery of rabbits in whom the femoral artery had been excised to induce unilateral hind limb ischemia. There was no significant difference in body weight between the rabbits treated with rhHGF and vehicle on day 40 after surgery (data not shown). A single administration of rhHGF into the ischemic limb on day 10 after surgery produced significant augmentation of collateral vessel development as assessed by angiography 30 days later in the critical limb ischemia model (vehicle: 120.1±4.0%; versus rhHGF: 180±5.8%, P<0.01), as shown in Figure 11. Serial angiograms revealed progressive linear extension of the collateral arteries of the origin stem artery to the distal point of the parent vessel reconstitution in HGF-treated animals (Figure 11A). Of importance, a single administration of rhHGF also reduced severe necrosis due to ischemia in rabbit muscle, whereas vehicle-treated rabbits demonstrated severe necrosis of muscle and the nail (Figure 11B, Table 1).

Discussion

In this study, we focused on 2 principle candidates as potential mediators of endothelial cell death by hypoxia: VEGF and HGF. Both VEGF and HGF are secreted from vascular cells and act on endothelial cells.7,8 Because addition of anti-HGF antibody attenuated endothelial cell growth, local HGF production in vascular cells may have a pathophysiological role in endothelial growth in an autocrine-
paracrine manner. Our study demonstrated the differential regulation of HGF and VEGF in response to hypoxia: VEGF was upregulated and HGF was downregulated by hypoxic treatment. Some stimuli capable of inducing the development of neovessels in vivo, specifically certain cytokines and hypoxia, fail to stimulate endothelial cell proliferation in vitro, suggesting a role for additional mediators and/or cell types. Decreased local HGF may abolish the mitogenic activity of the increased vascular VEGF expression. In the human heart, the degree of collateral growth is highly variable among individuals; in many, the functional capacity of collaterals is not sufficient to counterbalance myocardial functional derangement caused by coronary obstruction. Importantly, our study demonstrated that a single intra-arterial administration of rHGF was sufficient to induce angiogenesis in the rabbit hind limb ischemia model. Because development of collateral vessels in the ischemic porcine heart may require several weeks, neovascularization therapy using recombinant and/or gene transfer of angiogenic growth factors might be considered.

What are the molecular mechanisms of the downregulation of HGF in response to hypoxia? Upregulation of VEGF by hypoxia has been reported to be due to the presence of the homology sequence in the VEGF promoter, identified as a binding site for a hypoxia-specific transcription factor (HIF-1). In contrast, our previous study documented a marked reduction of local HGF production by TGF-β, severe ulcer or necrosis in localized area; +++, ulcer or necrosis in wide area.

Pathological Findings in Rabbit Critical Ischemia Model Treated With rHGF or Vehicle

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