Therapeutic Angiogenesis Induced by Human Hepatocyte Growth Factor Gene in Rat Diabetic Hind Limb Ischemia Model

Molecular Mechanisms of Delayed Angiogenesis in Diabetes

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Background—Because no study has documented the angiogenic properties of hepatocyte growth factor (HGF) in a diabetes model, we examined the feasibility of gene therapy using HGF to treat peripheral arterial disease in diabetes.

Methods and Results—Because intramuscular injection of luciferase plasmid by the hemagglutinating virus of Japan (HVJ)–liposome method had much higher efficiency than injection of naked plasmid, we used the HVJ-liposome method to transfect the human HGF gene into the rat diabetic hindlimb model. As expected, transfection of human HGF vector resulted in a significant increase in blood flow as assessed by laser Doppler imaging and capillary density, even in the diabetes model, accompanied by the detection of human HGF protein. Interestingly, the degree of natural recovery of blood flow was significantly greater in nondiabetic rats than in diabetic rats. Thus, in an in vitro culture system, we further studied the molecular mechanisms of how diabetes delayed angiogenesis. Importantly, high-β-glucose treatment of endothelial cells resulted in a significant decrease in matrix metalloproteinase (MMP)-1 protein and ets-1 expression in human aortic endothelial cells. Similarly, high β-glucose significantly decreased mRNA and protein of HGF in endothelial cells. Downregulation of MMP-1 and ets-1 by high β-glucose might be due to a significant decrease in HGF, because HGF stimulated MMP-1 production and activated ets-1.

Conclusions—Overall, intramuscular injection of human HGF plasmid induced therapeutic angiogenesis in a rat diabetic ischemic hindlimb model as a potential therapy for peripheral arterial disease. The delay of angiogenesis in diabetes might be due to downregulation of MMP-1 and ets-1 through a decrease in HGF by high β-glucose. (Circulation. 2001;104:2344-2350.)

Key Words: peripheral vascular disease ▪ endothelium ▪ angiogenesis ▪ gene therapy ▪ growth substances

Critical limb ischemia is estimated to develop in 500 to 1000 individuals per million per year.1 In a large proportion of these patients, the anatomic extent and the distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization. Thus, the disease frequently follows an inexcusable downhill course.2,3 Of importance, there is no optimal medical therapy for critical limb ischemia.1 Therefore, novel therapeutics are necessary to treat these patients. Recently, the efficacy of therapeutic angiogenesis by use of vascular endothelial growth factor (VEGF) gene transfer has been reported in human patients with critical limb ischemia and myocardial ischemia.4–8 Thus, the strategy for therapeutic angiogenesis should be considered for the treatment of patients with critical limb ischemia or myocardial infarction. In addition to VEGF, we and others have reported the angiogenic property of hepatocyte growth factor (HGF) in a rabbit ischemia model.9,10 Diabetes mellitus (DM) is characterized by the premature development of microvascular and macrovascular disease.11 Nevertheless, it is not clear whether therapeutic angiogenesis by HGF after transfection occurs in a diabetes model. This issue may be most critical in the case of arterial gene therapy, because a third of patients with peripheral arterial disease are diabetic. Thus, it is preferable to stimulate angiogenesis from an actively expressing transgene in muscle, not only in normal rats but also in diabetic rats. In this study, we examined the feasibility of gene therapy using HGF to treat peripheral arterial disease in a diabetes model. Here,
we performed preclinical studies that demonstrated that injection of HGF plasmid induced therapeutic angiogenesis to treat peripheral arterial disease in a diabetic rat hindlimb ischemia model, with the goal of progression to human clinical trials. In addition, we especially focused on the matrix-degrading pathway, including matrix metalloproteinase (MMP)-1 (interstitial collagenase), a zinc enzyme responsible for degradation of extracellular fibers composed of collagen types I, II, and III, and ets-1, an essential transcription factor for angiogenesis.\(^{12-15}\) The present studies demonstrated that high glucose decreased ets-1 and MMP-1 in endothelial cells, suggesting their role in delayed angiogenesis in diabetes. In addition, HGF is known to activate ets-1,\(^{16}\) suggesting that HGF may stimulate MMP-1 production through ets-1 activation.

**Methods**

**Experiment 1: Rat Diabetic Hindlimb
Ischemia Model**

**Construction of Plasmids**

To produce an HGF expression vector, human HGF cDNA (2.2 kb) was inserted into a simple eukaryotic expression plasmid that uses the cytomegalovirus (CMV) promoter/enhancer.\(^{17}\) The vector used as a control was the CMV expression vector plasmid, which does not contain HGF cDNA. We obtained luciferase gene expression vector driven by SV40 promoter from a commercial source (Promega Corp).

**In Vivo Gene Transfer by Direct Injection Approach**

Diabetes was induced by intraperitoneal injection of streptozotocin (100 μg/g body wt in 50 mmol/L citric acid buffer, pH 4.5) once to Sprague-Dawley rats (400 to 500 g; Charles River Breeding Laboratories, Wilmington, Mass). Then, 1 week later, blood was obtained periorbitally after 8 hours of fasting. Only rats with blood glucose values >300 mg/dL were kept in the protocol and randomized for experiments. An ischemia model was created as in previous reports.\(^{18,19}\) Consequently, blood flow to the ischemic limb was dependent on collateral vessels developing from the internal iliac artery.

Design 1: “Naked” luciferase vector (200 μg/animal) or hemaglutinating virus of Japan (Sendai virus; HVJ)–liposome complex containing luciferase vector (20 μg/animal) was carefully injected directly into the ischemic limb of rats with a 27-gauge needle (Terumo) at 10 days after surgery (day 10). Four separate injections of human HGF vector locally (intramuscularly into the ischemic limb near both the proximal and distal arterial stumps) were given. The average blood flow in each histogram was calculated for 5 seconds after addition of sample. The counting lasted for 10 seconds, and the counts in 10 seconds were used for calculation of luciferase activity.

**Measurement of HGF Concentration in Hindlimb**

To document successful transfection of HGF vector into the hindlimb, we examined the production of human immunoreactive HGF\(^{22,23}\) at 4 days after transfection. The concentration of HGF in the hindlimb was determined by enzyme immunoassay with an anti–human HGF antibody.\(^{22,23}\) The antibody against human HGF reacts only with human HGF, and not with rat HGF.\(^{22}\) Rat immunoreactive HGF in the rat hindlimb was also measured by enzyme immunoassay with an anti–rat HGF antibody, because the antibody against rat HGF reacts with only rat HGF, and not with human HGF.\(^{22}\)

**Measurement of Blood Flow by Laser Doppler Image and Capillary Density**

Measurement of blood flow with a laser Doppler imager has been described previously.\(^{6,24}\) Because it was clearly demonstrated that laser Doppler flow velocity correlates well with capillary density,\(^{16,24}\) we measured the cardiac blood flow by means of a laser Doppler blood flowmeter (Laser Doppler Imager, Moor Instruments). Consecutive measurements were obtained over the same regions of interest (leg and foot). Laser Doppler imaging uses a 12-mW helium-neon laser beam that sequentially scans a 5×5-cm surface area with extremely high speed to be able to measure the blood flow in the ischemic hindlimb. The perfusion signal is subdivided into 14 different intervals, and each interval is displayed in a separate color. 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.

**Experiment 2: In Vitro Experiments**

**Measurement of MMP-1, ets-1, and HGF**

Human aortic endothelial cells (passage 5) and human aortic vascular smooth muscle cells (passage 3) were obtained from Clonetics Corp and cultured in the standard fashion. Human endothelial cells were seeded on 6-well plates (Corning) at a density of 5×10\(^4\) cells/cm\(^2\) and cultured for 24 hours. After the medium had been replaced with fresh defined serum-free medium supplemented with insulin (5×10\(^{-7}\) mol/L), transferrin (5 mg/mL), and ascorbate (0.2 mmol/L)\(^{25}\) and after culture for 24 hours, the concentration of MMP-1 in the medium was determined by enzyme immunoassay (MMP-1 Biotrack; Amersham). The concentration of HGF in the medium of endothelial cells was also determined by enzyme immunoassay using anti–human HGF antibody.\(^{22,23}\) Northern blotting was also performed for analysis of ets-1 mRNA. Northern blotting was performed in the standard manner, and RNA was hybridized with \(^{32}\)P–end-labeled ets-1 cDNA.

**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 Intramuscular Injection of HGF Plasmid in Diabetes Model

Initially, we measured rat endogenous HGF concentration in the hindlimb of nondiabetic and diabetic rats. As shown in Figure 1A, HGF concentration in the ischemic hindlimb of nondiabetic rats was significantly decreased compared with that in the nonischemic hindlimb of nondiabetic rats as control. Consistent with the present data, a significant decrease in blood vessels of patients with peripheral arterial disease has been documented.9 Of importance, endogenous HGF concentration was further reduced in the ischemic hindlimb of diabetic rats compared with that of nondiabetic rats (P<0.01, Figure 1A). The decrease in local HGF production in the ischemic hindlimb of diabetic rats might be due to activation of transforming growth factor-β by high glucose, which is a strong suppressor of vascular HGF in vitro as well as in vivo.23 Given the significant decrease in endogenous HGF production in the ischemic limb of diabetic rats, we hypothesized that transfection of human HGF vector into the ischemic limb of diabetic rats might result in a beneficial effect in hypoxia as “supplemental” cytokine therapy. Therefore, HGF plasmid was transfected intramuscularly into the ischemic hindlimb of rats in which the femoral artery was excised to induce unilateral hindlimb ischemia. Initially, we compared the transfection efficiency of luciferase gene by naked plasmid injection with that by the HVJ-liposome method. As expected, as shown in Figure 1B, luciferase activity was much higher in the hindlimb of rats transfected by the HVJ-liposome method (20 μg/animal) than by naked plasmid DNA (200 μg/animal) (P<0.01). Thus, we used the HVJ-liposome method to test the feasibility of therapeutic angiogenesis by HGF gene transfer.

Next, we measured human HGF concentration in the ischemic hindlimb transfected with human HGF or control vector by the HVJ-liposome method. As expected, human immunoreactive HGF was readily detected in the hindlimb transfected with human HGF vector, but not control vector, at 4 days after transfection (Figure 2, P<0.01). After an increase in human HGF concentration, injection of human HGF vector into the ischemic hindlimb resulted in a significant increase in blood flow from 3 weeks after transfection to 5 weeks after transfection as assessed by laser Doppler imaging (P<0.01), as shown in Figures 3 and 4. Moreover, transfection of human HGF vector significantly increased capillary density in the ischemic hindlimb of nondiabetic rats around the injection site compared with hindlimb transfected with control vector (Figure 4, P<0.01). These results clearly demonstrated that transfection of human HGF vector into the ischemic hindlimb induced therapeutic angiogenesis, which could be applied for the treatment of peripheral arterial disease. Interestingly, the recovery of blood flow was significantly diminished in diabetic rats compared with nondiabetic rats.

Figure 1. A, Endogenous rat HGF concentration in muscle of nonischemic and ischemic rats with and without DM. Nonischemic indicates muscle from nonischemic hindlimb of nondiabetic rats; Ischemic, muscle from ischemic hindlimb of nondiabetic rats; and DM-Ischemic, muscle from ischemic hindlimb of diabetic rats. Each group contains 7 or 8 animals. B, Comparison of transfection efficiency using luciferase gene between intramuscular injection of naked plasmid DNA and HVJ-liposome method. Control indicates intramuscular injection of control naked plasmid; Naked-plasmid, intramuscular injection of control naked plasmid; Naked-plasmid, intramuscular injection of naked plasmid of luciferase gene; HVJ-liposome, intramuscular injection of plasmid of luciferase gene by HVJ-liposome method; and N.D., not detected. Each group contains 5 to 7 animals.

Figure 2. Human immunoreactive HGF in muscle transfected with control or human HGF gene by HVJ-liposome method. Control indicates rats transfected with control vector; HGF/HVJ-liposome, rats transfected with human HGF vector; and N.D., not detected. Control group contains 5 animals; HGF group, 7 animals.

Figure 3. Effect of intramuscular injection of human HGF plasmid in ischemic limb of nondiabetic rats: quantitative analysis of blood flow in ischemic hindlimb at 5 weeks after transfection. Control indicates blood flow in ischemic hindlimb transfected with control vector (20 μg); HGF/HVJ, blood flow in ischemic hindlimb transfected with HGF vector (20 μg/animal). Each group contains 7 or 8 animals.
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Figure 4. Effect of transfection of HGF vector on vascular formation in nondiabetic rats at 5 weeks after transfection. Bottom, Representative cross sections (×200). Control indicates muscle from rats transfected with control vector (20 μg); HGF/HVJ, muscle from rats transfected with human HGF vector (20 μg). Top, Effect of transfection of human HGF vector on number of vessels. Each group contains 7 or 8 animals.

Figure 5. Effect of intramuscular injection of human HGF plasmid in rat ischemic limb model: quantitative analysis of blood flow in right hindlimb. Control indicates blood flow in ischemic hindlimb transfected with control vector (20 μg); DM/HGF-HVJ, blood flow in ischemic hindlimb transfected with HGF vector (20 μg/animal) in diabetic rats; 0, before treatment; and 1W, 3W, and 5W, 1, 3, or 5 weeks after transfection. *P<0.01 vs control; #P<0.01 vs DM. Each group contains 7 or 8 animals.

Figure 6. Effect of transfection of HGF vector on vascular formation in diabetic rats. Bottom, Representative cross sections (×200): Control indicates muscle from rats transfected with control vector (20 μg) in diabetic rats; DM/HGF, muscle from rats transfected with human HGF vector (20 μg) in diabetic rats. Top, Effect of transfection of human HGF vector on number of vessels. Each group contains 7 or 8 animals.

Molecular Mechanisms of Impairment of Angiogenesis in Diabetic Rats

Given the impairment of angiogenesis in diabetic rats, we examined further how diabetes, especially high D-glucose, diminished angiogenesis. As previously reported, treatment of endothelial cells with a high concentration of D-glucose, but not mannitol or L-glucose, resulted in a significant decrease in cell number.25 Consistent with the previous report,25 a decrease in local HGF production by high glucose was also confirmed by the present study, in which endogenous HGF concentration was significantly lower in the hindlimb of diabetic rats than that of nondiabetic rats (Figure 1A). In this study, we especially focused on the matrix-degrading pathway, including MMP-1. Interestingly, in human aortic endothelial cells, high D-glucose significantly decreased the production of MMP-1 (Figure 8A, P<0.05). In addition, HGF significantly attenuated the decrease in MMP-1 protein induced by high D-glucose (Figure 8A, P<0.01). To further confirm the impairment of angiogenesis in diabetes, an essential transcription factor for angiogenesis, ets-1, was analyzed. As shown in Figure 8B, high D-glucose treatment significantly decreased ets-1 mRNA as assessed by Northern blotting (P<0.01). In contrast, RhG significantly attenuated the decrease in ets-1 mRNA induced by high D-glucose (P<0.01, Figure 8B). These results demonstrated that high D-glucose treatment diminished the degradation pathway of extracellular matrix, accompanied by a significant decrease in local HGF production.

Discussion

Recently, the clinical utility of gene therapy using the VEGF gene has been reported for the treatment of critical limb ischemia and myocardial ischemia.4–8 A novel therapeutic strategy using angiogenic growth factors to expedite and/or augment collateral artery development has recently entered the realm of treatment of ischemic diseases, although there is no pharmacological treatment for patients with critical limb ischemia. The present study raises the possibility of a new strategy, therapeutic angiogenesis using HGF as gene therapy, in addition to VEGF, for the treatment of patients with
Although the feasibility of therapeutic angiogenesis using HGF has been reported in experimental models, no report has examined the potential utility of HGF gene transfer to stimulate angiogenesis in a diabetes model. Therefore, we examined the feasibility of therapeutic angiogenesis using the HGF gene in a rat diabetes ischemia model, because the most common complication of diabetes is peripheral vascular disease. Because we reported a high transfection efficiency of the HVJ-liposome method into rat intact hearts, we used the HVJ-liposome method rather than naked plasmid transfection. As expected, the transfection efficiency of the HVJ-liposome method was much greater than that of naked plasmid DNA. Using the HVJ-liposome method, we demonstrated direct in vivo evidence of therapeutic angiogenesis induced by HGF gene transfer in an ischemia model. Moreover, it is noteworthy that repeated injection of the HGF gene by the HVJ-liposome method demonstrated further induction of angiogenic activity, suggesting that viral gene transfer using HVJ-liposome vector is not limited by inflammation/immunological reactions, consistent with the previous reports. In future, therapeutic angiogenesis using HVJ-based gene transfer might be useful to treat cardiovascular disease.

In patients with critical limb ischemia, because there is no pharmacological treatment, amputation, despite its associated morbidity, mortality, and functional implications, is often recommended as a solution to the disabling symptoms, in particular excruciating ischemic rest pain. Because diabetic patients often present with advanced forms of coronary and peripheral artery disease, it is important to know whether compensatory mechanisms for vascular ischemia are affected in this condition in developing human gene therapy. Accordingly, we sought to determine whether cytokine-induced therapeutic neovascularization is feasible in a diabetes model. In diabetic rats, measurement of the Doppler flow ratio between the ischemic and normal limbs indicated that restorative effects of HGF on MMP-1 protein in human aortic endothelial cells. N=8 per group calculated from 8 independent experiments. Control indicates vehicle under 5 mmol/L D-glucose; HGF(+) rHGF 100 ng/mL; and D-glucose, 25 or 50 mmol/L D-glucose. #P<0.01 vs control, *P<0.01 vs HGF(-). B, Top, Typical example of ets-1 mRNA in human aortic endothelial cells treated with HGF. Left, Control, vehicle under 5 mmol/L D-glucose; middle, glucose, vehicle under 25 mmol/L D-glucose; and right, HGF+glucose, rHGF (100 ng/mL) under 25 mmol/L D-glucose. Bottom, Effects of high glucose on ets-1 mRNA in human aortic endothelial cells treated with HGF. N=5 to 8 per group calculated from 5 independent experiments. Control indicates vehicle under D-glucose 5 mmol/L; Glucose, vehicle under 25 mmol/L D-glucose; and HGF+Glucose, rHGF (100 ng/mL) under 25 mmol/L D-glucose.
ration of perfusion in the ischemic hindlimb was significantly impaired. This impairment of blood flow recovery persisted throughout the duration of the study. Alkaline phosphatase staining confirmed the laser Doppler data by showing a significant reduction in capillary density in the diabetic rats after surgery. Previous reports have documented similar findings.\textsuperscript{29–31} Notably, a single intramuscular injection of HGF plasmid was sufficient to induce therapeutic angiogenesis even in the rat diabetic hindlimb ischemia model.

Next, we studied how diabetes could impair new collateral vessel formation in response to tissue ischemia. Although Rivard et al.\textsuperscript{29} reported a reduction in VEGF in diabetic ischemic conditions, we also found that the reduction in neovascularization in the diabetic rats was a result of a lower level of HGF in the diabetic tissues. We confirmed the pivotal role of HGF by showing that an increase in neovascularization could be achieved in diabetic rats that had been supplemented with HGF via intramuscular injection of HGF vector. Because HGF has been reported to stimulate VEGF expression,\textsuperscript{10} VEGF might have contributed to the angiogenesis induced by HGF in a diabetes model. In addition to the decrease in local HGF production, our present study demonstrated that high D-glucose treatment of endothelial cells resulted in a significant decrease in MMP-1, accompanied by a significant decrease in local HGF production. Because members of the ets family play important roles in regulating gene expression in response to multiple developmental and mitogenic signals,\textsuperscript{13–16} we also examined the effects of high D-glucose on ets-1 activity. Importantly, high D-glucose treatment decreased ets-1 mRNA expression in human endothelial cells. Because previous reports suggest that the ets family may activate the transcription of genes encoding MMP-1, stromelysin 1, and urokinase plasminogen activator,\textsuperscript{29–34} downregulation of ets-1, which takes part in regulating angiogenesis by controlling the transcription of these genes necessary for the migration of endothelial cells from preexisting capillaries, might play a pivotal role in the impairment of angiogenesis in diabetes. Interestingly, HGF stimulated ets-1 mRNA and activity in human endothelial cells. Thus, the decrease in local HGF production by high D-glucose might decrease ets-1 activity.

Overall, the present studies suggest a novel therapeutic strategy that might reduce the symptoms of critical limb ischemia, using the angiogenic properties of HGF gene transfer, in a rat model of diabetes. Impairment of endogenous neovascularization of ischemic tissues in diabetes might be due to reduction in an essential transcription factor for angiogenesis, ets-1, by high D-glucose, resulting in the suppression of necessary genes for the migration of endothelial cells, such as MMP-1.

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