Angiogenesis and Antifibrotic Action by Hepatocyte Growth Factor in Cardiomyopathy

Yoshiaki Taniyama, Ryuichi Morishita, Motokuni Aoki, Kazuya Hiraoka, Keita Yamasaki, Naotaka Hashiya, Kunio Matsumoto, Toshikazu Nakamura, Yasufumi Kaneda, Toshio Ogihara

Abstract—Impairment of cardiac function in cardiomyopathy has been postulated to be related to decreased blood flow and increased collagen synthesis. Therefore, a therapeutic approach to alter the blood flow or fibrosis directly by means of growth factors may open a new therapeutic concept in dilated cardiomyopathy. From this viewpoint, hepatocyte growth factor (HGF) is a unique growth factor with antifibrosis and angiogenesis effects. Using the hereditary cardiomyopathic Syrian hamster as a model of genetically determined cardiomyopathy and heart failure, the effects of overexpression of HGF on fibrosis and microvascular dysfunction were examined. HGF gene or control vector was injected by the Hemagglutinating Virus of Japan–liposome method into the anterior heart of cardiomyopathic hamsters (Bio 14.6) under echocardiography once a week, from 12 to 20 weeks of age (total, 8 times). Blood flow, as assessed by a laser Doppler imager score, and the capillary density in hearts, as assessed by alkaline phosphatase staining, were significantly increased in hamsters transfected with HGF gene compared with control-vector-transfected hamsters ($P<0.01$). In contrast, the fibrotic area was significantly decreased in hamsters transfected with HGF gene compared with control ($P<0.01$). Overall, in vivo experiments demonstrated that transfection of HGF gene into the myocardium of cardiomyopathic hamsters stimulated blood flow through the induction of angiogenesis and reduction of fibrosis. These results suggest that HGF gene transfer may be useful to protect against myocardial injury in cardiomyopathy through its cardioprotective effects such as antifibrosis and angiogenesis actions. (Hypertension. 2002;40:47-53.)

Key Words: cardiomyopathy ■ fibrosis ■ angiogenesis ■ gene therapy ■ hepatocyte growth factor

In patients with clinically manifest heart failure, primary cardiomyopathy is diagnosed in 2% to 15% of patients, whereas in recent large-scale therapeutic trials, the proportion of patients with nonischemic heart failure ranged from 18% to 53%.1 The functional consequence of myocardial damage in nonischemic heart failure is a global rather than localized abnormality of ventricular contractility. It is not the quantity but rather the quality of myocardium that accounts for pathologic hypertrophy and predisposes to ventricular dysfunction and arrhythmia, which, in turn, confer increased risk of adverse cardiovascular events. Herein, factors regulating the growth of these compartments are involved in promoting adverse remodeling of intramyocardial coronary arteries and arterioles by fibrous tissue.2 Therefore, a therapeutic approach to alter the fibrosis directly by means of growth factors may open a new therapeutic concept in dilated cardiomyopathy. Recent advances in cardiovascular molecular biology have provided new insights into how the structure and function of adult cardiac myocytes can be changed in relation to the compensatory and secondary phenomena that occur during the pathogenesis of cardiomyopathy in vivo.3 From this viewpoint, hepatocyte growth factor (HGF) should be the center of interest as an antifibrotic growth factor. HGF is a mesenchyme-derived pleiotropic factor that regulates cell growth, motility, and morphogenesis of various types of cells and is thus considered a humoral mediator of epithelial-mesenchymal interactions responsible for morphogenetic tissue interactions.4,5 Moreover, HGF is a unique growth factor to prevent fibrosis, as administration of human recombinant HGF prevented and/or regressed fibrosis in liver and pulmonary injury models.6–8 Thus, HGF may also play an important role in the pathogenesis of fibrotic cardiovascular disease, ie, cardiomyopathy. Although the characterization of gene mutations in cardiomyopathy has led to the development of therapies specifically targeting the defective protein or the pathway in which it is involved, replacement of the defective gene with a functional one (gene therapy) seems to be impossible using the present techniques. Another promising therapeutic strategy to treat cardiomyopathy is to inhibit fibrosis using antifibrotic genes such as HGF. Thus, in the present study, we examined the feasibility of
gene therapy to inhibit the progression of cardiac fibrosis by overexpression of HGF in the cardiomyopathic hamster.

Methods

Experiment 1: Cardiomyopathic Hamster Model

Hemagglutinating Virus of Japan (HVJ)-lipoosome complex (10 μL) containing human HGF cDNA driven by the cytomegalovirus promoter/enhancer,9 luciferase vector driven by SV 40 promoter (Promega, Madison, WI), or control vector (10 μg/mL in liposomes) was carefully injected directly into the myocardium of cardiomyopathic hamsters (12 weeks of age; Baa 146, Charles River, Osaka, Japan) with a 27-g needle under endocardiography. In the present study, we used a high-efficiency transfection system using HVJ liposomes.10,11 To identify the transfectable area, we used sonicated ioxaglate to enhance the echo contrast on injection of HVJ-liposome complex. Four days after transfection, the concentration of HGF in the myocardium was determined by enzyme-immunoassay using antihuman HGF antibody12,13 to document successful transfection of human HGF vector into the myocardium. The antibody against human HGF reacts with only human HGF and not with hamster HGF.12,13

As it was clearly demonstrated that laser Doppler flow velocity correlates well with capillary density,14,15 we measured the cardiac blood flow by means of a laser Doppler blood flowmeter (Laser Doppler Imager, Moor Instruments). The animals were coded so that the analysis was performed without any knowledge of which treatment each individual animal had received. Alkaline phosphatase staining was also used as a specific marker of endothelial cells in paraffin-embedded sections.14,15 To analyze the number of vessels in the myocardium, 3 individual sections from the middle of transfected myocardium were analyzed. The number of vessels was counted under a light microscope (x100) in a blinded manner. The total number of vessels in each section was summed and expressed as number per section. At least 10 individual sections were evaluated in each heart. The areas in which the number of vessels was quantified were randomly selected in the injected site and around the injected site.

Experiment 2: In Vitro Experiments

After reaching 80% confluence of human fibroblast cells (passage 5; Clonetics Corp, San Diego, CA), phosphorothioate oligodeoxynucleotides (ODN) encapsulated in HVJ-liposomes (15 μmol/L encapsulated ODN) were transfected. For measurement of ets-1, MMP-1, uPA, and TGF-β, human fibroblasts were cultured for 24 hours. After replacing the medium with fresh defined serum-free medium and after culture for 24 hours, the concentrations of MMP-1, uPA, and HGF in the medium were determined by enzyme-immunoassays (MMP-1; Biotrack, Amersham, uPA; Cosmo Co). The sequence of phosphorothioate ODN was as follows: antisense ets-1 (5’-AGATC-GACGGCCGCCTTCAT-3’) and sense ets-1 (5’-ATGAAGG-CGGCCGCTCAGCTC-3’). Northern blotting was also performed for analysis of ets-1 and TGF-β mRNA. Western blotting was also performed for analysis of c-met protein using a monoclonal antibody to c-met (1:500; PharMingen). Amounts of loaded proteins were confirmed to be equal by staining with Coomassie brilliant blue R (Sigma), and Western blotting of tubulin using antitubulin antibody (antihuman mouse IgG, 1:100; Oncogene).

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 of P<0.05 were considered significant.

Results

Angiogenic and Antifibrotic Actions Induced by Intramuscular Injection of Human HGF Gene in Hamster Model

First, we measured endogenous HGF concentration in the myocardium of hamsters. Interestingly, HGF concentration in the myocardium of the cardiomyopathic hamster was significantly decreased compared with that in the normal hamster at 12 weeks of age (noncardiomyopathic hamster, 3.8±0.8 ng/g tissue, versus cardiomyopathic hamster, 1.7±0.5 ng/g tissue; P<0.01). Downregulation of the endogenous HGF system may be related to the pathogenesis of cardiomyopathy, because HGF has shown an angiogenic action in myocardium.15,18 The decrease in local HGF production in the myocardium might be caused by angiotensin II or transforming growth factor-β (TGF-β), which are strong suppressors of HGF.13 Given the significant decrease in endogenous HGF production in the cardiomyopathic heart, we hypothesized that transfection of human HGF vector into the cardiomyopathic heart might result in a beneficial effect. Therefore, HGF plasmid was directly intramuscularly transfected into the myocardium of cardiomyopathic hamsters.

First, we transfected luciferase gene into the myocardium of cardiomyopathic hamster to examine the feasibility of gene transfer using echo-guided direct injection, as shown in Figure 1A. To identify the transflectable area, we used sonicated ioxaglate to enhance echo contrast. As shown in Figure 1B, echocardiography using sonicated ioxaglate clearly demonstrated the feasibility of direct injection of therapeutic genes into the myocardium, especially the anterior area. Consistent with echocardiographic findings, luciferase activity could be detected in myocardium transfected with luciferase vector at 5 days after transfection, whereas no luciferase activity was detected (Figure 1C; P<0.01). Then, we measured human HGF concentration in the myocardium transfected with human HGF or control vector. Expectedly, human immunoreactive HGF was readily detected in the myocardium transfected with human HGF vector, but not control vector at 4 days after transfection using a specific antibody against human HGF (Figure 1D; P<0.01).

One of the favorable features of echo-guided gene transfection is to enable repeated transfection into the myocardium. No evidence of severe toxicity of direct injection under echo-guided gene transfection was observed. Different from adenoviral vector, HVJ-liposome method is less immunogenic. Although the details of mechanisms have not yet understood, the previous reports documented the repeated injection of HVJ-liposome did not cause cytotoxic T lymphocytes in vivo, thus allowing the equivalent transfection efficiency at the repeated injection.19,20 Thus, to treat cardiomyopathy, we used repeated injection of human HGF vector into the myocardium. As the HVJ-liposome method could transfete genes by repeated injection,19,20 we directly intramuscularly transfected human HGF vector once a week for 8 weeks. After an increase in human HGF concentration, injection of human HGF vector into the myocardium resulted in a significant increase in blood flow at 8 weeks, as assessed by laser Doppler imaging, compared with control vector (P<0.01), as shown in Figure 2A. The laser Doppler imaging score of myocardium of cardiomyopathic hamsters transfected with control vector was not different from that in nontransfected hamsters. Moreover, transfusion of human HGF vector significantly increased capillary density, as demonstrated by alkaline phosphatase (a marker of endothelial cells) staining in the myocardium of cardiomyopathic
hamsters compared with control vector (Figure 2B, \(P < 0.01\)). In contrast, there was no significant change in the number of capillary density between noncardiomyopathic hamster and untransfected cardiomyopathic hamster, whereas the tendency to decrease the number of capillary density was observed in cardiomyopathic hamster. These results demonstrate that transfection of human HGF vector into the myocardium of cardiomyopathic hamsters induced therapeutic angiogenesis.

In addition to angiogenesis, previous reports demonstrated that overexpression of HGF showed an antifibrotic action in various models.\(^7\) Our previous in vitro studies using cardiac fibroblasts revealed that HGF stimulated the degradation of extracellular matrix through activation of MMP-1 and uPA, and inhibited the synthesis of extracellular matrix through the inhibition of TGF-\(\beta\) expression.\(^{16}\) Thus, we measured fibrotic area in the myocardium after HGF gene transfection. Expectedly, cardiomyopathic hamster demonstrated the increase in fibrosis compared with that of the nonmyopathic control group. Importantly, a significant decrease was observed in the myocardium of hamsters transfected with human HGF vector compared with control after 8 weeks of transfection (\(P < 0.01\), Figure 2C). These results demonstrate that the angiogenic and antifibrotic actions induced by gene transfer of HGF would be beneficial in treating cardiomyopathy. Notably, human HGF could not be detected in plasma after 3 days and 1, 2, and 4 weeks of transfection. Therefore, the alteration of cardiac structure seems to be caused by local effects of HGF.

**Molecular Mechanisms of Antifibrotic Action of HGF**

Given the antifibrotic action of overexpression of human HGF gene, we further explored the molecular mechanisms of the antifibrotic actions of HGF. Especially, we focused on the role of a transcription factor for angiogenesis, ets-1. Consistent with previous reports,\(^{16}\) we confirmed the presence of the specific receptor for HGF, c-met, in cultured human fibroblasts. The c-met protein was clearly detected in cultured fibroblasts. Unexpectedly, HGF did not stimulate the growth of fibroblasts (data not shown). Thus, to examine the role of c-met in fibroblasts, we determined the expression of ets-1 mRNA after HGF stimulation. As shown in Figure 3A, ets-1 mRNA in fibroblasts was significantly increased after 1 hour of HGF stimulation and continued at least up to 6 hours. Probably, upregulation of ets-1 mRNA may activate antifibrotic genes such as MMP-1, because the promoter region of MMP-1 contains the ets binding site.\(^{21}\) We used an antisense approach to dissect out the role of ets-1 in the antifibrotic action of HGF. The inhibitory effect of antisense ODN on ets-1 was confirmed by the observation that transfection of antisense ets-1, but not sense, ODN resulted in significant inhibition of ets-1 mRNA induced by HGF (Figure 3B), consistent with a previous report.\(^{17}\) Therefore, we examined the effect of rHGF on MMP-1 expression. As shown in Figure 3A, ets-1 mRNA in fibroblasts was significantly increased after 1 hour of HGF stimulation and continued at least up to 6 hours. Probably, upregulation of ets-1 mRNA may activate antifibrotic genes such as MMP-1, because the promoter region of MMP-1 contains the ets binding site.\(^{21}\) We used an antisense approach to dissect out the role of ets-1 in the antifibrotic action of HGF. The inhibitory effect of antisense ODN on ets-1 was confirmed by the observation that transfection of antisense ets-1, but not sense, ODN resulted in significant inhibition of ets-1 mRNA induced by HGF (Figure 3B), consistent with a previous report.\(^{17}\) Therefore, we examined the effect of rHGF on MMP-1 expression. As shown in Figure 4A, rHGF stimulated the production of MMP-1 in fibroblasts (\(P < 0.01\)), whereas transfection of antisense ets-1, but not sense, ODN significantly inhibited the induction of MMP-1 (\(P < 0.01\)). Because both uPA and c-met gene promoters contain the ets binding site,\(^{22,23}\) upregulation of both genes by HGF might be owing to ets-1 activation. Indeed, the production of uPA was stimulated by rHGF in fibroblasts (\(P < 0.01\)), whereas transfection of antisense ets-1, but not sense, ODN significantly inhibited the induction of uPA by...
HGF (P<0.01; Figure 4B). In addition, c-met protein was also increased by rHGF (P<0.01; Figure 4C). Related to the blockade of extracellular matrix production, we confirmed that rHGF inhibited TGF-β production and attenuated the increase in TGF-β by angiotensin II in cultured human fibroblasts (P<0.01; Figure 5).

Discussion
Numerous morphological changes can be observed in the human failing myocardium due to dilated cardiomyopa-
thy. These changes include (1) an enlarged extracellular space, due to fibrosis, which contains increased amounts of different matrix proteins, cellular debris, and numerous macrophages and fibroblasts; (2) degenerative changes in myocytes, consisting of nuclei of varying size and shape, lack of contractile material, and disorganization of the cytoskeleton; and (3) the occurrence of hypertrophied and atrophied myocytes and of cells of normal size. The reduction in the number and function of myocardial myocytes induces hypertrophy and leads to interstitial fibrosis. Indeed, previous reports demonstrated that the mRNA levels of types I and III collagen were significantly increased in the left ventricular tissue of experimental cardiomyopathic hamster, and inhibited the collagen synthetic pathway as discussed below. The present study demonstrated that transfection of HGF gene into myocardium resulted in a significant decrease in the fibrotic area, accompanied by a marked increase in HGF expression, capillary density and blood flow. Because in dilated cardiomyopathy, a dense endomyosial woven network consisting of fine fibrils was associated predominantly with collagen, a decrease in collagen may participate in the improvement of cardiac function.

The antifibrotic actions of HGF depend on (1) inhibition of collagen synthesis through inhibition of TGF-β expression, and (2) degradation of collagen through activation of MMP-1 and uPA, etc. In the present study, we further explored how HGF stimulated collagen-degrading enzymes such as MMP-1. We focused on the ets family, which contains essential transcription factors for angiogenesis and vasculogenesis, because members of the ets family play important roles in regulating gene expression in response to multiple developmental and mitogenic signals. The ets family has a DNA-binding domain in common that binds a core GGA(A/T) DNA sequence. Previous reports suggest that 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. As our previous study demonstrated that HGF upregulated ets-1 mRNA expression in fibroblasts. Using an antisense strategy, we demonstrated that HGF stimulated MMP-1 and uPA through the activation of ets. In contrast, downregulation of TGF-β by HGF is consistent with the results of previous reports. However, little is known about how HGF inhibited TGF-β expression. In addition, the molecular mechanisms of the angiogenic activity of HGF seem to be also largely dependent on the ets pathway, because the ets family activates the transcription of genes encoding collagenase 1, stromelysin 1 and urokinase plasminogen activator, which are proteases involved in extracellular matrix degradation. As our previous study demonstrated that HGF upregulated ets activity and ets-1 protein in a myocardial infarction model, the activation of ets may regulate the both angiogenic and anti-fibrotic actions of HGF.
Perspectives

Overall, the present study suggests a novel therapeutic strategy that might reduce the symptoms of cardiomyopathy, by using the angiogenic and antifibrotic properties of HGF gene transfer. In addition, the stimulation of new vessel formation and decrease in cardiac fibrosis by HGF is likely to create new therapeutic options in fibrotic conditions such as myocardial infarction, chronic heart failure, and pulmonary fibrosis. Nevertheless, the clinical utility of gene therapy in the myocardium is still enigmatic. Although early studies using gene therapy vectors were promising, translating studies in animals to viable therapeutic options for humans has remained problematic. In the present study, we transfected the human HGF gene using echo-guided direct injection. Using echo contrast such as sonicated ioxaglate, direct injection of therapeutic genes into the myocardium using echo-guided direct injection provides a new less invasive technique to treat cardiac diseases. To consider human gene therapy, the transfection using naked plasmid DNA or adenoviral vector might be applied, because HVJ-liposome method is not currently available. What is the difference between HGF and VEGF? We believe that one of the distinguishing features of HGF is the presence of its specific receptor, c-met, in the cardiac fibroblasts, although the receptors of VEGF were not existed in fibroblasts. Thus, HGF inhibited collagen synthesis through TGF-β and stimulated collagen degradation through upregulation of MMP-1 and uPA, although VEGF dose not have these unique characters. Thus, the improvement of cardiac structure may be occurred in case of HGF gene therapy rather than VEGF. In addition to antifibrotic action of HGF, HGF may have the different actions on vascular smooth muscle cells because the c-met receptor is also existed in smooth muscle cells, although further studies might be necessary to examine the effects of various angiogenic growth factors—including HGF, VEGF, and FGF—in cardiac function.

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