Potential Contribution of a Novel Antifibrotic Factor, Hepatocyte Growth Factor, to Prevention of Myocardial Fibrosis by Angiotensin II Blockade in Cardiomyopathic Hamsters

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Background—Because hepatocyte growth factor (HGF) prevented and/or regressed fibrosis in liver and pulmonary injury models, HGF may play an important role in the pathogenesis of fibrotic cardiovascular disease. Because angiotensin (Ang) II significantly decreased local HGF production, we performed (1) in vitro experiments using fibroblasts and (2) administration of an ACE inhibitor (temocapril) and an Ang II type 1 receptor antagonist (CS-866) to cardiomyopathic hamsters.

Methods and Results—In human fibroblasts, HGF significantly increased the production of matrix metalloprotease-1 (MMP-1) and urokinase plasminogen activator, whereas HGF also significantly attenuated the reduction of MMP-1 activity induced by Ang II. In contrast, HGF significantly decreased transforming growth factor (TGF)-β mRNA stimulated by Ang II, whereas HGF also decreased basal TGF-β protein level without affecting growth. Similarly, in rat cardiac fibroblasts, HGF inhibited the expression and production of TGF-β, whereas HGF upregulated its specific receptor, c-met. Conversely, in vivo experiments revealed that administration of temocapril and CS-866 to cardiomyopathic hamsters resulted in a significant decrease in fibrotic area and increase in cardiac HGF concentration and mRNA (\(P \leq 0.01\)), whereas cardiac concentration and mRNA of HGF were significantly decreased in cardiomyopathic hamsters. In contrast, mRNA expression of collagen III was markedly decreased by treatment with temocapril and CS-866.

Conclusions—Here, we demonstrated that Ang II blockade prevented myocardial fibrosis in the cardiomyopathic hamster, accompanied by a significant increase in cardiac HGF. Overall, increase in local HGF expression may participate in the prevention of myocardial injury by Ang II blockade through its antifibrotic action. (Circulation. 2000;102:246-252.)

Key Words: fibrosis ■ metalloproteinases ■ collagen ■ remodeling ■ extracellular matrix

Hepatocyte 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 morphogenic tissue interactions.1–3 Moreover, HGF is a unique growth factor to prevent fibrosis, because previous studies demonstrated that administration of human recombinant HGF (rHGF) prevented and/or regressed fibrosis in liver and pulmonary injury models.4–6 For example, administration of human rHGF or gene transfer of human HGF to rats with hepatic fibrosis/cirrhosis caused by dimethylnitrosamine prevented the onset and progression of hepatic fibrosis/cirrhosis.5,7 Thus, HGF may also play an important role in the pathogenesis of fibrotic cardiovascular disease, ie, cardiomyopathy. However, it is not clear how HGF acts as an antifibrotic factor. Therefore, in this study, we elucidated the molecular mechanisms of the antifibrotic action of HGF.

In cardiomyopathy, the contribution of growth factors and cytokines has been reported.8–10 Transforming growth factor (TGF)-β, which is a well-known growth factor that stimulates fibrosis through the accumulation of extracellular matrix, was upregulated in myocardial infarction and cardiomyopathy.8–10 In addition, activation of angiotensin (Ang) II is also believed to play an important role in the pathogenesis of fibrosis in such cardiovascular disease.11–14 Indeed, blockade of Ang II, such as with an ACE inhibitor or an Ang II type 1 receptor antagonist, prevents fibrosis in fibrotic cardiovascular disease.15,16 Interestingly, TGF-β and Ang II are strong negative regulators of local HGF production in various cells.17,18

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Nevertheless, we found no reports investigating the relationship among TGF-β, Ang II, and HGF in fibrotic cardiovascular disease, such as cardiomyopathy. Thus, knowledge of local HGF regulation by those growth factors would be important in understanding the pathophysiology of cardiovascular diseases. To clarify the role of HGF in the inhibitory effects of Ang II blockade on myocardial injury, we also examined the effects of Ang II blockade on cardiac fibrosis and local HGF production in the cardiomyopathic hamster. Therefore, in this study, we addressed the following specific questions: (1) How does HGF prevent fibrosis in in vitro human cultured fibroblasts? and (2) Does Ang II blockade affect local HGF production and cardiac fibrosis in the cardiomyopathic hamster in vivo?

Methods

In Vitro Experiment

Cell Culture
Human primary fibroblasts (passage 5) were obtained from Clonetics Corp.19 Rat cardiac fibroblasts and myocytes were prepared by a previously reported method.20 All the cells were used within passages 5 to 9. In the preparation of experiments for determination of cell count, the cells were grown to subconfluence. After cells reached 80% confluence, the media were changed to fresh defined serum-free (DSF) medium supplemented with insulin (5×10⁻⁶ mol/L), transferrin (5 mg/mL), and ascorbate (0.2 mmol/L).21 On day 4, an index of cell proliferation was determined with a WST cell counting kit (Wako).22

Measurement of MMP-1, MMP-1 Activity, uPA, TGF-β, and HGF
Fibroblasts were seeded at a density of 5×10⁴ cells/cm² and cultured for 24 hours. After the medium had been replaced with fresh DSF and the culture followed up for 24 hours, the concentrations of MMP-1, MMP-9, and urokinase-type plasminogen activator (uPA) in the medium were determined by enzyme immunoassays (EIAs; MPP-1 Biotrack and MPP-9 Biotrack, Amersham; uPA, Cosmo Co Ltd). In addition, MMP-1 activity was also evaluated by a collagenease type I activity test kit (Yagai Co Ltd). ELISA for immunoreactive TGF-β1 in the supernatant was performed with an ELISA kit (Amersham). The antibody against TGF-β1 cross-reacts with rat active TGF-β1, TGF-β2, or TGF-β3. After conversion of TGF-β3 from the inactive to the active form by the addition of protease, measurement of latent TGF-β3 was performed by ELISA.23 The concentrations of HGF in the medium of rat cardiac fibroblasts and myocytes were also determined by EIA using anti-rat HGF antibody.24 Northern blotting was performed for analysis of TGF-β mRNA in the standard manner, and RNA was hybridized with 3²P-end-labeled TGF-β primer (Clontech).

Western Blot for Analysis of c-met Protein
Fibroblasts were grown to confluence and made quiescent by incubation in DSF medium. After 24 hours of HGF treatment, the cells were fixed with 10% trichloroacetic acid in saline. Samples containing 100 μg protein were incubated with a monoclonal antibody to c-met (1:500; Pharmingen) at 4°C overnight. Amounts of loaded proteins were confirmed to be equal by staining with Coomassie brilliant blue R (Sigma). Staining with Coomassie brilliant blue revealed identical amounts of protein in all samples of Western blotting. Western blotting of tubulin with anti-tubulin antibody (anti-human mouse IgG; 1:100; Oncogene) was also performed to confirm equal amounts of loaded proteins.

In Vivo Experiment

Experimental Design
Male cardiomyopathic hamsters (Bio 14.6) (12 weeks old; Charles River Breeding Laboratories, Tokyo) were divided into 3 groups and treated until 20 weeks old with vehicle (distilled water), ACE inhibitor (temocapril; 20 mg ⋅ kg⁻¹ ⋅ d⁻¹), Ang II type I receptor antagonist (CS-866; 1 or 10 mg ⋅ kg⁻¹ ⋅ d⁻¹), or hydralazine (8 mg ⋅ kg⁻¹ ⋅ d⁻¹) (n=10, respectively) by gavage. Control Bio F1B hamsters were also given vehicle. Systolic blood pressure was measured directly. Gene expression of HGF and of collagen III was also measured by Northern blotting. The filter was hybridized to cDNAs of human HGF, collagen III (donated by Dr Y. Yamada, Harvard Medical School, Boston, Mass), or G3PDH (Clontech) labeled by 3²P end-labeling for Northern blotting. Tissue HGF concentration was assayed with a recently developed EIA (HGF EIA kit, Tokushumeneki Research Center).17,18,24,25 Tissue HGF concentration was determined by EIA using anti-rat HGF antibody, because antibody against rat HGF recognizes murine and hamster HGF but not human HGF.17,18,24,25

For histological analyses, hearts were fixed with 4% paraformaldehyde and subsequently processed. Cardiac fibrotic area was measured on a digitizing tablet (Mac Scope, Mitani Corp Image System Development) after Masson staining.16 At least 3 individual sections from the middle of the hearts were analyzed. Animals were coded so that the analysis was performed without knowledge of which treatment each individual animal had received.

Materials
Basic fibroblast growth factor and dexamethasone were obtained from Sigma. Temocapril, CS-866, and hydralazine were donated by Sankyo Pharmaceutical Co.

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

In Vitro Experiments

To investigate the molecular mechanisms of the antifibrotic actions of HGF, we initially focused on the production of MMP-1 (interstitial collagenase), a zinc enzyme responsible for degradation of extracellular fibers composed of collagen types II, III, and I. In human fibroblasts, human HGF (100 ng/mL) significantly increased the production of MMP-1 (Figure 1, P < 0.05). Interestingly, Ang II significantly decreased MMP-1 activity (Figure 1b, P < 0.01), whereas Ang II did not affect MMP-1 protein in human fibroblasts. Of importance, HGF significantly attenuated the reduction of MMP-1 activity induced by Ang II (P < 0.01). In contrast, no detectable amount of MMP-9 was observed in the conditioned medium of human fibroblasts. In addition, production of uPA, which also degrades extracellular matrix, was also significantly increased by rHGF in human fibroblasts (Figure 1c, P < 0.01). These results demonstrated that rHGF stimulated the degradation pathway of extracellular matrix.

Next, we focused on the effect of HGF on the synthetic pathway of extracellular matrix, especially on TGF-β expression. Importantly, rHGF significantly decreased Ang II–stimulated TGF-β mRNA expression at 6 hours after addition of rHGF in human fibroblasts (vehicle, 14.9 ± 2.1 ng/mL; HGF 100 ng/mL, 10.8 ± 1.5 ng/mL; P < 0.05). These findings are extremely important, because TGF-β significantly reduced the activity of MMP-1 (vehicle, 2.9 ± 0.2 U/mL; TGF-β 100 ng/mL, 2.2 ± 0.1 U/mL; P < 0.05). However, rHGF did not affect the growth of human fibroblasts (data not shown). Similarly, rHGF significantly decreased Ang II–stimulated TGF-β mRNA expression at 6 hours after addition of rHGF (Figure 2a) and significantly attenuated TGF-β protein induced by Ang II in rat cardiac fibroblasts (Figure 2b, P < 0.05).

To confirm the action of HGF, we analyzed the expression of the specific receptor of HGF, c-met, in fibroblasts. As shown in Figure 3a, the presence of c-met was clearly demonstrated by Western blotting, whereas the amount of c-met protein in human fibroblasts was obviously lower than that in endothelial cells (fibroblasts, 100%; endothelial cells, 492 ± 42%; P < 0.01). Interestingly, rHGF significantly upregulated c-met protein in human fibroblasts (Figure 3a). In addition, rHGF significantly upregulated c-met protein in rat cardiac fibroblasts as well (Figure 3b). Finally, we examined the secretion of endogenously produced HGF in rat cardiac fibroblasts and cardiac myocytes. Interestingly, immunoreactive HGF could be detected in the conditioned medium of cardiac fibroblasts (0.88 ± 0.19 ng · 24 hours⁻¹ · 10⁶ cells⁻¹) and cardiac myocytes (0.33 ± 0.18 ng · 24 hours⁻¹ · 10⁶ cells⁻¹). However, these levels of secreted HGF were quite low compared with that of the conditioned medium of vascular smooth muscle cells (11.3 ± 0.2 ng · 24 hours⁻¹ · 10⁶ cells⁻¹). Overall, these results demonstrated that rHGF upregulated the degradation pathway of extracellular matrix through the induction of MMP-1 and uPA and inhibited the synthetic pathway through the decrease in TGF-β expression via its specific receptor, c-met, without affecting the growth of fibroblasts.
In Vivo Experiments

Because Ang II is a strong negative regulator of local HGF production, we further examined the effect of Ang II on local cardiac HGF production in the cardiomyopathic hamster, because Ang II makes a significant contribution in the pathogenesis of cardiac fibrosis in this model. Indeed, a marked reduction of cardiac HGF mRNA was observed in the cardiomyopathic hamster compared with normal hamster (Figure 4). Consistent with the change in mRNA, cardiac HGF concentration was significantly decreased in the cardiomyopathic hamster compared with normal hamster (P<0.01, Figure 4c). A decrease in cardiac HGF production at 20 weeks of age, the noncompensatory phase, might contribute to the development of cardiac fibrosis.

Therefore, cardiomyopathic hamsters were treated with temocapril, CS-866, or vehicle. Treatment of cardiomyopathic hamsters with temocapril or CS-866 for 8 weeks decreased blood pressure (P<0.05) compared with cardiomyopathic

| Effect of Antihypertensive Drugs on Body Weight, Blood Pressure, and Heart Rate in Hamsters |
|----------------------------------|-------|------|-------|-------|------|------|
| BW at 20                        |       |      |       |       |      |      |
| wk, g                           |       |      |       |       |      |      |
| Normal (Bio F1b)                | 150±3 | 174±5| 123±4 | 140±3 | 366±14|
| Control (Bio 14.6)              | 130±2*| 156±6| 116±5 | 129±4 | 336±16|
| Bio 14.6+temocapril             | 129±2*| 129±3†| 98±6  | 109±4†| 338±15|
| Bio 14.6+CS-866 1 mg/kg         | 129±2*| 130±4†| 89±5‡ | 103±4‡| 333±21|
| Bio 14.6+CS-866 10 mg/kg        | 129±2*| 116±6‡| 81±5‡ | 92±4‡ | 333±21|

BW indicates body weight; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; and HR, heart rate.

*P<0.01 vs normal hamster (Bio F1b); †P<0.05 vs control hamster (Bio 14.6); ‡P<0.01 vs control hamster (Bio 14.6)
hamsters treated with vehicle (Table). Heart rate did not differ among all the groups (Table). Interestingly, administration of temocapril or CS-866 resulted in significant inhibition of myocardial fibrotic area compared with vehicle (Figure 5, \( P < 0.01 \)). In contrast, hydralazine treatment did not affect fibrotic area (vehicle, 16.7 ± 3.6% versus hydralazine, 14.5 ± 4.1%, \( P = \text{NS} \)), although hydralazine treatment decreased blood pressure similarly to other drug treatment (mean blood pressure, hydralazine, 99 ± 3 mm Hg). Similarly, hydralazine treatment did not alter cardiac HGF concentration (vehicle, 1.9 ± 0.3 ng/g tissue versus hydralazine, 2.4 ± 0.5 ng/g tissue, \( P = \text{NS} \)). Therefore, the reduction in fibrotic area observed in this study is most likely due to a direct action of HGF by inhibition of the Ang II pathway rather than due to a reduction in blood pressure.

In contrast, cardiac HGF mRNA expression and HGF concentration were significantly increased in cardiomyopathic hamsters treated with temocapril or CS-866 compared with vehicle (Figure 4, \( P < 0.01 \)). To confirm the antifibrotic action of HGF, we also measured mRNA level of collagen III. Of importance, mRNA expression of collagen III was markedly decreased by treatment with temocapril or CS-866 compared with vehicle, whereas cardiac collagen III mRNA was significantly increased in cardiomyopathic hamsters compared with F1b control hamsters (Figure 6). In contrast, there was no significant change in GAPDH mRNA among all groups.

**Discussion**

Numerous morphological changes can be observed in the human failing myocardium due to dilated cardiomyopathy. These changes include (1) an enlarged extracellular space, that is, 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 various sizes and shapes, lack of contractile material, and disorganization of the cytoskeleton; and (3) the occurrence of hypertrophied and atrophied myocytes as well as cells of normal size. Thus, it is hypothesized that an interaction exists between myocytes and the extracellular matrix. 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 hamsters. Increased collagen synthesis may impair cardiac function in the development of cardiomyopathy. Therefore, a therapeutic approach to alter the fibrosis directly by means of growth factors may open a new therapeutic concept in dilated cardiomyopathy.

From this viewpoint, HGF should be the center of interest, because the previous studies and the present study clearly demonstrated that HGF acted as an antifibrotic growth factor. Thus, increased local HGF results in elevated levels of cell-associated...
We have previously reported that Ang II and TGF-β inhibited TGF-β protein and mRNA). HGF and c-met in the normal or cardiomyopathic hearts. Further study is necessary to characterize cellular localization of the conditioned medium of cardiac fibroblasts and myocytes, and also inhibited MMP-1 activity, etc. and inhibition of matrix-producing pathway (decrease in TGF-β protein and mRNA).

matrix-degrading enzymes and enhanced plasmin-generating activity in these cells. These studies link HGF/SF-Met signaling to the activation of proteases that mediate dissolution of the extracellular matrix basement membrane, an important property for the inhibition of fibrosis. More importantly, HGF is secreted by mesenchymal cells as an inactive precursor (pro-HGF). Interestingly, uPA activates pro-HGF, and activation of pro-HGF involves the formation of a stable complex between pro-HGF and uPA, suggesting that the biological effects of HGF can be titrated by the level of uPA activity. Locally, induction of uPA by HGF may condition the tissue microenvironment by rendering HGF bioavailable to its target cells. Importantly, rhHGF also upregulated its specific receptor, c-met. The autoloop between HGF and c-met may initiate a chain reaction of antifibrotic actions. Although HGF could be detected in the conditioned medium of cardiac fibroblasts and myocytes, further study is necessary to characterize cellular localization of HGF and c-met in the normal or cardiomyopathic hearts.

How did HGF stimulate MMP-1 and inhibit TGF-β? From this viewpoint, the Ets family, essential transcription factors for angiogenesis and vasculogenesis, would be interesting. 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 MMP-1, stromelysin 1, and uPA. Conversely, our preliminary results demonstrated that HGF activated Ets activity in endothelial cells. HGF probably stimulated MMP-1 as well as uPA through the activation of Ets. In contrast, downregulation of TGF-β by HGF is consistent with the previous reports. However, little is known about how HGF inhibited TGF-β expression.

Given that local HGF production in cardiac cells may have a pathophysiological role in fibrosis in an autocrine-paracrine manner, the regulation of local HGF production is important. We have previously reported that Ang II and TGF-β are strong negative regulators of local HGF production. This phenomenon raises the interesting hypothesis that disruption of the autocrine-paracrine local HGF system, which regulates fibrosis, by TGF-β and Ang II may result in abnormal accumulation of extracellular matrix. Negative regulation of HGF by Ang II and TGF-β has also been reported in the hypertrophied heart of spontaneously hypertensive rats. However, the contribution of HGF to cardiac fibrosis has not yet been clarified. Therefore, we next focused on the interaction of Ang II and TGF-β with the HGF system in the cardiomyopathic hamster, because activation of the renin-angiotensin system and TGF-β was increased in the fibrotic lesions of cardiomyopathy. Our present study documented a marked reduction of local HGF mRNA and concentration in the myocardium of cardiomyopathic hamsters. As expected, blockade of Ang II by temocapril or CS-966 significantly stimulated local HGF expression and production, accompanied by inhibition of myocardial fibrosis. Of importance, the increased local HGF production by Ang II blockade may participate in the inhibition of fibrosis, because HGF stimulated the degradation pathway of extracellular matrix and inhibited the collagen synthetic pathway (Figure 7). Indeed, the present study demonstrated that blockade of Ang II resulted in a significant decrease in the fibrotic area and expression of collagen type III, accompanied by a marked increase in HGF expression. Because in dilated cardiomyopathy a dense endomysial woven network consisting of fine fibrils was associated predominantly with collagen types I and III, a decrease in collagen type III may participate in the improvement of cardiac function. It is noteworthy that administration of an ACE inhibitor decreased the mortality and mobility of the patients with myocardial infarction and cardiomyopathy, fibrotic cardiovascular diseases, in human subjects. Increased local HGF production may participate in the improvement of cardiac function through the inhibition of fibrosis observed in those cases treated by blockers of Ang II, in addition to the blockade of Ang II–mediated cardiac hypertrophy. Alternatively, an increase in locally produced bradykinin via ACE inhibition by temocapril or Ang II type 2 receptor stimulation by CS-866 might affect cardiac fibrosis, because ACE is a rate-limiting step in the bradykinin pathway and Ang II type 2 receptor induces bradykinin action. Further studies are necessary to elucidate the role of bradykinin in the cardiac fibrosis.

Overall, the present studies demonstrated that Ang II blockade prevented myocardial fibrosis in cardiomyopathic hamsters, accompanied by a significant increase in cardiac HGF production. Together with in vitro data that HGF stimulated matrix-degrading pathway and inhibited matrix-producing pathway, an increase in local HGF expression may participate in the prevention of myocardial injury by Ang II blockade through its antifibrotic actions. Negative regulation of local HGF production by Ang II and TGF-β may have physiological roles in the fibrotic cardiovascular diseases.

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