Hepatocyte Growth Factor Ameliorates the Progression of Experimental Autoimmune Myocarditis
A Potential Role for Induction of T Helper 2 Cytokines

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Abstract—Hepatocyte growth factor (HGF) plays a role in cell protection, antiapoptosis, antifibrosis, and angiogenesis. However, the role of HGF in the immune system is not well defined. We examined the influence of HGF on T cells and the effects of HGF therapy in acute myocarditis. Lewis rats were immunized on day 0 with cardiac myosin to establish experimental autoimmune myocarditis (EAM). Human HGF gene with hemagglutinating virus of the Japan-envelope vector was injected directly into the myocardium on day 0 or on day 14 (two groups of treated rats). Rats were killed on day 21. Expression of c-Met/HGF receptor in splenocytes and myocardial infiltrating cells was confirmed by immunohistochemical staining or FACS analysis. Myocarditis-affected areas were smaller in the treated rats than in control rats. Cardiac function in the treated rats was markedly improved. An antigen-specific T cell proliferation assay was done with CD4-positive T cells isolated from control rats stimulated with cardiac myosin. HGF suppressed T cell proliferation and production of IFN-γ and increased production of IL-4 and IL-10 secreted from CD4-positive T cells in vitro. Additionally, TUNEL assay revealed that HGF reduced apoptosis in cardiomyocytes. HGF reduced the severity of EAM by inducing T helper 2 cytokines and suppressing apoptosis of cardiomyocytes. HGF has potential as a new therapy for myocarditis. (Circ. Res. 2005;96:823-830.)

Key Words: hepatocyte growth factor ▪ myocarditis ▪ Th1/Th2 cytokines ▪ gene therapy ▪ immune system

Myocarditis is a major cause of dilated cardiomyopathy, and the clinical course of giant cell myocarditis, in comparison to that of lymphocytic myocarditis, is more fulminant.1,2 Immunosuppressive therapy is considered to be efficacious, but it is still controversial; thus, treatment of myocarditis in humans is a major clinical problem.3 The development of myocarditis includes infiltration of mononuclear cells into the myocardium,3 and autoimmunity plays an important role.4 Autoimmunity is particularly associated with giant cell myocarditis. Giant cell myocarditis could, in fact, be caused by autoimmune mechanisms. It is distinguished from lymphocytic myocarditis, which is mainly induced by viral infection. Thus, giant cell myocarditis is a unique and highly fatal form of inflammatory heart disease. Experimental autoimmune myocarditis (EAM), induced in rats by T cell activation,5 is characterized by severe myocardial damage and the appearance of multinucleated giant cells. Therefore, it can be used as a model of human giant cell myocarditis.5 In this study, we examined the role of hepatocyte growth factor (HGF) in myocarditis, especially the relation between HGF and T cell activation, which plays an important role in EAM.

HGF, originally purified and cloned as a potent mitogen for hepatocytes,6,7 has mitogenic, motogenic, morphogenic, antifibrotic, and antiapoptotic activities in various cell types.8,9 These biological activities of HGF are initiated by autophosphorylation of the proto-oncogene c-Met, the receptor tyrosine kinase for HGF.10 Several studies have proposed a role for HGF in immunity,11,12 but that role has not yet been established, and the relation between HGF and T cell activation has not been investigated. Additionally, HGF is a pivotal factor in induction of tolerance, angiogenesis, antiapoptotic, and antifibrosis in several cardiovascular diseases represented by the heart transplant model, the ischemic hind limb model, and the acute and chronic myocardial infarction models.13-16 However, there have been no reports regarding the role of HGF in autoimmune diseases.

In the present study, we investigated the pathophysiological role of HGF in T cell activation and whether HGF gene transfection reduces the development of giant cell myocarditis in rats. We found that HGF is a key regulator in myocarditis. HGF gene transfer inhibited the development of myocarditis by inducing T helper (Th) 2 cytokines and suppressing apoptosis in cardiomyocytes.
Materials and Methods

Animals
Male Lewis rats (7 weeks old, 200 to 250 g) were purchased from Sankyo Laboratories (Tokyo, Japan). They were given a standard diet and water and were maintained in compliance with the animal welfare guidelines of the Institute of Experimental Animals, Tokyo Medical and Dental University.

Antigen and Immunization
Purified porcine cardiac myosin (Sigma Chemical Co) was dissolved in 0.01 mol/L phosphate-buffered saline and emulsified with an equal volume of complete Freund’s adjuvant (Difco) supplemented with Mycobacterium tuberculosis H37RA (Difco). On day 0, rats were injected subcutaneously in the footpad with 0.2 mL emulsion, which yielded an immunizing dose of 1.0 mg cardiac myosin per rat.17

Reagents
FITC-conjugated anti-rat CD4 antibody, FITC-conjugated anti-rat CD8 antibody, streptavidin-PE, isotype-matched control IgG, and biotinylated anti-rabbit IgG antibody were purchased from PharMingen. Anti-rat c-Met antibody was obtained from Santa Cruz Biotechnology, Inc. Plasmid humanized monster green fluorescent protein (phMGFP) was purchased from Promega.

Preparation of Human Recombinant HGF
Human Recombinant HGF (hrHGF) was purified from culture media of Chinese hamster ovary cells transfected with an expression vector containing human HGF cDNA, as previously described.7

Immunohistochemistry
Frozen sections of hearts (5 μm) were incubated with 10% normal serum at room temperature. Sections were then incubated with anti-rat CD4 antibody, anti-rat CD8 antibody, or isotype-matched control IgG overnight at 4°C. Sections were incubated with secondary antibody (biotinylated anti-rabbit IgG) at room temperature for 30 minutes. Antigen-antibody conjugates were detected with avidin-biotin-horseradish peroxidase complex (Nichirei) according to the manufacturer’s instructions. CD4- or CD8-positive cells per high-power field were counted in 5 random fields, and the counts were averaged.

FACS Analysis
Splenocytes and myocardial infiltrating cells were isolated from rats with myocarditis on day 21 as described previously.18 Briefly, cardiac tissue was minced and rocked in Hanks balanced salt solution (Sigma) with 2.5% FCS and collagenase. Dead lymphocytes and red blood cells were removed by centrifugation through Percoll (Amersham), and the resulting interface lymphocytes were washed. Cells were incubated with anti-rat c-Met antibody or isotype-matched control IgG. After incubation with biotinylated anti-rabbit IgG, cells were incubated with FITC-conjugated anti-rat CD4 or anti-rat CD8 antibody and streptavidin-PE. Cells were then analyzed as described previously.19

In Vivo Gene Transfer
Hemagglutinating virus of Japan (HVJ)-envelope vector kit was provided by Ishihara Sangyo Kaisha and prepared as previously described.20 HVJ-envelope vector (200 μL) containing the phMGFP gene (20 μg) or human HGF gene (20 μg), which was inserted into the NotI site of the pUC-SKα expression vector,21 was transfected directly into the hearts after thoracotomy. We transfected directly the HVJ-envelope containing the human HGF gene into rat hearts at 4 sites (each site: 50 μL) on day 0 (early phase; group HGF-E) or on day 14 (late phase; group HGF-L). For control, empty vector was transfected into the hearts (group Vect-E and group Vect-L). Another group of rats received no transfection (group UT). There were 7 rats in each of the 5 groups.

Histological Examination
Hearts were harvested immediately after rats were killed on day 21. We obtained transverse sections for histological examination. Slices were stained with hematoxylin and eosin (HE). The area of myocardium and surrounding tissue affected by myocarditis (consisting of inflammatory cell infiltration and myocardial necrosis) was determined by means of a computer-assisted analyzer (Scion Image beta 4.02, Scion Corporation). The area ratio (affected/entire area expressed as a percentage) was calculated as described previously.18

Echocardiography
A transthoracic echocardiogram was obtained on day 21. Left ventricular fractional shortening (LVFS) was calculated as described previously.18

Apoptotic Assay
Apoptotic cells were detected by TUNEL assay in sections embedded in paraffin for histological examination. TUNEL assay was performed as described previously.22 TUNEL-positive cells per high-power field were counted in 5 random fields, and the counts were averaged.23

Figure 1. HGF expression in rats with myocarditis from 0 to 21 days after immunization. A, Plasma HGF concentration determined by ELISA. B, HGF concentration in cardiac tissues determined by ELISA. HGF concentrations levels in both plasma and cardiac tissues increased significantly after immunization. P<0.05, vs day 0.
CD4-Positive T Cell Proliferation Assay
CD4-positive T cells were isolated from splenocytes in rats with myocarditis on day 18 with a FACS Vantage (Becton Dickinson). We confirmed by FACS that the purity of sorted cells was 95% to 99%. Cells (5 × 10^5 per well) were cultured as previously described in 96-well culture plates with 50 μg/mL purified porcine heart myosin and splenic antigen-presenting cells inactivated by mitomycin C (Sigma Chemical Co) from normal rats.24 Cells were incubated at 37°C under 5% CO2 at various concentrations of hrHGF for 5 days. T cell proliferation was estimated with the Cell Counting Kit-8. Cell proliferation was expressed as an optical density.

Enzyme-Linked Immunosorbent Assay
The concentration of rat or human HGF (rHGF or hHGF) in cardiac tissue or plasma was measured with an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Institute of Immunology) for rats, which reacts with rat HGF but not with human HGF, or an ELISA kit (Institute of Immunology) for humans, which is specific for human HGF (each day: n = 5). Supernatant was collected from cultures used for the T cell proliferation assay. Concentrations of IFN-γ, IL-4, and IL-10 in cardiac tissue or supernatant were determined with an ELISA kit (BioSource International) according to the manufacturer’s instructions.

Statistical Analysis
Values are given as mean±SD. Schéffe ANOVA was used for between-group comparisons (Stat View, SAS Institute, Inc). Differences were considered statistically significant at P < 0.05.

Results
Expression of HGF in Rats With Myocarditis
The plasma HGF concentration increased significantly and reached a peak 7 days after immunization (Figure 1A). The HGF concentration in cardiac tissue of rats with myocarditis on day 18 after immunization was significantly increased...
Additionally, the numbers of infiltrating CD4- or CD8-positive cells increased significantly from day 14.

Expression of c-Met in Splenocytes and Inflammatory Cells
Expression of c-Met in splenocytes and myocardial infiltrating cells was assessed by flow cytometry. We detected expression of c-Met in CD4-positive splenocytes on day 0, and the population of positive cells had increased by day 21 (Figure 2A and 2B). However, we barely detected c-Met expression in CD8-positive splenocytes on day 0 (Figure 2C), and on day 21, a minority of CD8-positive splenocytes were positive for c-Met (Figure 2D). In myocardial infiltrating cells, we detected expression of c-Met in CD4-positive cells, but expression of c-Met in CD8-positive cells was barely detectable (Figure 2E and 2F).

In Vivo Gene Transfer Into the Heart With HVJ Envelope
Injection of the GFP gene resulted in limited expression at the site of injection, as detected by fluorescence microscopy 3 days after transfection (Figure 3A). In contrast, the negative
control, treated only with HVJ-envelope and not the GFP gene, showed no fluorescence (Figure 3B). We measured the HGF protein concentration in the transfected hearts by ELISA. Both the exogenous HGF level, which were hHGF protein concentration, and the endogenous HGF level, which were rHGF protein concentration, increased significantly (Figure 3C).

**In Vivo Effect of HGF on EAM**

The heart weight to body weight ratios in groups HGF-E (treatment group in early phase) and HGF-L (treatment group in late phase) were lower than those of the control groups (Figure 4A). The weight ratios in group Vect-E and group HGF-E were 7.70±1.19 and 4.22±0.74, respectively (P<0.05); the weight ratios in group Vect-L and group HGF-L were 7.90±1.49 and 5.39±1.74, respectively (P<0.05). Severe myocardial lesions were observed in the hearts of all control rats on day 21 (Figure 4B). These lesions showed extensive necrosis and infiltration by mononuclear cells and polymorphonuclear neutrophils (Figure 4C). However, myocardial lesions were rarely observed in hearts of rats treated with HGF (Figure 4D). In the groups treated with HGF, there was little infiltration of inflammatory cells or myocardial necrosis (Figure 4E). The myocarditis-affected area rates in groups HGF-E and HGF-L were lower than those of the control groups (Figure 4F). Area ratios in group Vect-E and group HGF-E were 51.9±10.6% and 15.5±13.3%, respectively (P<0.05); area ratios in group Vect-L and group HGF-L were 55.1±5.9% and 24.5±5.7%, respectively (P<0.05). On day 21, LVFS in the control groups was only 20% (Figure 4G). HGF improved LVFS up to 40% in the treatment groups (Figure 4H). LVFS in groups HGF-E and HGF-L showed better improvement than that of either control group (Figure 4I). LVFS improved more in group HGF-E than in group Vect-E and more in group HGF-L than in group Vect-L: 40.0±6.2% versus 22.7±4.1% (P<0.05) and 37.5±5.8% versus 23.7±5.8% (P<0.05), respectively. The heart weight to body weight ratios, myocarditis-affected areas, and cardiac function in group Vect-E and group Vect-L did not differ significantly from those in group UT (each group: n=7).

**Inhibition of CD4- or CD8-Positive Cells Expression by HGF**

We confirmed the expression of CD4- or CD8-positive cells in control groups on day 21. Either CD4- or CD8-positive cells were fewer in treatment groups than in control groups on day 21 (Figure 5).

**HGF Suppressed Myocyte Apoptosis In Vivo**

Apoptosis detected by TUNEL was observed in group Vect-E (Figure 6A). However, we could barely detect apoptotic cardiomyocytes in group HGF-E (Figure 6B). The HGF gene therapy resulted in a significant reduction of the incidence of apoptotic cardiomyocytes (Figure 6C).

**HGF Effect on Cytokines in Hearts**

ELISA was used to examine expression of cytokines in the myocardium on day 21. Levels of IFN-γ in groups HGF-E and HGF-L were significantly decreased in comparison to levels in control groups (n=4, each). In contrast, levels of IL-4 and IL-10 in groups HGF-E and HGF-L were significantly increased (Figure 7).

**Antigen-Specific CD4-Positive T Cell Proliferation Assay**

We previously confirmed the strongest response to cardiac myosin on day 18, so we used specimens on day 18 for cell proliferation assay to examine the effect of HGF on antigen-induced CD4-positive T cell proliferation. Antigen-induced CD4-positive T cell proliferation was suppressed by HGF (Figure 8A). ELISA analysis of supernatants after incubation of antigen-induced CD4-positive T cells with cardiac myosin revealed that production of IFN-γ was suppressed significantly by HGF and that production of IL-4 and IL-10 was increased significantly by HGF (Figure 8B through 8D).

**Discussion**

HGF plays important roles in a variety of cardiovascular diseases through its angiogenesis, antifibrosis, and antiapoptotic activities. Several studies have defined the role of HGF with regard to immunity in acute and chronic renal rejection after transplantation and acute graft-versus-host disease models. However, the role of HGF in T cell-
mediated autoimmune disease has not been reported, and relations between HGF and T cell activation have not been elucidated. To the best of our knowledge, we are the first to find that HGF plays an important role in T cell activation and that HGF gene therapy suppresses the development of autoimmune myocarditis.

We investigated HGF concentrations in plasma and cardiac tissues in relation to myocarditis by using an EAM model. HGF concentration in both plasma and cardiac tissues increased rapidly in response to immunization. Similar rapid induction of HGF was noted in the case of ischemia/reperfusion, acute hepatic, and renal injuries. The increased HGF concentration may reflect defensive reactions. In addition to the increase in HGF concentration, expression of c-Met receptor was upregulated in splenocytes and myocardial infiltrating cells. Expression of c-Met in splenocytes and in myocardial infiltrating cells has not been reported. We detected upregulation of c-Met receptor in both CD4-positive and CD8-positive cells after immunization, and these findings support the hypothesis that the increase in c-Met expression is related to autoinduced expression triggered by HGF. Additionally, expression of c-Met after immunization was greater in CD4-positive cells than in CD8-positive cells. The intense expression of c-Met in CD4-positive T cells indicates that HGF plays an important role in EAM because the development of EAM involves autoreactive CD4-positive T cell proliferation.

We transfected the HGF gene directly into cardiomyocytes using the HVJ-envelope method to examine whether HGF would reduce the development of acute myocarditis. The simultaneous GFP expression and detection of hHGF showed that human HGF gene transfer into cardiomyocytes by the HVJ-envelope method resulted in efficient transfection of the gene into the rat hearts. Additionally, we found that levels of endogenous HGF were increased by human HGF gene transfection. These observations are consistent with the previous reports that exogenous HGF enhances autolooped positive feedback on endogenous HGF production.

Our in vivo studies revealed that treatment by HGF gene transfer on day 0 inhibits the development of EAM by day 21. Exogenous HGF produced through HGF gene transfection may cooperate with endogenous HGF and suppress CD4- or CD8-positive cells infiltration. In addition, we applied the therapy on day 14 to examine its effect on established disease. HGF gene therapy was effective not only in preventing development of the disease but also in attenuating established inflammation. This result is important for clinical treatment because human myocarditis is usually diagnosed after onset.

Figure 6. Reduction of apoptosis by HGF gene transfer. Apoptosis of cardiomyocytes in the control group (A) and HGF-treated group (B) on day 21. Graph shows counts of TUNEL-positive cells (C).

Figure 7. Expression of cytokines in the heart. ELISA was used to examine production of cytokines in hearts on day 21. Levels of cytokine for IFN-γ in treatment groups decreased significantly (A). However, levels of cytokine for IL-4 and IL-10 in the treatment groups significantly increased (B and C).
Inhibition of antigen-specific T cell proliferation can be a therapeutic strategy for retarding the development of myocarditis, but it is still unclear whether HGF affects antigen-specific T cell proliferation. We are the first to provide evidence that HGF suppresses secondary T cell responses, especially those of CD4-positive T cells, after myosin immunization. HGF reduced myosin-specific CD4-positive T cell proliferation in EAM. T cell proliferation was modulated by the Th1-Th2 balance and suppressed by activation of Th2 cytokines. Th2 cytokines mainly inhibit the number of CD4 cells, which secrete Th1 cytokines. Previous studies showed that dysfunction of the immune system associated with autoimmune disease is related to the imbalance between Th1 and Th2 cells. The altered Th1-Th2 balance regulates the clinical course of EAM and IL-10 plays a protective role(s) during the development of myocarditis. Thus, we examined production of Th1 cytokines, IFN-γ, and Th2 cytokines, IL-4 and IL-10, by myosin-specific CD4-positive T cells in response to cardiac myosin. We found that production of IFN-γ was reduced and that production of IL-4 and IL-10 was increased by HGF; these findings show that HGF promotes the shift from Th1 to Th2. Similar changes in cytokines were found in vivo. These results are supported by previous findings that the Th1-to-Th2 shift prevented the development of EAM. HGF inhibits Th1 cytokines, such as IFN-γ, in an acute graft-versus-host disease model; however, the mechanism is unknown. Thus, our results suggest that Th2 cytokines induced by HGF suppress production of Th1 cytokines and the development of EAM.

Our results show that suppression of the antigen-specific immune responses by HGF contributed to therapeutic outcomes during the progression of EAM, but the effect was partial and not enough to completely block the immune mechanisms. Of interest, we found during the natural course of myocarditis that surviving cardiomyocytes strongly express c-Met/HGF receptor (data not shown). This expression in cardiomyocytes is compatible with findings in other cardiovascular models. We showed that the number of TUNEL-positive cardiomyocytes was significantly less in HGF-treated groups than in vector control groups. Given that HGF is a potent antipapoptotic factor with respect to cardiomyocytes, a direct antipapoptotic effect toward the heart parenchymal cells should be considered.

It has been reported that c-Met and HGF are involved in cardiac stem cell biology. Bone marrow–derived mesenchymal stem cells expressed high level of HGF. Also, HGF administration enhanced the efficacy of cellular cardiomyoplasty. However, the role of cardiac stem cells in myocarditis is still not clear. Further studies are needed to investigate the role of cardiac stem cells and the relation between HGF and cardiac stem cells in myocarditis.

The present study revealed crucial roles of HGF in the development of T cell–mediated immune disease. Exogenous administration of HGF attenuated the development of EAM through induction of endogenous HGF, which is associated with reduction in myocyte apoptosis, suppression of Th1 cytokines, and increase in Th2 cytokines. These findings indicate that gene therapy targeting HGF has potential as a treatment strategy for clinical myocarditis.

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