Postinfarction Treatment With an Adenoviral Vector Expressing Hepatocyte Growth Factor Relieves Chronic Left Ventricular Remodeling and Dysfunction in Mice

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Background—Hepatocyte growth factor (HGF) is implicated in tissue regeneration, angiogenesis, and antiapoptosis. However, its chronic effects are undetermined on postinfarction left ventricular (LV) remodeling and heart failure.

Methods and Results—In mice, on day 3 after myocardial infarction (MI), adenovirus encoding human HGF (Ad.CAG-HGF) was injected into the hindlimb muscles (n = 13). As a control (n = 15), LacZ gene was used. A persistent increase in plasma human HGF was confirmed in the treated mice: 1.0 ± 0.2 ng/mL 4 weeks later. At 4 weeks after MI, the HGF-treated mice showed improved LV remodeling and dysfunction compared with controls, as indicated by the smaller LV cavity and heart/body weight ratio, greater % fractional shortening and LV +dP/dt, and lower LV end-diastolic pressure. The cardiomyocytes near MI, including the papillary muscles and trabeculae, were greatly hypertrophied in the treated mice. The old infarct size was similar between the groups, but the infarct wall was thicker in the treated mice, where the density of noncardiomyocyte cells, including vessels, was greater. Fibrosis of the ventricular wall was significantly reduced in them. Examination of 10-day-old MI revealed no proliferation or apoptosis but showed augmented expression of c-Met/HGF receptor in cardiomyocytes near MI, whereas a greater proliferating activity and smaller apoptotic rate of granulation tissue cells in the HGF-treated hearts was observed compared with controls.

Conclusions—Postinfarction HGF gene therapy improved LV remodeling and dysfunction through hypertrophy of cardiomyocytes, infarct wall thickening, preservation of vessels, and antifibrosis. These findings imply a novel therapeutic approach against postinfarction heart failure. (Circulation. 2003;107:2499-2506.)

Key Words: congestive heart failure ■ gene therapy ■ hepatocyte growth factor ■ myocardial infarction

Hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes,1,2 was reported to have mitogenic, angiogenic, antiapoptotic, and antifibrotic activities in various cells, preferentially in most epithelial and endothelial cells.3,4 Recent studies, however, reported cardioprotective effects of HGF: HGF protected cardiomyocytes from acute ischemic death during acute myocardial infarction (MI)5,6 and enhanced survival of cardiomyocytes subjected to oxidant stress.6,7 These studies focused on the effects of HGF on cardiomyocytes under acute stress.

However, MI subsequently causes left ventricular (LV) remodeling when the infarct size is large, which is characterized by ventricular dilatation, diminished cardiac performance, and poor recovery of function.8 Patients with large-sized MI have a high risk of developing heart failure, even if they escape acute-phase death. Actually, the patients with postinfarction heart failure, one of the most serious clinical problems, constitute 44% of candidates for cardiac transplantation.9 The extent of cardiomyocyte death during the acute phase of MI is indeed a critical determinant of the subsequent ventricular remodeling with eventual heart failure, but the complex process of remodeling is not limited to the size of the acute infarct10,11; hypertrophic responses occur in cardiomyocytes in the surviving portion of the ventricle, and ventricular dilatation follows because of an architectural rearrangement of the myocardium, including cardiomyocytes and interstitial cells. Moreover, in clinical settings, preinfarction treatments...
are not practical. Recently, Miyagawa et al.\textsuperscript{12} injected hemagglutinating virus of Japan (HVJ)-liposomes bearing the human HGF gene directly into the postinfarction rat heart but found no beneficial effects on ventricular remodeling or dysfunction. They started the therapy 2 weeks after MI, which we assume may have been too late to be effective. Meanwhile, Taniyama et al.\textsuperscript{13} recently reported beneficial effects of HGF on cardiac function in an animal model of cardiomyopathy through its angiogenic and antifibrotic actions. The hypothesis in the present study is that the postinfarction treatment with HGF may improve chronic heart failure by affecting the LV remodeling process. In the present study, we started adenovirus-mediated transduction of the HGF gene into mouse hindlimbs (systemic transfection) on day 3 after MI, a time at which the therapy does not effect acute ischemic death of cardiomyocytes, and examined its effects on postinfarction heart failure during the chronic stage.

Methods

Experimental MI of Mice

The study was approved by our Institutional Animal Research Committee and conformed to the animal care guidelines of the American Physiological Society. MI was created in 55 male 10-week-old C57BL/6J mice (Chubu Kagaku, Nagoya, Japan) by ligation of the left coronary artery.\textsuperscript{14} On day 3 after MI, 38 mice survived and were entered the study.

Recombinant Adenoviral Vectors

Adenoviral vector plasmid pAd-HGF, which comprises cystemegalo-virus immediate-early enhancer, a modified chicken β-actin promoter, and human HGF cDNA (Ad.CAG-HGF), was constructed by the in vitro ligation method (a kind gift from Dr Mark A. Kay, Stanford University School of Medicine) as described previously.\textsuperscript{15} Control Ad-LacZ was prepared as described previously.\textsuperscript{16}

On day 3 of MI, Ad.CAG-HGF (1×10\textsuperscript{9} pfu/mouse) was injected into the hindlimbs muscles of mice (n=13). As a control, adenovirus encoding LacZ gene was injected similarly to the other 15 mice. These groups were followed up until 4 weeks after MI. In the other experiment, 10 mice on day 3 of MI were divided into the HGF- and LacZ-gene–treated groups (n=5 each), and the survivors (n=4 of the HGF group and n=3 of the LacZ group) were examined at day 10 after MI. The assignment of the animals into groups was performed randomly.

Measurement of Human HGF Level in the Plasma

The plasma concentration of human HGF was measured by use of an ELISA kit (Institute of Immunology).

Biological Assay of Human HGF on Mice

The biological activity of human HGF in mice was assessed from the proliferating activity of hepatocytes. On day 3 after virus injection, injection of bromodeoxyuridine (BrdU, 50 mg · kg\textsuperscript{-1} · d\textsuperscript{-1} IP) was started and continued for 4 days until the animals were killed. In the liver sections, the number of BrdU-positive hepatocytes was counted.

Physiological Studies

Echocardiograms were recorded with an echocardiographic system (Aloka) equipped with a 7.5-MHz imaging transducer at 4 weeks after MI. After cardiac echocardiography, the right carotid artery was cannulated with a micromanometer-tipped catheter (SPR 407, Millar Instruments) and advanced into the aorta and then into the left ventricle for recording pressures and ±dP/dt.

Histological Analysis

After measurements, all surviving mice were killed, and the hearts were removed. The hearts were cut into 2 transverse slices, and the basal specimens were fixed with 10% buffered formalin and embedded in paraffin. Sections 4 μm thick were stained with hematoxylin-eosin and Sirius red F3BA (0.1% solution in saturated aqueous picric acid) (Aldrich).\textsuperscript{17} Quantitative assessments including cell size, cell population, and fibrotic area were performed with a multipurpose color image processor, LUZEX F (Nireco).

Immunohistochemical Analysis

The ABC kit (Dako) was used for immunohistochemistry. The sections were incubated with primary antibodies against BrdU (Dako) at a dilution of 1:100, c-Met (sc-162, Santa Cruz) at 1:100, Flk-1 (Sigma) at 1:100; and anti-macrophage (ED-1, Serotec) at 1:100. Immunostains were visualized by use of diaminobenzidine hydrochloride.

In Situ Nick End-Labeling

Terminal dUTP nick end-labeling (TUNEL) assay was performed in deparaffinized sections 4 μm thick with an ApopTag kit (Intergen).

Double Immunohistochemistry

Sections were stained first with anti-BrdU antibody or TUNEL as described above. Then the sections were stained with the second primary antibody against Flk-1, which was visualized with VIP substrate (Vector) as previously described.\textsuperscript{18}

Immunoprecipitation and Western Blotting for c-Met

An immunoprecipitation assay of the lysate of heart tissues was performed with Ultra-Link Biosupport medium (Pierce) with anti-c-Met antibody (sc-162, Santa Cruz). Subsequently, the isolated protein was analyzed by Western blotting using the same antibody. Five hearts from each group (normal hearts, 10-day-postinfarction hearts treated with LacZ, and 10-day-postinfarction hearts treated with HGF) and 3 normal livers were subjected to the assay.

Statistical Analysis

Values are shown as mean±SEM. Analyses of survival after day 3 after MI were performed by the Kaplan-Meier method with the log-rank Cox-Mantel method. The significance of differences was evaluated by Student’s t test, and a difference at P<0.05 was considered significant.

Results

Plasma Levels of Human HGF

Plasma human HGF levels reached 1.9±0.16 ng/mL at 10 days after the onset of MI (1 week after the viral transfection), and 4 weeks later, it was 1.0±0.24 ng/mL in the HGF-treated mice (Figure 1A). No human HGF was detected in the LacZ-treated mice at any time.

Bioactivity of Human HGF on Mice

To check the bioactivity of human HGF in mice, we examined the proliferating activity of hepatocytes on the basis of BrdU uptake index of the liver tissue. One week after the treatments, BrdU-positive hepatocytes were >3-fold greater in the HGF-treated mice than in the controls (P<0.05), confirming effective bioactivity of human HGF in mice (Figure 1B).
Mice at 4 Weeks After MI

Survival Rate
Within 1 week after MI, 4 (27%) of the controls and 3 (23%) of the HGF-treated mice were dead. Of note, an additional 2 mice (13%) were dead in the controls, whereas all mice survived during the subsequent 3 weeks. In total, the survival rate was 60% in the control and 77% in the HGF-treated groups at 4 weeks after MI ($P=NS$) (Figure 2A).

Physiological Studies
According to echocardiography and cardiac catheterization at 4 weeks after MI, the LacZ-treated mice showed severe LV remodeling, with a marked enlargement of the LV cavity and signs of decreased cardiac function (Figure 2): decreased LV percent fractional shortening and $\pm dP/dt$ and an increased LV end-diastolic pressure. Compared with the controls, the HGF gene therapy significantly improved each of these conditions, indicating improvements of postinfarction remodeling and cardiac function.

Pathological Studies
There was no significant difference in heart weight and in the ratio of heart to body weight between the groups. Although the control hearts with 4-week-old MI showed marked LV dilatation with a thin infarct segment, the HGF-treated hearts presented a smaller LV cavity (Figure 3, A and B). Both the absolute area of the old infarct and the percentage of the old infarct area to the whole LV area were similar between the controls and HGF-treated mice (Figure 3, C and D). However, the thickness of the old infarct wall, but not of the noninfarct wall, was greater in HGF-treated mice than in the controls (Figure 3, E and F). In the LV remote from the old infarct, surviving cardiomyocytes measured at the transverse diameter showed no significant difference between the groups, whereas in the LV near the old infarct, it was significantly greater in the HGF-treated mice ($16.8\pm0.4 \mu m$) than in the controls ($14.1\pm0.3 \mu m$) (Figure 3G). Both values were significantly greater than the data with the same areas in age-matched intact hearts without MI ($11.1\pm0.2 \mu m, n=5$ mice). Of note, the LV papillary muscles and trabeculae were markedly developed in the HGF-treated mice, the area of which was significantly greater than that of the controls (Figure 3H).

The old infarct area of control mice was replaced by fibrous scar tissue containing scanty fibroblasts and vessels. However, that of the HGF-treated mice contained not only collagen fibers and fibroblasts but also abundant vessels. Populations of noncardiomyocytes and vessels in the old infarct area were significantly greater in the HGF-treated mice (450\/HPF and 253\/HPF) compared with those in the controls (306\/HPF and 21\/HPF).
185±36/HPF) (Figure 4, A and B). However, macrophages were rarely preserved in either group, and their population was similar between the groups (data not shown). Fibrosis of the old infarct area assessed in Sirius red-stained sections appeared attenuated in the HGF-treated mice compared with the controls. The amount of fibrosis was also significantly reduced in the noninfarct LV walls of the HGF-treated mice (percent collagen fiber area, 2.6±0.2% versus 4.9±0.45% in LacZ, P<0.001) (Figure 4C).

There was no significant difference in body weight between the groups at 4 weeks after MI. Liver weight of the HGF-treated mice (1.79±0.010 g) was found to be significantly greater than that of the control mice (1.32±0.05 g, P<0.05), with no histological abnormalities such as congestion or tumor formation. Despite the weight of the lungs not being significantly different between the groups, pulmonary congestion was apparently less slight in the HGF-treated mice. The weights of the other extracardiac organs, such as kidneys, spleen, and testes, were not significantly different between the groups, and no histological abnormality was found in them in any group.

Mice at 10 Days After MI

To clarify the mechanisms responsible for the improvement of LV remodeling and dysfunction in the hearts treated with
the HGF gene during the chronic stage of MI, we examined mouse hearts at 10 days after MI (1 week after gene transfection).

**Expression of c-Met**
According to immunohistochemistry, c-Met was slightly stained in vessels alone, not in cardiomyocytes in normal myocardium. However, the immunohistochemical expression showed specific localization in the hearts at 10 days after MI (Figure 5A): the immunostain was markedly intense in the surviving cardiomyocytes near the infarct and in those of papillary muscles, whereas the myocardium remote from the infarct was only slightly stained. In the granulation tissue of the infarct area, positive immunoreaction was observed primarily in capillary endothelial cells, vascular smooth muscle cells, and myofibroblasts.

Western blot analysis followed by immunoprecipitation revealed apparently enhanced expression of HGF receptor/c-Met in the heart tissues at 10 days after MI from both LacZ- and HGF-treated groups compared with intact mouse hearts (Figure 5B). The expression level was greater in the HGF-treated hearts compared with the LacZ-treated hearts.

**Proliferation and Apoptosis**
BrdU-positive cells occupied 4.9±2.0% of granulation tissue cells (noncardiomyocytes) of control hearts with MI, whose incidence was significantly greater in the HGF-treated hearts (18.0±2.0%) (Figure 6A). In contrast, BrdU-positive cardiomyocytes were not observed in hearts of each group.

Apoptosis indicated by TUNEL was observed in granulation tissue cells from the infarct area (apoptotic index, 7.9±1.4% in the controls). The HGF gene therapy resulted in a significant reduction of the incidence of TUNEL-positive cells (2.6±1.0%, P<0.05) (Figure 6A). Cardiomyocyte apoptosis, however, was substantially absent in both control and HGF-treated groups in TUNEL-stained preparations.

According to double immunohistochemistry for Flk-1 and BrdU or TUNEL, the HGF gene therapy resulted in a significant increase in proliferation of vascular endothelial cells and conversely, a significant decrease in their apoptosis (Figure 6B).
Discussion

The present study revealed that postinfarction HGF gene therapy, started on day 3 after the onset of MI, alleviated LV remodeling and dysfunction during the chronic stage.

Mechanisms of Beneficial Effects of HGF on Postinfarction Heart Failure

The mechanisms responsible for the beneficial effects of HGF on postinfarction heart failure seemed very complicated, probably reflecting the multiple functions of HGF. The biological effects of HGF, such as cell proliferation, angiogenesis, and antifibrosis, were already reported in previous studies. Moreover, HGF induced angiogenic and antifibrotic effects in a model of cardiomyopathy and heart failure. The present study confirmed these facts. However, the most remarkable finding in the present study was cardiomyocyte hypertrophy at the edges of the old infarct, including papillary muscles and trabeculae, accompanied by overexpression of c-Met: perhaps one of the most important mechanisms contributing to the beneficial effects of HGF on postinfarction cardiac function. Furthermore, we would like to point out the importance of the significant thickening of the infarcted wall of the HGF-treated hearts as one mechanism for preventing the vicious circle of postinfarction ventricular remodeling: in general, wall stress, which accelerates ventricular dilatation, is more markedly augmented in the thinner wall, according to Laplace’s law. These are mechanistic considerations of the beneficial effects of HGF that are newly proposed in the present study.

The above hypertrophy of cardiomyocytes in the HGF-treated hearts could not be explained simply by the compensatory response, because it was more markedly exaggerated in the HGF-treated hearts, in which heart failure was less severe than in the LacZ-treated hearts. In addition, in the hypertrophied cardiomyocytes, c-Met/HGF receptor was markedly expressed, which is compatible with previous

Figure 5. A, c-Met immunohistochemistry. A1, Normal myocardium. Arrow indicates a small vessel. A2 and A3, Border zone between infarct area (MI) and surviving myocardium of heart treated with LacZ (A2) or with HGF (A3). A4, Infarct area of HGF-treated heart at 10 days after MI containing papillary muscle (double arrows). Magnification: A2, ×100; A1, A3 and A4, ×200. B, Immunoprecipitation followed by Western blotting for c-Met.

Figure 6. A, Graph showing comparisons of percentages of BrdU-positive or TUNEL-positive cells between LacZ- and HGF-treated mice 10 days after MI. B, Graph showing comparisons of densities of BrdU-positive or TUNEL-positive vascular endothelial cells between LacZ- and HGF-treated mice 10 days after MI.
findings6,20: the increase in c-Met may related to the autoinduction of gene expression triggered by endogenous HGF.6 Cardiomyocyte hypertrophy is one of the compensatory responses in the failing heart. However, it is generally accepted that pathological hypertrophy of cardiomyocytes is deleterious to cardiac function. A longer follow-up of the animals would be necessary in future to rule out the possible adverse effects of this cardiomyocyte hypertrophy.

The thickened infarcted wall of the HGF-treated hearts consisted of ~50% greater cell population than in the 4-week-old infarct scar of the control hearts. Those cells were destined to disappear via apoptosis during the natural course of infarct healing.18 Double immunohistochemistry revealed that the HGF gene therapy significantly prevented apoptosis of vascular endothelial cells during the subacute stage of MI. Moreover, it also revealed that HGF increased the proliferative activity of those cells. Thus, both deceleration of apoptosis and acceleration of proliferation during the subacute stage of MI might have accounted for the increase in the vascular endothelial cell population of old infarcts in the HGF-treated hearts during the chronic stage.

Because the HGF gene therapy was started on day 3 after MI in the present study, it is unlikely that the therapy influenced the prevention of cardiomyocyte apoptosis during the acute phase of MI. It is also unlikely that the therapy affected the survival of cardiomyocytes through apoptosis inhibition at the subacute and chronic stages of MI, because cardiomyocyte apoptosis was insubstantial at these stages, being inconsistent with earlier findings.10

In the present study, cardiomyocytes with positive BrdU, a marker of proliferative activities, were not observed in either of the LacZ-treated and HGF-treated hearts. In addition, the heart weights, old infarct areas, and noninfarct areas of the left ventricle were similar between the groups, although cardiomyocyte size was similar or greater in the HGF-treated hearts. This does not suggest that there was much increase in the number of cardiomyocytes in the HGF-treated hearts; that is, the HGF treatment may have not induced translocation of such a large number of cardiomyocytes from bone marrow stem cells, as shown by colony-stimulating factors.24 Further investigation is warranted.

Study Limitations

Cytokines other than HGF were not examined in the present study. However, several cytokines are known to influence cardiac function and may play important roles particularly in pathological situations, such as MI.22,23 The possibility would be great that HGF critically modulates the postinfarction process through interaction with the other cytokines.

Miyagawa et al12 injected HVJ liposomes bearing the HGF gene directly into the postinfarction rat heart but found no beneficial effects on ventricular remodeling or dysfunction. That report seems to be in conflict with our present data. However, there are major differences in the methods between that study and ours: (1) the animal species was different: Miyagawa et al used rats; (2) they transfected HGF gene not with adenovirus but rather with HVJ liposomes, not systemically but rather by direct injection into the hearts; and (3) they injected the HGF gene 2 weeks after MI, not on day 3 of MI, when the infarct had already begun to transition into scar tissue. Considering the effects of HGF on granulation tissue cells as revealed by our study, 2 weeks after MI might be too late for the treatment. Therefore, those different factors might have a strong bearing on the ineffectiveness of HGF reported in that study.

Clinical Implications

Rapid recanalization of the occluded coronary artery, which results in salvaging of the ischemic myocardial cells, would be the best clinical approach for acute MI at present. Unfortunately, most patients actually lose their chance of coronary reperfusion therapy, because it should be performed within hours after the onset of infarction.24 The present findings may imply a novel therapeutic strategy against chronic progressive heart failure in patients with large MI, which can be performed even in patients who have lost their chance for coronary reperfusion during the acute stage.

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