Gene transfection of hepatocyte growth factor attenuates the progression of cardiac remodeling in the hypertrophied heart

Keiji Iwata, MD,^a Yoshiki Sawa, MD,^a Satoru Kitagawa-Sakakida, MD,^a Naomasa Kawaguchi, MD,^c Nariaki Matsuura, MD,^c Toshikazu Nakamura, MD,^b and Hikaru Matsuda, MD^a

Objectives: Hepatocyte growth factor plays a significant role in angiogenesis, anti-apoptosis, and anti-transforming growth factor- β 1-mediated fibrosis in several organs. In this study, we investigated the effect of transfection of the hepatocyte growth factor gene in attenuation of cardiac remodeling in the hypertrophied heart.

Methods: Two weeks after banding the ascending aorta of male Sprague-Dawley rats, a hemagglutinating virus of Japan-liposome complex with (H group) or without (C group) human hepatocyte growth factor cDNA was transfected into the left ventricle wall by direct injection. The hepatocyte growth factor, c-Met, and transforming growth factor- β 1 mRNA levels in the left ventricle were then analyzed by real-time quantitative reverse-transcriptase polymerase chain reaction.

Results: Two weeks after transfection, the expression of transforming growth factor- β 1 mRNA was significantly attenuated in the H group compared with the C group ($P < .01$). Myocardial collagen content after 4 weeks of banding was significantly lower in the H group (5.0 ± 0.6 mg/g tissue) than in the C group (7.4 ± 0.5 mg/g tissue, $P < .01$). Left ventricular diastolic function (E/A ratios quantified by Doppler echocardiography) showed a significant increase in the H group (1.9 ± 0.1) compared with the C group (1.1 ± 0.1 , $P < .01$).

Conclusions: Our results demonstrated that gene transfection of hepatocyte growth factor attenuated left ventricular diastolic dysfunction and cardiac fibrosis in association with a decrease in transforming growth factor- β 1 in the rat heart subjected to pressure overload. Thus, the transfection of the hepatocyte growth factor gene into the hypertrophied heart may be a strategy for the hypertrophied and failing heart even for cardiac surgery.

From the Division of Cardiovascular Surgery, Department of Surgery E1,^a Division of Biochemistry, Department of Oncology, Biomedical Research Center B7,^b and Department of Molecular Pathology,^c School of Allied Health Science, Faculty of Medicine, Osaka University Graduate School of Medicine, Osaka, Japan.

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Address for reprints: Keiji Iwata, MD, Department of Surgery, Osaka University Graduate School of Medicine (E1), 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan (E-mail: iwata@surg1.med.osaka-u.ac.jp).

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Left ventricular hypertrophy (LVH) is most commonly induced by pressure overload as a consequence of aortic valve stenosis in cardiovascular disease. The development of LVH seems to be an appropriate and beneficial adaptation to compensate for high left ventricular (LV) pressure. However, if the increase in LV pressure persists, various alterations such as myocardial fibrosis and depressed contractility occur, which can lead to heart failure. Such inappropriate LVH has been associated with high perioperative morbidity and mortality.¹

A number of growth factors, including insulin-like growth factor-I and transforming growth factor (TGF)- β 1, have recently been reported to regulate cardiomyocyte hypertrophy.^{2,3} TGF- β 1 has also been reported to regulate the expression of collagen genes and extracellular matrix component synthesis in the heart and to simultaneously block matrix degradation by decreasing the synthesis of proteases and increasing the levels of protease inhibitors.^{4,5} Thus, TGF- β 1 plays a key role in the development of cardiac fibrosis.

Hepatocyte growth factor (HGF), which was originally identified and cloned as a potent mitogen for hepatocytes,^{6,7} has been shown to play a significant role in mitogenic and angiogenic activities for a wide variety of cells,^{8,9} and moreover to inhibit fibrogenesis^{10,11} and apoptosis^{12,13} in several organs. All of the biologic effects of HGF are mediated by the c-Met receptor.¹⁴ We previously reported a role of HGF in ischemia-reperfusion injury in the heart, and c-Met expression was detected in the heart. Therefore, HGF seems to be a significant growth factor for myocytes. However, no study has proven that HGF plays a role in the hypertrophied heart.

TGF- β 1 suppresses the expression of HGF in several types of cells, whereas an anti-TGF- β 1 antibody markedly improves impaired HGF production. Therefore, TGF- β 1 is a potent negative regulator of local HGF production in various cells.¹⁵ Thus, it is reasonable to speculate that the inhibition of TGF- β 1 production by enhancement of HGF expression may attenuate the progression of fibrosis in the hypertrophied heart.

In the present study, we first analyzed the expression of endogenous HGF and the c-Met/HGF receptor compared with the expression of TGF- β 1 in the hypertrophied heart. We next determined whether overexpressing HGF in the heart might attenuate the increase in TGF- β 1 expression that plays an essential role in the progression of fibrosis leading to heart failure.

Methods

Aortic Banding Model

LVH was induced in Sprague-Dawley rats (200 g in body weight) by aortic banding.¹⁶ Rats were anesthetized with sodium pentobarbital intraperitoneally and ventilated, and a right parasternal thoracotomy was performed to expose the ascending aorta. A 5-0 polypropylene suture ligature was tied around the ascending aorta against an 18-gauge needle, and the needle was then removed. Six rats in each group were killed at 1, 2, 4, 7, 14, and 28 days after surgery. In addition, unoperated rats were used as day 0 (prebanding) controls. All animals received proper care in compliance with the institution's guidelines.

Expression of Hepatocyte Growth Factor, c-Met, and Transforming Growth Factor- β 1 mRNA in the Hypertrophied Heart

Heart tissue was frozen immediately in liquid N₂ for total RNA isolation. mRNA transcripts were quantified by the dual-labeled fluorogenic probe method for real-time reverse-transcriptase polymerase chain reaction (RT-PCR), using a Prism 7700 thermal cycler and sequence detector (Perkin-Elmer/ABI, Norwalk, Conn). The RT-PCR primers and probes were the following: rat HGF (GeneBank accession number D90102) sense, 5'-AAGAGTGGCATCAAGTGCCAG-3' and antisense, 5'-CTGGATTGCTTGTGAAACACC-3', and 5'-(FAM)-TGATCCCCCATGAACACAGCTTTTGTG-(TAMRA)-3'; human HGF (GeneBank accession number X16323) sense, 5'-TGCCCTATTCTCGTTGTGAAG-3' and antisense, 5'-TGTCGTGCAGTAAGAACCCAAC-3', and 5'-(FAM)-

CCATCCCGTAATATCTTGCGCCAAAAC-(TAMRA)-3'; rat c-Met (GeneBank accession number Y00671) sense, 5'-GTACGGTGTCTCCAGCATTTTT-3' and antisense, 5'-AGAGACCACCTGCATGAAG-3', and 5'-(FAM)-ACCACGAGCACTGTTTCAATAGGACCC-(TAMRA)-3'; rat TGF- β 1 (GeneBank accession number X52498) sense, 5'-GCTGAACCAAGGAGACGGAATA-3' and antisense, 5'-GCCGTACACAGCAGTTCTTCTCT-3', and 5'-(FAM)-CATGACATGAACCGACCCTTCCTGCT-(TAMRA)-3'; rat GAPDH, as a constitutive control (GeneBank accession number X02231) sense, 5'-CCATCACTGCCACTCAGAAGAC-3' and antisense, 5'-TCATACTTGGCAGGTTTCTCCA-3', and 5'-(FAM)-CGTGTTCCTACCCCCAATGTATCCGT-(TAMRA)-3'. The PCR data are reported as the number of transcripts per number of rat GAPDH transcripts.

Transfection of the Human Hepatocyte Growth Factor Gene Into the Heart

The human HGF cDNA was inserted into the Not I site of the pUC-SR α expression vector. The preparation of the liposome complex with hemagglutinating virus of Japan (HVJ) has been described.¹⁷ Two weeks after banding, rats were anesthetized and ventilated, a left anterolateral thoracotomy was performed to visualize the LV free wall, and approximately 0.5 mL of the HVJ-liposome complex with human HGF cDNA, with 35 μ g of cDNA (H group) or without human HGF cDNA (C group), was transfected into the LV free wall by direct injection at several points.

Enzyme-Linked Immunosorbent Assay for Rat and Human Hepatocyte Growth Factor

The concentrations of rat HGF in the LV were determined by enzyme-linked immunosorbent assay (ELISA) using an anti-rat HGF monoclonal antibody (Institute of Immunology, Tokyo, Japan). The concentrations of human HGF in the transfected LV were also determined by ELISA using an anti-human HGF monoclonal antibody (Tokusymeneki Research Center, Tokyo, Japan). The human HGF ELISA system specifically detects human HGF but not rat HGF.¹⁸

Measurement of Left Ventricular Collagen Content

A portion of the LV free wall was homogenized in cold saline, and the collagen content was measured by the quantitative dye-binding method with the Sircol collagen assay kit (Biocolor Ltd, Belfast, Ireland).

Echocardiography

Anesthetized and ventilated rats were assessed by a Sonos 5500 ultrasound system (Agilent Technologies, Palo Alto, Calif) with a 12-MHz transducer. With the rat in the left lateral decubitus position, the transducer was placed on the left hemithorax. Care was taken to avoid excessive pressure, which can induce bradycardia. With the parasternal short axis and/or long axis imaging plane as a guide, an LV M-mode tracing was obtained close to the papillary muscle level, and pulse Doppler tracings of the mitral inflow velocities were obtained in a modified parasternal long-axis view at a sweep speed of 150 mm/s.¹⁹ Measurements of the ventricular septal thickness, LV internal dimension (LVID), and LV posterior wall thickness (LVPW) were made

Figure 1. Results of quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis in the hypertrophied rat heart. The vertical axis denotes the amount of hepatocyte growth factor (HGF) mRNA (A), c-Met mRNA (B), and transforming growth factor (TGF)- β 1 mRNA (C) normalized to the amount of GAPDH mRNA. Values represent relative amplification (day 0 control values were set at 1.0, and the remaining values were adjusted accordingly) and are means $\pm$ standard error of mean (SEM) for $n = 6$ per group. Black star, $P < .05$ vs day 0 control.

from 2-dimensional direct M-mode images of the LV in both systole and diastole. Fractional shortening (FS), a measure of systolic function, was calculated as $FS (\%) = [(LVIDd - LVIDs) / LVIDd] \times 100$ where d indicates diastole and s indicates systole. Doppler measurements of the peaks of the E (early diastolic transmitral velocities) and A (late diastolic transmitral velocities) waves were recorded as the average of 6 beats.

Statistical Analysis

All values were expressed as the mean $\pm$ standard error of mean (SEM). Statistical comparisons between groups were made by analysis of variance, which was followed by an unpaired Student t test when a significant difference was detected.

Results

Expression of Endogenous HGF and the c-Met/HGF Receptor Compared With the Expression of TGF- β 1 in the Hypertrophied Heart

The level of rat HGF mRNA was significantly increased, 6-fold, after 2 days of banding ($P < .05$ vs prebanding) and then declined gradually to 2 times the control level after 4 weeks of banding (Figure 1, A). The level of c-Met mRNA was increased 2.6-fold after 2 days of banding ($P < .05$ vs prebanding) and maintained at this level after 2 weeks of banding (Figure 1, B). The level of TGF- β 1 mRNA was increased 1.5-fold after 1 week of banding ($P < .05$ vs prebanding) and increased gradually to 2.7 times the control level after 4 weeks of banding (Figure 1, C).

The expression of endogenously produced HGF protein in the rat LV was significantly increased until 4 days after banding (55.2 ± 4.0 ng/g tissue, $P < .01$ vs control; 11.9 ± 1.4 ng/g tissue) and then gradually decreased.

In Vivo Hepatocyte Growth Factor Gene Transfection Into the Hypertrophied Heart

Human HGF protein content in the transfected hearts was measured with ELISA by using an anti-human HGF monoclonal antibody. The level of human HGF in the H group was 3.38 ± 0.18 ng/g tissue at 1 day after transfection, 2.31 ± 0.48 ng/g tissue at 4 days after transfection, and 0.22 ± 0.01 ng/g tissue at 7 days after transfection. In contrast, human HGF protein was undetectable in the C group (Figure 2, A). Rat HGF protein content in the transfected hearts was not measured by ELISA in our second study.

Moreover, to analyze the expression of the human HGF transgene, total RNA was prepared from the transfected LV free wall at 1, 2, 4, and 14 days after transfection, and human HGF mRNA expression was then analyzed by real-time RT-PCR using a primer set that specifically detects human but not rat HGF mRNA. The expression of human HGF mRNA was specifically detected in the H group, even 14 days after transfection (Figure 2, B). In contrast, human HGF mRNA was undetectable in the C group.

Figure 2. A, Expression of human HGF protein in the rat left ventricle (LV) transfected with human HGF. Human HGF concentrations were measured by enzyme-linked immunosorbent assay (ELISA) as described in "Methods." Values are means $\pm$ SEM for $n = 6$ per group. B, Expression of human HGF mRNA in the rat LV transfected with human HGF, measured by quantitative RT-PCR as described in "Methods." The vertical axis denotes the logarithm of human HGF mRNA normalized to rat GAPDH mRNA. Values are means $\pm$ SEM for $n = 6$ per group. hHGF, human hepatocyte growth factor.

Figure 3. Expression of TGF- β 1 mRNA in the rat LV transfected with (H group) and without (C group) human HGF, measured by quantitative RT-PCR as described in "Methods." The vertical axis denotes the amount of TGF- β 1 mRNA normalized to that of GAPDH mRNA. Values represent the relative amplification (the prebanding values were set at 1.0, and the remaining values were adjusted accordingly) and are means $\pm$ SEM for $n = 6$ per group. White star, $P < .05$ versus prebanding. Black star, $P < .05$ versus C group. TGF, transforming growth factor.

Preventive Effects of Hepatocyte Growth Factor Transfection on Cardiac Fibrosis in the Hypertrophied Heart

After 2 weeks of transfection, the expression of TGF- β 1 mRNA was significantly lower in the H group (2.05 ± 0.16 fold vs prebanding) than in the C group (2.69 ± 0.25 -fold vs prebanding; $P < .01$) (Figure 3).

The myocardial collagen content of the LV free wall after 4 weeks of banding was significantly lower in the H group (5.0 ± 0.6 mg/g tissue) than in the C group (7.4 ± 0.5 mg/g tissue, $P < .01$) (Figure 4).

Effects of Hepatocyte Growth Factor Transfection on Cardiac Function by Echocardiography

In the M-mode echocardiographic measurements, there were no significant differences in the HR, LVID, ventricular septal thickness, LVPW, and FS between the C and H groups (Table 1). To assess the LV diastolic function, we evaluated the peak early components of ventricular filling (E velocity) and the E/A ratio by pulse Doppler echocardiography. The C group showed a significant decrease in both the E velocity and the E/A ratio compared with the prebanding control value (E velocity, C group: 53.9 ± 7.8 cm/s, prebanding: 86.5 ± 10.8 cm/s, $P < .01$; E/A ratio, C group: 1.1 ± 0.1 , prebanding: 1.6 ± 0.1 , $P < .01$), whereas the H group did not show a significant decrease. The H group showed a significant increase in both the E velocity and E/A ratio compared with the C group (E velocity, 85.3 ± 3.1 cm/s, $P < .01$ vs the C group; E/A ratio, 1.9 ± 0.1 , $P < .01$ vs the C group). There was no significant difference in A velocity between the C group (50.1 ± 2.9 cm/s) and the H group (44.7 ± 0.3 cm/s) (Figure 5).

Discussion

The present study demonstrated for the first time the different patterns of HGF, c-Met, and TGF- β 1 mRNA expression during the development of pressure-overload hypertrophy in rat heart. After aortic banding, HGF and c-Met mRNA increased significantly as early as 2 days after the operation, and then decreased gradually toward the control levels, whereas TGF- β 1 mRNA increased significantly as early as 7 days after the operation and continued to increase during the development of pressure-overload-induced LVH.

Myocardial TGF- β 1 is thought to regulate cell differentiation and proliferation, control protein synthesis, and regulate the degradation of extracellular matrix components, and is correlated with myocardial hypertrophy.^{3,5} In vitro studies demonstrated that TGF- β 1 expression increases collagen gene expression and concomitantly reduces matrix metalloproteinase (MMP) expression, and stimulates extracellular matrix deposition from cultured cardiac fibroblasts.²⁰ These correlative observations have led to the suggestion that TGF- β 1 plays a pivotal role in cardiac fibrosis. This coincided with our results that TGF- β 1 mRNA was persistently overexpressed during the development of pressure-overload-induced LVH, and cardiac fibrosis progressed concomitantly, whereas HGF mRNA expression in the hypertrophied heart gradually decreased after its initial induction by pressure overload.

It has been reported that TGF- β 1 is a strong negative regulator of local HGF production, whereas HGF potentially suppresses the expression of TGF- β 1 in various organs.^{11,15} Although the mechanisms through which HGF inhibited TGF- β 1 synthesis are not clear, HGF expression may down-regulate TGF- β 1. Thus, a reciprocal balance may exist between the expression of HGF and TGF- β 1.²¹ Previous studies showed that the administration of human recombinant HGF or the gene transfer of human HGF into rats with hepatic fibrosis/cirrhosis induced by dimethylnitrosamine attenuates the progression of the hepatic fibrosis/cirrhosis by inhibiting TGF- β 1 expression.¹⁰ In our study, TGF- β 1 mRNA was persistently overexpressed during the development of pressure-overload-induced LVH, and cardiac fibrosis progressed concomitantly, whereas HGF mRNA expression in the hypertrophied heart gradually decreased after its initial induction by pressure overload. On the basis of these findings, a potential therapy to prevent cardiac fibrosis may be to supply growth factors that act as an antifibrotic factor, such as HGF.

Moreover, HGF reduces the extracellular matrix accumulation and promotes matrix degradation by increasing MMP expression and reducing the levels of specific inhibitors, such as tissue inhibitors of MMP, in a coordinated manner.²² A previous study demonstrated that HGF upregulated Ets activity and Ets-1 protein in a myocardial infarction model.²³ Ets-1 is a transcription factor that positively regulates the expression of urokinase-type plasminogen activator (uPA), MMP-1, MMP-3, and MMP-9.²⁴ HGF induces Ets-1 transcription,²⁵ whereas TGF- β attenuates the transactivation activity of Ets-1 by inducing a protein that interferes with Ets-1 binding to its DNA site.²⁶ HGF seems to have biologic activities that are the opposite of those of TGF- β 1. Therefore, the antifibrotic actions of HGF depend on inhibition of collagen synthesis through inhibition of TGF- β 1 expression and degradation of collagen through activation of MMP and uPA.

Figure 4. Changes in collagen content in the rat LV transfected with (H group) and without (C group) human HGF, measured by a quantitative dye-binding collagen assay as described in "Methods." Values are means $\pm$ SEM for $n = 6$ per group. **White star,** $P < .01$ versus prebanding. **Black star,** $P < .01$ versus C group.

In our second study, it was approximately a 20% increase over basal endogenous HGF levels at the highest exogenous HGF. We did not measure endogenous HGF at the time of assay for human HGF. However, previous study has shown that endogenous rat HGF was further increased by transduction with exogenous human HGF in a rat liver cirrhosis model through induction of Ets activity.²⁷ Therefore, the relatively small amount of exogenous HGF could have such an impact on cardiac remodeling. We showed in our second study that an increase in HGF production induced by transfecting the HGF gene into rat hypertrophied heart significantly inhibited TGF- β 1 expression and prevented cardiac fibrosis. In the present study, we did not demonstrate that antifibrotic actions of HGF depend on degradation of collagen through upregulation of MMP and uPA. Although little is known about how the overexpressed HGF inhibited TGF- β 1 expression in the hypertrophied heart, an increase in HGF expression in the LV may participate in the stimulation of a matrix-degrading pathway and in the inhibition of a TGF- β 1-regulated matrix-producing pathway. These findings indicate that HGF in the hypertrophied heart may play an important role in attenuating the progression of cardiac remodeling.

We evaluated LV function by pulse Doppler echocardiographic interrogation of transvalvular flows. Transmittal Doppler waveform analysis provides insight into the temporal distribution of LV filling. Impaired LV relaxation and reduced ventricular compliance are reflected in characteristic diastolic filling patterns. Transaortic Doppler waveform analysis can be used to estimate LV systolic function, but in this study it was difficult to assess the transaortic flow because the ascending aortic banding

TABLE 1. Echocardiographic findings in the hypertrophied rat heart

	Prebanding (n = 6)	14 days after banding (n = 6)	28 days after banding	
			C group (n = 6)	H group (n = 6)
HR (beats/min)	415 ± 24	379 ± 31	331 ± 11	350 ± 16
LVIDd (mm)	5.15 ± 0.33	4.94 ± 0.27	4.32 ± 0.21	3.42 ± 0.38
LVIDs (mm)	2.52 ± 0.15	2.23 ± 0.28	1.51 ± 0.03	1.26 ± 0.18
VSTd (mm)	1.41 ± 0.10	1.94 ± 0.20	3.05 ± 0.13*	2.67 ± 0.31*
LVPWd (mm)	1.63 ± 0.09	2.44 ± 0.14	3.37 ± 0.06*	3.14 ± 0.41*
FS (%)	51 ± 2	60 ± 4	65 ± 2	69 ± 5*

HR, Heart rate; LVIDd, diastolic left ventricular internal dimension; LVIDs, systolic left ventricular internal dimension; VSTd, diastolic ventricular septal thickness; LVPWd, diastolic left ventricular posterior wall thickness; FS, fractional shortening. Values are means ± standard error of mean. * $P < .05$ versus prebanding.

Figure 5. Efficacy of the gene transfection of HGF for LV diastolic function. Changes in the E velocity, A velocity (A), and E/A ratio (B) in rat LV transfected with (H group) and without (C group) human HGF, measured by pulsed-wave Doppler analysis as described in "Methods." Values are means ± SEM for n = 6 per group. White star, $P < .01$ versus prebanding. Black star, $P < .01$ versus C group.

portion just above the aortic valve interfered with reading. We found that the LV diastolic function was impaired, as indicated by the decrease in the peak early components (E velocity) and the early to late diastolic velocity ratio (E/A) of the ventricular filling during the development of LVH and cardiac fibrosis. It is generally believed that increased extracellular matrix content contributes to diastolic stiffness and that this process ultimately promotes ventricular dysfunction. The present study demonstrated that the collagen content of the LV free wall was markedly decreased by HGF overexpression, which therefore attenuated the significant decrease in LV diastolic function compared with the untreated heart.

We have shown that intracoronary infusion using the HVJ-liposome method resulted in the efficient transduction of a gene into the entire rat heart.²⁸ In this study, the

HVJ-liposome was transfected into the myocardium by direct injection. The expression of human HGF mRNA in the LV was detected by RT-PCR for 14 days after a single injection of the HGF HVJ-liposome. The duration of HGF overexpression induced using this method may be at least a few weeks. Therefore, we consider this HVJ-liposome direct injection method to be efficient, simple, and safe for in vivo gene transfection into the myocardium. For clinical applications in the field of cardiovascular surgery, this direct injection of HVJ-liposome seems to be a good method performed during aortic valve replacement, which is the only effective treatment for severe aortic valve stenosis with concentric LV hypertrophy. With the HVJ-liposome method the efficient period of gene transfection is limited. Therefore, this method may be applied by means of intermittent and repeated administration of HVJ-liposome using echo-guided direct or cardiac catheter injection.

Conclusion

The overexpression of HGF significantly decreased the expression of TGF- β 1 and myocardial collagen content, and attenuated the significant decrease in LV diastolic function in the rat heart subjected to pressure overload. Overall, the present study demonstrated that an increase in HGF in association with a decrease in TGF- β 1 may attenuate cardiac remodeling in the hypertrophied heart. Thus, transfection of the HGF gene into the hypertrophied heart may have a role in the management of the hypertrophied and failing heart.

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