Treatment with an adenoviral vector encoding hepatocyte growth factor mitigates established cardiac dysfunction in doxorubicin-induced cardiomyopathy

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The neoplastic drug doxorubicin is highly effective against a broad spectrum of hematogenous and solid human malignancies, but its clinical use is limited by its adverse side effects: irreversible degenerative cardiomyopathy and congestive heart failure (29, 32). Much effort has gone into the search for treatments able to reduce or eliminate the risk of doxorubicin-induced cardiomyopathy and congestive heart failure (11, 31, 33), but so far the ability of these treatments to protect the heart from damage has been varied and limited.

Hepatocyte growth factor (HGF), which was originally identified and cloned as a potent mitogen for hepatocytes (25, 26), has been shown to exert mitogenic, angiogenic, antiapoptotic, and antifibrotic effects in various cell types, especially in epithelial and endothelial cells (5, 14). Moreover, HGF also reportedly exhibits cardioprotective effects. For instance, HGF protected cardiomyocytes from acute ischemic death during myocardial infarction (27, 36), and it enhanced survival among cardiomyocytes subjected to oxidative stress (13, 36). In addition to its beneficial effects on cardiomyocytes under acute stress, recent research has demonstrated that HGF also exerts beneficial effects on cardiac function in animal models of chronic heart diseases, including ischemic cardiomyopathy following old myocardial infarction and hereditary cardiomyopathy (18, 28, 34). In those cases, the main mechanisms appeared to be a hypertrophic effect on cardiomyocytes as well as angiogenic and antifibrotic actions. More recently, Iwasaki et al. (12) reported that HGF prevents cardiac dysfunction in an animal model of doxorubicin-induced cardiomyopathy. In that study, however, HGF was administered as the protein form, and the delivering method of HGF was very specific (intravenous injection of HGF particles/mouse) was injected into the hindlimb muscles; LacZ gene served as the control. Left ventricular dilatation and dysfunction normally seen 4 wk after doxorubicin administration were significantly mitigated in HGF-treated mice, as were the associated cardiomyocyte atrophy/degeneration and myocardial fibrosis. Myocardial expression of GATA-4 and a sarcomeric protein, myosin heavy chain, was downregulated by doxorubicin, but the expression of both was restored by HGF treatment. The protective effect of HGF against doxorubicin-induced cardiomyocyte atrophy was confirmed in an in vitro experiment, which also showed that neither cardiomyocyte apoptosis nor proliferation plays significant roles in the present model. Upregulation of c-Met/HGF receptor was noted in HGF-treated hearts. Among the mediators downstream of c-Met, the activation of extracellular signal-regulated kinase (ERK) was reduced by doxorubicin, but the activity was restored by HGF. Levels of transforming growth factor-β1 and cyclooxygenase-2 did not differ between the groups. Our findings suggest the HGF gene delivery exerts therapeutic antitrophic/degenerative and antifibrotic effects on myocardium in cases of established cardiac dysfunction caused by doxorubicin. These beneficial effects appear to be related to HGF-induced ERK activation and upregulation of c-Met, GATA-4, and sarcomeric proteins.

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MATERIALS AND METHODS

Recombinant adenoviral vectors. The adenoviral vector plasmid pAd-HGF, which is comprised of the cytomegalovirus immediate early enhancer, a modified chicken β-actin promoter, rabbit β-globin polya (CAG) and hHGF cDNA (Ad.CAG-HGF), was constructed using the in vitro ligation method described previously (18, 21). Control Ad-LacZ (Ad.CAG-LacZ) was prepared as described previously (7).

Measurement of hHGF levels. hHGF levels in plasma and tissues (n = 3 to 4/group) were measured using an ELISA kit (Institute of Immunology) as previously reported (18).

Experimental protocols. This study was approved by our Institutional Animal Research Committee and conformed to the animal care guidelines of the American Physiological Society. Cardiomyopathy was induced in 10-wk-old male C57BL/6J mice (Chubu Kagaku, Nagoya, Japan) by a single intraperitoneal injection of doxorubicin (doxorubicin hydrochloride, Kyowa Hakko, Tokyo, Japan) at a dose of 15 mg/kg. We previously confirmed both functionally and histologically that, at that dosage, doxorubicin induces cardiomyopathy in all mice not receiving a therapeutic intervention (16). In sham-operated mice, the same volume of saline was injected in a similar manner.

In the first set of experiments (protocol 1), treatment with adenoviral vectors was started 2 wk after saline or doxorubicin injection; Ad.CAG-HGF or Ad.CMV-LacZ at 1 × 10^9 particles/mouse was injected into the hindlimb muscles of 10-wk-old male C57BL/6J mice. At that time, mice were assigned to receive saline alone (n = 11), doxorubicin plus LacZ gene (n = 10), or doxorubicin plus hHGF gene (n = 9). Before this assignment, echocardiography was done to reduce any bias among the groups. After an additional 2 wk (4 wk after doxorubicin administration), all mice received a physiological examination and were then euthanized with an overdose of pentobarbital sodium. Cardiomyocytes were then collected and subjected to histological, immunohistochemical, and molecular biological analyses.

In a second set of experiments (protocol 2), we evaluated the role played by the extracellular signal-regulated protein kinase (ERK) signaling pathway in mediating the effects of the hHGF gene therapy. PD-98059 (Cell Signaling), a MEK1-p42/p44 mitogen-activated protein kinase (MAPK)-specific inhibitor (8), was administered intraperitoneally at a dose of 0.5 mg·kg⁻¹·day⁻¹ for 2 wk to mice given saline or doxorubicin plus hHGF (n = 7 each), after which the mice were examined as described in protocol 1.

In vitro study. Cardiomyocytes were isolated from 1-day-old neonatal C57BL/6J mice as previously reported (3) and plated on laminin-coated dishes or in slide glass chambers and incubated in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% FBS (Sigma) for 48 h at 37°C. Doxorubicin was then added to the medium to a final concentration of 0.1 μmol/l. Simultaneously, recombinant hHGF (Wako) was added to a concentration of 0, 0.02, 0.2, 2, or 10 ng/ml. For the controls, doxorubicin and/or HGF was replaced with the same volume of saline. Twenty-four hours later, the cells were collected for morphometric and biochemical analyses.

The cardiomyocytes in slide glass chambers were fixed in 4% paraformaldehyde, permeabilized with 0.05% Triton X, and stained with rhodamine phalloidin and Hoechst 33342 (both from Molecular Probes). Digital images captured using a laser-confocal microscope system (LSM510, Zeiss) were employed for morphometric analysis using Photoshop 7.0 (Adobe Systems). Proteins extracted from cardiomyocytes on dishes were used for Western blot analysis.

Physiological studies. Echocardiography and cardiac catheterization were carried out as described previously with modifications (16). Animals were anesthetized with halothane (induction, 2%; maintenance, 0.5%) in a mixture of N₂O and O₂ (0.5 l/min each) via a nasal mask. Echocardiograms were recorded using an echocardiographic system (Vevo770, Visualsonics) equipped with a 45-MHz imaging transducer before treatment and at death. Following echocardiography, the right carotid artery was cannulated with a micromanometer-tipped catheter (SPR 671, Millar Instrument) that was advanced into the aorta and then into the left ventricle for recording pressure and maximal and minimal first derivative of pressure (±dP/dt).

Histological analysis. Following echocardiography, each heart was removed and cut into three transverse slices. Of those, the middle slice was fixed with 10% buffered formalin and embedded in paraffin, after which 4-μm-thick sections were stained with hematoxylin-eosin or Sirius red F3BA (0.1% solution in saturated aqueous picric acid) (Aldrich). Quantitative assessments, including cell size and cell number, were carried out in randomly chosen high-power fields (HPFs) in each section using a multipurpose color image processor (LUZEX F, Nireco). The fibrotic area was measured by searching the entire ventricle. Cardiomyocyte size (expressed as the transverse diameter of myocytes cut at the level of the nucleus) and cell populations were assessed in 20 randomly chosen HPFs in each section.

Immunohistochemistry. After deparaffinization, the 4-μm-thick sections were incubated with a primary antibody against c-Met/HGF receptor (Santa Cruz), endothelial cells (Flk-1, Santa Cruz), leuko...
cytes (CD45, Pharmingen), or Ki-67 (Santa Cruz). A Vectastain Elite ABC system (Vector) was then used to immunostain the sections; dianinobenzidine served as the chromogen, and the nuclei were counterstained with hematoxylin. Quantitative assessments, including the number or area of the immunopositive cells, were made in 20 randomly chosen HPFs using the multipurpose color image processor.

In situ terminal dUTP nick-end labeling (TUNEL) assays were carried out with sections using an ApopTag kit (Chemicon) according to the supplier’s instructions. Mouse mammary tissue served as a positive control.

Electron microscopy. Cardiac specimens were immersion fixed overnight in phosphate-buffered 2.5% glutaraldehyde (pH 7.4), post-fixed for 1 h with 1% osmium tetroxide, dehydrated through a graded ethanol series, and embedded in Epon medium. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in an electron microscope (H700, Hitachi, Tokyo, Japan).

Western blot analysis. Heart tissue lysates were used for Western blot analysis. Proteins were separated and transferred to membranes using standard protocols, after which they were probed using antibodies against GATA-4 and myosin heavy chain (MHC) (both from Santa

Fig. 2. Effects of hHGF gene delivery on cardiac remodeling and function assessed 4 wk after Dox injection (protocol 1). The indicated parameters were measured using echocardiography (A) and cardiac catheterization (B). LVDd, left ventricular (LV) end-diaelastic diameter; %LVFS, %LV fractional shortening; LVSP, LV peak systolic pressure; bpm, beats/min; +dP/dt, maximum and minimum first derivative of pressure. *P < 0.05 vs. sham group; #P < 0.05 vs. Dox + LacZ group.

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Cruz), transforming growth factor-β1 (TGF-β1, Promega), cyclooxygenase-2 (Santa Cruz), and Akt or ERK (both from Cell Signaling). Activation of Akt and ERK was assessed using antibodies against the phosphorylated form of Akt (p-Akt) and p-ERK (both from Cell Signaling), respectively. Pro-caspase-3 and the activated form of caspase-3 were evaluated using anti-caspase-3 antibody (Santa Cruz). Western blot analysis of hHGF was performed using anti-human IgG antibody (DAKO). Three to five hearts from each group were subjected to the blotting. The blots were visualized by means of enhanced chemiluminescence (Amersham), and the signals were quantified by densitometry. α-Tubulin (analyzed using an antibody from Santa Cruz) served as the loading control.

**Immunoprecipitation and Western blot analysis for c-Met.** Heart tissue lysates were subjected to immunoprecipitation assays carried out with Ultra-Link Biosupport medium (Pierce) using anti-c-Met antibody (Santa Cruz). Thereafter, the immunoprecipitate was analyzed by Western blot analysis using the same antibody. Three to five hearts from each group and three normal livers were subjected to the assay.

**Fig. 3.** Effects of hHGF gene delivery on cardiac histology in mice 4 wk after Dox administration (protocol 1). A: photomicrographs of histological [hematoxylin-eosin (HE) and Sirius red stained] and immunohistochemical (Flk-1 and CD45) preparations of heart specimens from the indicated groups. Arrows point to immunopositive cells; scale bars, 20 μm. B: morphometric data. *P < 0.05 vs. sham group; #P < 0.05 vs. Dox + LacZ group. C: electron photomicrographs of doxorubicin-induced cardiomyopathy treated with LacZ or hHGF gene (bar, 1 μm).
Statistical analysis. Values are shown as means ± SE. Survival was assessed by constructing Kaplan-Meier curves, which were analyzed using the log-rank Cox-Mantel method. The significance of differences between groups was evaluated using one-way ANOVA with a post hoc Newman-Keuls multiple comparisons test. Values of \( P < 0.05 \) were considered significant.

Fig. 4. c-Met expression in the heart. Western blot (A) and immunohistochemical (B) analyses. *\( P < 0.05 \) vs. sham group; #\( P < 0.05 \) vs. Dox + LacZ group. Arrows indicate intramyocardial vessels. Bars, 20 μm.

Fig. 5. Western blot analysis of sarcomere-related (A) and fibrosis-related (B) proteins. A: myocardial expression of GATA-4 and myosin heavy chain (MHC). B: myocardial expression of transforming growth factor-β1 (TGF-β1) and cyclooxygenase-2 (COX-2). Graphs are not shown for TGF-β1 and COX-2 since there were no significant differences among the groups. *\( P < 0.05 \) vs. sham group; #\( P < 0.05 \) vs. DOX + LacZ group.
RESULTS

hHGF in plasma and tissues. In the hindlimb receiving the adenoviral vector (Ad.CAG-HGF), hHGF levels peaked at 4.35 ± 0.03 ng/mg 3 days after injection; no hHGF was detected in the hindlimbs of LacZ-treated mice (Fig. 1A). Plasma hHGF also peaked 3 days after injection of Ad.CAG-HGF (3.25 ± 0.85 ng/ml), and significant levels were sustained for an additional 9 days thereafter (Fig. 1B). Myocardial hHGF levels showed a similar pattern (Fig. 1C).

Effects of hHGF gene delivery on cardiac function and pathology. All mice in each group remained alive 4 wk after doxorubicin administration. Echocardiography and cardiac catheterization showed that, compared with the saline-treated controls, mice receiving doxorubicin had significant deterioration of left ventricular (LV) function characterized by an enlargement of the LV cavity and decreased LV fractional shortening and ±dP/dt (Fig. 2). The delivery of the hHGF gene significantly attenuated the doxorubicin-induced impairment of cardiac function.

No significant difference was observed in the heart weight-to-body weight ratios among the groups (saline, 3.78 ± 0.01; doxorubicin with LacZ, 3.87 ± 0.01; and doxorubicin with hHGF, 3.71 ± 0.01 mg/g). On the other hand, an examination of transverse sections of hearts stained with hematoxylin-eosin revealed that the sizes of cardiomyocytes (expressed as the transverse diameter) from the group receiving doxorubicin plus LacZ were significantly smaller than those in the saline group (11.5 ± 0.22 vs. 13.8 ± 0.37 μm, P < 0.05) and that hHGF delivery exerted a significant protective effect against such doxorubicin-induced cardiomyocyte atrophy (transverse diameter, 13.4 ± 0.18 μm) (Fig. 3). Similarly, when we assessed myocardial fibrosis using Sirius red-stained sections, we found significantly greater fibrosis in the group receiving doxorubicin plus LacZ than in groups receiving saline (0.99 ± 0.05% vs. 0.55 ± 0.04%, P < 0.05) or doxorubicin plus hHGF (0.58 ± 0.04%) (Fig. 3). Myocardial capillary density, which we assessed based on Flk-1 immunostaining, was unaffected by either doxorubicin or hHGF treatment (Fig. 3). Immunohistochemical analysis also revealed that CD45-positive leukocyte infiltration did not differ among the groups (Fig. 3).

Degenerative changes within cardiomyocytes caused by doxorubicin were clearly evident under an electron microscope, which confirmed previously described findings in doxorubicin-induced cardiomyopathy (16, 30). These changes were characterized by myofibrillar derangement and disruption and by increases in the volume of subcellular organelles such as mitochondria (Fig. 3). These degenerative changes were significantly mitigated by hHGF gene transfer. No apoptotic cells were ever detected by electron microscopic observation of cardiac tissue from any of the groups.

TUNEL-positive cardiomyocytes were detected, though very rarely, and the incidence was not affected by either doxorubicin administration or hHGF gene transfer (saline, 0.04 ± 0.03%; doxorubicin plus LacZ, 0.06 ± 0.03%; and doxorubicin plus hHGF, 0.05 ± 0.04%). Consistent with that finding, the active (cleaved) form of caspase-3 was not detectable in hearts from any of the groups by Western blot analysis (data not shown). The absence of apoptotic cells in the present model confirms earlier studies (16, 17). In addition, proliferating cardiomyocytes, as indicated by the presence of Ki-67, were never detected (data not shown).

Expression of c-Met/HGF receptor. The HGF receptor has been identified as c-Met, the product of the c-Met proto-oncogene (5, 6). Western blot analysis revealed that the expression of the c-Met/HGF receptor was significantly down-regulated in doxorubicin-treated hearts but was greatly enhanced by hHGF gene transfer (Fig. 4A). Consistent with this finding, the active (cleaved) form of caspase-3 was not detectable in hearts from any of the groups.

Fig. 6. In vitro experiments. A: confocal micrographs and a graph showing the atrophic degeneration of cardiomyocytes exposed to Dox and its prevention by recombinant hHGF. Bars, 10 μm. *P < 0.05 vs. sham group; #P < 0.05 vs. Dox + LacZ group. B: Western blot analysis of the effect of hHGF on the Dox-mediated reduction of MHC in cultured cardiomyocytes. *P < 0.05 vs. control group; #P < 0.05 vs. group treated with Dox alone.
finding, immunohistochemical analysis showed c-Met to be expressed on cardiomyocytes and to be more strongly expressed in hHGF-treated hearts (Fig. 4B).

Expression of GATA-4 and MHC. GATA-4 is a key transcriptional factor regulating expression of sarcomeric proteins in the heart (22, 23). Myocardial levels of GATA-4 were significantly reduced by doxorubicin, confirming earlier reports (4). This reduction was significantly reversed by hHGF gene transfer (Fig. 5A). Likewise, the level of MHC was significantly reduced by doxorubicin, and this inhibitory effect was also significantly reversed by hHGF gene therapy (Fig. 5A).

Expression of TGF-β1 and cyclooxygenase-2. Doxorubicin had no significant effect on the expression of TGF-β1 or cyclooxygenase-2 in hearts 4 wk after administration, and neither was affected by hHGF gene transfer (Fig. 5B, data not shown).

In vitro effect of hHGF on cardiomyocytes. Doxorubicin exerted a significant atrophic/degenerative effect on cultured neonatal mouse cardiomyocytes, but this effect was largely reversed by an application of recombinant hHGF (Fig. 6A). hHGF affected the cardiomyocytes in a dose-dependent manner. Western blot analysis revealed that doxorubicin significantly reduced expression of MHC in cultured cardiomyocytes, but the expression was restored by the addition of hHGF to the cultures (Fig. 6B).

ERK activity. ERK/MAPK and phosphatidylinositol 3-kinase (PI3K)/Akt are known to be components of major signaling pathways downstream of c-Met/HGF receptor (9, 24). Neither doxorubicin-induced cardiomyopathy nor the effects of hHGF gene transfer was found to be related to the activation (phosphorylation) of Akt in the heart 4 wk after doxorubicin treatment (Fig. 7A). In contrast, ERK phosphorylation, and thus its activation, was markedly diminished by doxorubicin,
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and that effect was significantly attenuated by hHGF treatment (Fig. 7A).

To further examine the role played by ERK activation in mediating the cardioprotective effects of hHGF, we next tested the effect of inhibiting ERK activation using the MEK1-p42/p44 MAPK-specific inhibitor PD-98059 (protocol 2). When administered to mice along the hHGF gene, PD-98059 suppressed the hHGF-mediated reversal of doxorubicin’s inhibition of GATA-4 expression (Fig. 7B). Moreover, PD-98059 significantly suppressed the hHGF-mediated improvement in cardiac function and histology, i.e., the increase in cardiomyocyte size and the reduction in myocardial fibrosis (Table 1). This suggests that the ERK pathway is critically involved in the protective effect exerted by hHGF against doxorubicin-induced cardiomyopathy.

**DISCUSSION**

The present study provides clear evidence of the beneficial effects of HGF gene delivery on the cardiac dysfunction associated with doxorubicin-induced cardiomyopathy, a non-ischemic cardiomyopathy. The principal pathological findings were that HGF prevented doxorubicin-induced atrophic degeneration of cardiomyocytes and myocardial fibrosis. The mechanism of action of HGF in this model differs from that seen in cases of myocardial infarction, where HGF reportedly enhances the survival of ischemic cardiomyocytes (27, 36). Notably, HGF exerted its therapeutic effects despite the fact that the cardiomyopathy was well established.

**Mechanisms underlying the cardioprotective effects of HGF.**

Our findings suggest that several factors contribute to the cardioprotective effects of HGF against doxorubicin-induced cardiomyopathy. The first is that HGF mitigates the evoked atrophic degeneration of cardiomyocytes. The sarcomeric protein MHC is important for the structural integrity and function of cardiomyocytes, and its myocardial expression is reportedly downregulated by doxorubicin (11), an effect we confirmed in the present study. Our new finding is that HGF significantly restored the doxorubicin-induced decrease in c-Met/HGF receptor expression in the heart, which is compatible with previous findings (18, 27): the increase in c-Met may be related to the autoinduction of gene expression triggered by HGF (27). However, immunohistochemistry showed cytoplasmic staining although c-Met is a membrane protein. One possible explanation for this discrepancy is the thickness of the sections (4 μm) relative to myocyte size (12 μm). A second possible explanation is the diffusion of diaminobenzidine products during the staining procedure. It is also possible that cytoplasmic staining is not an artifact but rather represents an abnormal distribution of excessive protein. Thus further studies are desirable in the future on the subcellular localization of c-Met in cardiomyocytes at the electron microscopic level.

Recent findings suggest that apoptosis among cardiomyocytes is a leading cause of cardiac dysfunction in doxorubicin-induced cardiomyopathy (13, 36). This hypothesis remains controversial, however, because the cardiomyocytes in question do not show the typical apoptotic morphology (16, 17, 30, 38). Seeking evidence of doxorubicin-induced apoptosis/cell death, we previously conducted a series of TUNEL assays, myocardial expression of MHC and troponin I (22, 23) and to be depleted in doxorubicin-induced cardiotoxicity (4). Our results not only confirm those earlier findings but also demonstrate that HGF restores GATA-4 expression, even in the presence of doxorubicin.

c-Met/HGF receptor signaling is known to activate ERK/MAPK and PI3K/Akt signaling pathways (9, 24), both of which are implicated in myocardial hypertrophy (2, 5). Our findings suggest that altered signaling via ERK, but not Akt, is involved in doxorubicin-induced cardiomyopathy, which is consistent with a recent study showing that ERK activation is significantly diminished during the chronic stage of doxorubicin-induced cardiomyopathy (3 wk after doxorubicin administration) (20). Given that another study, in which isolated rat heart was subjected to excessive LV wall stress (induced by balloon inflation), showed MAPK (p38 and ERKs) to be involved in the activation of GATA-4 binding to DNA (35), we suggest that HGF exerts its cardioprotective effects by restoring activity in ERK/MAPK signaling pathway.

The HGF gene therapy significantly restored the doxorubicin-induced decrease in c-Met/HGF receptor expression in the heart, which is compatible with previous findings (18, 27): the increase in c-Met may be related to the autoinduction of gene expression triggered by HGF (27). However, immunohistochemistry showed cytoplasmic staining although c-Met is a membrane protein. One possible explanation for this discrepancy is the thickness of the sections (4 μm) relative to myocyte size (12 μm). A second possible explanation is the diffusion of diaminobenzidine products during the staining procedure. It is also possible that cytoplasmic staining is not an artifact but rather represents an abnormal distribution of excessive protein. Thus further studies are desirable in the future on the subcellular localization of c-Met in cardiomyocytes at the electron microscopic level.

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**Table 1. Effects of inhibiting ERK activity with PD-98059 on LV function and histology 4 wk after administering saline or doxorubicin followed by LacZ or human HGF gene therapy: protocol 2**

<table>
<thead>
<tr>
<th>Function</th>
<th>Sham (protocol 1)</th>
<th>Sham + PD-98059</th>
<th>Dox + HGF (protocol 1)</th>
<th>Dox + HGF + PD-98059</th>
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<tbody>
<tr>
<td></td>
<td>11</td>
<td>7</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>LVDd, mm</td>
<td>3.78 ± 0.12</td>
<td>3.79 ± 0.03</td>
<td>3.77 ± 0.10</td>
<td>3.93 ± 0.09</td>
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<td>LVFS, %</td>
<td>29.2 ± 1.61</td>
<td>30.7 ± 0.42</td>
<td>25.2 ± 1.08</td>
<td>20.9 ± 0.96*</td>
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<tr>
<td>+dP/dt, mmHg/s</td>
<td>7.08 ± 845</td>
<td>6.596 ± 1.075</td>
<td>8.27 ± 936</td>
<td>5.012 ± 607*</td>
</tr>
<tr>
<td>−dP/dt, mmHg/s</td>
<td>−6.568 ± 364</td>
<td>−6.355 ± 976</td>
<td>−8.524 ± 718</td>
<td>−5.434 ± 779*</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>80.5 ± 2.21</td>
<td>73.5 ± 5.40</td>
<td>93.9 ± 4.36</td>
<td>70.4 ± 5.85*</td>
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<tr>
<td>Heart rate, beats/min</td>
<td>512 ± 37</td>
<td>523 ± 60</td>
<td>520 ± 36</td>
<td>492 ± 24</td>
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| Histology                |                   |                 |                       |                       |
| Myocyte size, μm         | 13.8 ± 0.37       | 13.5 ± 0.20     | 13.4 ± 0.18           | 12.4 ± 0.32*          |
| Fibrosis, %              | 0.55 ± 0.04       | 0.48 ± 0.04     | 0.58 ± 0.04           | 0.69 ± 0.01*          |
| Fk-1 vessels/HPF         | 279 ± 37.9        | 272 ± 23.2      | 306 ± 60.4            | 272 ± 31.8            |
| CD45* cells/HPF          | 0 ± 0             | 0 ± 0           | 0.02 ± 0.04           | 0 ± 0                 |

Values are means ± SE; n, number of animals/group. Dox, doxorubicin; HGF, hepatocyte growth factor; LVDd, left ventricular (LV) end-diastolic diameter; LVFS, LV fractional shortening; ±dP/dt, maximum and minimum first derivative of pressure; LVSP, LV peak systolic pressure; HPF, high-power field. *P < 0.05 vs. corresponding group without PD-98059 treatment.
electron microscopic examinations, and analyses of myocardial caspase-3 activation in the same animal model, but we detected no effect of doxorubicin on the incidence of apoptosis/cell death (17). We have now confirmed those findings. In the present study, mice received a single dose of doxorubicin, and the survival rate was 100% in all groups. This suggests the doxorubicin insult may have been too weak to induce cardiac cell death and weaker than the insults induced in earlier models. This may also hold true for our in vitro model.

HGF has been reported to be angiogenic (18, 28, 34), but we did not detect any doxorubicin-induced reduction in capillary density, nor did HGF promote capillary outgrowth, indicating that angiogenesis likely plays a mechanism role in doxorubicin-induced cardiomyopathy or the cardioprotective effects of HGF.

**Limitations of the study.** We observed that doxorubicin stimulates the development of myocardial fibrosis and that HGF suppresses this pathological process. Although TGF-β1 is a potent stimulator of fibrosis in the failing heart, its involvement in doxorubicin-induced cardiomyopathy was challenged in a recent report (19). Consistent with that report, we found no significant doxorubicin-induced changes in the expression of TGF-β1. Therefore, although several studies suggest the mechanism underlying the anti-fibrotic effect of HGF is related, at least in part, to the inhibition of TGF-β1 secretion (28, 34), in the case of doxorubicin-induced cardiomyopathy, HGF appears to diminish fibrosis via a different mechanism. It is also known that doxorubicin induces cardiac expression of cyclooxygenase-2 (1), which occupies a central position in the biosynthesis of proinflammatory prostaglandin E₂, prostacyclin and thromboxane A₂, and that inhibition of cyclooxygenase-2 improves cardiac function in a model of doxorubicin-induced cardiomyopathy (10). Actually, we previously observed expression of cyclooxygenase-2 to be upregulated 2 wk after doxorubicin injection, but that is a more acute stage than the one studied here (16, 17). We did not see greater expression of cyclooxygenase-2 in the present 4-wk model, where significant infiltration of inflammatory cells also was not seen. Still, we cannot exclude the possibility that cyclooxygenase-2 contributes to the etiology of myocardial fibrosis in doxorubicin-induced cardiomyopathy. Our results also indicate that ERK inhibition blocks the anti-fibrotic effect of HGF in the present model; thus, further investigation will be needed to precisely define the mechanisms operating.

HGF reportedly exerts myocardial regeneration by mobilizing bone marrow-derived cells to the myocardium (15), and cardiac stem cells reportedly express c-Met/HGF receptors (12, 37). Although we did not directly evaluate the contribution made by cardiomyocyte regeneration (either from bone marrow cells or stem cells) to the beneficial effects of HGF, our immunohistochemical analysis of Ki-67, which showed an absence of cardiomyocyte proliferation, suggests that it is unlikely that cardiomyocyte regeneration plays a role in the present model. This result of ours seems to be in contrast with the previous study by Iwasaki et al. (12), which reported enhanced cardiomyocyte proliferation and increased Scal-positive cardiac progenitor cells in doxorubicin-induced cardiomyopathy by a specific delivering method of HGF (intra-venous injection of HGF delivered by ultrasound-mediated destruction of microbubbles). In addition, the peak plasma HGF concentration should have been widely different between the studies. Iwasaki et al. (12) intravenously gave 10 µg of HGF per animal (~20 g body wt), whereas in our study the plasma HGF concentration attained 3 days after gene delivery was 3.25 ± 0.85 ng/ml. These methodological differences might have a strong bearing on the different observations between the studies. Further studies are needed to focus specifically on the biological effect of HGF on stem cells.

**Conclusion.** The present study provides the first evidence of the beneficial effects of HGF gene transfer in doxorubicin-induced cardiomyopathy. These effects include the attenuation of atriotic degeneration of cardiomyocytes and the reduction of myocardial fibrosis, accompanied by the restoration of myocardial expression of GATA-4 and sarcomeric proteins. Our findings also suggest that HGF-mediated ERK activation is associated with these beneficial effects and may thus underlie the cardioprotection provided by HGF gene transfer.

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