A

c-Met

Tubulin

(Sham, DOX+LacZ, DOX+HGF)

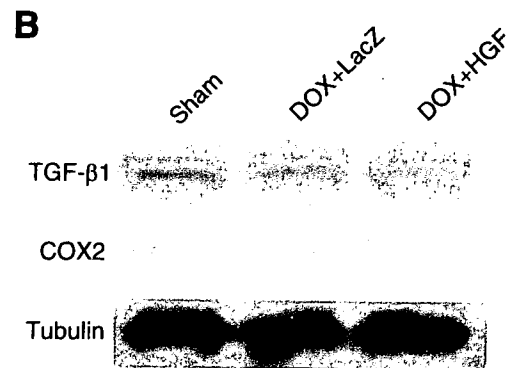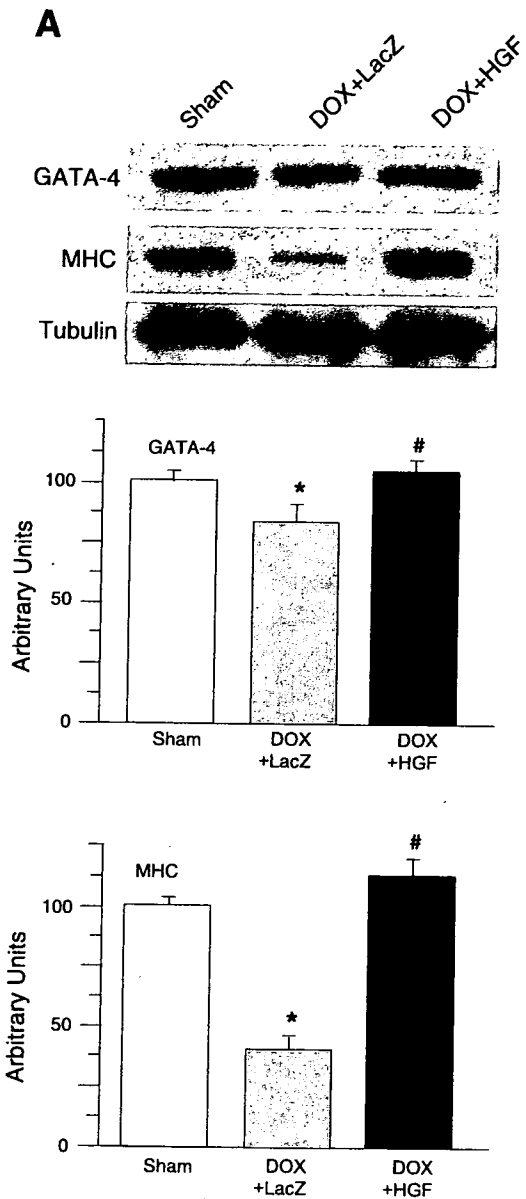Arbitrary Units / Sham, DOX+LacZ, DOX+HGF

B

Sham

DOX+LacZ   DOX+HGF

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.

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. 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.

A

GATA-4

MHC

Tubulin

(Sham, DOX+LacZ, DOX+HGF)

GATA-4 — Arbitrary Units (Sham, DOX+LacZ, DOX+HGF)

MHC — Arbitrary Units (Sham, DOX+LacZ, DOX+HGF)

B

TGF-β1

COX2

Tubulin

(Sham, DOX+LacZ, DOX+HGF)

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) functionality characterized by an enlargement of the LV cavity and decreased LV fractional shortening and ±dP/d*t* (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 diameters) 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, prolifer-

ating 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 downregulated in doxorubicin-treated hearts but was greatly enhanced by hHGF gene transfer (Fig. 4A). Consistent with this
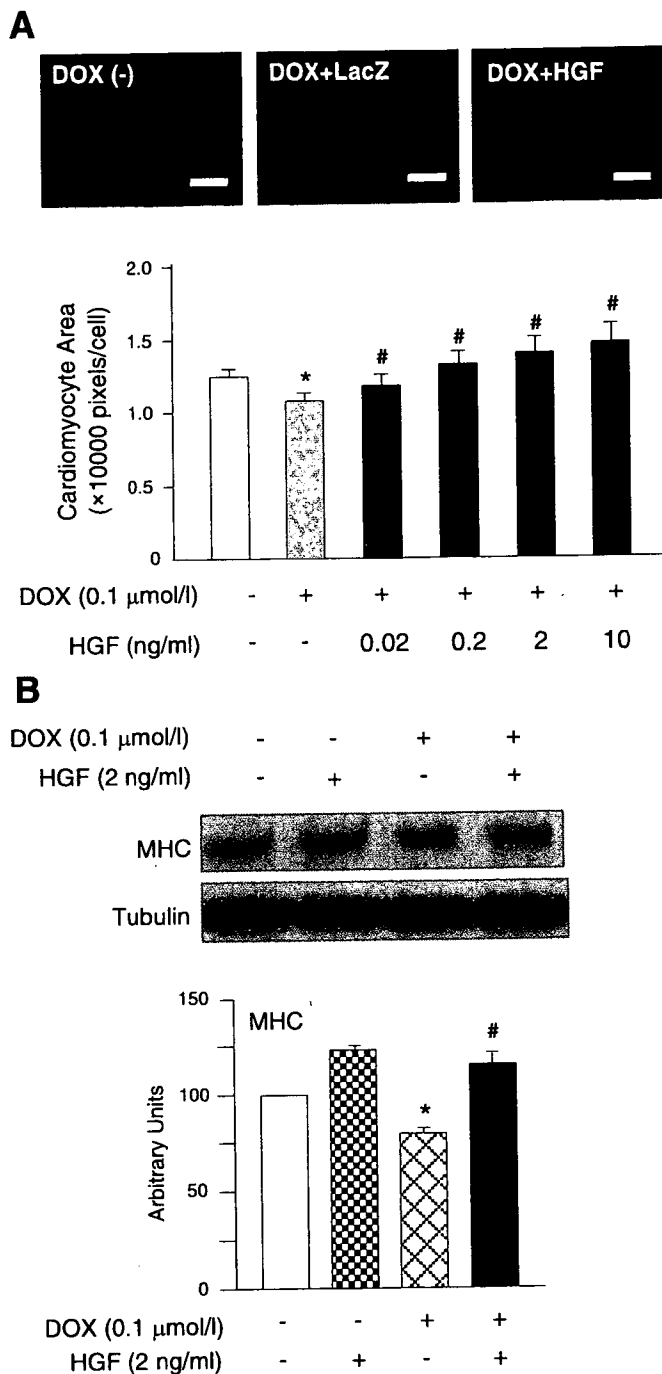

Fig. 6. In vitro experiments. *A*: confocal micrographs and a graph showing the atrophic degeneration of cardiomyocytes exposed to Dox and its prevention by recombinant hHGF. Bars, 10 μm. *$P < 0.05$ vs. sham group; #$P < 0.05$ vs. Dox + LacZ group. *B*: Western blot analysis of the effect of hHGF on 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,
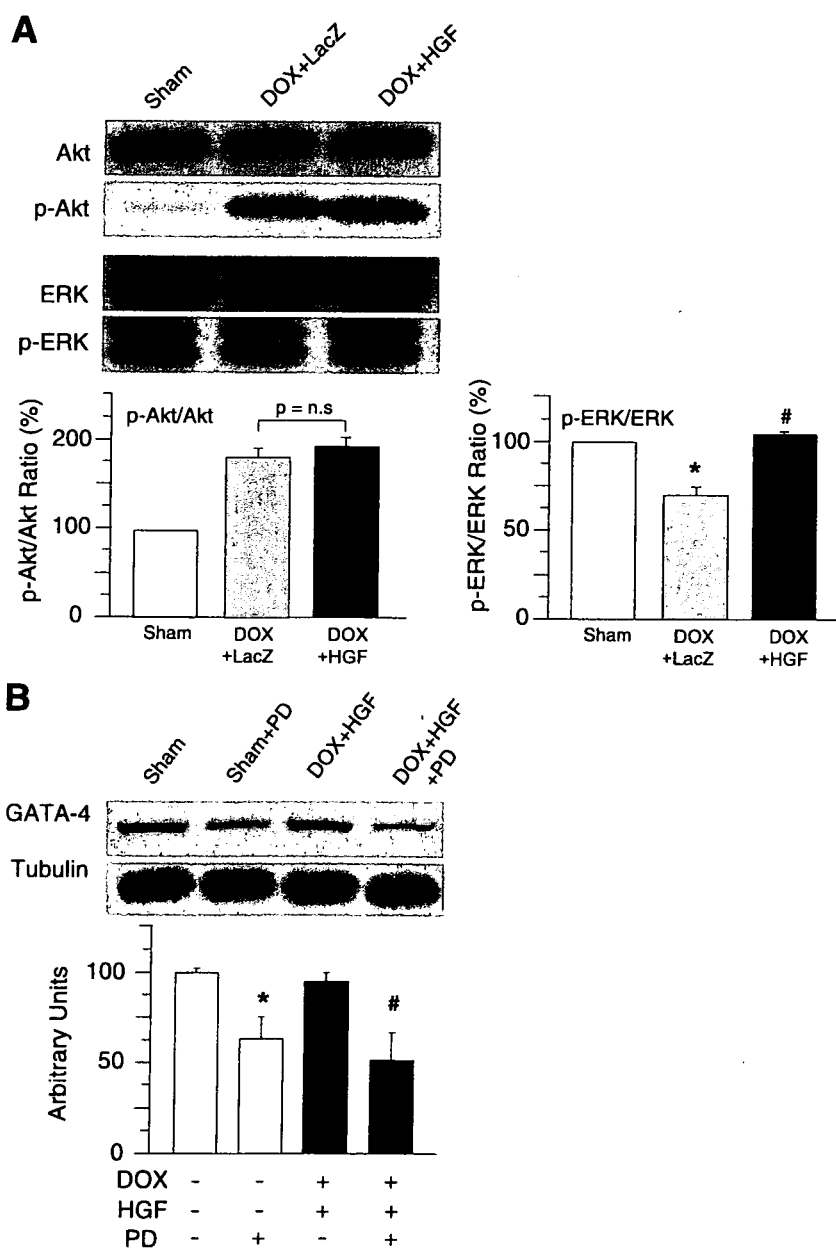

Fig. 7. *A*: Western blot analysis of the effects of hHGF gene transfer on myocardial expression of phosphorylated (p)-Akt and p-ERK. Activity of Akt and ERK is expressed, respectively, as the p-Akt-to-total Akt and p-ERK-to-total ERK ratio. *P < 0.05 vs. sham group; #P < 0.05 vs. the Dox + LacZ group. *B*: effect of the p42/p44 MAPK inhibitor PD-98059 (PD) on Dox-induced cardiomyopathy (*protocol 2*). Western blot and densitometric analyses of the effect of PD on myocardial expression of GATA-4. *P < 0.05 vs. control group; #P < 0.05 vs. Dox + HGF group.

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 HGF-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 expression of both MHC and GATA-4 in the presence of doxorubicin. We suggest that GATA-4 is crucially involved in the antiatrophic effect of HGF, since GATA-4 is known to be a key regulator of heart development, to regulate

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.

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,

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*

	Sham (protocol 1)	Sham + PD-98059	Dox + HGF (protocol 1)	Dox + HGF + PD-98059
n	11	7	9	7
Function				
LVDd, mm	3.78±0.12	3.79±0.03	3.77±0.10	3.93±0.09
LVFS, %	29.2±1.61	30.7±0.42	25.2±1.08	20.9±0.96*
+dP/dt, mmHg/s	7,708±845	6,596±1,075	8,27±936	5,012±607*
−dP/dt, mmHg/s	−6,568±364	−6,355±976	−8,524±718	−5,434±779*
LVSP, mmHg	80.5±2.21	73.5±5.40	93.9±4.36	70.4±5.85*
Heart rate, beats/min	512±37	523±60	520±36	492±24
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*
Flk-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 detected no doxorubicin-induced reduction in capillary density, nor did HGF promote capillary outgrowth, indicating that angiogenesis likely plays no mechanistic 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 antifibrotic 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 E_2, prostacyclin and thromboxane A_2, 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 antifibrotic 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 cardiac 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 Sca1-positive cardiac progenitor cells in doxorubicin-induced cardiomyopathy by a specific delivering method of HGF (intravenous 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 $\pm$ 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 atrophic 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.

REFERENCES

1. **Adderley SR, Fitzgerald DJ.** Oxidative damage of cardiomyocytes is limited by extracellular regulated kinases 1/2-mediated induction of cyclooxygenase-2. *J Biol Chem* 274: 5038–5046, 1999.
2. **Akiyama Y, Ashizawa N, Seto S, Ohtsuru A, Kuroda H, Ito M, Yamashita S, Yano K.** Involvement of receptor-type tyrosine kinase gene families in cardiac hypertrophy. *J Hypertens* 17: 1329–1337, 1999.
3. **Aoyama T, Takemura G, Maruyama R, Kosai K, Takahashi T, Koda M, Hayakawa K, Kawase Y, Minatoguchi S, Fujiwara H.** Molecular mechanisms of non-apoptosis by Fas stimulation alone versus apoptosis with an additional actinomycin D in cultured cardiomyocytes. *Cardiovasc Res* 55: 787–798, 2002.
4. **Aries A, Paradis P, Lefebvre C, Schwartz RJ, Nemer M.** Essential role of GATA-4 in cell survival and drug-induced cardiotoxicity. *Proc Natl Acad Sci USA* 101: 6975–6980, 2004.
5. **Birchmeier C, Gherardi E.** Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. *Trends Cell Biol* 8: 404–410, 1998.
6. **Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude GF, Aaronson SA.** Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science* 251: 802–804, 1991.
7. **Chen SH, Chen XH, Wang Y, Kosai K, Finegold MJ, Rich SS, Woo SL.** Combination gene therapy for liver metastasis of colon carcinoma in vivo. *Proc Natl Acad Sci USA* 92: 2577–2581, 1995.
8. **Cuenda A, Alessi DR.** Use of kinase inhibitors to dissect signaling pathways. *Methods Mol Biol* 99: 161–175, 2000.
9. **Day RM, Cioce V, Breckenridge D, Castagnino P, Bottaro DP.** Differential signaling by alternative HGF isoforms through c-Met: activation of both MAP kinase and PI 3-kinase pathways is insufficient for mitogenesis. *Oncogene* 18: 3399–3406, 1999.
10. **Delgado RM 3rd, Nawar MA, Zewail AM, Kar B, Vaughn WK, Wu KK, Aleksic N, Sivasubramanian N, McKay K, Mann DL, Willerson JT.** Cyclooxygenase-2 inhibitor treatment improves left ventricular function and mortality in a murine model of doxorubicin-induced heart failure. *Circulation* 109: 1428–1433, 2004.
11. **Ito H, Miller SC, Billingham ME, Akimoto H, Torti SV, Wade R, Gahlmann R, Lyons G, Kedes L, Torti FM.** Doxorubicin selectively inhibits muscle gene expression in cardiac muscle cells in vivo and in vitro. *Proc Natl Acad Sci USA* 87: 4275–4279, 1990.
12. **Iwasaki M, Adachi Y, Nishiue T, Minamino K, Suzuki Y, Zhang Y, Nakano K, Koike Y, Wang J, Mukaide H, Taketani S, Yuasa F, Tsubouchi H, Gohda E, Iwasaka T, Ikehara S.** Hepatocyte growth factor delivered by ultrasound-mediated destruction of microbubbles induces proliferation of cardiomyocytes and amelioration of left ventricular contractile function in doxorubicin-induced cardiomyopathy. *Stem Cells* 23: 1589–1597, 2005.
13. **Kitta K, Day RM, Ikeda T, Suzuki YJ.** Hepatocyte growth factor protects cardiac myocytes against oxidative stress-induced apoptosis. *Free Radic Biol Med* 31: 902–910, 2001.

14. Kosai K, Matsumoto K, Funakoshi H, Nakamura T. Hepatocyte growth factor prevents endotoxin-induced lethal hepatic failure in mice. *Hepatology* 30: 151–159, 1999.

15. Kucia M, Dawn B, Hunt G, Guo Y, Wysoczynski M, Majka M, Ratajczak J, Rezzoug F, Ildstad ST, Bolli R, Ratajczak MZ. Cells expressing early cardiac markers reside in the bone marrow and are mobilized into the peripheral blood after myocardial infarction. *Circ Res* 95: 1191–1199, 2004.

16. Li L, Takemura G, Li Y, Miyata S, Esaki M, Okada H, Kanamori H, Khai NC, Maruyama R, Ogino A, Minatoguchi S, Fujiwara T, Fujiwara H. Preventive effect of erythropoietin on cardiac dysfunction in doxorubicin-induced cardiomyopathy. *Circulation* 113: 535–543, 2006.

17. Li L, Takemura G, Li Y, Miyata S, Esaki M, Okada H, Kanamori H, Ogino A, Maruyama R, Nakagawa M, Minatoguchi S, Fujiwara T, Fujiwara H. Granulocyte colony-stimulating factor improves left ventricular function of doxorubicin-induced cardiomyopathy. *Lab Invest* 87: 440–455, 2007.

18. Li Y, Takemura G, Kosai K, Yuge K, Nagano S, Esaki M, Goto K, Takahashi T, Hayakawa K, Koda M, Kawase Y, Maruyama R, Okada H, Minatoguchi S, Mizuguchi H, Fujiwara T, Fujiwara H. Postinfarction treatment with an adenoviral vector expressing hepatocyte growth factor relieves chronic left ventricular remodeling and dysfunction in mice. *Circulation* 107: 2499–2506, 2003.

19. Lou H, Danelisen I, Singal PK. Cytokines are not upregulated in adriamycin-induced cardiomyopathy and heart failure. *J Mol Cell Cardiol* 36: 683–690, 2004.

20. Lou H, Danelisen I, Singal PK. Involvement of mitogen-activated protein kinases in adriamycin-induced cardiomyopathy. *Am J Physiol Heart Circ Physiol* 288: H1925–H1930, 2005.

21. Mizuguchi H, Kay AM. A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. *Hum Gene Ther* 10: 2013–2017, 1999.

22. Molkentin JD, Kalvakolanu DV, Markham BE. Transcription factor GATA-4 regulates cardiac muscle-specific expression of the alpha-myosin heavy-chain gene. *Mol Cell Biol* 14: 4947–4957, 1994.

23. Murphy AM, Thompson WR, Peng LF, Jones L 2nd. Regulation of the rat cardiac troponin I gene by the transcription factor GATA-4. *Biochem J* 322: 393–401, 1997.

24. Nakagami H, Morishita R, Yamamoto K, Taniyama Y, Aoki M, Matsumoto K, Nakamura T, Kaneda Y, Horiuchi M, Ogihara T. Mitogenic and antiapoptotic actions of hepatocyte growth factor through ERK, STAT3, and AKT in endothelial cells. *Hypertension* 37: 581–586, 2001.

25. Nakamura T, Nawa K, Ichihara A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem Biophys Res Commun* 122: 1450–1459, 1984.

26. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K, Shimizu S. Molecular cloning and expression of human hepatocyte growth factor. *Nature* 342: 440–443, 1989.

27. Nakamura T, Mizuno S, Matsumoto K, Sawa Y, Matsuda H, Nakamura T. Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. *J Clin Invest* 106: 1511–1519, 2000.

28. Nakamura T, Matsumoto K, Mizuno S, Sawa Y, Matsuda H, Nakamura T. Hepatocyte growth factor prevents tissue fibrosis, remodeling, and dysfunction in cardiomyopathic hamster hearts. *Am J Physiol Heart Circ Physiol* 288: H2131–H2139, 2005.

29. Olson RD, Mushlin P. Doxorubicin cardiotoxicity: analysis of prevailing hypotheses. *FASEB J* 4: 3076–3086, 1990.

30. Rosenoff SH, Olson HM, Young DM, Bostick F, Young RC. Adriamycin-induced cardiac damage in the mouse: a small-animal model of cardiotoxicity. *J Natl Cancer Inst* 55: 191–194, 1975.

31. Simpson C, Herr H, Courville KA. Concurrent therapies that protect against doxorubicin-induced cardiomyopathy. *Clin J Oncol Nurs* 8: 497–501, 2004.

32. Singal PK, Iliskovic N. Doxorubicin-induced cardiomyopathy. *N Engl J Med* 339: 900–905, 1998.

33. Sparano JA. Use of dexrazoxane and other strategies to prevent cardiomyopathy associated with doxorubicin-taxane combinations. *Semin Oncol* 125, Suppl 10: 66–71, 1998.

34. Taniyama Y, Morishita R, Aoki M, Hiraoka K, Yamasaki K, Hashiya N, Matsumoto K, Nakamura T, Kaneda Y, Ogihara T. Angiogenesis and antifibrotic action by hepatocyte growth factor in cardiomyopathy. *Hypertension* 40: 47–53, 2002.

35. Tenhunen O, Sarman B, Kerkela R, Szokodi I, Papp L, Toth M, Ruskoaho H. Mitogen-activated protein kinases p38 and ERK 1/2 mediate the wall stress-induced activation of GATA-4 binding in adult heart. *J Biol Chem* 279: 24852–24860, 2004.

36. Ueda H, Nakamura T, Matsumoto K, Sawa Y, Matsuda H, Nakamura T. A potential cardioprotective role of hepatocyte growth factor in myocardial infarction in rats. *Cardiovasc Res* 51: 41–50, 2001.

37. Urbanek K, Rota M, Cascapera S, Bearzi C, Nascimbene A, De Angelis A, Hosoda T, Chimenti S, Baker M, Limana F, Nurzynska D, Torella D, Rotatori F, Rastaldo R, Musso E, Quaini F, Leri A, Kajstura J, Anversa P. Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival. *Circ Res* 97: 663–673, 2005.

38. Zhang J, Clark JR Jr, Herman EH, Ferrans VJ. Doxorubicin-induced apoptosis in spontaneously hypertensive rats: differential effects in heart, kidney and intestine, and inhibition by ICRF-187. *J Mol Cell Cardiol* 28: 1931–1943, 1996.

Basic Science and Experimental Studies

In Vivo Hepatocyte Growth Factor Gene Transfer Reduces Myocardial Ischemia-Reperfusion Injury Through Its Multiple Actions

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ABSTRACT

Background: Hepatocyte growth factor (HGF) is reported to protect the heart against ischemia-reperfusion injury. However, whether in vivo adenovirus-mediated HGF gene transfer before ischemia is protective against ischemia-reperfusion and its precise mechanisms are still unknown.

Methods and Results: By using a rabbit model of ischemia-reperfusion injury, we demonstrate that HGF gene transfer is cardioprotective through its multiple beneficial actions, such as angiogenesis, Bcl-2 overexpression, and decreasing hydroxyl radicals, deoxyuride-5'-triphosphate biotin nick end labeling (TUNEL)-positive myocytes, and fibrotic area. After HGF gene transfer, the rabbits underwent 30 minutes of coronary occlusion and 30 minutes, 4 hours, 48 hours, and 14 days of reperfusion. The infarct size at 48 hours of reperfusion was significantly reduced in the HGF group ($13.4\% \pm 2.3\%$) compared with that in the LacZ group ($36.5\% \pm 2.0\%$) and saline group ($40.3\% \pm 3.2\%$). At 14 days of reperfusion, HGF gene transfer improved left ventricular ejection fraction and fractional shortening, reduced the fibrotic area, and increased the capillary density in the risk area. At 4 hours of reperfusion, Bcl-2 protein was overexpressed and the incidence of TUNEL-positive myocytes was significantly decreased in the risk area in the HGF group compared with the LacZ and saline groups. The myocardial interstitial 2,5-dihydroxybenzoic acid level, an indicator of hydroxyl radical, increased during 30 minutes of ischemia and 30 minutes of reperfusion in the LacZ and saline groups, and was significantly inhibited in the HGF group.

Conclusion: HGF gene therapy may be a novel therapeutic strategy against unstable angina pectoris or severe angina pectoris, which may progress to acute myocardial infarction. (*J Cardiac Fail 2007;13:874–883*)

Key Words: Angiogenesis, apoptosis, cardiac function, free radical, hepatocyte growth factor, infarct size.

Hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes,[1,2] was reported to have multiple actions such as mitogenic, angiogenic,

antiapoptotic, and antifibrotic activities in various cells, preferentially in most epithelial and endothelial cells.[3,4] Recent studies, however, reported that human recombinant HGF is cardioprotective: HGF protected cardiomyocytes from acute ischemic death during acute myocardial infarction[5,6] and enhanced the survival of cardiomyocytes subjected to oxidative stress.[7,8] Taniyama et al.[9] recently reported the beneficial effects of HGF on cardiac function in an animal model of cardiomyopathy through its angiogenic and antifibrotic actions. Furthermore, we reported that postinfarction treatment with an adenoviral vector expressing HGF relieves chronic left ventricular remodeling and dysfunction in mice.[10] However, whether in vivo HGF gene transfer before ischemia reduces myocardial infarct size and improves left ventricular

dysfunction and its precise mechanisms are still unknown. The hypothesis in the present study is that in vivo HGF gene transfer may reduce the myocardial infarct size and improve cardiac function via the antioxidant, angiogenic, antiapoptotic, and antifibrotic actions of HGF.

Methods

In this study, all rabbits received humane care in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the U.S. National Institutes of Health (NIH publication 8523, revised 1985). The study protocol was approved by the Ethical Committee of Gifu University School of Medicine, Gifu, Japan.

Animal Selection

Japanese white male rabbits, each weighing approximately 2.0 to 2.5 kg, were used. None of the rabbits had any clinically evident infections.

Surgical Preparation

Rabbits were anesthetized with an intravenous injection of sodium pentobarbital (30–40 mg/kg), and additional doses were given when required throughout the experiment. They were orally intubated and ventilated with room air supplemented with a low flow of oxygen by mechanical ventilation (tidal volume 25–35 mL, respiratory rate 20–30 breaths/min) (Shimano, model SN-480-5, Tokyo, Japan). Serial blood gas analysis was performed, and ventilatory conditions were adjusted to keep the arterial blood gas within the physiologic range. Surgery was performed under sterile conditions. The carotid artery and jugular veins were cannulated to monitor the peripheral arterial pressure and to administer drugs. Next, the rabbits were systemically heparinized (500 U/kg). A thoracotomy was performed in the left third intercostal space, and the heart was exposed after excising the pericardium. A 4-0 silk suture on a small curved needle was passed through the myocardium beneath the middle segment of the large arterial branch coursing down the middle segment of the anterolateral surface of the left ventricle (LV). A small vinyl tube was passed into both ends of the silk suture, and the coronary branch was occluded by pulling the snare, which was fixed by clamping the tube with a mosquito hemostat. Myocardial ischemia was confirmed by ST-segment elevation on the electrocardiogram and regional cyanosis of the myocardial surface. Reperfusion was confirmed by myocardial blush over the risk area after releasing the snare. All rabbits were allowed 20 minutes after completion of the surgical preparation to reach a steady state before starting the protocol.

Recombinant Adenoviral Vectors

Adenoviral vector plasmid pAd-HGF, which comprises cytomegalovirus immediate early enhancer, a modified chicken β-actin promoter, and human HGF cDNA (Ad.CAG-HGF), was constructed using the in vitro ligation method (a gift from Mark A. Kay, MD, Stanford University School of Medicine) as described previously.[11] Control Ad-LacZ was prepared as described previously.[12]

Measurement of Human HGF Level in the Plasma and Cardiac Tissues

The plasma and cardiac tissue levels of human HGF were measured using an enzyme-linked immunosorbent assay kit (Institute of Immunology, Tokyo, Japan). The detection threshold was 5 pg/mL.

Expression of HGF in the Heart Detected by Immunoblotting

After 48 hours of myocardial infarction, rabbit hearts were divided into ischemic and nonischemic areas, and homogenized in lysis buffer on ice. Proteins (100 μg) were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. Membranes were incubated with antibodies, antihuman HGF (R&D Systems Inc., Minneapolis, Minnesota), and β-actin (Sigma Chemical Co., St. Louis, Missouri), and subsequently with horseradish peroxidase-conjugated anti-goat immunoglobulin-G or anti-mouse immunoglobulin-G antibody (Dako Cytomation Inc., Carpinteria, California). Immunoreactive bands were detected using the chemiluminescent substrate (Amersham Biosciences, Piscataway, New Jersey) according to the manufacturer's protocol.

Protocol 1

After a thoracotomy was performed in the left third intercostal space and the heart was exposed after excising the pericardium, Ad.CAG-HGF (1×10^9 pfu/rabbit) was injected into the myocardium (HGF group). In the LacZ group, adenovirus encoding the LacZ gene was similarly injected into the myocardium. In the saline control group, saline was similarly injected into the myocardium. Three days after viral infection or saline injection, the rabbits underwent 30 minutes of coronary occlusion and 4 hours, 48 hours, or 14 days of reperfusion. In the sham group, rabbits received only a thoracotomy without induction of infarction. Hemodynamic parameters (systolic blood pressure, diastolic blood pressure, and heart rate) were monitored throughout the experiment. After the experiment, the chest was closed and the rabbits were allowed to recover from anesthesia for 48 hours or 14 days survival. The incidence of deoxyuride-5'-triphosphate biotin nick end labeling (TUNEL)-positive myocytes and the expression of Bcl-2 protein were detected after 4 hours of reperfusion. The myocardial infarct size was measured at 48 hours of reperfusion. The left ventricular function and the incidence of microvessels with positive CD31 were assessed after 14 days of reperfusion.

Physiologic Studies. Echocardiograms were recorded with an echocardiographic system (Aloka, Tokyo, Japan) equipped with a 7.5-MHz imaging transducer 14 days after myocardial infarction. After cardiac echocardiography, a micromanometer-tipped catheter (SPR 407, Millar Instruments, Houston, Texas) was inserted into the LV for recording $\pm$dP/dt.

Infarct Size Measurement. Forty-eight hours after reperfusion, the rabbits were heparinized (500 μ/kg) and killed with an overdose of pentobarbital. The heart was excised and mounted on a Langendorff apparatus. The coronary branch was reoccluded, and Evans blue dye (4%, Sigma Chemical Co.) was injected into the aorta at 80 mm Hg. The area at risk was defined as the area without blue staining. The LV was sectioned into seven slices parallel to the atrioventricular ring. Each slice was weighed, incubated in a 1% solution of triphenyl tetrazolium chloride at 37°C to visualize the infarct area, and photographed. The risk and infarct

areas were traced on each LV slice and multiplied by the slice's weight, then expressed as a fraction of the risk area or LV for each heart.

Histologic Analysis. The rabbits were killed at 4 hours, 48 hours, and 14 days after infarction. The hearts were removed and cut into seven transverse slices, and each slice was fixed with 10% buffered formalin and embedded in paraffin; 4-μm–thick sections were stained with hematoxylin-eosin and Masson's trichrome. Quantitative assessment of the fibrotic area as a percentage of the LV was performed using a multipurpose color image processor, LUZEX F (Nireco, Kyoto, Japan). The fibrotic area was measured within the infarct region.

Immunohistochemical Analysis. By using an indirect immunoperoxidase method, immunohistochemical staining was performed with monoclonal mouse antihuman CD31, endothelial cell antibody (Dako), and monoclonal mouse antihuman Bcl-2 (Dako), which cross-react with rabbit tissues. Morphometric analyses were performed by two persons blinded to the treatment. Hematoxylin-eosin staining was also performed in each slice, and localization was made on the basis of hematoxylin-eosin staining. The capillary density and Bcl-2–positive cardiomyocytes were counted in the ischemic area of the ventricle. There were 20 high-power fields (400×) for one slide. The capillary density was shown as the number of capillaries per high-power field, and the Bcl-2–positive cardiomyocytes were shown as a percentage of the total counts.

TUNEL. TUNEL assay was performed in deparaffinized 4-μm–thick sections with an ApopTag kit (Intergene, Purchase, New York). We counted 10,000 myocytes per heart. In the present study, we focused on the TUNEL-positive myocytes because we previously reported that TUNEL-positive myocytes are frequently observed at 4 hours of reperfusion after 30 minutes of ischemia in rabbits.[13]

Protocol 2

Measurement of Myocardial Interstitial Hydroxyl Radicals. Fourteen rabbits were used to investigate the effect of HGF gene transfer on the amount of myocardial interstitial hydroxyl radicals during ischemia-reperfusion. A microdialysis probe (PNF 1700; Asahi Medical, Tokyo, Japan; 20 mm in length, 0.31 mm OD, 0.2 mm ID; transverse type, 50,000 MW cutoff) for dialysate sampling was implanted in the risk region of the myocardium, which was served by the anterolateral coronary artery along the axis of the ventricular fibers and reached from the epicardial outer layer to the endocardial inner layer of the myocardium. Probe placement was confirmed at autopsy. The microdialysis probe was perfused with 1 mmol/L of salicylic acid dissolved in Ringer's solution at a rate of 10 μL/min. After a 60-minute rest following the completion of instrumentation, the dialysate was sampled during 30 minutes of pre-ischemia, 30 minutes of ischemia, and 30 minutes of reperfusion with intervals of 10 minutes in the saline (n = 7), LacZ (n = 7), and HGF (n = 7) groups. Dialysate samples were frozen at −83°C until further analysis. The measurement of the hydroxyl radical is based on the reaction between salicylic acid and hydroxyl radical; 1 mmol/L salicylic acid can trap approximately 10% of the theoretically generated hydroxyl radical, producing 2,3-dihydroxybenzoic acid (DHBA), 2,5-DHBA, and catechol as the derivatives in proportions of 49%, 40%, and 11%, respectively.[14] In the present study, we used 2,5-DHBA as an indicator of hydroxyl radical production because of its high specificity for hydroxyl radical.[15] The column used in the present study was an MCM C18 column (6 × 250;

5-120A; MC Medical Inc., Tokyo, Japan). The 2,5-DHBA, an indicator of hydroxyl radicals, was measured using high-performance liquid chromatography coupled with electrochemical detection, as described previously[14,15] with slight modifications.

Statistical Analysis

Data are expressed as the group mean ± standard error of the mean. To compare the group means of hydroxyl radical levels, area at risk, infarct size, and hemodynamic parameters, one-way analysis of variance (ANOVA) was performed. If the ANOVA result was significant, a modified unpaired t test was performed to assess which group was significantly different. The effects of treatments on hemodynamics were analyzed with one-way repeated-measures ANOVA. A post-ANOVA adjustment was made by the Bonferroni method. Differences with a P value less than .05 were considered statistically significant.

Results

Mortality and Animal Exclusion

Initially, 92 rabbits were enrolled in protocol 1. Among these rabbits, eight died of ventricular fibrillation during ischemia-reperfusion (Table 1). Of the remaining rabbits, eight died after the first day of the experiment. Thus, the experiments were completed in the remaining 76 rabbits, and the data from these animals were used for the analysis.

X-gal Staining

Figure 1 shows X-gal staining of the LV infected with Ad-LacZ. Hematoxylin-eosin staining showed that myocytes were stained blue, revealing the adenovirus-infected myocytes.

Plasma and Cardiac Tissue Levels of Human HGF

The cardiac tissue level of human HGF level reached 263 ng/g tissue 3 days after the viral transfection in the HGF-treated rabbits but not in the LacZ-treated rabbits. No human HGF was detected in the plasma, both in the LacZ-treated and HGF-treated rabbits.

Table 1. Survival Rate

Group	No.	VF	Premature Death	Survivors (%)
Sham	5	0	0	5/5 (100)
Saline 4 h	6	1	0	5/6 (83)
LacZ 4 h	7	1	0	6/7 (85)
HGF 4 h	6	0	0	6/6 (100)
Sham	5	0	0	5/5 (100)
Saline 48 h	10	1	1	8/10 (80)
LacZ 48 h	10	1	1	8/10 (80)
HGF 48 h	8	0	1	7/8 (87)
Sham	5	0	0	5/5 (100)
Saline 14 d	10	1	2	7/10 (70)
LacZ 14 d	10	2	1	7/10 (70)
HGF 14 d	10	1	2	7/10 (70)

VF, ventricular fibrillation; HGF, hepatocyte growth factor.

X-gal Staining
(macroscopic image)

Hematoxylin Eosin Staining
+ X-gal Staining
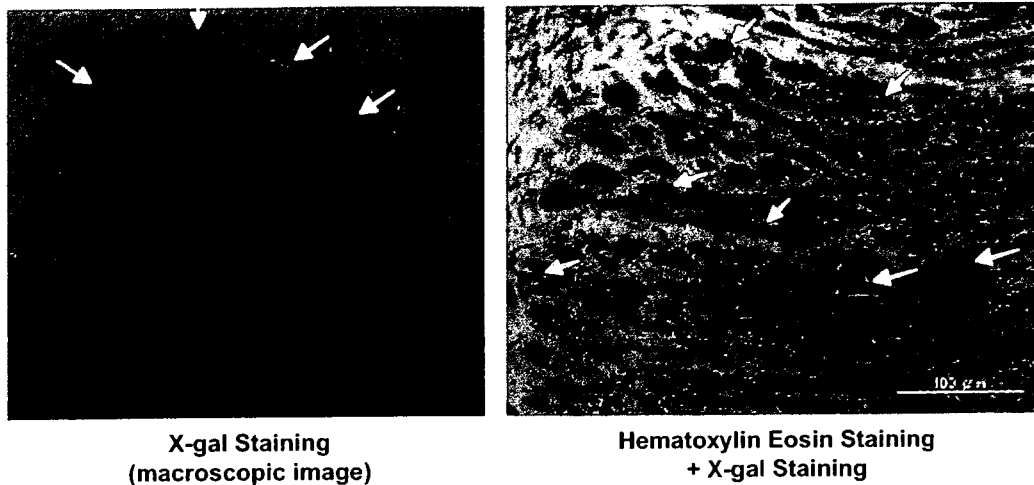
Fig. 1. X-gal staining of LV infected with Ad-LacZ. Macroscopic image (*left*). Cardiac tissues were stained blue, suggesting the infection of adenovirus. Myocytes were stained blue, suggesting the infection of adenovirus (*right*). Infection of adenovirus (*arrows*). Cardiac tissue was obtained 48 hours after myocardial infarction.

Immunoblot Analysis

As shown in Figure 2, the expression of human HGF protein was confirmed in the ischemic area in the HGF group but not in the LacZ group.

Physiologic Studies

According to echocardiography and cardiac catheterization 14 days post-myocardial infarction, the saline-treated and LacZ-treated rabbits showed decreased cardiac function, decreased LV ejection fraction percentage, LV fractional shortening percentage, and ±dP/dt (Fig. 3). HGF gene therapy significantly improved each of these conditions, indicating the improvements of post-infarct cardiac function. There was no difference in systolic and diastolic blood pressures and heart rate among the saline, LacZ, and HGF groups before, during, and after 30 minutes of ischemia.

Pathologic Studies

Infarct Size. There was no significant difference in the area at risk as a percentage of the LV between the LacZ and HGF groups. The infarct size as a percentage of the area at risk was significantly reduced in the HGF group (13.4% ± 2.3%) compared with the LacZ group (36.5% ± 2.0%) and saline group (40.3% ± 3.2%) 48 hours after infarction (Fig. 4).

TUNEL-Positive Myocytes. HGF gene therapy resulted in a significant reduction of the incidence of TUNEL-positive myocytes (2.6% ± 1.0%, $P < .05$) compared with the saline group (10.8% ± 1.9%) and LacZ group (11.1% ± 2.2%) (Fig. 5).

Immunohistochemical Staining. The capillary density in the infarcted area assessed by immunostaining of CD31 was significantly higher in the HGF group than in the saline and LacZ groups 14 days after infarction (Fig. 6). The expression of Bcl-2 was significantly

increased in the risk area after 4 hours of reperfusion in the HGF group compared with the saline and LacZ groups (Fig. 7).

Fibrotic Area. Fibrotic area as a percentage of the LV was significantly reduced in the HGF group compared with the saline and LacZ groups (Fig. 8).

Effect on Myocardial Interstitial 2,5-DHBA Levels. As shown in Figure 9, the myocardial interstitial levels of 2,5-DHBA, an indicator of the hydroxyl radical, were significantly increased at 10 and 20 minutes after the start of coronary occlusion and peaked at 10 minutes after the start of reperfusion compared with the preischemic period in the saline and LacZ groups. However, HGF gene therapy significantly attenuated the increase of the myocardial interstitial 2,5-DHBA levels during ischemia and reperfusion periods.

Discussion

The present study revealed that in vivo adenovirus-mediated HGF gene transfer before ischemia reduced the

Fig. 2. Expression of HGF in the heart detected by immunoblotting. The expression of human HGF protein was observed in the ischemic area of the LV in the HGF group but not in the LacZ group. The expression of human HGF was not observed in the remote area of the LV in the HGF group. Cardiac tissue was obtained 48 hours after myocardial infarction. HGF, hepatocyte growth factor.
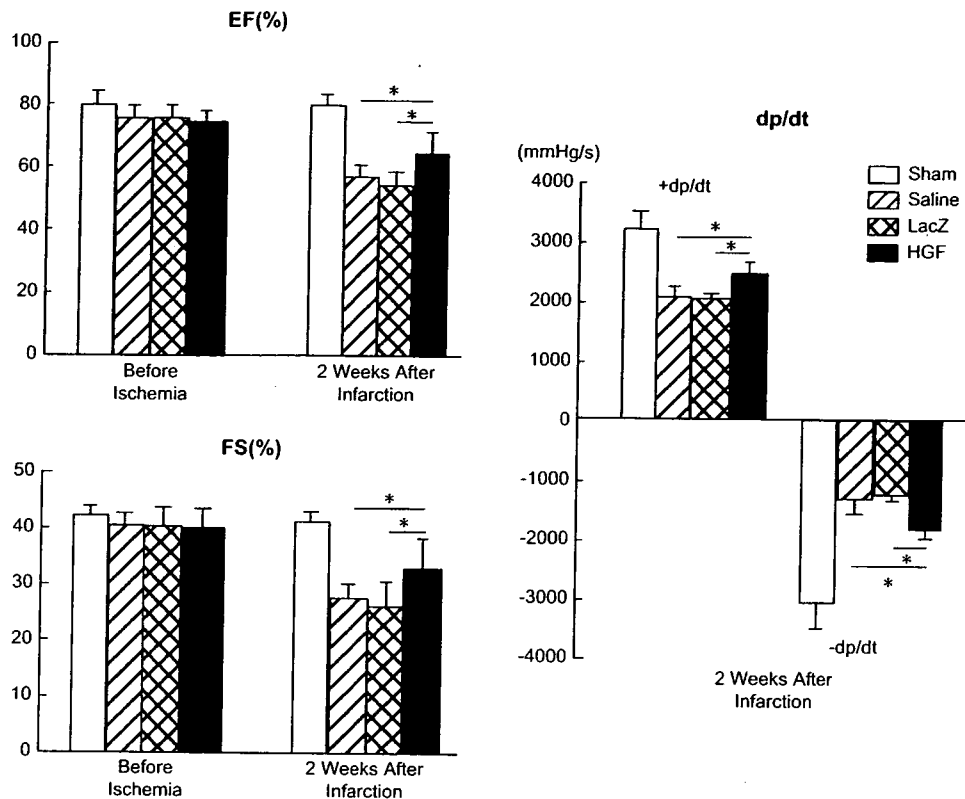
Fig. 3. Hemodynamic parameters assessed by echocardiography and cardiac catheterization 14 days after infarction. Note the improvement of ejection fraction, fractional shortening, and ± dp/dt in the HGF group. EF, ejection fraction; FS, fractional shortening; HGF, hepatocyte growth factor. *$P < .05$.

myocardial infarct size and improved left ventricular function via multiple beneficial actions, such as antioxidant, antiapoptotic, and antifibrotic actions, and enhancement of Bcl-2 expression and angiogenesis.

Expression of Human HGF

With the use of enzyme-linked immunosorbent assay and Western blot analysis, we detected human HGF in the cardiac tissue of HGF-treated rabbits but not in the LacZ-treated rabbits. We could not detect human HGF in the plasma or cardiac tissue in the LacZ group. However, Nakamura et al.[5] reported increased plasma levels of HGF after myocardial ischemia-reperfusion. This discrepancy may be explained by the fact that we used adenoviral vector plasmid, which produces human HGF, and antihuman HGF antibody (R&D Systems, Inc., Minneapolis, Minnesota) to assess whether human HGF was produced in the plasma and cardiac tissue of the rabbit, whereas Nakamura et al. used anti-rat HGF antibody to detect the plasma level of HGF in a rat model of myocardial infarction.

In the present study, we did not examine how long the expression of "exogenous" HGF continued in the myocardium of the rabbits. However, we previously reported that adenoviral vector-mediated transfer of HGF increased plasma levels of human HGF persistently for 4 weeks in mice.[16]

Mechanisms of Beneficial Effects of HGF on Infarct Size and Left Ventricular Function

The mechanisms responsible for the beneficial effects of HGF on the infarct size and left ventricular function seemed to be complicated, probably reflecting multifunctions of HGF. It has been reported that recombinant human HGF administered immediately after reperfusion after 20 minutes of ischemia reduced the infarct size and improved cardiac function through antiapoptotic effects in rats.[5] This is consistent with the result of the present study, although Nakamura et al.[5] used recombinant human HGF and we used adenovirus-mediated HGF gene transfer. In vitro studies showed that HGF has an antioxidant effect.[7,8] Gene transfer of human cDNA HGF has been reported to improve cardiac function in isolated rat hearts.[6] Moreover, it has been reported that HGF induces angiogenic and antifibrotic effects in a model of cardiomyopathy and heart failure.[9] The results of the present study demonstrated that gene transfer of HGF before ischemia 1) reduced the production of hydroxyl radical during ischemia and reperfusion, 2) reduced the incidence of TUNEL-positive myocytes, 3) enhanced the expression of Bcl-2 protein, 4) increased angiogenesis, 5) reduced the fibrotic area, 6) reduced the infarct size, and 7) improved LV dysfunction.

As mentioned above, the present study demonstrated that in vivo gene transfer of HGF before ischemia showed

Area At Risk as a Percent of Left Ventricle **Infarct Size as a Percent of Area At Risk**
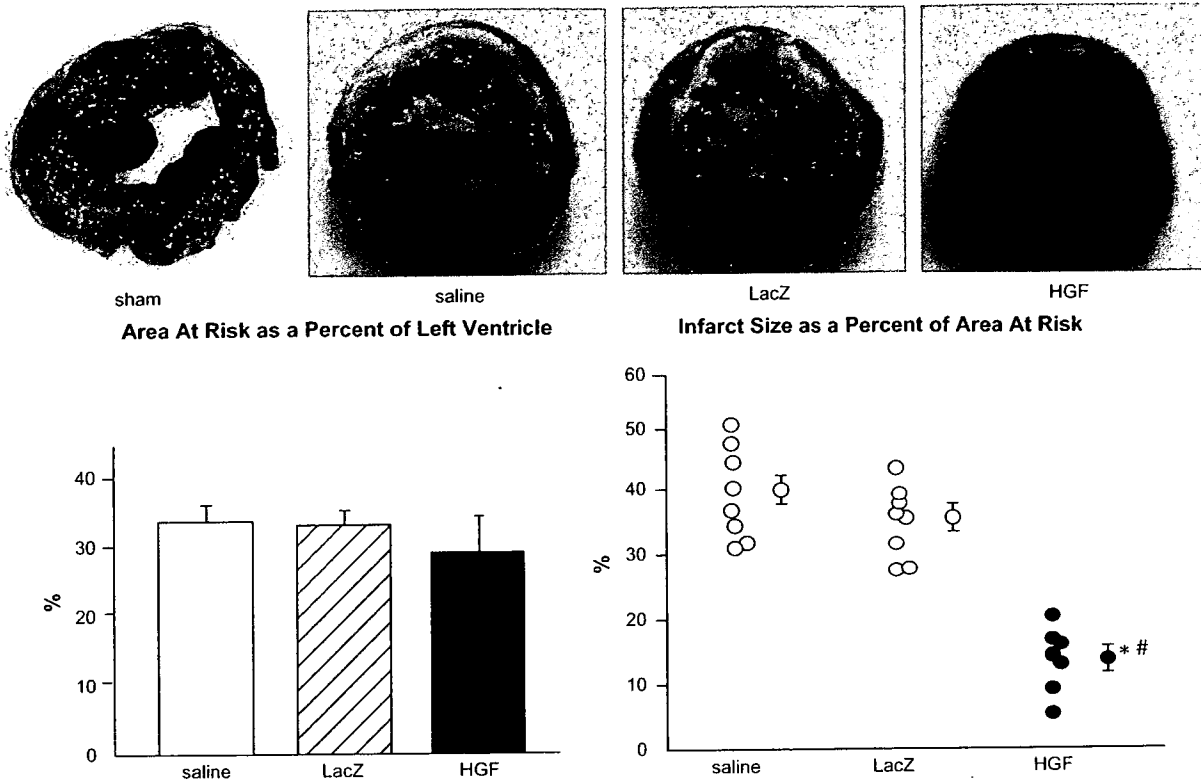
Fig. 4. Infarct size as a percentage of the LV. Typical photographs of the LV stained with triphenyl tetrazolium chloride and Evans blue dye (top). Note that the infarct area is smaller in the HGF group than in the LacZ group. Area at risk as a percentage of LV (left). Infarct size as percentage of area at risk (right). Note that the infarct size was significantly reduced in the HGF group than in the LacZ group. Cardiac issue was obtained 48 hours after myocardial infarction. HGF, hepatocyte growth factor. * $P < .05$ versus control. $\#P < .05$ versus LacZ group.
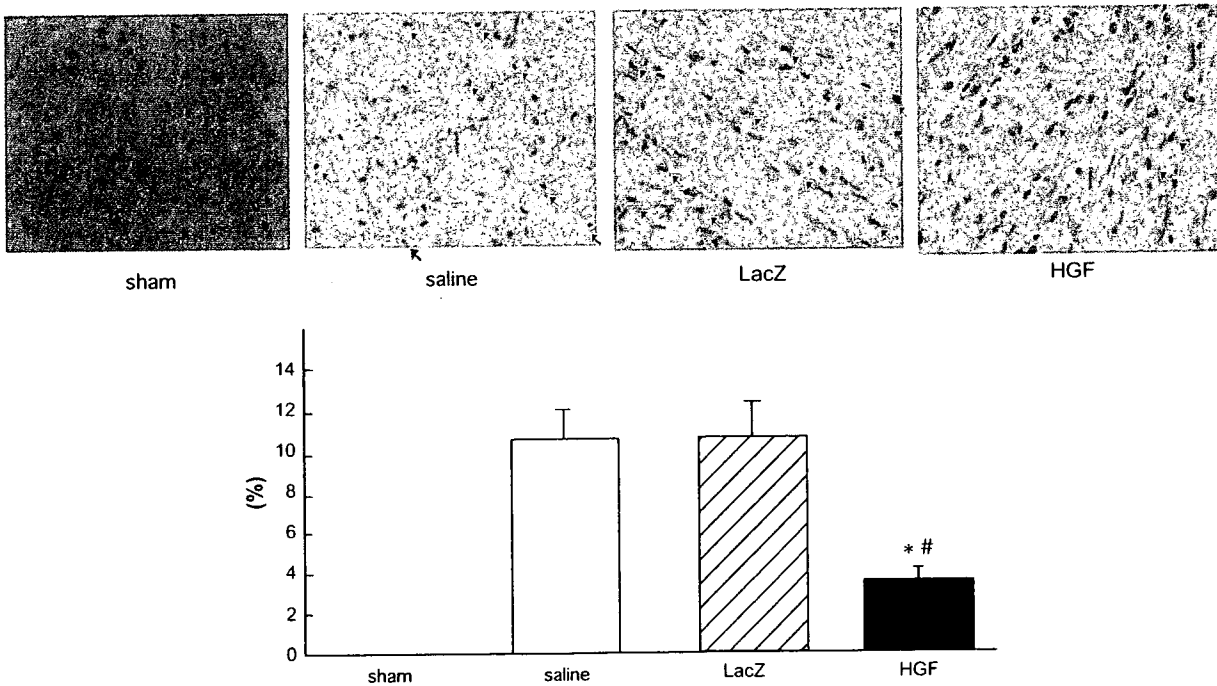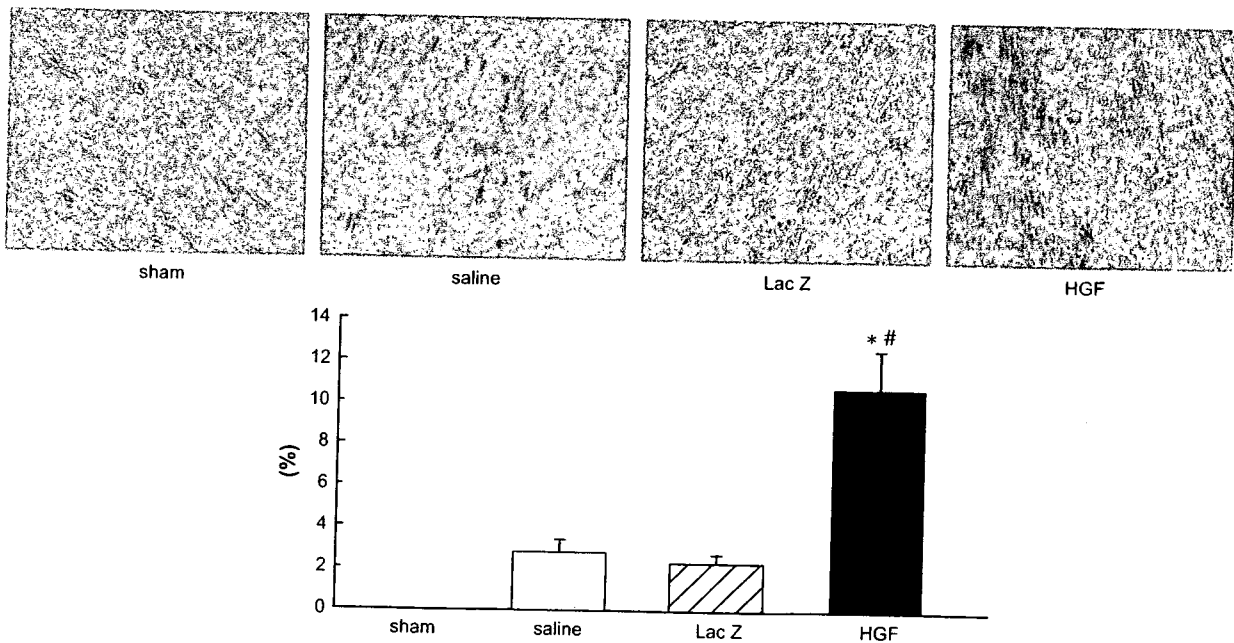

Fig. 5. TUNEL-positive myocytes in the infarcted myocardium. Photomicrograph (light microscopic TUNEL analysis) of myocardium from the LacZ and HGF groups subjected to 30 minutes of ischemia followed by 4 hours of reperfusion (×400) (top). Brown TUNEL-positive nuclei are seen in the infarcted area. Incidence of TUNEL-positive myocytes in the LacZ and HGF groups (bottom). *$P < .05$ versus LacZ group. $\#P < .05$ saline group. HGF, hepatocyte growth factor.
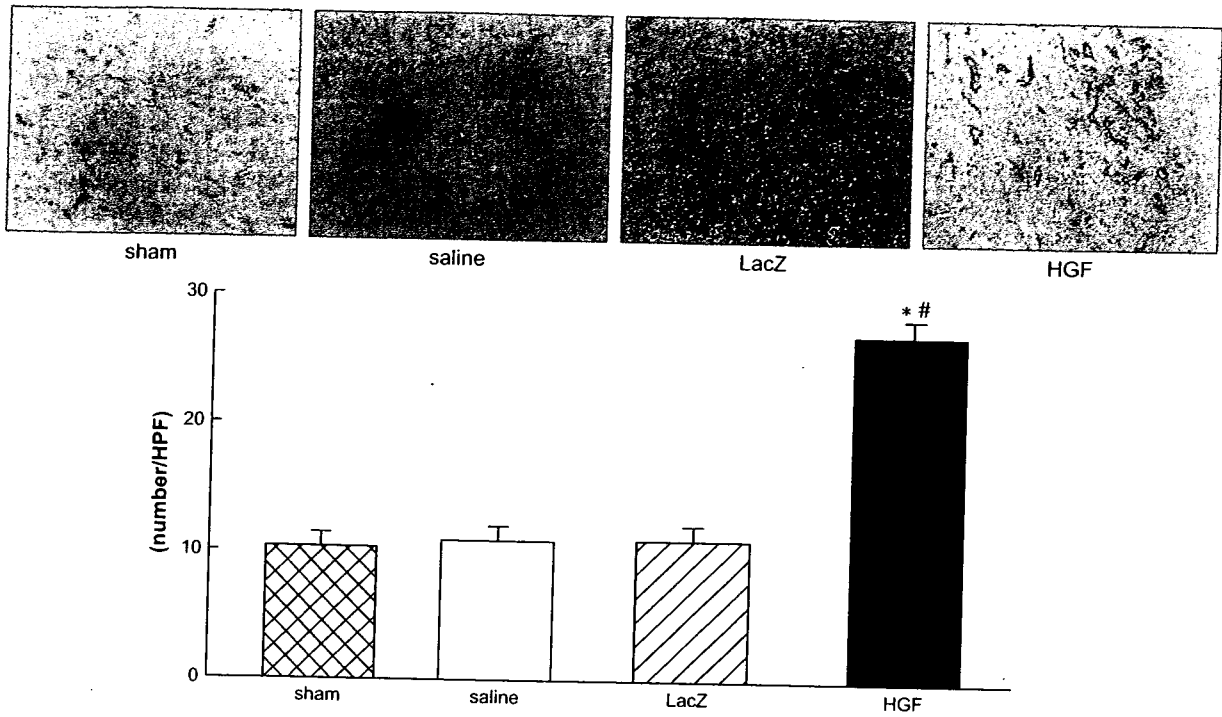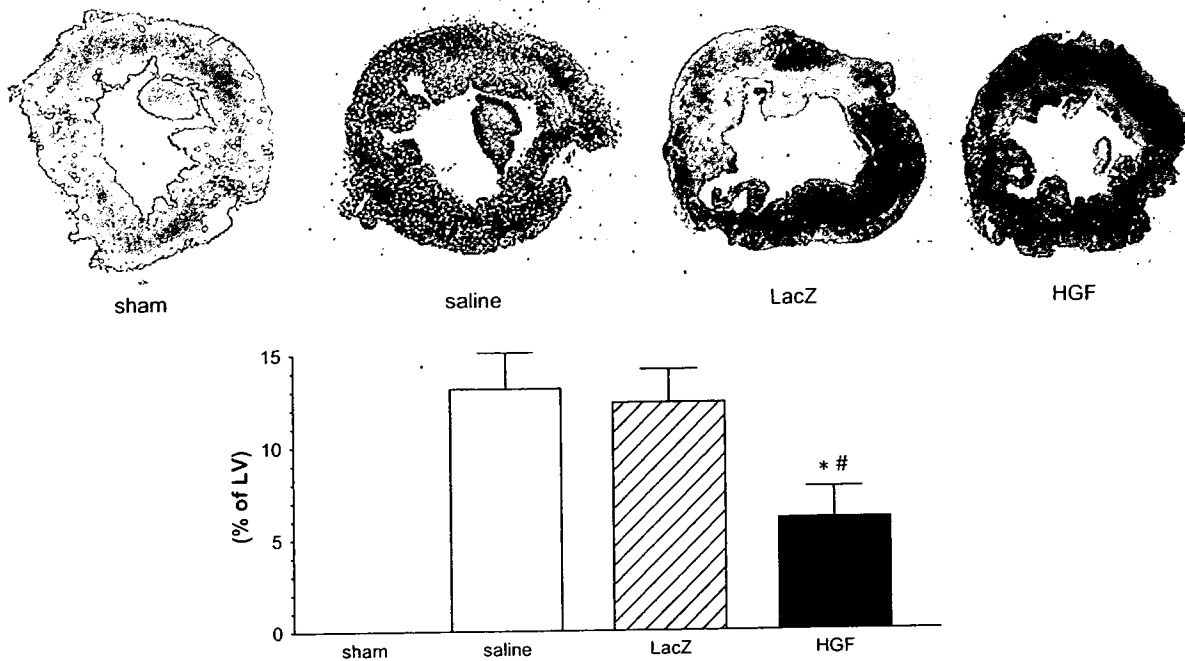
Fig. 6. Capillary density assessed by immunostaining of CD31. Photomicrograph of myocardium immunostained with CD31 from the LacZ and HGF groups subjected to 30 minutes of ischemia followed by 14 days of reperfusion (×400) (*top*). Brown stained microvessels are seen in the infarcted area. Incidence of CD31-positive microvessels in the LacZ and HGF groups (*bottom*). *P < .05 versus LacZ group. #P < .05 saline group. HPF, high-power field; HGF, hepatocyte growth factor.

multiple beneficial actions, and all of these effects might contribute to the reduction in the infarct size and the improvement of left ventricular dysfunction after infarction. Among the multiple effects of HGF, the most remarkable

finding in the present study was that HGF gene therapy strikingly decreased hydroxyl radical during ischemia-reperfusion. Reactive oxygen species, such as superoxide and hydroxyl radicals, have been suggested to be

Fig. 7. Expression of Bcl-2 protein in the infarcted myocardium. Photomicrograph of myocardium immunostained with Bcl-2 from the LacZ and HGF groups subjected to 30 minutes of ischemia followed by 14 days of reperfusion (×400) (*top*). Brown stained myocytes are seen in the infarcted area. Incidence of Bcl-2—positive myocytes in the LacZ and HGF groups (*bottom*). Bcl-2 protein was predominantly expressed at the border of infarcted and intact areas. *P < .05 versus LacZ group. #P < .05 saline group. HGF, hepatocyte growth factor.

Fig. 8. Fibrosis area determined by Masson's trichrome staining. Photomicrograph of myocardium with Masson's trichrome staining from the LacZ and HGF groups subjected to 30 minutes of ischemia followed by 14 days of reperfusion (×400) (*top*). Blue stained areas are seen in the infarcted area. The fibrosis area as a percentage of LV in the LacZ and HGF groups (*bottom*). *P < .05 versus LacZ group. #P < .05 saline group. LV, left ventricle; HGF, hepatocyte growth factor.

predominant mediators of ischemia-reperfusion injury.[17] Among the reactive oxygen species, the hydroxyl radical is highly reactive and plays a critical role in post-ischemic myocardial damage during reperfusion.[18] We investigated whether HGF gene therapy reduces the myocardial interstitial levels of 2,5-DHBA, an indicator of hydroxyl radical, using a microdialysis technique. We found that hydroxyl radicals were generated in the interstitium during both ischemia and reperfusion and that HGF gene therapy reduced the level of hydroxyl radicals in the myocardium during ischemia-reperfusion. This suggests that one of the most important mechanisms of HGF gene therapy for reducing the infarct size may be related to the reduction of hydroxyl radical production during ischemia-reperfusion. Indeed, HGF directly induces glutation, a radical scavenger,[19] whereas HGF inhibits neutrophil influx in vivo.[20]

In a strict sense, it may be impossible to determine whether HGF gene transfer is a cause of, or an effect of, the infarct limitation. However, in an in vivo study it is difficult to determine which is the cause and effect. We previously reported that decreased production of hydroxyl radicals during reperfusion by a free radical scavenger reduced the infarct size in a rabbit model of myocardial infarction.[21] In addition, it has been reported that HGF by itself has an anti-oxyradical effect.[7,8] Therefore, it may be possible that HGF gene therapy, which produced human HGF in the ischemic area, attenuated the level of hydroxyl radicals during ischemia-reperfusion and led to the infarct limitation.

It has been reported that the reactive oxygen species released from myocytes after ischemia-reperfusion may trigger both necrosis and apoptosis.[22] Free radical scavengers have been reported to inhibit the appearance of apoptosis, which suggests reactive oxygen species as triggers of apoptosis.[23,24] In the present study, the incidence of TUNEL-positive myocytes at 4 hours of reperfusion was significantly decreased in the HGF group compared with the LacZ group. Therefore, it is likely that HGF gene therapy inhibited TUNEL-positive
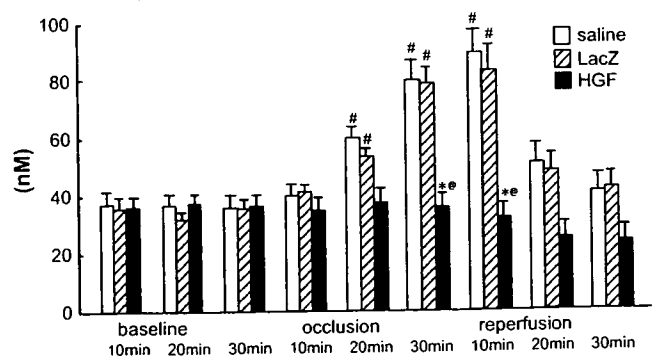
Fig. 9. Effect of HGF gene transfer on myocardial interstitial 2,5-DHBA levels, an indicator of hydroxyl radicals before, during 30 minutes of ischemia, and 30 minutes of reperfusion at an interval of 10 minutes. *P < .05 versus LacZ group. #P < .05 versus baseline value. @P < .05 versus saline group. HGF, hepatocyte growth factor.

myocytes by reducing the burst of oxygen free radicals such as hydroxyl radicals.

Apoptosis is governed by a member of the regulating genes mediated by apoptotic signals. Bcl-2 is one of the members of the Bcl-2 family, such as Bcl-2, Bcl-XL, and Bcl-w, which act as inhibitors of apoptosis. Cytochrome C released from the mitochondria has been reported to bind to Apaf-1 to activate caspase 9, which can cleave and activate caspase 3, leading to the formation of various death substrates that produce apoptosis.[25] Bcl-2 can interfere with cytochrome C release and suppress the actions of Apaf-1 and provide protection from apoptosis.[26] It has been reported that ischemia followed by reperfusion induced a time-dependent reduction in the expression of Bcl-2 protein.[27] In the present study, HGF gene therapy significantly enhanced the expression of Bcl-2 protein in the ischemic area. Therefore, the antiapoptotic effect of HGF gene therapy may be attributed to the up-regulation of Bcl-2 expression. HGF has been shown to activate GATA4,[28] and GATA-4 can regulate Bcl-2 expression;[29] thus, GATA-4 may be involved in the overexpression of Bcl-2.

It has been reported that HGF treatment enhances angiogenesis as assessed by vessel density[10,30] after myocardial infarction. In the present study, we also found that the incidence of CD31-positive microvessels in the infarcted area was significantly higher in the HGF group than in the LacZ group, suggesting that HGF gene transfer increased the angiogenesis in the infarcted area. This might have contributed to the reduction in the infarct size and the improvement of cardiac function via an increase of myocardial regional blood flow. HGF has also been reported to have an antifibrotic effect in several organs, such as the liver, kidney, lung, and heart.[10,30–34] We also demonstrated that the fibrosis area determined by Masson's trichrome staining 14 days after myocardial infarction was significantly smaller in the HGF group than in the LacZ group, suggesting that HGF gene transfer has an antifibrotic effect on post-infarction myocardium. However, reduced fibrosis and preserved function may be an effect of reduced infarct size. The precise cellular mechanism by which HGF reduces the infarct size still remains to be investigated. However, one of the likeliest candidates for the infarct size-reducing effect by HGF may be an effect of decreasing oxyradicals, such as hydroxyl radicals, during ischemia-reperfusion because free radical scavengers that scavenge hydroxyl radicals have been reported to reduce the infarct size.[21,35]

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 pathologic situations such as acute myocardial infarction.[36,37] It is possible that HGF modulates the ischemia-reperfusion injury and post-infarct process through interaction with other cytokines. In the present study, we did not perform an experiment on survival. Whether the protective effects of HGF gene therapy translate into survival benefits still remains to be elucidated.

Clinical Implications

The present findings demonstrate a novel therapeutic strategy against unstable angina pectoris or severe angina pectoris, which may progress into acute myocardial infarction despite conventional therapy.

Acknowledgment

References

1. Nakamura T, Nawa K, Ichihara A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. Biochem Biophys Res Commun 1984;122:1450–9.
2. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, et al. Molecular cloning and expression of human hepatocyte growth factor. Nature 1989;342:440–3.
3. Birchmeier C, Gherardi E. Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. Trends Cell Biol 1998;8:404–10.
4. Kosai K, Matsumoto K, Funakoshi H, Nakamura T. Hepatocyte growth factor prevents endotoxin-induced lethal hepatic failure in mice. Hepatology 1999;30:151–9.
5. Nakamura T, Mizuno S, Matsumoto K, Sawa Y, Matsuda H, Nakamura T. Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. J Clin Invest 2000;106:1511–9.
6. Ueda H, Sawa Y, Matsumoto K, Sawa Y, Matsuda H, Nakamura T. Gene transfection of hepatocyte growth factor attenuates reperfusion injury in the heart. Ann Thorac Surg 1999;67:1726–31.
7. Ueda H, Nakamura T, Matsumoto K, Sawa Y, Matsuda H, Nakamura T. A potential cardioprotective role of hepatocyte growth factor in myocardial infarction in rats. Cardiovasc Res 2001;51: 41–50.
8. Kitta K, Day RM, Ikeda T, Suzuki YJ. Hepatocyte growth factor protects cardiac myocytes against oxidative stress-induced apoptosis. Free Radic Biol Med 2001;31:902–10.
9. Taniyama Y, Morishita R, Auki M, Hiraoka K, Yamasaki K, Hashiya N, et al. Angiogenesis and antifibrotic action by hepatocyte growth factor in cardiomyopathy. Hypertension 2002;40:47–53.
10. Li Y, Takemura G, Kosai K, Takahashi T, Okada H, Miyata S, et al. Postinfarction treatment with an adenoviral vector expressing hepatocyte growth factor relieves chronic left ventricular remodeling and dysfunction in mice. Circulation 2003;107:2499–506.
11. Mizuguchi H, Kay AM. A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. Hum Gene Ther 1999;10:2013–7.
12. Chen SH, Chen XH, Wang Y, Kosai K, Finegold MJ, Rich SS, et al. Combination gene therapy for liver metastasis of colon carcinoma in vivo. Proc Natl Acad Sci U S A 1995;92:2577–81.
13. Ohno M, Takemura G, Ohno A, Misao J, Hayakawa Y, Minatoguchi S, et al. "Apoptotic" myocytes in infarcted area in rabbit hearts may be oncotic myocytes with DNA fragmentation analysis by immunogold electron microscopy combined with in situ nick end-labeling. Circulation 1998;98:1422–30.
14. Grootveld M, Halliwell B. Aromatic hydroxylation as a potential measure of hydroxyl radical formation in vivo. Identification of hydroxylated derivatives of salicylate in human body fluids. Biochem J 1986; 237:499–504.

15. Takemura G, Onodera T, Ashraf M. Quantification of hydroxyl radical and its lack of relevance to myocardial injury during early reperfusion after graded ischemia in rat hearts. Circ Res 1992;71:96—105.

16. Li Y, Takemura G, Kosai K, Yuge K, Nagano S, Esaki M, et al. Post-infarction treatment with an adenoviral vector expressing hepatocyte growth factor relieves chronic left ventricular remodeling and dysfunction in mice. Circulation 2003;107:2499—506.

17. Bolli R. Oxygen-derived free radicals and postischemic myocardial dysfunction ("stunned myocardium"). J Am Coll Cardiol 1988;12: 239—49.

18. Portz SJ, Lesnefsky EJ, Vanbenthuysen KM, Mcmurtry IM, Horowitz LD. Dimethylthiourea, but not dimethylsulfoxide, reduces canine infarct size. Free Radic Biol Med 1989;7:53—8.

19. Tsuboi S. Elevation of glutathione level in rat hepatocytes by hepatocyte growth factor via induction of gamma-glutamylcysteine synthetase. J Biochem 1999;126:815—20.

20. Mizuno S, Nakamura T. Prevention of neutrophil extravasation by hepatocyte growth factor leads to attenuations of tubular apoptosis and renal dysfunction in mouse ischemic kidneys. Am J Pathol 2005;166:1895—905.

21. Hashimoto K, Minatoguchi S, Hashimoto Y, Wang N, Qiu X, Yamashita K, et al. Role of protein kinase C, KATP channels and DNA fragmentation in the infarct size-reducing effects of the free radical scavenger T-0970. Clin Exp Pharmacol Physiol 2001;28:193—9.

22. Hare JH. Oxidative stress and apoptosis in heart failure progression. Circ Res 2001;89:198—200.

23. Yang JJ, Kettritz R, Falk RJ, Jennette JC, Gaido ML. Apoptosis of endothelial cells induced by the neutrophil serine proteases proteinase 3 and elastase. Am J Pathol 1996;149:1617—23.

24. Ambrosio G, Zwier JL, Becker LC. Apoptosis is prevented by administration of superoxide dismutase in dogs with reperfused myocardial infarction. Basic Res Cardiol 1998;93:94—6.

25. Sabbah HN. Apoptotic cell death in heart failure. Cardiovasc Res 2000;45:704—12.

26. Goldstein P. Controlling cell death. Science 1997;275:1081—2.

27. Zhao ZQ, Velez DA, Wang NP, Hewan-Lowe KO, Nakamura M, Guyton RA, et al. Progressively developed myocardial apoptotic cell death during late phase of reperfusion. Apoptosis 2001;6:279—90.

28. Kitta K, Day RM, Kim Y, Torregroza I, Evans T, Suzuki YJ. Hepatocyte growth factor induces GATA-4 phosphorylation and cell survival in cardiac muscle cells. J Biol Chem 2003;278:4705—12.

29. Kobayashi S, Lackey T, Huang Y, Bisping E, Pu WT, Boxer LM, et al. Transcription factor gata4 regulates cardiac BCL2 gene expression in vitro and in vivo. FASEB J 2006;20:800—2.

30. Wang Y, Ahmad N, Wani MA, Ashraf M. Hepatocyte growth factor prevents ventricular remodeling and dysfunction in mice via Akt pathway and angiogenesis. J Mol Cell Cardiol 2004;37:1041—52.

31. Lin Y, Xie WF, Chen YX, Zhang X, Zeng X, Qiang H, et al. Treatment of experimental hepatic fibrosis by combinational delivery of urokinase-type plasminogen activator and hepatocyte growth factor genes. Liver Int 2005;25:796—807.

32. Mizuno S, Matsumoto K, Li MY, Nakamura T. HGF reduces advancing lung fibrosis in mice: a potential role for MMP-dependent myofibroblast apoptosis. FASEB J 2005;19:580—2.

33. Chatziantoniou C, Dussaule JC. Insights into the mechanisms of renal fibrosis: is it possible to achieve regression. Am J Physiol Renal Physiol 2005;289:F227—34.

34. Nakamura T, Matsumoto K, Mizuno S, Sawa Y, Matsuda H, Nakamura T. Hepatocyte growth factor prevents tissue fibrosis, remodeling, and dysfunction in cardiomyopathic hamster hearts. Am J Physiol Heart Circ Physiol 2005;288:H2131—9.

35. Rajesh KG, Sasaguri S, Suzuki R, Maeda H. Antioxidant MCI-186 inhibits mitochondrial permeability transition pore and upregulates Bcl-2 expression. Am J Physiol Heart Circ Physiol 2003;285:H2171—8.

36. Lee SH, Wolf PL, Escudero R, Deutsch R, Jamieson SW, Thistlethwaite PA. Early expression of angiogenesis factors in acute myocardial ischemia and infarction. N Engl J Med 2000;342:626—33.

37. Ono K, Matsumori A, Shioi T, Furukawa Y, Sasayama S. Enhanced expression of hepatocyte growth factor/c-Met by myocardial ischemia and reperfusion in a rat model. Circulation 1997;95:2552—8.

Journal of Joint Surgery

関節外科

基礎と臨床

別 刷

増殖制御型アデノウイルスによる遺伝子治療

　癌はいまだわが国を含む多くの先進諸国の最多死因であり，わが国だけでも毎年約32万人が癌で死亡している。つまり近年に治療成績の向上がみられる早期癌とは対照的に，多発性遠隔転移の進行癌にはいまだ効果的な治療法は確立されておらず，そのため遺伝子治療のような革新的治療法の開発が切望されている。

　遺伝子治療の臨床成績は，まず90年代にレトロウイルスベクターによる*ex vivo*遺伝子治療（切除癌に*in vitro*の培養下でサイトカインなどの治療遺伝子を導入し，放射線で増殖不能化したのちに体内に戻す）が行われた。しかしこれは不十分な治療効果に加え，多大な労力に伴う治療の不確実性と高額の経費の問題もあり，一般医薬化には至っていない。

　90年代後半より，*in vivo*遺伝子導入（直接癌部にベクター注入）を可能とする非増殖型アデノウイルス（ADV）ベクターが，癌遺伝子治療の中心ベクターとなり，臨床試験数も増加してきた[1]。現在まで世界で約1,300の遺伝子治療の臨床プロトコールが発表されているが，その2/3は癌であり，使用されているベクターはADVが最多である。このような臨床試験（研究）での結論は，「遺伝子治療は癌には安全な一般医薬となりうるが，治療効果は当初期待されたレベルには達していない」ということである。この原因の1つは，「非」増殖型のベクターでは*in vivo*で体内の全癌細胞にもれなく治療遺伝子を導入することは「物理的に」不可能であるため，癌の再発が起こりえるという問題である。

　この問題を克服するため，現在世界中で盛んに研究が進められているのが，癌特異的増殖型ADV（CRA）である。CRAは，ウイルス増殖が正常細胞では阻止され，癌細胞内では旺盛に起こるように遺伝子改変されたADVで，生体内で効率的かつ癌細胞特異的な遺伝子導入が可能とな

る[2]。またさらにCRA自身が，増幅したウイルス（蛋白）により癌細胞を特異的に殺す「溶解性ウイルス」医薬となる利点も併せもつ。その原理は，非増殖型ADVではウイルス増殖に必須のE1領域を欠損させて治療遺伝子に置換していたが，CRAではE1領域を改変（一部欠失変異化と，内因性プロモーターの置換の2戦略あり）することで，ウイルス増殖が癌のみで起こるようにするというものである[2]。CRAは基礎研究，臨床研究の両方でその有用性が示されている一方で，完全に理想的なCRAを開発するためには，2つの問題が残っていた。第1には，このような一因子で癌と正常の細胞を完全に識別するレベルの癌特異化は困難ということである。また最大の問題は，このような一因子制御のCRAでさえ，効率的・標準化作製技術が確立されていないため，研究開発がきわめて非効率ということであった。

　著者らはこの問題を克服するため，従来の単一因子のCRAとは一線を画く「多数」の異なる癌特異化因子で，精密なウイルス増殖制御が可能なCRAであるm-CRAを，迅速・効率的に作製可能な「標準化」作製技術を初めて開発した[3]。その原理と作製法の詳細は拙著[2,3]に譲るが，これにより7因子以上の癌特異化因子を挿入する次世代のm-CRAが作製可能となった。

　著者らはこの独自技術でさまざまな癌治療m-CRA医薬を作製，評価しているが，本稿ではサバイビン依存性m-CRAを紹介する[4]。サバイビンはIAP（inhibitor of apoptosis）ファミリーとして同定されたが，その後，骨軟部腫瘍を含むほとんどの種類の癌で高発現している一方，分化した正常細胞では発現が認められないことがわかり，現在は癌治療のターゲット分子としても注目されている。著者らはこのサバイビン遺伝子プロモーターでADVのE1を発現制御する

Surv.m-CRAを開発した。

　Surv.m-CRAは調べた全種類の癌細胞で，癌細胞特異的なウイルス増殖と細胞死を誘導し，さらに骨肉腫の動物モデルで高い治療効果を示した。さらに既報告のCRAのなかでは最良のテロメラーゼ(TERT)依存性m-CRA(Tert.m-CRA)と詳細な比較実験をしたところ，Surv.m-CRAはTert.m-CRAを，癌治療効果と癌特異性(安全性)の両面でしのぐという有望な成果が得られた[2),4)]。このように骨軟部腫瘍はもとより，ほぼ全種類の癌を効率よく安全に治療でき，既存のm-CRAより優れたSurv.m-CRAは，早期の臨床応用化が期待される。また，癌特異性(安全性)も癌治療効果もさらに増殖した高度m-CRA化の種々の改良型Surv.m-CRA，あるいは第二，第三弾の新規m-CRAの開発も進めている。

　将来のわが国の国民福祉と経済の向上につながるのは，「基盤技術からわが国で開発し，基本特許の知財を確保した医薬」であるため，著者らのm-CRAの医薬化は重要であると思われる。遺伝子治療の臨床化の公的支援の体制が十分でないわが国では困難もあるが，著者ら自身でのm-CRA医薬開発と併せて，わが国全体のm-CRA研究開発の発展に寄与できる体制づくりや産業化も計画している。

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□文　　献
1)Chen SH, Chen XH, Wang Y, Kosai K, et al：Combination gene therapy for liver metastasis of colon carcinoma *in vivo*. Proc Natl Acad Sci USA,1995; 92：2577-2581, 1995.
2)室伏善照, 神薗純一, 小賊健一郎：新世代癌遺伝子治療のための多因子で増殖制御/癌特異化するアデノウイルスの作製法. 細胞工学, 25：60-66, 2005.
3)Nagano S, Oshika H, Fujiwara H, Komiya S, Kosai K, et al：An efficient construction of conditionally replicating adenoviruses that target tumor cells with multiple factors. Gene Ther, 2005.
4)Kamizono J, Nagano S, Murofushi Y, Komiya S, Kosai K, et al：Survivin-responsive conditionally replicating adenovirus exhibits cancer-specific and efficient viral replication. Cancer Res, 65：5284-5291, 2005.

5

教授就任記念講演
先端医学開発の研究と医学教育

小　戝　健一郎