Methods

This study included 15 (12 men and 3 women) patients with chronic CHF (Table 1); 6 with idiopathic dilated cardiomyopathy, 1 with dilated phase of hypertrophic cardiomyopathy, 7 with old myocardial infarction, and 1 with valvular heart disease. Age of the patients ranged from 34 to 75 (mean 62 ± 15) years. Of these patients, 3 and 12 were in New York Heart Association (NYHA) functional class II and III, respectively. This study excluded patients with (1) acute myocardial infarction or unstable angina; (2) uncontrollable tachycardic or bradycardic arrhythmia; (3) acute pulmonary edema; (4) cardiogenic shock; (5) uncontrollable hypertension; (6) systemic disorders such as severe hepatic, renal, hematologic, and malignant diseases; (7) diabetes mellitus under insufficient control; (8) active inflammation (serum C-reactive protein >2.0 mg/mL); (9) anemia (hemoglobin concentration <8.0 mg/mL); or (10) hypoxia (arterial oxygen saturation <70 %).

All patients were in stable clinical condition before this study in our hospital on maintenance doses of conventional medications for chronic CHF, including renin-angiotensin system inhibitor (angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers), β-blocker, diuretics, and digitalis.[13] Of the 15 patients, all had a renin-angiotensin system inhibitor and furosemide, 9 had spironolactone, and 11 had digitalis (Table 1). Although 5 patients had a β-blocker, β-blockers were not used in the remaining 10 patients because of intolerable bradycardia or hypotension. Their medications were not changed during the study. This study complied with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of Kurume University, and written informed consent was obtained from each patient before participation.

Sauna Bathing

A far infrared-ray sauna system (Olympia Co, Miyazaki, Japan) was used. Patients were placed in the sitting position in a 60°C dry sauna for 15–20 minutes, and then rested on a reclining chair with sufficient warmth provided by blankets in a temperature-controlled room at 25°C for 30 minutes.[11] Patients were weighed before and after sauna bathing, and oral hydration with water (100–200 mL) was supplied to compensate for water loss.

Study Protocol

Patients underwent 60°C sauna bathing once per day, 5 days per week, for 4 weeks. Exercise tolerance, plasma levels of neurohumoral factors, echocardiogram, and chest roentgenogram were evaluated just before and immediately after 4-week bathing. Fasting blood samples were drawn from the antecubital vein early in the morning. Plasma epinephrine, norepinephrine and brain natriuretic peptide (BNP) were measured at a commercial available laboratory (SRL, Fukuoka, Japan). The left ventricular (LV) and left atrial dimensions were measured on the M-mode echocardiograms, and LVEF was calculated by the modified Simpson's method.[14]

Assessment of Clinical Symptoms

Clinical symptoms (shortness of breath, fatigue, appetite loss, insomnia, and constipation) were evaluated by a self-assessment quality-of-life questionnaire.[12] After 4 weeks of sauna bathing, questionnaire sheets were given to patients and they were asked to choose 1 from 4 grades: "remarkably improved," "improved," "no change," or "worsened," as compared with the baseline, for each symptom. Based on the results, patients were categorized to the following 3 groups: improved patients with "remarkable improvement" or "improvement" of more than 2 items, worsened patients with "worsening" of at least more than 1 item, and the others.

Exercise Tolerance Tests

In all patients, a 6-minute walk testing was carried out and the total walking distance was determined. For further quantitative evaluation for exercise tolerance, in the last 5 consecutive patients (Patients 11–15), cardiopulmonary exercise testing was performed

Table 1. Patient Profile

Patient Number	Age (y)	Gender	Diagnosis	NYHA Class	Diabetes Mellitus	HC	Medication				
							ACEI or ARB	β-Blocker	Furosemide	Spironolacton	Digitalls
1	61	Male	DCM	III	−	−	+	−	+	−	+
2	63	Female	dHCM	III	+	−	+	−	+	−	+
3	34	Male	DCM	III	+	−	+	+	+	+	+
4	53	Male	OMI	III	+	+	+	−	+	+	+
5	38	Male	DCM	III	−	−	+	−	+	+	+
6	74	Male	OMI	III	−	−	+	−	+	+	+
7	74	Female	DCM	III	−	−	+	+	+	−	+
8	70	Female	OMI	III	+	+	+	−	+	+	+
9	47	Male	OMI	II	−	+	+	+	+	+	+
10	71	Male	VHD	II	−	−	+	−	+	−	+
11	68	Male	OMI	III	+	+	+	−	+	+	−
12	77	Male	OMI	III	+	−	+	−	+	+	+
13	72	Male	DCM	III	−	−	+	−	+	−	−
14	62	Male	OMI	II	+	+	+	+	+	+	−
15	75	Male	DCM	III	−	−	+	+	+	−	−

DCM, dilated cardiomyopathy; dHCM, dilated phase of hypertrophic cardiomyopathy; OMI, old myocardial infarction; VHD, valvular heart disease; ACEI: angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker.

Diabetes mellitus was defined as hemoglobin A1c greater than 5.8%.

Hypercholesterolemia (HC) was defined as total cholesterol greater than 220 mg/mL.

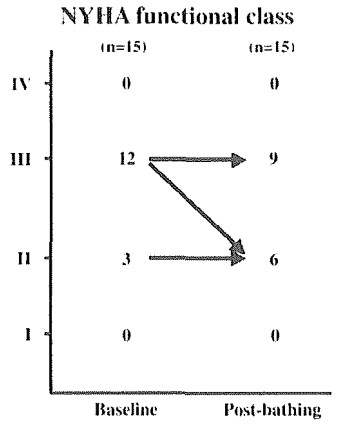

Fig. 1. Changes in New York Heart Association (NYHA) functional class.

using a bicycle ergometer. The peak respiratory oxygen uptake (peak $\dot{V}O_2$) was calculated, and the anaerobic threshold was determined by the V-slope method. The patient profile of the subgroup was similar to that of the whole patients studied.

To confirm that the patients were in stable condition and to evaluate the consistency of repeated measurements of exercise tolerance, 6-minute walking test and cardiopulmonary exercise testing were repeated 1–2 weeks and just before the entry of the study protocol.

Statistical Analysis

Values are mean ± SD. Paired- and unpaired *t*-tests were performed for comparisons between the baseline and postbathing and between 2 groups, respectively. For exercise tolerance tests, Kruskal-Wallis test was used. A value of $P < .05$ was considered statistically significant.

Results

All patients enrolled in this study completed 4-week repeated sauna bathing without any adverse symptoms and events. Of 12 patients with NYHA functional class III, 3 improved to functional class II (Fig. 1). As shown in Table 2, the self-assessment quality-of-life questionnaire revealed that 13 of 15 patients were categorized as the improved group. Improvements of shortness of breath and fatigue were most evident. Body weight did not change (Table 3). Repeated sauna bathing decreased systemic blood pressure without changes in heart rate, resulting in significant decrease in the rate-pressure product. And LVEF improved from 30 ± 11 to 34 ± 11%. Cardiothoracic ratio was slightly, yet significantly, decreased.

Exercise Tolerance

As shown in Fig. 2, the patients were stable before sauna bathing. Just before sauna bathing, 6-minute walking distance was 388 ± 110 m. Four-week sauna bathing improved 6-minute walking distance in each patient, and the average was significantly increased to 448 ± 118 m. Baseline examination revealed markedly reduced peak $\dot{V}O_2$ and anaerobic threshold (13.3 ± 1.8 and 9.4 ± 1.2 mL/kg/min, respectively). In each patient, repeated sauna bathing improved peak VO_2 and anaerobic threshold, and the averages were significantly increased to 16.3 ± 2.1 and 11.5 ± 1.9 mL/kg/min, respectively.

Neurohumoral Factors

At baseline, plasma catecholamines and BNP were elevated (Table 3). Repeated sauna bathing markedly reduced plasma epinephrine and norepinephrine levels. Although sauna bathing tended to decrease plasma BNP levels, the effects were inconsistent and the changes did not reach the statistical significance.

Hospital Admission for CHF

During the 12-month follow-up, 2 of 15 patients died of CHF. In the remaining 13 patients, the number of hospital admission for CHF during 1 year was compared before

Table 2. Changes of Symptoms

Patient Number	Self-Assessment Quality-of-Life Questionaere						New York Heart Association Functional Class	
	Shortness of Breath	Fatigue	Appetite Loss	Insomnia	Constipation	Overall Assessment	Baseline	Posttreatment
1	1 +	1 +	0	1 +	1 +	Improved	III	III
2	1 +	1 +	0	1 +	1 +	Improved	III	III
3	0	0	0	0	1 +	Unchanged	III	III
4	1 +	2 +	1 +	0	1 +	Improved	III	III
5	1 +	1 +	0	0	0	Improved	III	III
6	1 +	1 +	1 +	2 +	1 +	Improved	III	III
7	1 +	1 +	1 +	0	1 +	Improved	III	III
8	1 +	1 +	1 +	0	1 +	Improved	III	III
9	1 +	1 +	0	0	0	Improved	II	II
10	1 +	1 +	0	0	1 +	Improved	II	II
11	1 +	1 +	1 +	1 +	0	Improved	III	II
12	2 +	0	0	2 +	0	Improved	III	II
13	1 +	1 +	0	0	1 +	Improved	III	II
14	0	1 +	0	0	0	Unchanged	II	II
15	2 +	2 +	1 +	1 +	0	Improved	III	III

2 +, remarkably improved; 1 +, improved; 0, unchanged; 1 −, worsened.

Table 3. Effects of 4-Week Sauna Treatment

	Baseline	Postbathing	
Body weight (kg)	60 ± 11	60 ± 11	NS
Systolic blood pressure (mm Hg)	101 ± 13	98 ± 14	$P < .05$
Heart rate (/min)	73 ± 12	72 ± 12	NS
Rate-pressure product	6811 ± 1323	6292 ± 1093	$P < .01$
Echocardiographic findings			
Left atrial dimension (mm)	48 ± 12	46 ± 12	NS
Left ventricular end-diastolic dimension (mm)	68 ± 7	68 ± 9	NS
Left ventricular ejection fraction (%)	30 ± 11	34 ± 11	$P < .05$
Chest roentogenographic finding			
Cardiothoracic ratio (%)	59 ± 7	58 ± 7	$P < .05$
Neurohumoral factors			
Epinephrine (pg/mL)	40 ± 42	21 ± 23	$P < .05$
Norepinephrine (pg/mL)	633 ± 285	443 ± 292	$P < .01$
Brain natriuretic peptide (pg/mL)	456 ± 469	399 ± 393	NS

and after sauna bathing (Fig. 3). In each patient, sauna bathing decreased the number of admission for CHR.

Discussion

The present study demonstrated for the first time that repeated 60°C sauna bathing for 4 weeks improved exercise tolerance and clinical symptoms in patients with chronic systolic CHF on conventional treatments. Moreover, LVEF was improved, and elevated plasma epinephrine and norepinephrine were decreased. No apparent adverse events were experienced. During the follow-up, sauna bathing reduced the number of hospital admission for CHF.

Most of patients were subjectively in NYHA functional class III (mean NYHA class = 2.8 ± 0.4). At baseline, plasma BNP and norepinephrine were greater than 450 pg/mL and 600 pg/mL, respectively, indicating that the patients were in NYHA class more than III.[19] Echocardiography revealed LV dilation with LV end-diastolic dimension of 68 mm in association with reduced LVEF of 30%, indicating remarkably impaired LV function. Moreover, average 6-minute walking distance was less than 400 m, associated with low peak $\dot{V}O_2$ <14 mL/kg/min. Thus the enrolled patients were subjectively or objectively in severe CHF. Nonetheless, sauna bathing was safely performed. Conventional hot (80°C) sauna bathing is contraindicated for severe CHF patients because thermal stress increases cardiac workload and sympathetic activity.[12–14] The present study suggests that repeated 60°C sauna bathing can be safely performed even in chronic systolic CHF patients.

The symptoms were improved in 13 of 15 patients, and no patients experienced worsening of symptoms (Table 2). Therefore, repeated sauna bathing was effective for improvement of subjective well-being in chronic CHF patients on conventional treatments. However, because only 3 patients had improvement in NYHA functional class, the subjective improvement may have been placebo effects. Thus we evaluated exercise tolerance such as 6-minute walking distance and peak $\dot{V}O_2$, which are better and more objective indices of improvement. They all had prolongation of 6-minute walking distance, and 5 had improved cardiopulmonary exercise tolerance. Although we did not have the control group, patients had the stable exercise tolerance before sauna bathing (Fig. 2), indicating that the effects were not "warm-up" effects. Thus sauna bathing was effective for improvements of symptoms and exercise tolerance.

Several mechanisms whereby repeated sauna bathing improved exercise tolerance are considered. The most likely one was improvement of cardiac performance; LVEF was improved from 30% to 34% and the cardiac silhouette got

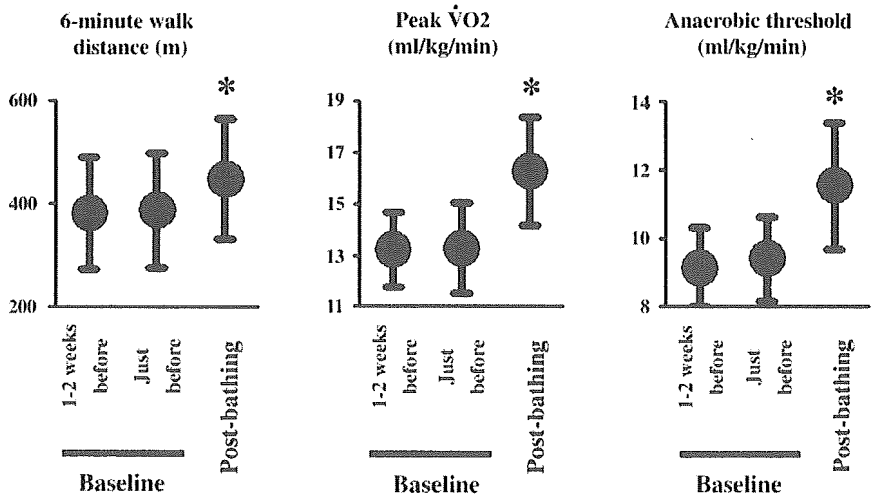

Fig. 2. Four-week repeated sauna bathing improved 6-minute walking distance in all patients (left, bar = 1 × SD [n = 15]). In a subgroup (Patients 11–15), the peak $\dot{V}O_2$ (middle) and anaerobic threshold (right) were improved after 4 weeks in each patient (bar = 1 × SD [n = 5]). At baseline, exercise tolerance tests were repeated 1–2 weeks and just before sauna bathing. *$P < .05$ versus just before sauna bathing.

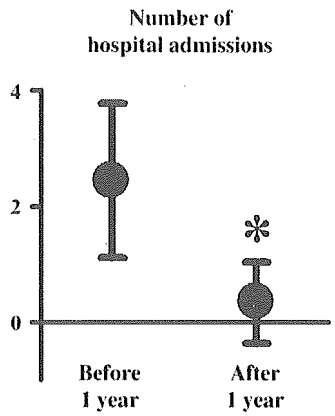

Fig. 3. Number of hospital admission for chronic heart failure in the 12-month period before (before) and after sauna bathing (after). Bar = 1 × SD (n = 13). $^*P < .01$ versus before.

smaller. The increased cardiac function was not due to decrease in preload (water loss), because the body weight did not differ before and after repeated bathing. Although we did not examine it in this study, reduction in afterload (peripheral vascular resistance) could have been responsible because blood pressure decreased (Table 3) and because Tei et al have shown that acute sauna bathing greatly decreases peripheral vascular resistance,[15] and that 2-week sauna bathing improves endothelial function.[16] The decreased plasma catecholamine levels indicate attenuated sympathetic nerve activity by sauna bathing. Although we were not able to determine whether the attenuated sympathetic nerve activity was directly caused by sauna bathing or because of improvement of heart failure, decreased sympathetic nerve activity is a good prognostic sign. It remains unclear why BNP levels did not decrease despite clinical improvement.

In the present study, 4-week sauna bathing reduced the number of hospital admission for CHF. Although the number of patients studied was too small, the observation may raise the possibility that the beneficial effects remain longer even after the cessation of sauna bathing. The mechanisms of the long lasting effects are unknown.

The small number of patients studied limits our interpretations and discussion in this study. Assessment of long-term survival rate was not an objective of the study. Another important limitation is the lack of the control group who did not receive sauna bathing, because blinded study was not feasible. Instead, we had an internal control as described previously.

Conclusions

Repeated 60°C sauna bathing for 4 weeks improved not only well-being but also exercise tolerance in chronic systolic CHF patients on conventional treatments. It is suggested that repeated sauna bathing is a safe and effective adjunctive therapy for chronic CHF.

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Selective disruption of MMP-2 gene exacerbates myocardial inflammation and dysfunction in mice with cytokine-induced cardiomyopathy

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Selective disruption of MMP-2 gene exacerbates myocardial inflammation and dysfunction in mice with cytokine-induced cardiomyopathy

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[1]*Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka;* [3]*Department of Cardiovascular Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan; and* [2]*Department of Medicine, Jefferson Medical College, Philadelphia, Pennsylvania*

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Matsusaka, Hidenori, Masaki Ikeuchi, Shouji Matsushima, Tomomi Ide, Toru Kubota, Arthur M. Feldman, Akira Takeshita, Kenji Sunagawa, and Hiroyuki Tsutsui. Selective disruption of MMP-2 gene exacerbates myocardial inflammation and dysfunction in mice with cytokine-induced cardiomyopathy. *Am J Physiol Heart Circ Physiol* 289: H1858–H1864, 2005. First published June 3, 2005; doi:10.1152/ajpheart.00216.2005.—Tumor necrosis factor-α (TNF-α) plays a pathophysiological role in the development and progression of heart failure. Matrix metalloproteinase (MMP)-2 is involved in extracellular matrix remodeling. Recent evidence suggests a protective role for this protease against tissue inflammation. Although MMP-2 is upregulated in the failing heart, little is known about its pathophysiological role. We thus hypothesized that ablation of the MMP-2 gene could affect cardiac remodeling and failure in TNF-α-induced cardiomyopathy. We crossed transgenic mice with cardiac-specific overexpression of TNF-α (TG) with MMP-2 knockout (KO) mice. Four groups of male and female mice were studied: wild-type (WT) with wild MMP-2 (WT/MMP$^{+/+}$), WT with MMP-2 KO (WT/MMP$^{-/-}$), TNF-α TG with wild MMP-2 (TG/MMP$^{+/+}$), and TG with MMP-2 KO (TG/MMP$^{-/-}$). The upregulation of MMP-2 zymographic activity in TG/MMP$^{+/+}$ mice was completely abolished in TG/MMP$^{-/-}$ mice, and other MMPs and tissue inhibitors of metalloproteinase were comparable between groups. Survival was shorter for male TG/MMP$^{-/-}$ than TG/MMP$^{+/+}$ mice. Female TG/MMP$^{-/-}$ mice were more severely affected than TG/MMP$^{+/+}$ mice with diminished cardiac function. Myocardial TNF-α and other proinflammatory cytokines were increased in TG/MMP$^{+/+}$ mice, and this increase was similarly observed in TG/MMP$^{-/-}$ mice. The extent of myocardial infiltrating cells including macrophages was greater in TG/MMP$^{-/-}$ than in TG/MMP$^{+/+}$ mice. Selective ablation of the MMP-2 gene reduces survival and exacerbates cardiac failure in association with the increased level of myocardial inflammation. MMP-2 may play a cardioprotective role in the pathogenesis of cytokine-induced cardiomyopathy.

metalloproteinases; heart failure; tumor necrosis factor

THE DYNAMIC SYNTHESIS and breakdown of extracellular matrix (ECM) proteins play an important role in myocardial remodeling and failure. In particular, increased expression and activation of matrix metalloproteinases (MMPs) have been implicated in heart failure (4, 8, 20, 23, 26, 27). The activity of MMPs is controlled by transcription, activation of the latent proenzymes, and inhibition of MMPs by tissue inhibitors of MMPs (TIMPs). Although transcriptional regulation is essential for MMP production, activation of latent enzymes by proteolytic cleavage is required for matrix degradation. In addition, TIMPs modulate ECM deposition through inhibition of activated MMPs. Together, activation of latent MMP and inhibition of MMPs by TIMPs contribute to myocardial remodeling and failure. Among the known MMPs, MMP-2 is ubiquitously distributed in cardiac myocytes and fibroblasts (2) and has been shown to be upregulated in heart failure (1, 8). Recent studies have shown that MMP-2 has diverse cellular function, such as attenuation of the tissue inflammatory response, independent of its action on the ECM (3, 13, 29). Therefore, the ultimate effects of MMPs may include modulation of ECM proteins, as well as modification of cellular functions, including cell migration.

Myocardial production of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), plays an important role in the pathogenesis of heart failure (5, 22). Transgenic (TG) mice that overexpress TNF-α specifically in the heart developed myocardial inflammation, with premature death from heart failure in association with ECM remodeling (16, 17). Activation of MMPs, i.e., MMP-2 and MMP-9, has been demonstrated in this model (17, 18). Furthermore, expression of MMP-2 can be regulated by the inflammatory cytokines (19). Therefore, MMP-2 could influence the progression of inflammation by affecting the function of mediators such as cytokines/chemokines and could play an important role in myocardial remodeling. In the present study, we evaluated the effects of a targeted deletion of the MMP-2 gene on cardiac structural and functional alterations in this type of heart failure. To ensure selective and long-term complete inhibition of MMP-2, we crossed TNF-α TG mice with MMP-2 knockout (KO) mice (8, 12). The most effective way to evaluate the contribution of a specific MMP and obtain direct evidence for a role of MMP is through gene manipulation instead of an MMP inhibitor.

MATERIALS AND METHODS

Animal model. We used TG mice with cardiac-specific overexpression of TNF-α, which have been well characterized as a model of cytokine-induced cardiomyopathy (16). To ensure complete inhibition of MMP-2 activity, we crossed TG mice with MMP-2 KO (MMP$^{-/-}$) mice. There were no detectable differences in cardiac size and structure between MMP$^{-/-}$ and MMP$^{+/+}$ mice either macroscopically or microscopically (8). The original breeding pairs used to develop the mice for this study were obtained from Dr. Shigeyoshi Itohara (Laboratory for Behavioral Genetics, RIKEN). Because

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TNF-α TG (FVB) and MMP-2 KO (C57BL/6J) mice arise from different genetic backgrounds, we generated all mice as mixed genetic background (1:1 ratio of C57BL/6J to FVB) to minimize genetic heterogeneity of the mice, as described previously (6, 10). Briefly, mating male TG mice with female $MMP^{-/-}$ mice yielded TG or wild-type (WT) mice with $MMP^{+/-}$ (F1). Then we mated TG and WT mice with $MMP^{+/-}$ mice to obtain TG or WT mice with $MMP^{+/+}$, $MMP^{+/-}$, and $MMP^{-/-}$ (F2). To minimize the effect of genetic background, littermates were studied in each analysis. The study was approved by our Institutional Animal Research Committee and conformed to the animal care guidelines of the American Physiological Society.

MMPs and TIMPs. Myocardial MMP levels, including MMP-2 and MMP-9, were determined in the left ventricle (LV) from 12-wk-old female mice by gelatin zymography (8). The LV myocardial samples were homogenized (~30-s bursts) in 1 ml of an ice-cold extraction buffer containing cacodylic acid (10 mmol/l), NaCl (0.15 mol/l), $ZnCl_2$ (20 mmol/l), NaN_2 (1.5 mmol/l), and 0.01% Triton X-100 (pH 5.0). The homogenate was then centrifuged (4°C, 10 min, 10,000 *g*), and the supernatant was decanted and saved on ice. The pH levels of the samples were adjusted to 7.5 with Tris (1 mol/l). The final protein concentration of the myocardial extracts was determined using a standardized colorimetric assay. The extracted samples were aliquoted and stored at −80°C until the time of assay. The myocardial extracts were then directly loaded onto electrophoretic gels (SDS-PAGE) containing 1 mg/ml gelatin under nonreducing conditions. The myocardial extracts at a final protein content of 5 μg were loaded onto the gels using a 3:1 sample buffer (10% SDS, 4% sucrose, 0.25 mol/l Tris·Cl, and 0.1% bromphenol blue, pH 6.8). The gels were run at 15 mA/gel through the stacking phase (4%) and at 20 mA/gel for the separating phase (10%), while the running buffer temperature was maintained at 4°C. After SDS-PAGE, the gels were washed twice in 2.5% Triton X-100 for 30 min each, rinsed in water, and incubated for 24 h in a substrate buffer at 37°C (50 mmol/l Tris·HCl, 5 mmol/l, $CaCl_2$, and 0.02% NaN_3, pH 7.5). After incubation, the gels were stained with Coomassie brilliant blue R-250. The zymograms were digitized, and the size-fractionated bands, which indicated MMP proteolytic levels, were measured by integrated optical density in a rectangular region of interest.

The mRNA levels of myocardial MMPs, including MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9, as well as TIMPs, including TIMP-1, TIMP-2, TIMP-3, and TIMP-4, were determined by multiprobe ribonuclease protection assay (RiboQuant, PharMingen). Each value was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each template set as an internal control and then calculated as a ratio to $WT/MMP^{+/+}$.

Survival. Survival was analyzed in male and female $WT/MMP^{+/+}$, $WT/MMP^{-/-}$, $TG/MMP^{+/+}$, and $TG/MMP^{-/-}$ mice. During the study period, the cages were inspected daily to identify any deceased animals. All deceased mice were examined for the presence of pleural effusion (serous fluid within the chest wall cavity) in the postmortem examination. Because most of the male TG mice died earlier, we used 12-wk-old female mice for the subsequent analyses.

Echocardiographic and hemodynamic measurements. Echocardiographic studies were performed under light anesthesia with tribromoethanol-amylene hydrate (Avertin, 2.5% wt/vol, 8 μl/g ip) and spontaneous respiration as described previously (24). A two-dimensional parasternal short-axis view of the LV was obtained at the level of the papillary muscles. In general, the best views were obtained with the transducer lightly applied to the midportion of the upper left anterior chest wall. The transducer was then gently moved cephalad or caudad and angulated until desirable images were obtained. After confirmation that the imaging was on axis (on the basis of roundness of the LV cavity), two-dimensional targeted M-mode traces were recorded at a paper speed of 50 mm/s. Then a 1.4-Fr micromanometer-tipped catheter (Millar) was inserted into the right carotid artery and advanced into the LV to measure LV pressures. Our previous validation study showed that intra- and interobserver variabilities of our echocardiographic measurements for LV cavity dimensions and fractional shortening were small and that measurements made in the same animals on separate days were highly reproducible (24).

Histopathology. After in vivo echocardiographic and hemodynamic studies, the heart was excised and dissected into right ventricle and LV, including the septum. From the mid-LV transverse sections, 5-μm sections were cut and stained with hematoxylin and eosin and Masson's trichrome for determination of myocyte cross-sectional area and collagen volume fraction. They were stained also with picrosirius red for visualization of the interstitial collagen fibers.

Myocardial infiltration was quantified in hematoxylin-and-eosin-stained sections by determination of nuclear density (nuclei/mm^2) according to methods described previously (15, 21). Because it is difficult to differentiate inflammatory cells from myocytes and/or fibroblasts, all nuclei were included. In each animal, five independent high-power fields were analyzed. To further determine the number of macrophages, an immunohistochemical analysis using a specific antibody against mouse Mac-3 (BD Pharmingen) was performed.

Cytokine gene expression. To determine the myocardial gene expression of TNF-α as well as other proinflammatory cytokines, including regulated on activation, normal T cell expressed and secreted (RANTES), interleukin (IL)-6, IL-1β, transforming growth factor (TGF)-β, and monocyte chemoattractant protein (MCP)-1, ribonuclease protection assay was performed with 5 μg of total RNA isolated from LV tissue samples according to methods described previously (11).

Plasma cytokine and MMP levels. Plasma levels of TNF-α, as well as other cytokines, were measured with commercially available ELISA kits for mouse TNF-α, IL-6, IL-1β, and MCP-1 (Quantikine, R & D Systems). Plasma MMP-2 was also measured with commercially available ELISA kits (Quantikine). All assays were done in duplicate. Results were analyzed spectrophotometrically at a wavelength of 450 nm with a microtiter plate reader.

Statistical analysis. Values are means ± SE. A survival analysis was performed by the Kaplan-Meier method, and between-group difference in survival was tested by the log-rank test. Between-group comparisons of the means were performed by one-way ANOVA followed by *t*-tests. Bonferroni's correction was done for multiple comparisons of the means.

RESULTS

MMPs and TIMPs. Zymographic MMP-2 levels increased in TNF-α $TG/MMP^{+/+}$ compared with $WT/MMP^{+/+}$ mice (Fig. 1). As expected, MMP-2 activity was not detected in $WT/MMP^{-/-}$ and $TG/MMP^{-/-}$ mice. Importantly, the MMP-9 zymographic levels, even though they were faint in $WT/MMP^{+/+}$ mice, did not increase in $WT/MMP^{-/-}$ mice. They significantly increased in the TNF-α TG groups; however, no difference was seen between $TG/MMP^{+/+}$ and $TG/MMP^{-/-}$ mice.

Again, the MMP-2 mRNA levels significantly increased in TNF-α $TG/MMP^{+/+}$ compared with $WT/MMP^{+/+}$ mice (Fig. 2). This increase was completely prevented in $TG/MMP^{-/-}$ mice. The MMP-9 mRNA levels also increased in the TNF-α TG groups; however, no difference was seen between $TG/MMP^{+/+}$ and $TG/MMP^{-/-}$ mice. These results were consistent with those observed in gelatin zymography (Fig. 1). Other MMPs, including MMP-1, MMP-3, and MMP-8, were not altered in these mice (Fig. 2). The changes in TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) were comparable between $TG/MMP^{+/+}$ and $TG/MMP^{-/-}$ mice.

Survival. Survival rate was shorter for male $TG/MMP^{-/-}$ than $TG/MMP^{+/+}$ mice (Fig. 3). Although MMP-2 gene ab-

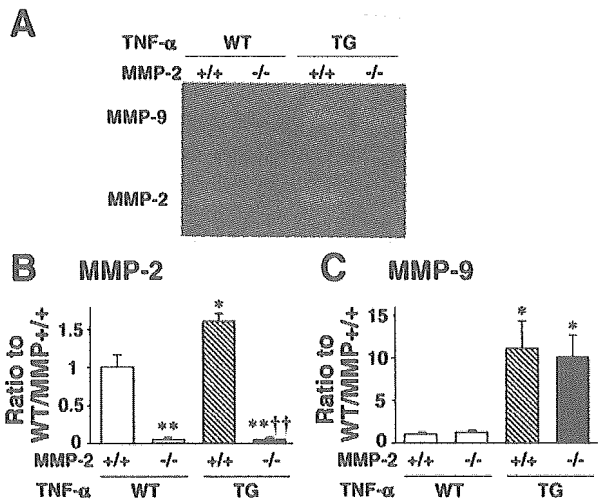

Fig. 1. *A*: representative gelatin zymography of left ventricle (LV) from wild-type (WT) mice with wild matrix metalloproteinase-2 (WT/MMP$^{+/+}$), WT mice with MMP-2 knockout (KO) (WT/MMP$^{-/-}$), TNF-α transgenic (TG) mice with MMP-2 (TG/MMP$^{+/+}$), and TNF-α TG mice with MMP-2 KO (TG/MMP$^{-/-}$). *B* and *C*: densitometric analysis of MMP-2 and MMP-9 zymographic activity (n = 3/group). Samples from WT/MMP$^{+/+}$ mice were run concurrently on the same gel. Values are means ± SE. *P < 0.05; **P < 0.01 vs. WT/MMP$^{+/+}$. ††P < 0.01 vs. TG/MMP$^{+/+}$.

lation was also observed in female mice, it has less effect on survival. These observations of gender differences in survival were consistent with results from a previous study (14), which demonstrated that such gender differences might be due to higher expression of TNF-α receptors in the myocardium of male TG mice, because the extent of myocardial expression of TNF-α was comparable in both genders (14). All the TG mice that died spontaneously developed cardiac dilatation and pleural effusion, suggesting that they died of heart failure. Because most of the male TG mice died earlier, we used surviving 12-wk-old female mice for the subsequent analyses.

Echocardiography and hemodynamics. The echocardiographic and hemodynamic data of the surviving 12-wk-old

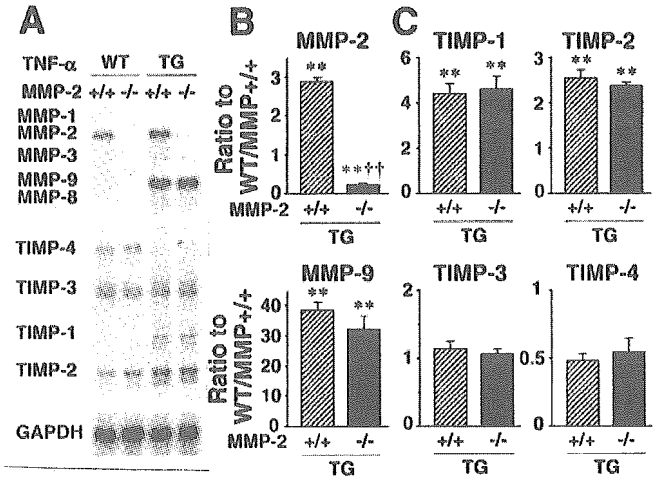

Fig. 2. *A*: representative image of myocardial gene expression of MMPs and tissue inhibitors of MMPs (TIMPs). *B* and *C*: densitometric analysis of MMP and TIMP gene expression in TG/MMP$^{+/+}$ (n = 7) and TG/MMP$^{-/-}$ (n = 7) mice. Each value was normalized to that of GAPDH in each template set as an internal control and expressed as ratio to WT/MMP$^{+/+}$ (n = 6). Values are means ± SE. **P < 0.01 vs. WT/MMP$^{+/+}$. ††P < 0.01 vs. TG/MMP$^{+/+}$.

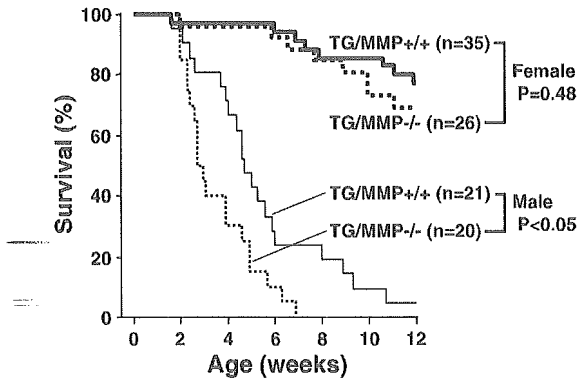

Fig. 3. Kaplan-Meier survival analysis of survival in male TG/MMP$^{+/+}$ (n = 21) and TG/MMP$^{-/-}$ (n = 20) and female TG/MMP$^{+/+}$ (n = 35) and TG/MMP$^{-/-}$ (n = 26) mice.

female mice are shown in Table 1. Presence or absence of the MMP-2 gene did not affect baseline echocardiographic parameters in WT mice. LV end-diastolic diameter was significantly greater, and fractional shortening was significantly less in TG/MMP$^{+/+}$ than in WT mice. TG/MMP$^{-/-}$ mice exerted more impaired LV contractile function than TG/MMP$^{+/+}$ mice.

There was no significant difference in heart rate or LV end-diastolic pressure (EDP) between WT/MMP$^{+/+}$ and WT/MMP$^{-/-}$ mice. LV EDP increased slightly, but significantly, in TG/MMP$^{+/+}$ mice and further increased in TG/MMP$^{-/-}$ mice. Consistent with LV EDP, pleural effusion was observed only in TG/MMP$^{-/-}$ mice. On the basis of these results, the exacerbation of heart failure might contribute to premature death in TG/MMP$^{-/-}$ mice.

Histopathology. We examined the histopathology of the heart from 12-wk-old female mice. LV weight was significantly increased in the TG groups compared with the WT groups (Table 1); however, LV weight did not differ between TG/MMP$^{+/+}$ and TG/MMP$^{-/-}$ mice. Furthermore, myocyte cross-sectional area and collagen volume fraction were increased in TNF-α TG groups (Fig. 4, *A* and *B*). However, the extent of these histopathological changes was comparable between TG/MMP$^{+/+}$ and TG/MMP$^{-/-}$ mice. Moreover, in picrosirius red-stained sections, the structure of the interstitial collagen fibers was similar between TG/MMP$^{+/+}$ and TG/MMP$^{-/-}$ mice (Fig. 4*C*), indicating that selective disruption of the MMP-2 gene did not alter collagen content or structure in this mouse model.

The number of infiltrating interstitial cells in the myocardium was greater in TNF-α TG than in WT mice (Fig. 5). The extent of infiltration was significantly greater in TG/MMP$^{-/-}$ than TG/MMP$^{+/+}$ mice. Macrophages infiltrated into the myocardium from TG mice, and, importantly, selective MMP-2 ablation further augmented their infiltration (Fig. 6).

Cytokine gene expression. TNF-α gene expression was significantly upregulated in the TNF-α TG myocardium (Fig. 7). In addition, overexpression of the TNF-α gene increased expression of other cytokines and chemokines, including RANTES, IL-6, IL-1β, and MCP-1, indicating that overexpression of TNF-α induced "downstream" cytokines and chemokines in this model. Importantly, upregulation of TNF-α was not altered by ablation of the MMP-2 gene. Similarly, MMP-2 gene ablation in TG mice had no significant effects on myo-

Table 1. *Characteristics of animal models*

	WT/MMP$^{+/+}$ (n = 15)	WT/MMP$^{-/-}$ (n = 15)	TG/MMP$^{+/+}$ (n = 15)	TG/MMP$^{-/-}$ (n = 17
Echocardiographic data				
Heart rate, beats/min	450±11	446±10	452±12	453±11
LV EDD, mm	3.4±0.1	3.3±0.1	4.3±0.1*	4.5±0.2*
Fractional shortening, %	37.4±0.8	36.8±0.7	26.7±0.9*	22.9±1.3*†
Hemodynamic data				
Heart rate, beats/min	471±10	469±10	460±9	459±8
LV EDP, mmHg	0.5±0.7	0.2±0.3	1.5±0.7*	7.2±2.5*†
Organ weight data				
Body wt, g	20.7±0.5	19.2±0.4	21.7±0.4	20.7±0.5
LV wt/body wt, mg/g	3.0±0.1	2.9±0.0	3.8±0.1*	3.9±0.2*
Pleural effusion, %			0	18

Values are means ± SE. WT/MMP$^{+/+}$, wild-type mice with matrix metalloproteinase-2 (MMP-2); WT/MMP$^{-/-}$, wild-type mice with MMP-2 knockout TG/MMP$^{+/+}$, transgene (TG) mice with cardiac overexpression of TNF-α with MMP-2; TG/MMP$^{-/-}$, TG mice with cardiac overexpression of TNF-α with MMP-2 knockout; LV, left ventricular; EDD, end-diastolic diameter; EDP, end-diastolic pressure. *$P < 0.01$ vs. WT/MMP$^{+/+}$. †$P < 0.05$ vs. TG/MMP$^{+/+}$

cardial mRNA levels of other cytokines/chemokines, indicating that the decline in survival and LV function in TG/MMP$^{-/-}$ mice was not due to enhancement of myocardial cytokine/chemokine expression by selective disruption of the MMP-2 gene.

Plasma cytokine and MMP levels. Plasma levels of TNF-α, as well as other cytokines, were below detection in the four groups of animals (Table 2), consistent with previous studies demonstrating that the TNF-α TG transcripts were limited to the heart (16). As expected, plasma levels of MMP-2 were very low in MMP-2 KO mice. Plasma MMP-2 was comparable between WT/MMP$^{+/+}$ and TG/MMP$^{+/+}$ mice.

DISCUSSION

In the present study, we demonstrated that selective disruption of the MMP-2 gene exacerbated survival and LV function in TG mice with cardiac-specific overexpression of TNF-α. Disruption of the MMP-2 gene did not alter myocardial hypertrophy and interstitial fibrosis but exacerbated inflammatory cell infiltration. These results indicate that MMP-2 plays a protective role against myocardial inflammation and dysfunction in cytokine-induced cardiomyopathy.

Consistent with previous studies (17, 18), MMP-2 mRNA and activities were upregulated in myocardium from animals with TNF-α-induced cardiomyopathy (Figs. 1 and 2). Although the mechanisms responsible for this activation remain to be determined, cellular constituents of cardiac muscle including fibroblasts, inflammatory cells, and myocytes, are known to be capable of expressing MMP-2 in response to specific stimuli (25). Activation of MMPs may be involved in the remodeling process of the failing heart by provoking alterations of the ECM (4, 8, 20, 23, 26, 27). Indeed, MMP-9 disruption reduced myocardial remodeling and improved LV function and survival rate after myocardial infarction (9). Similarly, MMP-2 ablation inhibited cardiac rupture and remodeling after myocardial infarction (8).

The present study investigated the long-term effects of selective MMP-2 gene disruption on development of TNF-α-induced cardiomyopathy. We employed KO mice, because selective MMP-2 inhibitors are not available and "selective" MMP-2 disruption is possible only with a KO mouse model. As expected, no MMP-2 expression was observed in myocardium from WT/MMP$^{-/-}$ and TG/MMP$^{-/-}$ mice (Figs. 1 and

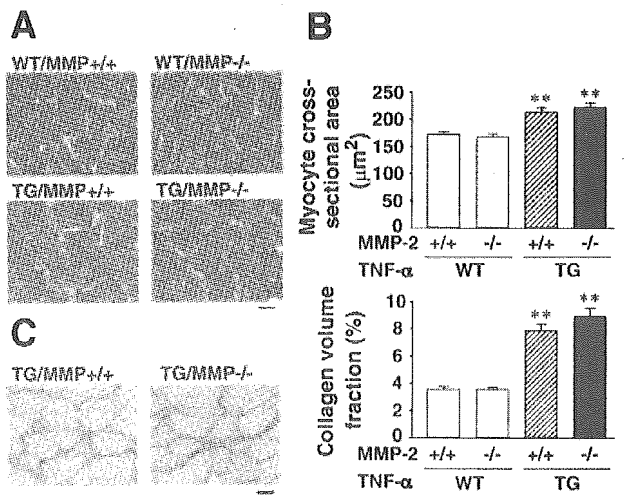

Fig. 4. *A*: representative high-power photomicrographs of Masson's trichrome-stained LV cross sections from WT/MMP$^{+/+}$, WT/MMP$^{-/-}$, TG/MMP$^{+/+}$, and TG/MMP$^{-/-}$ mice. Scale bar, 10 μm. *B*: summary data of myocyte cross-sectional area and collagen volume fraction by histopathological analysis of LV tissue sections (n = 6/group). Values are means ± SE. **$P < 0.01$ vs. WT/MMP$^{+/+}$. *C*: representative high-power photomicrographs of picrosirius red-stained LV cross sections from TG/MMP$^{+/+}$ and TG/MMP$^{-/-}$ mice. Scale bar, 10 μm.

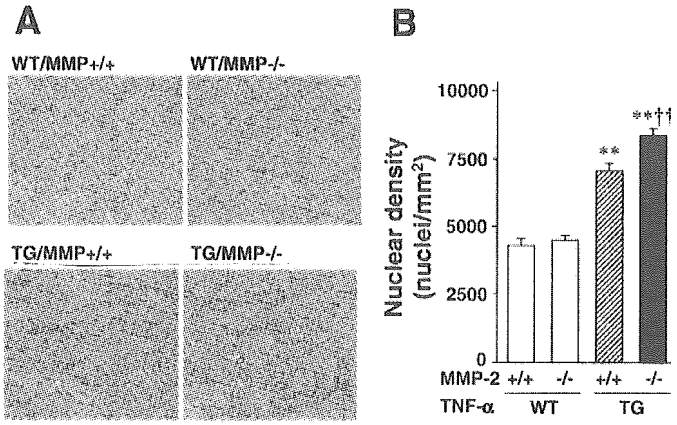

Fig. 5. *A*: representative photomicrographs of hematoxylin-and-eosin-stained LV sections from WT/MMP$^{+/+}$, WT/MMP$^{-/-}$, TG/MMP$^{+/+}$, and TG/MMP$^{-/-}$ mice. Scale bar, 100 μm. *B*: summary data for nuclear density of infiltrating cells (n = 6/group). Values are means ± SE. **$P < 0.01$ vs. WT/MMP$^{+/+}$, ††$P < 0.01$ vs. TG/MMP$^{+/+}$.

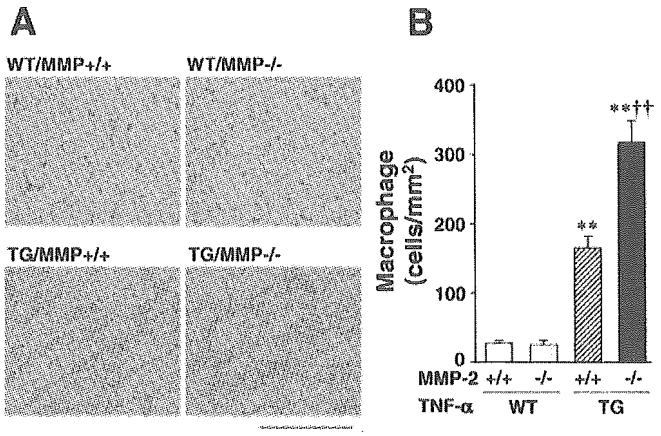

Fig. 6. *A*: representative photomicrographs of anti-Mac-3 antibody-stained LV sections from WT/MMP$^{+/+}$, WT/MMP$^{-/-}$, TG/MMP$^{+/+}$, and TG/MMP$^{-/-}$ mice. Scale bar, 100 μm. *B*: summary data for density of Mac-3-positive cells (n = 5/group). Values are means ± SE. $^{**}P < 0.01$ vs. WT/MMP$^{+/+}$. $^{††}P < 0.01$ vs. TG/MMP$^{+/+}$.

2). We had expected that MMP-2 disruption could ameliorate cardiac ECM remodeling and dysfunction in TNF-α TG mice and prolong survival. On the contrary, selective blockade of the MMP-2 gene exacerbated LV contractile dysfunction and failure (Table 1) and even shortened survival (Fig. 3), suggesting that myocardial induction of MMP-2 may play a protective role against the development of cytokine-induced cardiomyopathy.

The most striking finding of the present study was an increase in inflammatory cell recruitment into the myocardium seen in MMP-2 KO mice due to overexpression of TNF-α in the heart (Figs. 5 and 6). Even though the pathophysiological significance of cellular infiltrates in myocardial remodeling and failure remains mostly speculative in this model, the observed increase in inflammation might exacerbate myocardial contractile and structural defects, which could lead to premature death

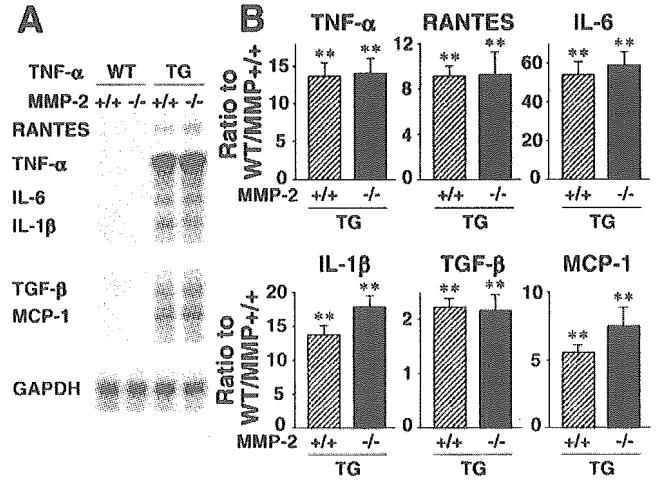

Fig. 7. *A*: myocardial gene expression of proinflammatory cytokines and chemokines from WT/MMP$^{+/+}$, WT/MMP$^{-/-}$, TG/MMP$^{+/+}$, and TG/MMP$^{-/-}$ mice. *B*: densitometric analysis of gene expression in myocardium from TG/MMP$^{+/+}$ (n = 7) and TG/MMP$^{-/-}$ (n = 6) mice. Each value was normalized to that of GAPDH in each template set as an internal control and expressed as ratio to WT/MMP$^{+/+}$ (n = 6). MCP, monocyte chemoattractant protein; TGF, transforming growth factor; RANTES, regulated on activation, normal T cell expressed and secreted. Values are means ± SE. $^{**}P < 0.01$ vs. WT/MMP$^{+/+}$.

Table 2. *Plasma levels of cytokines and MMPs*

	WT/MMP$^{+/+}$	WT/MMP$^{-/-}$	TG/MMP$^{+/+}$	TG/MMP$^{-/-}$
Cytokines/chemokines				
TNF-α, pg/ml	ND	ND	ND	ND
IL-6, pg/ml	9.0±0.2	9.0±0.8	9.0±0.6	10.4±2.5
IL-1β, pg/ml	ND	ND	ND	ND
MCP-1, pg/ml	20.7±10.2	8.3±3.2	17.3±3.3	24.9±6.3
MMP				
MMP-2, ng/ml	60.9±3.0	0.9±0.2*	63.2±2.5	0.8±0.2*

Values are means ± SE; n = 5. ND, not detectable; MCP, monocyte chemoattractant protein. *$P < 0.01$ vs. WT/MMP$^{+/+}$.

in TG/MMP$^{-/-}$ mice. Several potential mechanisms of MMP-2 deletion are responsible for exacerbating cellular inflammation in TNF-α TG hearts. One possible mechanism is a further increase in expression of the TNF-α gene in TG/MMP$^{-/-}$ mice and the resultant enhancement of inflammation. However, this possibility is less likely, because the gene expression of cytokines, TNF-α and MCP-1, was not altered by selective disruption of the MMP-2 gene (Fig. 7), perhaps in part because expression of TNF-α in TG mice is driven by α-myosin heavy chain promoter, which is supposed to be MMP-2 independent. Another possibility is that MMP-2 might alter the milieu of the ECM, which could accelerate the infiltration of cells. In the present study, no significant changes were observed in collagen deposition between TG/MMP$^{+/+}$ and TG/MMP$^{-/-}$ mice (Fig. 4). Therefore, exacerbation of myocardial inflammation in TG/MMP$^{-/-}$ mice was not due to impairment of interstitial collagen formation and/or structure.

Theoretically, an increase in MMP activity would result in a decrease in the MMP substrate, collagen, whereas inhibition of MMP-2 would result in an increase in collagen. However, in agreement with previous studies (18), the present study demonstrated that an increase in MMP-2 activity was accompanied by an increase in fibrosis in TNF-α TG mice (Fig. 4). Moreover, selective disruption of the MMP-2 gene did not alter these changes in interstitial fibrosis. The present study could not provide a definite explanation for these paradoxical findings, perhaps because total ECM collagen content is a complex function of synthesis and degradation.

Although the functional role of MMP-2 in this aspect and its significance in myocardial inflammation remain unknown, on the basis of a previous study by Heymans et al. (9), we could not exclude the possibility that deletion of the MMP-2 gene might alter ECM components other than collagens, disrupt the alignment of myocytes with the ECM or degrade the ECM surrounding the myocytes, and promote the further progression of inflammatory cell migration into the interstitial space. MMPs have been shown to facilitate inflammatory cell recruitment (7). However, the present study does not provide a direct proof for the cause-and-effect relation between the increase in cellular infiltration and the exacerbation of heart failure, and further investigation is clearly needed.

The results of the present study are consistent with those of previous studies of MMP-2 gene ablation in other models of tissue inflammation (3, 13). Disruption of the MMP-2 gene exacerbated allergic lung inflammation and increased lethal susceptibility to asphyxiation in a mouse model of asthma (3). Furthermore, Itoh et al. (13) demonstrated an increase in severity of arthritis in association with tissue inflammation in

MMP-2 KO mice. These results indicate that MMP-2 suppressed the development of inflammatory disease. Therefore, TNF-α overexpression and MMP-2 deletion might synergistically activate the cellular inflammatory response in the heart, which could further damage cardiac function.

The present study could not demonstrate the long-term beneficial effects of MMP-2 deletion on the decline in LV systolic function in TNF-α-induced cardiomyopathy. These findings are in contrast to those from a previous study, in which broad-spectrum MMP inhibition could ameliorate myocardial dysfunction in TNF-α TG mice (18). The MMP inhibitor batimastat was found to reduce myocardial hypertrophy and diastolic dysfunction. Even though it is difficult to compare the two studies directly because of the broad-spectrum action of the MMP inhibitor used by Li et al. (18), these studies suggest that MMP-2 has a complex role in maintaining the physiological function of the heart, and information about the action of individual MMPs with the use of such an inhibitor is limited. The most effective way to evaluate the contribution of the specific MMP and obtain direct evidence for a role of MMP in heart failure is through gene manipulation, as employed in the present study. Whatever the mechanisms are, MMP-2 could protect the heart by inhibiting acceleration of interstitial inflammatory infiltration in this model. The disparity between selective and nonselective inhibition of MMP-2 may have important implications in the development of pharmacological agents for the treatment of heart failure. These findings may further draw attention to treatment of heart failure by using the nonselective, broad-spectrum MMP-2 inhibitor.

There are several limitations to be acknowledged in this study. *1*) We examined only female mice for physiological, pathological, and biochemical analyses, because most male TG mice died before 12 wk of age, when these analyses were performed. As a result, we are not certain whether selective disruption of the MMP-2 gene exacerbates myocardial inflammation and dysfunction even in male TNF-α TG mice. *2*) Even though in vivo assessment of LV function with echocardiography is feasible and reproducible in the mouse, it might be difficult to interpret the indexes in dilated LV. However, our validation study has shown that intra- and interobserver variabilities of our echocardiographic measurements for LV cavity dimensions and fractional shortening were small and that measurements in the same animals on separate days were highly reproducible (24). Therefore, our technique could be considered to allow for a noninvasive assessment of LV structure. *3*) The heart rates in the present study (450–470 beats/min) were lower than those measured in conscious mice (600 beats/min). Therefore, the LV size and function results might be greatly influenced by differences in anesthetic regimens and experimental conditions, such as heart rate. *4*) Although the present study employed the conventional gelatin zymography method to analyze MMP activities, as in a previous study (8), two-dimensional zymography is essential, especially to ascertain the existence of plasmin in the 66-kDa MMP complex (28). *5*) We assessed TGF-β, which has been well established to be involved in ECM remodeling in heart failure, in the present study. Further studies on TGF-α are also needed, because it plays an important role in cell proliferation and differentiation.

In summary, selective disruption of the MMP-2 gene exacerbated myocardial inflammation and dysfunction in TNF-α-induced cardiomyopathy. Thus myocardial expression o MMP-2 may play a protective role in the development o congestive heart failure in cytokine-induced cardiomyopathy.

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Hepatocyte Growth Factor Ameliorates the Progression of Experimental Autoimmune Myocarditis

A Potential Role for Induction of T Helper 2 Cytokines

Hideki Futamatsu, Jun-ichi Suzuki, Shinya Mizuno, Noritaka Koga, Susumu Adachi, Hisanori Kosuge, Yasuhiro Maejima, Kenzo Hirao, Toshikazu Nakamura, Mitsuaki Isobe

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

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

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

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

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

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Materials and Methods

Animals

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

Antigen and Immunization

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

Reagents

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

Preparation of Human Recombinant HGF

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

Immunohistochemistry

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

FACS Analysis

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

In Vivo Gene Transfer

Hemagglutinating virus of Japan (HVJ)-envelope vector kit was provided by Ishihara Sangyo Kaisha and prepared as previously described.[20] HVJ-envelope vector (200 μL) containing the phMGFP gene (20 μg) or human HGF gene (20 μg), which was inserted into the *Not*I site of the pUC-SRα expression vector,[21] was transfected directly into the hearts after thoracotomy. We transfected directly the HVJ-envelope containing the human HGF gene into rat hearts at 4 sites (each site: 50 μL) on day 0 (early phase; group HGF-E) or on day 14 (late phase; group HGF-L). For control, empty vector was transfected into the hearts (group Vect-E and group Vect-L). Another group of rats received no transfection (group UT). There were 7 rats in each of the 5 groups.

Histological Examination

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

Echocardiography

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

Apoptotic Assay

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

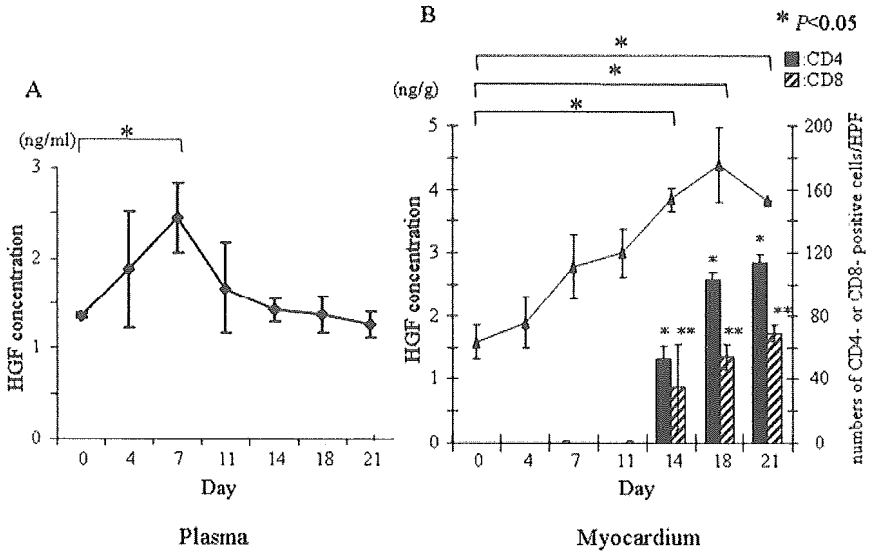

Figure 1. HGF expression in rats with myocarditis from 0 to 21 days after immunization. A, Plasma HGF concentration determined by ELISA. B, HGF concentration in cardiac tissues determined by ELISA. HGF concentrations levels in both plasma and cardiac tissues increased significantly after immunization. $P<0.05$, vs day 0.

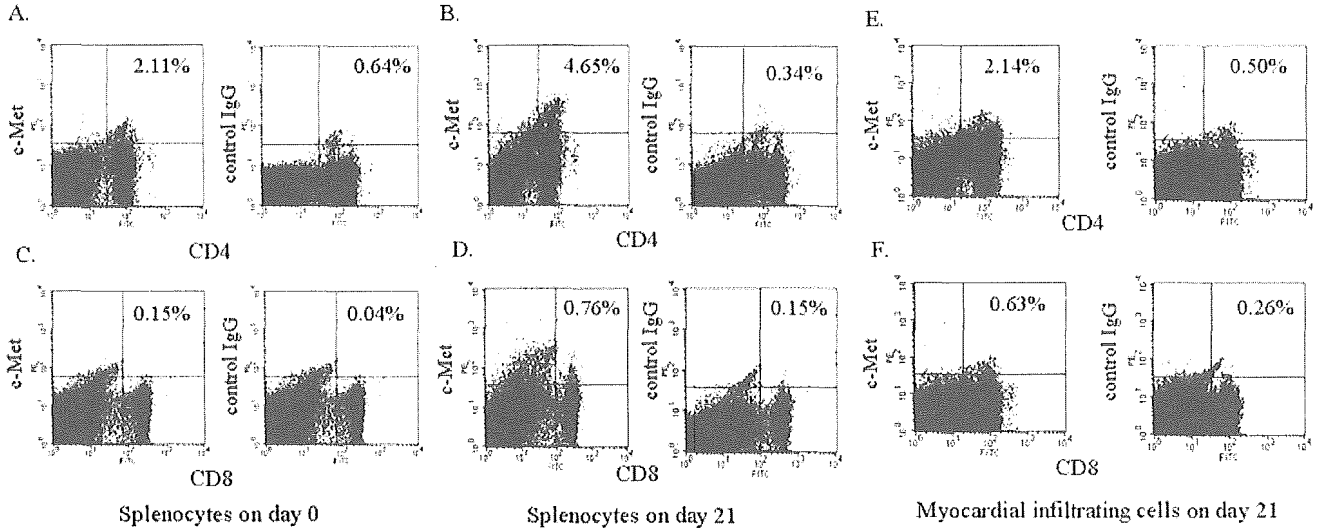

Figure 2. Expression of c-Met in splenocytes and myocardial infiltrating cells. FACS analysis of splenocytes on day 0 (A and C), splenocytes on day 21 (B and D), and myocardial infiltrating cells on day 21 (E and F). We detected upregulation of c-Met receptor in both CD4-positive and CD8-positive cells in splenocytes and myocardial infiltrating cells after immunization (A through F). Data are representative of three independent experiments.

CD4-Positive T Cell Proliferation Assay

CD4-positive T cells were isolated from splenocytes in rats with myocarditis on day 18 with a FACS Vantage (Becton Dickinson). We confirmed by FACS that the purity of sorted cells was >95% to 99%. Cells (5×10^5 per well) were cultured as previously described in 96-well culture plates with 50 μg/mL purified porcine heart myosin and splenic antigen-presenting cells inactivated by mitomycin C (Sigma Chemical Co) from normal rats.[24] Cells were incubated at 37°C under 5% CO_2 at various concentrations of hrHGF for 5 days. T cell proliferation was estimated with the Cell Counting Kit-8. Cell proliferation was expressed as an optical density.

Enzyme-Linked Immunosorbent Assay

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

Statistical Analysis

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

Results

Expression of HGF in Rats With Myocarditis

The plasma HGF concentration increased significantly and reached a peak 7 days after immunization (Figure 1A). The HGF concentration in cardiac tissue of rats with myocarditis on day 18 after immunization was significantly increased

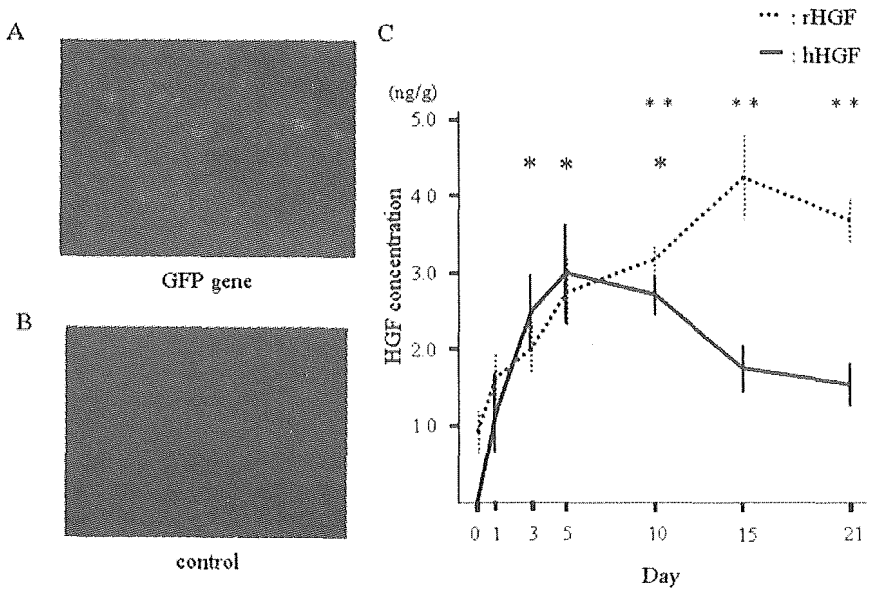

Figure 3. In vivo gene transfer into the heart. GFP expression in the injected area of the heart 3 days after transfection as detected by fluorescent microscopy. HVJ-E containing GFP plasmid (A) or HVJ-E only (B). Original magnification, ×400. HGF protein concentration in the transfected hearts as measured by ELISA on days 0 to 21 after immunization. Both exogenous and endogenous HGF levels increased significantly (C). *$P<0.05$, exogenous HGF (hHGF) levels, vs day 0. **$P<0.05$, endogenous HGF (rHGF) levels, vs day 0.

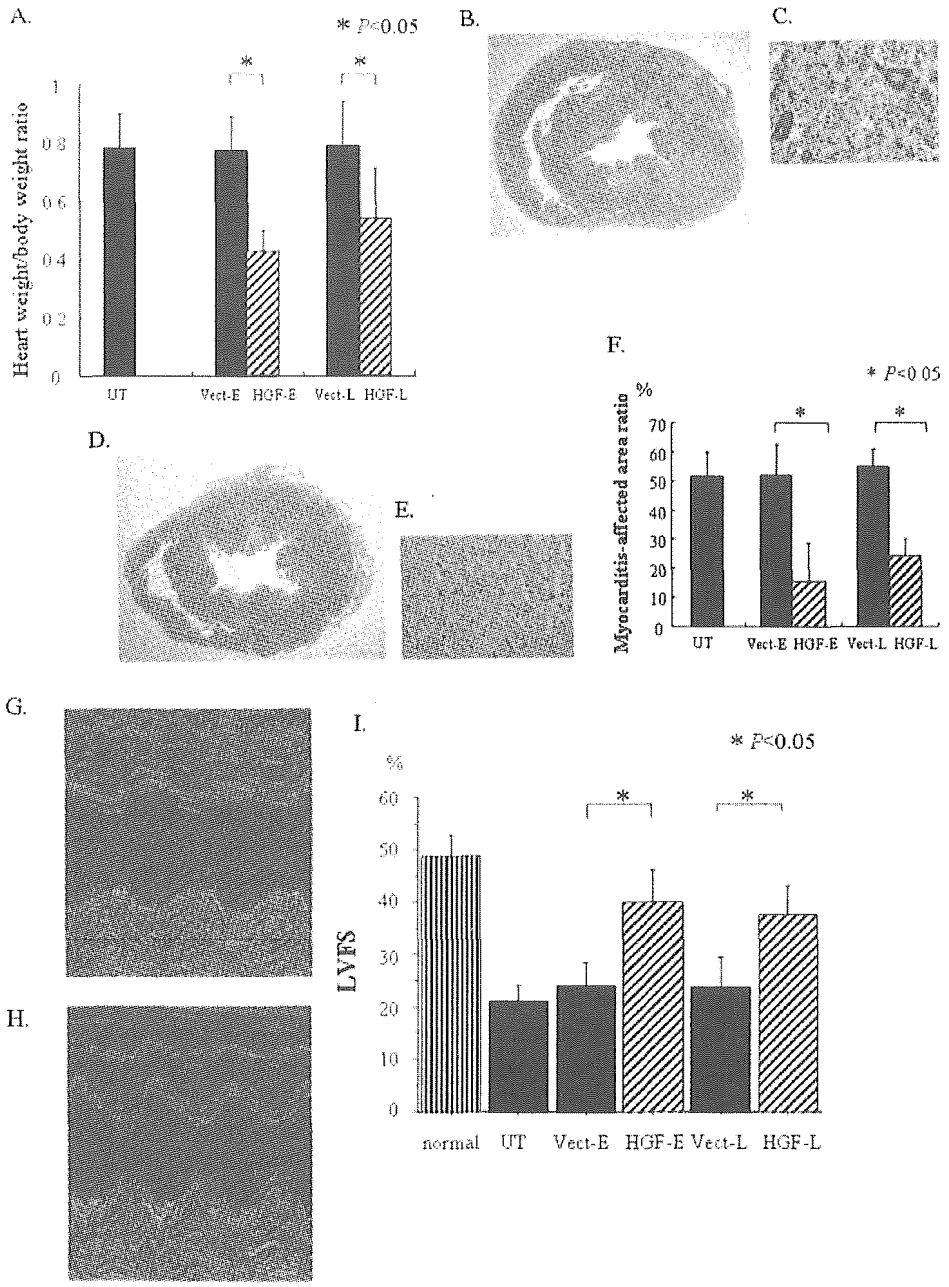

Figure 4. Clinical effect of HGF on EAM. Closed bar indicates the control group, and hatched bar indicates the HGF-treated group. A, Heart weight/body weight ratio in groups HGF-E and HGF-L were significantly lower than those in control groups. B through E, Hematoxylin and eosin. Representative cases of myocardial lesions on day 21 in the control group (B and C) and the HGF-treated group (D and E). F, Myocarditis-affected area ratios in the respective groups. Original magnification in B and D is ×10. Original magnification in C and E is ×400. M-mode echocardiograms in the control group (G) and the HGF-treated group (H). I, Effect of HGF on left ventricular fractional shortening (LVFS) on day 21.

(Figure 1B). Additionally, the numbers of infiltrating CD4- or CD8-positive cells increased significantly from day 14.

Expression of c-Met in Splenocytes and Inflammatory Cells

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

In Vivo Gene Transfer Into the Heart With HVJ Envelope

Injection of the GFP gene resulted in limited expression at the site of injection, as detected by fluorescence microscoy 3 days after transfection (Figure 3A). In contrast, the negative

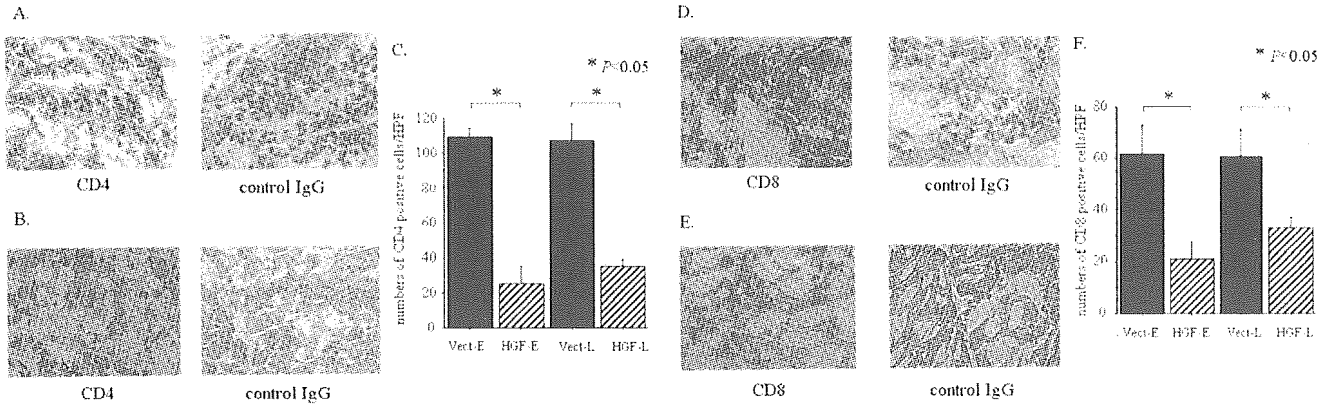

Figure 5. Inhibition of CD4- or CD8-positive cells expression. Immunohistochemical analysis with anti-CD4 antibody in control groups (A) and in treatment groups (B). C, Numbers of CD4-positive cells. CD8 staining in control groups (D) and in treatment groups (E). F, Numbers of CD8-positive cells. Original magnification, ×400.

control, treated only with HVJ-envelope and not the GFP gene, showed no fluorescence (Figure 3B). We measured the HGF protein concentration in the transfected hearts by ELISA. Both the exogenous HGF level, which were hHGF protein concentration, and the endogenous HGF level, which were rHGF protein concentration, increased significantly (Figure 3C).

In Vivo Effect of HGF on EAM

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

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

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

HGF Suppressed Myocyte Apoptosis In Vivo

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

HGF Effect on Cytokines in Hearts

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

Antigen-Specific CD4-Positive T Cell Proliferation Assay

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

Discussion

HGF plays important roles in a variety of cardiovascular diseases through its angiogenesis, antifibrosis, and antiapoptotic activities.[13-15] Several studies have defined the role of HGF with regard to immunity in acute and chronic renal rejection after transplantation and acute graft-versus-host disease models.[11,12] However, the role of HGF in T cell–

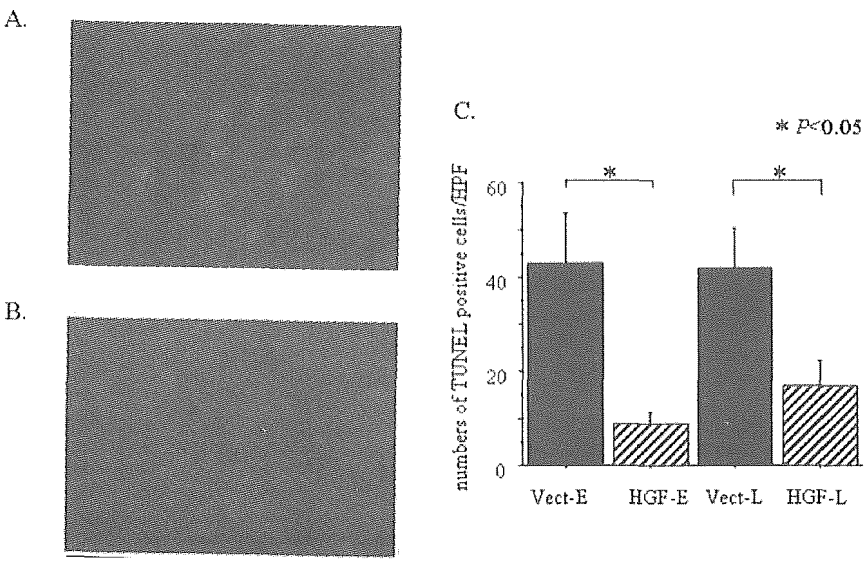

Figure 6. Reduction of apoptosis by HGF gene transfer. Apoptosis of cardiomyocytes in the control group (A) and HGF-treated group (B) on day 21. Graph shows counts of TUNEL-positive cells (C).

mediated autoimmune disease has not been reported, and relations between HGF and T cell activation have not been elucidated. To the best of our knowledge, we are the first to find that HGF plays an important role in T cell activation and that HGF gene therapy suppresses the development of autoimmune myocarditis.

We investigated HGF concentrations in plasma and cardiac tissues in relation to myocarditis by using an EAM model. HGF concentration in both plasma and cardiac tissues increased rapidly in response to immunization. Similar rapid induction of HGF was noted in the case of ischemia/reperfusion, acute hepatic, and renal injuries.[14,25,26] The increased HGF concentration may reflect defensive reactions.[14] In addition to the increase in HGF concentration, expression of c-Met receptor was upregulated in splenocytes and myocardial infiltrating cells. Expression of c-Met in splenocytes and in myocardial infiltrating cells has not been reported. We detected upregulation of c-Met receptor in both CD4-positive and CD8-positive cells after immunization, and these findings support the hypothesis that the increase in c-Met expression is related to autoinduced expression triggered by HGF.[27] Additionally, expression of c-Met after immunization was greater in CD4-positive cells than in CD8-positive cells. The intense expression of c-Met in CD4-positive T cells indicates that HGF plays an important role in EAM because the development of EAM involves autoreactive CD4-positive T cell proliferation.[28]

We transfected the HGF gene directly into cardiomyocytes using the HVJ-envelope method to examine whether HGF would reduce the development of acute myocarditis. The simultaneous GFP expression and detection of hHGF showed that human HGF gene transfer into cardiomyocytes by the HVJ-envelope method resulted in efficient transfection of the gene into the rat hearts. Additionally, we found that levels of endogenous HGF were increased by human HGF gene transfection. These observations are consistent with the previous reports that exogenous HGF enhances autolooped positive feedback on endogenous HGF production.[29,30]

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

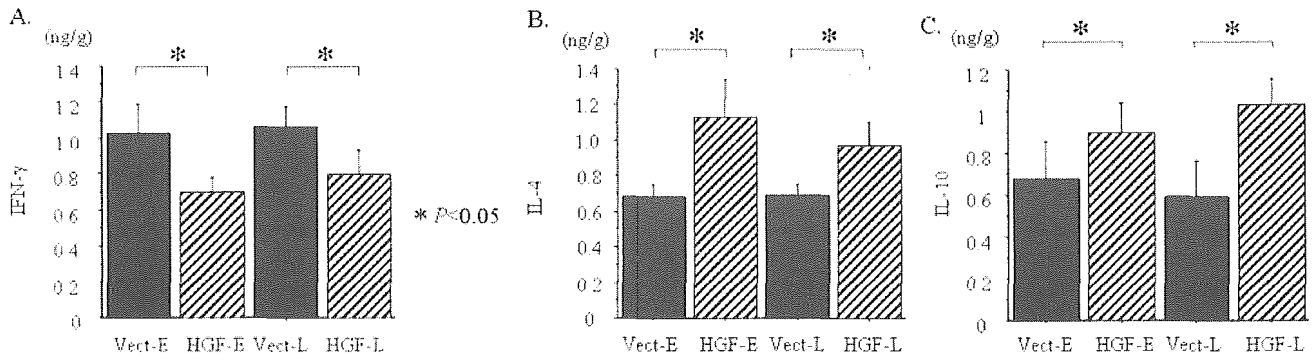

Figure 7. Expression of cytokines in the heart. ELISA was used to examine production of cytokines in hearts on day 21. Levels of cytokine for IFN-γ in treatment groups decreased significantly (A). However, levels of cytokine for IL-4 and IL-10 in the treatment groups significantly increased (B and C).

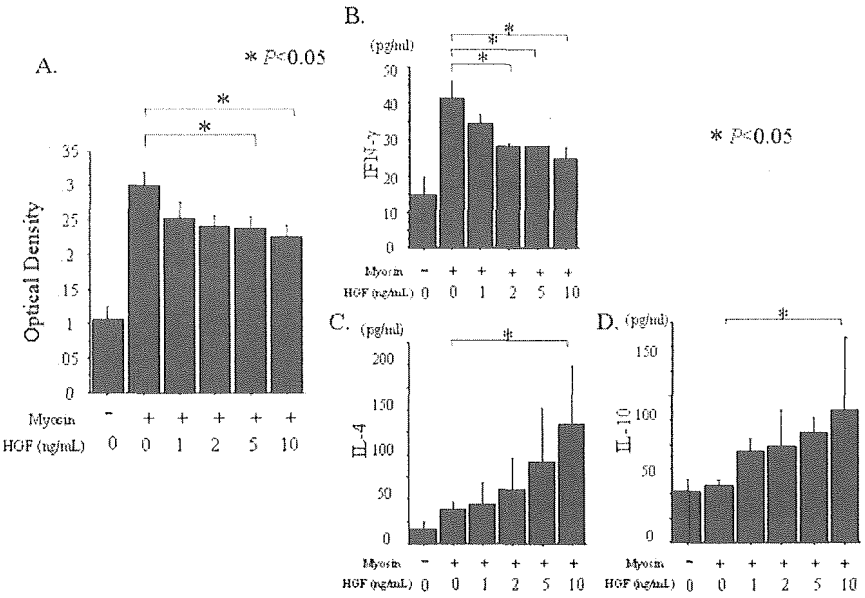

Figure 8. T cell proliferation assay. CD4-positive T cells isolated from splenocytes in rats with myocarditis on day 18 were stimulated by cardiac myosin. Cell proliferation was significantly suppressed by HGF (A). Production of IFN-γ was suppressed significantly by HGF, and production of IL-4 and IL-10 was increased significantly by HGF (B through D).

Inhibition of antigen-specific T cell proliferation can be a therapeutic strategy for retarding the development of myocarditis,[18] but it is still unclear whether HGF affects antigen-specific T cell proliferation. We are the first to provide evidence that HGF suppresses secondary T cell responses, especially those of CD4-positive T cells, after myosin immunization. HGF reduced myosin-specific CD4-positive T cell proliferation in EAM. T cell proliferation was modulated by the Th1-Th2 balance and suppressed by activation of Th2 cytokines. Th2 cytokines mainly inhibit the number of CD4 cells, which secrete Th1 cytokines. Previous studies showed that dysfunction of the immune system associated with autoimmune disease is related to the imbalance between Th1 and Th2 cells.[31] The altered Th1-Th2 balance regulates the clinical course of EAM[3] and IL-10 plays a protective role(s) during the development of myocarditis.[32] Thus, we examined production of Th1 cytokines, IFN-γ, and Th2 cytokines, IL-4 and IL-10, by myosin-specific CD4-positive T cells in response to cardiac myosin. We found that production of IFN-γ was reduced and that production of IL-4 and IL-10 was increased by HGF; these findings show that HGF promotes the shift from Th1 to Th2. Similar changes in cytokines were found in vivo. These results are supported by previous findings that the Th1-to-Th2 shift prevented the development of EAM.[28] HGF inhibits Th1 cytokines, such as IFN-γ, in an acute graft-versus-host disease model[12]; however, the mechanism is unknown. Thus, our results suggest that Th2 cytokines induced by HGF suppress production of Th1 cytokines and the development of EAM.

Our results show that suppression of the antigen-specific immune responses by HGF contributed to therapeutic outcomes during the progression of EAM, but the effect was partial and not enough to completely block the immune mechanisms. Of interest, we found during the natural course of myocarditis that surviving cardiomyocytes strongly express c-Met/HGF receptor (data not shown). This expression in cardiomyocytes is compatible with findings in other cardiovascular models.[15,33] We showed that the number of TUNEL-positive cardiomyocytes was significantly less in HGF-treated groups than in vector control groups. Given that HGF is a potent antiapoptotic factor with respect to cardiomyocytes,[14–16] a direct antiapoptotic effect toward the heart parenchymal cells should be considered.

It has been reported that c-Met and HGF are involved in cardiac stem cell biology.[34,35] Bone marrow–derived mesenchymal stem cells expressed high level of HGF. Also, HGF administration enhanced the efficacy of cellular cardiomyoplasty. However, the role of cardiac stem cells in myocarditis is still not clear. Further studies are needed to investigate the role of cardiac stem cells and the relation between HGF and cardiac stem cells in myocarditis.

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

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