Table 1. Laboratory Data on Admission at 19 years of age.

Blood cell count			
White blood cells	10,700/μL	Na	135 mEq/L
Neutrophils	73 %	K	4.1 mEq/L
Eosinophils	1 %	Cl	100 mEq/L
Monocytes	5 %	Calcium	8.3 mg/dL
Lymphocytes	21 %	Phosphate	3.1 mg/dL
Red blood cells	488×10 /μL	Total cholesterol	119 mg/dL
Hemoglobin	15.6/g/dL	Triglycerides	66 mg/dL
Platelet	24.8×10 /μL	Choline esterase	328 IU/L
ESR	7 mm/h	C-Reactive protein	1.6 mg/dL
		STS	(-)
Biochemistry			
Total protein	5.7 g/dL	**Coagulation**	
Albumin	3.3 g/dL	Prothrombin time	59.2 %
AST	149 IU/L	APTT	36.1 %
ALT	227 IU/L	Thrombo test	41.0 %
γ-GTP	29 IU/L	Fibrinogen	363 mg/dL
LDH	191 IU/L		
Alkaline phosphatase	228 IU/L	**Arterial blood gas**	
Total bilirubin	0.8 mg/dL	PO_2	67.3 torr
Blood urea nitrogen	6 mg/dL	PCO_2	27.1 torr
Creatinene	0.8 mg/dL	HCO_3	19.3 mEq/L
Uric acid	6.0 mg/dL	pH	7.46

APTT. Activated partial thromboplastin time; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ESR, erythrocyte sedimentation rate;γ-GTP, γ-glutamyltranspeptidase; LDH, lactate dehydrogenase; STS, serological test for syphilis

Table 2. Immunological data of the patient and his mother when he was 19 years old.

	Patient	Mother
ESR (mm/h)	7	26 *
CRP (mg/dL)	< 0.3	< 0.3
WBC (/μL)	6200	3700
RA test (IU/mL)	<20	
ANF homogeneous	(−)	× 320 *
ANF speckled	× 40 *	× 2560 *
Anti-SS-A	(−)	(+) *
Anti-SS-B	(−)	(+) *
Anti-RNP	(−)	(−)
Anti-Sm	(−)	(−)
Anti-Scl-70	(−)	(−)
C3 (mg/dL)	61	56
C4 (mg/dL)	20	19
CH50 (U/mL)	37.8	39.6
IgA (mg/dL)	347	747 *
IgG (mg/dL)	729 *	2036 *
IgM (mg/dL)	50	98

ANF, antinuclear antibody; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; WBC, white blood cells * abnormal value

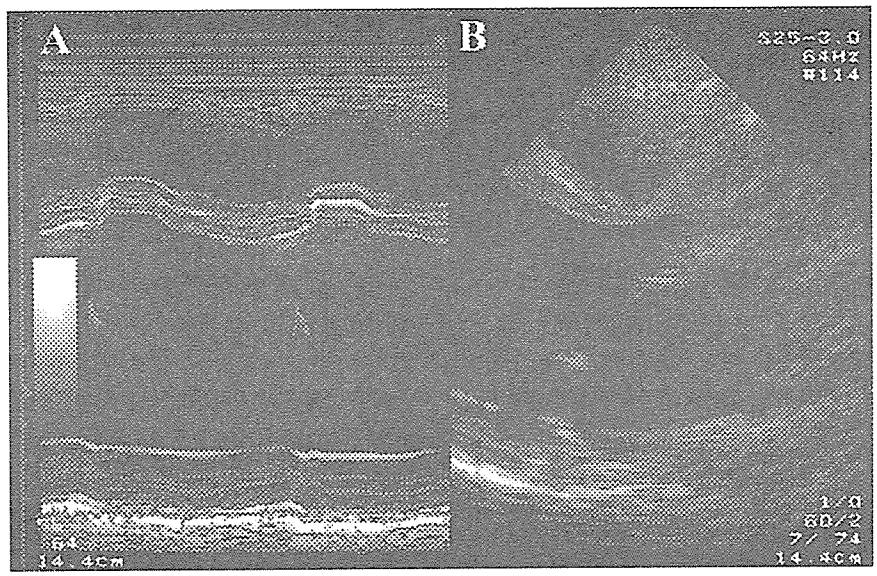

Figure 1. Echocardiography at the age of 27. (1997.1.24)
(Panel A) M-mode, (Panel B) 2D-mode. LVDd (left ventricular diastolic dimension) 74 mm; LVDs (left ventricular systolic dimension) 64 mm; EF (ejection fraction) 0.35; %FS (fractional shortening) 14 %; IVSth (interventricular septal thickness) 9 mm; PWth (posterior wall thickness) 8 mm; LAD (left atrial dimension) 44 mm; AoD (aortic dimension) 22 mm; Diffuse hypokinesis of LV were observed. Doppler echocardiography showed mild MR (mitral regurgitation) and mild TR (tricuspid regurgitation); RVSP (right ventricular systolic pressure) was 37 mmHg.

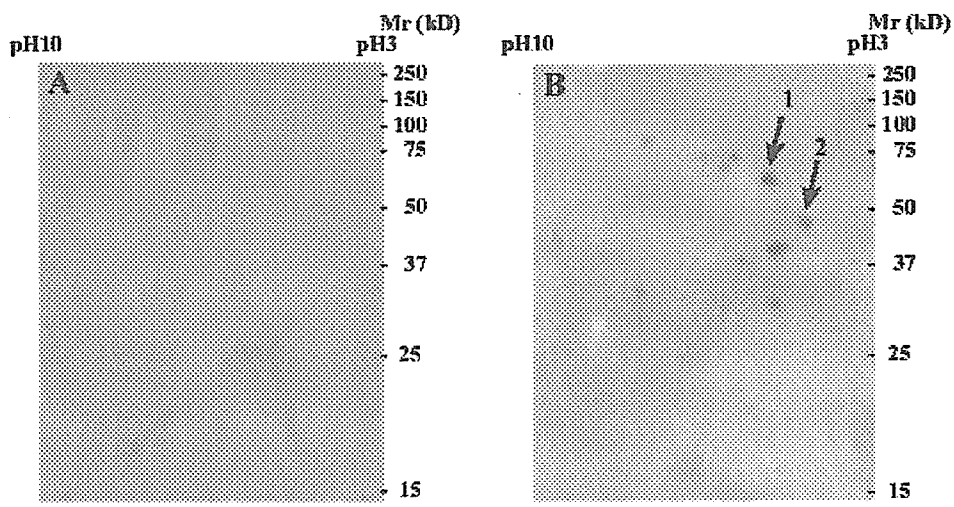

Figure 2. Western Blot of a Plasma Membrane Fraction From Cultured Rat Cardiac Myocytes With Sera From a Healthy Control Subject (panel A) and The Patient (panel B). Arrows 1 and 2 indicate protein spots, which were identified as annexin A6 and vimentin, respectively (panel B).

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Reduction of inflammatory cytokine expression and oxidative damage by erythropoietin in chronic heart failure

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Abstract

Objectives: Late treatment with erythropoietin (EPO), as well as the administration before the onset of or during the acute stage of myocardial infarction (MI), has recently been shown to mitigate post-MI heart failure. We investigated the mechanisms, including the downstream signaling pathways, for the beneficial effect of late treatment with EPO on chronic post-MI heart failure.
Methods and results: EPO (1500 U/kg, twice a week) was administered to mice beginning 6 weeks after induction of large MI. The EPO treatment for 4 weeks diminished left ventricular dilatation and improved function. It significantly reduced inflammatory cell infiltration and fibrosis, and increased vascular density in noninfarcted areas. The elevated levels of the inflammatory cytokines interleukin (IL)-1β, IL-6, tumor necrosis factor-α and transforming growth factor-β1 seen in the failing hearts were returned nearly to control levels by EPO treatment. Oxidative damage in surviving cardiomyocytes was also significantly attenuated by EPO. Expression of EPO receptor was upregulated in failing hearts, and EPO treatment led to myocardial activation of signal transducer and activator of transcription-3 (Stat3), Stat5, and Akt. These *in vivo* effects of EPO were confirmed *in vitro* in experiments that showed the anti-inflammatory and anti-oxidant effects of EPO to be mediated via Stat and Akt activation. Finally, the beneficial effects of EPO were found to persist for 4 weeks after discontinuing treatment.
Conclusions: It thus appears that Stat-mediated reduction of inflammation and cytokine production and Akt-mediated attenuation of oxidative stress accompany the beneficial effects of late treatment with EPO on chronic post-MI heart failure.

Keywords: Cytokines; Heart failure; Myocardial infarction; Myocardial inflammation

This article is referred to in the Editorial by Qingping Feng (pages 615–617) in this issue.

1. Introduction

Erythropoietin (EPO) is a hypoxia-induced hormone that is essential for normal erythropoiesis and has been broadly used in patients with anemias having a variety of etiologies. In addition, early studies in heart failure patients with anemia suggest that EPO therapy is a safe and effective method of reducing left ventricular hypertrophy, enhancing exercise performance and increasing ejection fraction [1,2]. Notably, however, expression of the EPO receptor (EPOR) within the cardiovascular system, including on cardiomyocytes and endothelial cells, suggests EPO exerts cardiovascular effects beyond hematopoiesis [3–5]. For instance, based on the observations that recombinant human EPO exerts cardioprotective effects in the infarcted heart and following ischemia–reperfusion injury of the myocardium – i.e., EPO administration either prior to or during ischemia significantly enhances functional recovery after reperfusion – it was suggested

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that one cellular mechanism contributing to this protective effect was inhibition of cardiomyocyte apoptosis, resulting in decreased infarct size within the area at risk [6,7].

Given that large myocardial infarctions (MIs) are a major cause of heart failure, it is noteworthy that van der Meer et al. [8] recently showed that EPO treatment started 3 weeks after the onset of MI (late treatment) improved cardiac function in a rat model of post-MI heart failure. This beneficial effect was accompanied by increases in the capillary density and the capillary-to-myocyte ratio and a shift in the expression of the myosin heavy chain phenotype. In addition, evidence from both animal and human studies suggests that increased inflammatory cytokines and oxidative stress are associated with a poor prognosis following MI and may play an important role in the pathogenesis and progression of heart failure: they influence heart contractility by inducing hypertrophy and promoting apoptosis or fibrosis, thereby contributing to the continuous myocardial remodeling process [9–13]. In neurons, on the other hand, EPO exerts anti-inflammatory [14,15] and anti-oxidant [16,17] effects by suppressing production of inflammatory cytokines and reactive oxygen species. A recent study has shown that EPO decreases inflammation during myocardial ischemia and reperfusion [18]. Similar effects of EPO have yet to be documented in chronic heart failure [5], however, and the molecular signaling pathways related to the effects of EPO in failing hearts remain largely unknown.

Our aims in the present study, therefore, were first to confirm the beneficial effect of late treatment with EPO on an established case of heart failure; second, to investigate whether the expression of inflammatory cytokines and oxidative stress is involved in its effect; and third, to begin to characterize the molecular signaling upstream of the outcome.

2. Methods

2.1. Experimental heart failure

This study was approved by our Institutional Animal Research Committee. MI was induced in 70 male 12-week-old C57BL/6 mice (Chubu Kagaku Shizai) by ligating the left coronary artery as previously described [19]. Six weeks after the operation, there were 40 surviving mice (survival rate, 57%). The surviving animals underwent echocardiographic examination and were entered into the *in vivo* studies. For sham-operated mice ($n=18$), the suture was passed around the artery but was not tied; all mice survived for 6 weeks after surgery.

2.2. In vivo treatment with EPO

2.2.1. Protocol-1

MI-bearing mice were randomly assigned to two groups, resulting in similar levels of echocardiography-based cardiac function in both. In one group ($n=10$) recombinant human EPO (Chugai Pharmaceutical Co.) was subcutaneously administered twice a week at a dosage of 1500 U/kg in saline, beginning 6 weeks post-MI. The dose of EPO was selected because it was within the range of known dosages providing organ protection [5,6]. The injections were continued for 4 weeks, until 10 weeks post-MI. The other group (control; $n=10$) received the same volume of saline alone over the same period. Sham-operated mice were treated with saline or EPO ($n=9$ each) in the same manner. At the end of the protocol, cardiac geometry and function were examined physiologically (see below), and necropsies were performed for histological and biochemical analyses.

2.2.2. Protocol-2

Continuous treatment with EPO such as described in Protocol-1 may be impossible in actual clinical situations because of various adverse effects (e.g., severe polycythemia). In another protocol, therefore, we also examined how long the effects of EPO persist after discontinuing treatment. Beginning 6 weeks post-MI, EPO or saline ($n=10$ each) was given for 4 weeks, as in Protocol-1, then after an additional 4 weeks (14 weeks post-MI) cardiac function was examined physiologically (see below).

2.3. Blood sampling

Blood used for hemocounts was drawn from the tail vein before and during treatment and from the interior vena cava at sacrifice.

2.4. Physiology studies

Physiological studies were carried out as previously described [19]. Animals were anesthetized with halothane (induction, 2%; maintenance, 0.5%) in a mixture of N_2O and O_2 (0.5 l/min each) via a nasal mask. Echocardiograms were recorded using an echocardiographic system (Aloka) equipped with a 7.5-MHz imaging transducer 6 weeks post-MI (before treatment) and at sacrifice. Following echocardiography, the right carotid artery was cannulated with a micromanometer-tipped catheter (SPR 671, Millar Instrument) and advanced into the aorta and then into the left ventricle (LV) for recording pressure and maximal and minimal dp/dt.

2.5. Histological analysis

After making the physiological measurements, the mice were killed and the hearts removed. The hearts were then cut into two transverse slices through the middle of the infarct area, and the apical specimens were quickly frozen in liquid nitrogen or used to prepare cryosections. The basal specimens were fixed with 10% buffered formalin and embedded in paraffin. Four-micrometer-thick deparaffinized sections were stained with hematoxylin-eosin, Masson's trichrome or Sirius red. Quantitative assessments of MI size, cell population, cardiomyocyte size and fibrotic area were made using a LUZEX F multipurpose color image processor (Nireco, Tokyo, Japan). The size of the MI and the fibrotic

area in the noninfarcted region was measured by searching the entire ventricle. Cardiomyocyte size (expressed as the transverse diameter of the myocyte cut at the level of the nucleus) and cell populations were assessed in 20 randomly chosen high-power fields in each section.

2.6. Immunohistochemistry

The 4-μm-thick deparaffinized sections and 8-μm-thick cryosections were incubated with a primary antibody against Flk-1 (Santa Cruz), F4/80 (Biomedicals AG), CD45 (Pharmingen) or 8-hydroxy-2′-deoxyguanosine (8-OHdG; Japan Institute of The Control of Aging, Shizuoka, Japan), which is the DNA base-modified product most commonly used for the evaluation of oxidative DNA damage [20]. An ABC kit (DAKO) with which DAB served as the chromogen was then used to immunostain the sections, after which they were observed under a light microscope. For all of the sections, the interpretations of the staining by two observers blinded to the specimens' group were in accord. The percentage of immunopositive cells present was calculated in 20 randomly chosen high-power fields in each section.

In addition, cultured cardiomyocytes were fixed with 4% paraformaldehyde, triple-stained with anti-8OHdG antibody followed by Alexa Fluor 488-conjugated secondary antibody (Molecular Probes), rhodamine-phalloidin (Molecular Probes) and Hoechst 33342. They were observed under a confocal microscope (LSM510, Zeiss).

2.7. In vitro treatment with EPO

Cardiomyocytes and cardiac fibroblasts were isolated from neonatal C57BL/6 mice and cultured separately in DMEM supplemented with 10% FBS as previously described [21]. The cells were subjected to oxidative stress by incubating them in 100 μmol/l H_2O_2 for 1 h or 6 h to induce inflammatory cytokine production and oxidative damage. To assess the capacity of EPO to attenuate cytokine production and oxidative damage, some cells were pretreated with EPO (0 to 10,000 U/ml) for 1 h prior to H_2O_2 exposure. In addition, to investigate the molecular signaling mediating the effects of EPO on cardiac cells exposed to H_2O_2, in some cases parthenolide (1 μg/ml; Sigma), an inhibitor of signal transducer and activator of transcription (Stat) signaling [22], or wortmannin (16 ng/ml; Sigma), a widely used inhibitor of phosphatidylinositol 3-kinase (PI3K)/Akt signaling, was added to the cells along with EPO. Cell death was evaluated by the 3-[4,5-2-yl]2,5-diphenyltetrazolium bromide (MTT) method.

2.8. Western analysis

Protein extracted from hearts or cultured cells was subjected to 10% polyacrylamide gel electrophoresis and then transferred to PVDF membranes, after which the membranes were probed using primary antibodies against EPO receptor (EPOR, M-20, Santa Cruz) and transforming growth factor (TGF)-β1 (Promega). The activation of Stat3 and Stat5, Akt, and extracellular signal-regulated protein kinase (ERK) was assessed using antibodies against their phosphorylated forms (p-Stat3, p-Stat5, p-Akt and p-ERK; all from Cell Signaling). Three to five hearts from each group were subjected to Western analysis. The blots were visualized using an ECL kit (Amersham), and the signals were quantified by densitometry. α-Tubulin (analyzed using an antibody from Sigma) served as the loading control.

2.9. ELISA

The levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β in myocardial tissue and cultured cells were quantified using ELISA kits (Quantikine M®, R&D Systems) according to the supplier's instructions.

2.10. Statistical analysis

Values are shown as means±SD. The significance of differences between means was evaluated using *t* tests followed by Newman–Keul's multiple comparison test. Values of $p<0.05$ were considered significant.

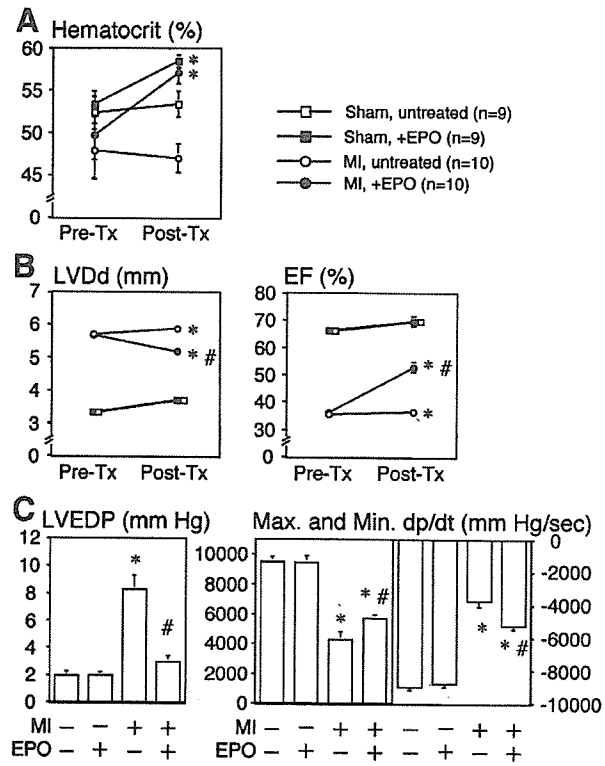

Fig. 1. Comparison of hematocrit levels and echocardiography- and cardiac catheterization-based cardiac geometry and function in untreated and EPO-treated mice 10 weeks after surgery (sham operation or induction of MI) in Protocol-1. $^{*}p<0.05$ vs. sham operated, $^{\#}p<0.05$ vs. the untreated MI group.

3. Results

3.1. Effect of late treatment with EPO on LV remodeling and function in failing hearts (In vivo experiment: Protocol-1)

Immediately prior to starting treatment with either vehicle (saline) or EPO (6 weeks post-MI), all of the mice bearing MIs showed significant LV dilatation and dysfunction, as compared to the sham-operated mice. Four weeks later (10 weeks post-MI), all of the mice in each group were still alive. The hematocrit level was significantly higher in the EPO-treated groups (Fig. 1A) and marked LV enlargement and the signs of diminished cardiac function – i.e., reduced LV ejection fraction (EF), LV developed pressure (LVDP), and maximal and

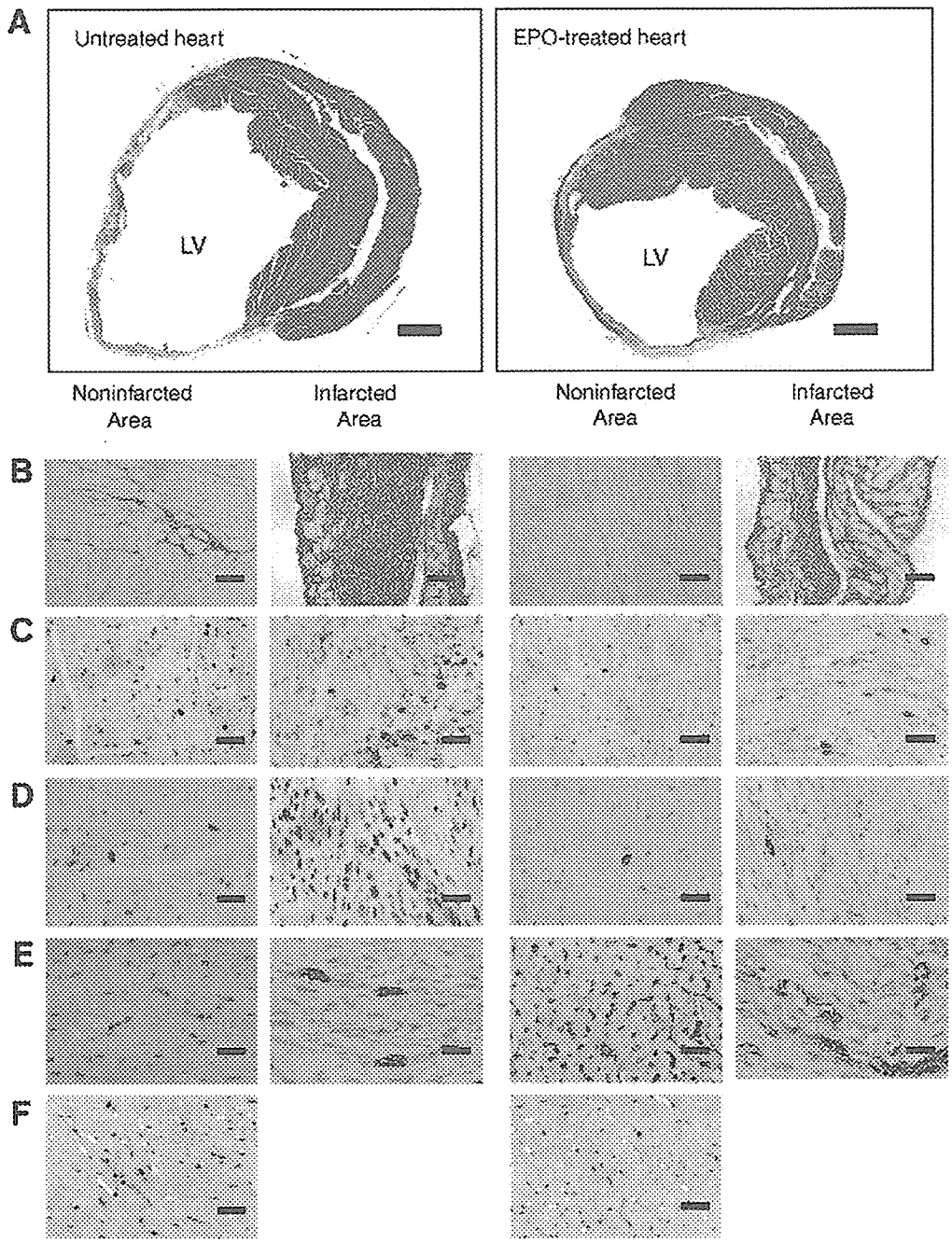

Fig. 2. Histological and immunohistochemical preparations of hearts from untreated and EPO-treated mice 10 weeks post-MI (Protocol-1). (A) Ventricular transverse slices stained with hematoxylin-eosin. (B) Sirius red stain showing fibrosis in noninfarcted and infarcted areas. (C) CD45 immunostaining showing leukocytes. (D) F4/80 immunostaining showing macrophages. (E) Flk-1 immunostaining showing endothelial cells. (F) 8-OHdG immunostaining showing oxidative DNA damage in surviving cardiomyocytes. Scale bars, 1 mm in panel A; 50 μm in panel B; and 20 μm in panels C–F.

minimal dp/dt and increased LV end-diastolic pressure (LVEDP) – persisted in the vehicle-treated mice. By contrast, all of these parameters were significantly improved by the EPO treatment (Fig. 1B–C). Systolic blood pressure was elevated by the EPO treatment in the sham-operated groups (EPO (−), 87±4 vs. EPO (+), 93±7 mm Hg, $p<0.05$), but it was similar in the groups with MI (EPO (−), 80±8 vs. EPO (+), 83±9 mm Hg, p=ns). The heart rate was similar among the groups. EPO had no effect on cardiac function except for systolic blood pressure in sham-operated mice.

3.2. Effects of EPO on the structure of failing hearts

At necropsy, LV dilatation was less marked in animals treated with EPO than untreated animals (Fig. 2A), and the heart-to-body (Fig. 3A) and lungs-to-body weight ratios (not shown) were significantly smaller. On the other hand, there was no difference in the size of the MI area relative to the total LV area in the untreated and EPO-treated groups (Fig. 3B). The size of the surviving cardiomyocytes was increased in MI-bearing mice, probably due to compensatory hypertrophy, and somewhat unexpectedly it did not differ between the two groups (Fig. 3C). Although the region of the 10-week-old infarct showed substantial fibrosis in both groups, the intensity of Sirius red staining appeared weaker in the EPO-treated group (Fig. 2B). Moreover, there was a significantly greater amount of interstitial fibrosis in the noninfarcted areas of the untreated hearts than in those of the EPO-treated hearts (Figs. 2B and 3D). The population of Flk-1-positive vessels did not differ in the infarcted areas of the untreated and EPO-treated hearts, but it was significantly greater in the noninfarcted areas of the EPO-treated hearts (Figs. 2E and 3F). CD45-positive leukocytes and F4/80-positive macrophages were found to have infiltrated both the infarcted and noninfarcted areas of hearts with 10-week-old MIs, but the populations were significantly smaller in the EPO-treated group (Figs. 2C, D and 3G, H).

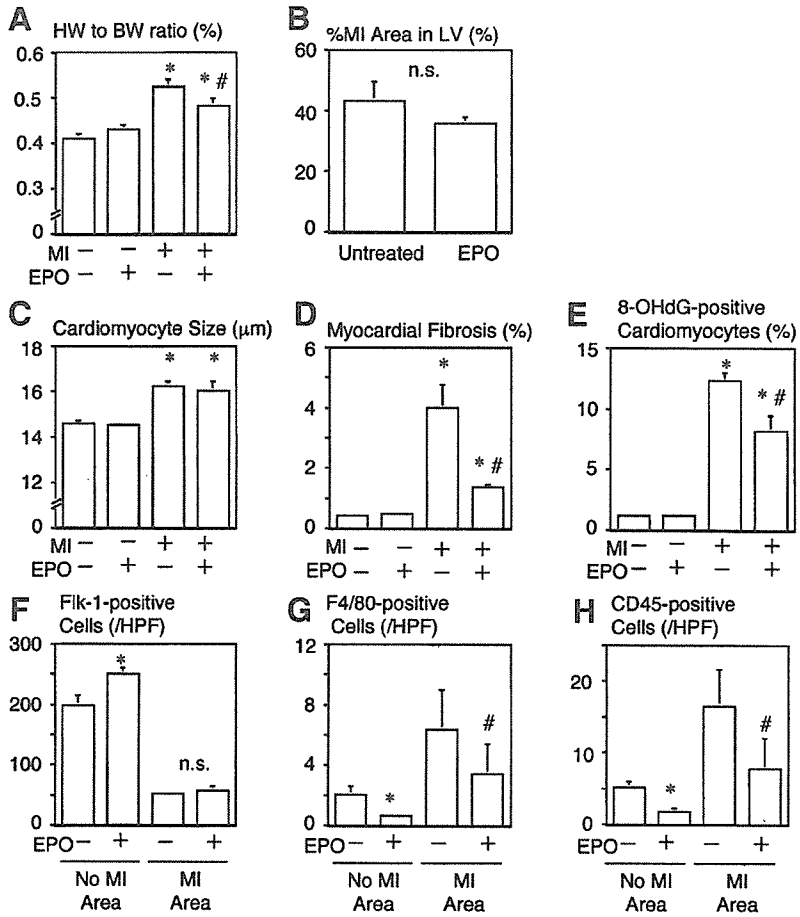

Fig. 3. Morphometric analysis of hearts collected from untreated and EPO-treated mice 10 weeks after sham operation or induction of MI (Protocol-1). (A) Heart-to-body weight ratios. (B) Infarct area expressed as a percentage of total LV area. (C) Cardiomyocyte size (transverse diameter through the nucleus). (D) Percentage fibrosis in the noninfarcted area. (E) Incidence of 8-OHdG positivity among cardiomyocytes. (F–H) Numbers of Flk-1 (F) CD45 (G) and F4/80-positive (H) cells in the noninfarcted and infarcted areas. N=9 each from sham operation groups and n=10 each from MI groups. In panels A–E, $^{*}p<0.05$ vs. the untreated sham group, $^{\#}p<0.05$ vs. the untreated MI group; in panels F–H, $^{*}p<0.05$ vs. the MI area, $^{\#}p<0.05$ vs. the untreated MI group.

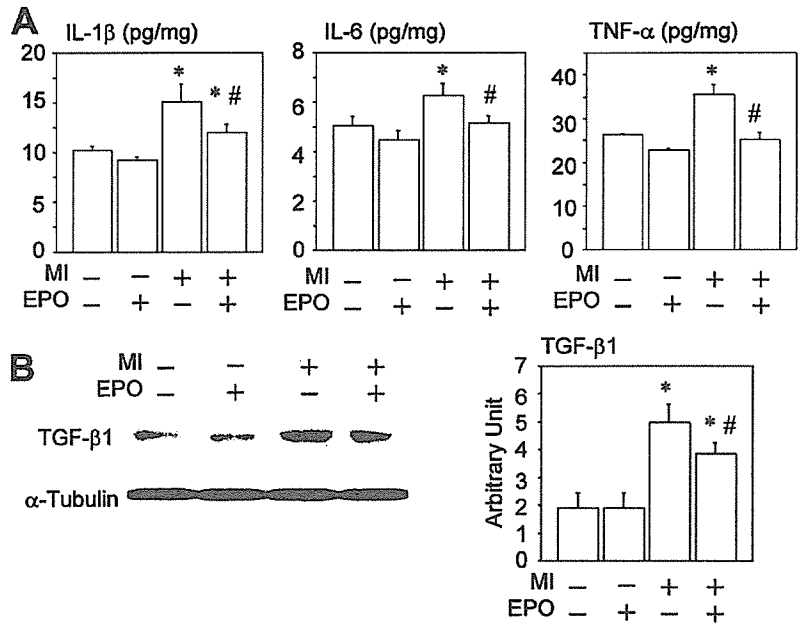

Fig. 4. ELISAs and Western analysis of the myocardium from untreated and EPO-treated mice carried out 10 weeks after sham operation or induction of MI (Protocol-1). (A) ELISAs for IL-1β, IL-6 and TNF-α. (B) Western blot of TGF-β1 and its densitometry. $N=6$ each from the groups. $^{*}p<0.05$ vs. the untreated sham group, $^{\#}p<0.05$ vs. the untreated MI group.

3.3. Effect of EPO on inflammatory cytokine expression and oxidative damage in failing hearts

ELISAs revealed that the myocardial levels of IL-1β, IL-6 and TNF-α were significantly higher in untreated mice 10-weeks post-MI than in sham-operated mice, and that levels were partially or nearly entirely restored to control by EPO treatment (Fig. 4A). In addition, Western analysis showed that myocardial expression of TGF-β1 was significantly upregulated in the untreated mice 10-weeks post-MI, but that expression was significantly attenuated in EPO-treated mice (Fig. 4B).

Cardiomyocytes positive for 8-OHdG (indicative of oxidative damage to DNA) were frequently found in the noninfarcted areas of hearts with 10-week-old MIs (12±0.7%), but significantly fewer were found in the hearts treated with EPO (8.2±1.2%, $p<0.05$), indicating that EPO mitigated the oxidative damage to the DNA (Figs. 2F and 3E).

3.4. EPOR expression and its downstream signaling in failing hearts

Western analysis revealed that myocardial expression of EPOR was significantly higher in hearts with 10-week-old MIs than in sham-operated hearts, and that EPO treatment further increased EPOR expression in MI-bearing hearts (Fig. 5). Receptor-associated Janus family tyrosine kinase (Jak)/Stat, PI3K/Akt, and mitogen-activated protein kinase (MAPK)/ERK are all known to be downstream mediators of EPOR signaling in cardiac cells both *in vitro* and *in vivo* [6]. It is therefore consistent with the observed increase in EPOR

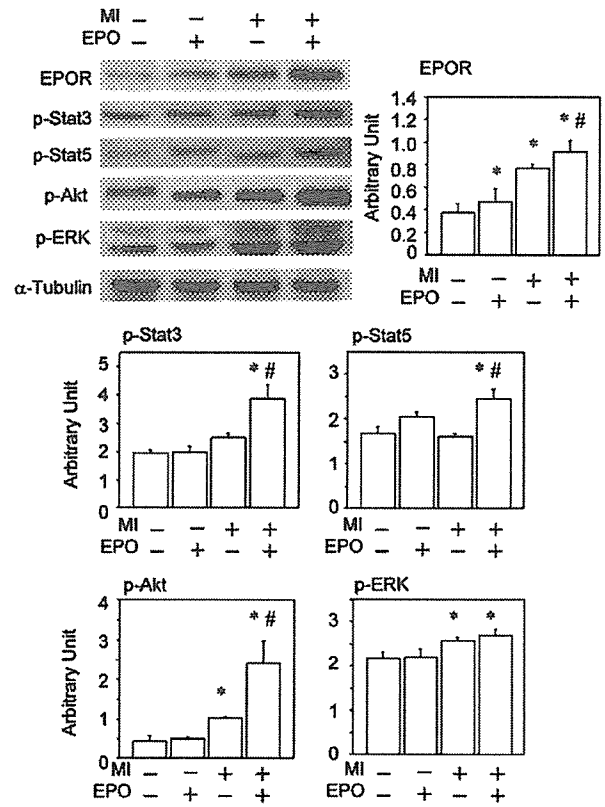

Fig. 5. Western analysis of EPOR, p-Stat3, p-Stat5, p-Akt and p-ERK expression in the myocardium of untreated and EPO-treated mice 10 weeks after sham operation or the induction of MI (Protocol-1). $N=6$ each from the groups. $^{*}p<0.05$ vs. the untreated sham group, $^{\#}p<0.05$ vs. the untreated MI group.

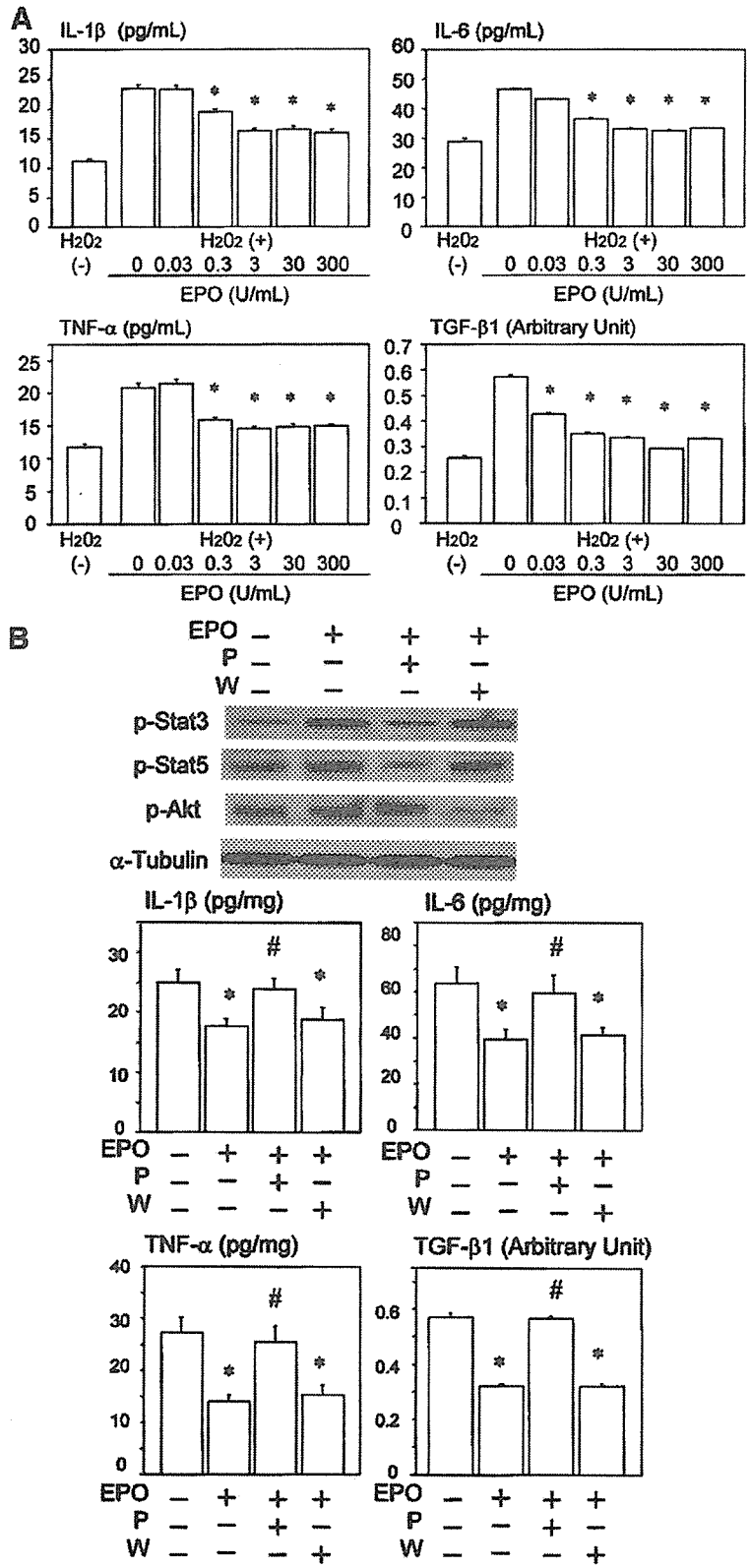

Fig. 6. (A) Dose-dependent effect of EPO on H_2O_2-induced expression of the indicated inflammatory cytokine in cultured cardiac fibroblasts. (B) Parthenolide (P) but not wortmannin (W) inhibits the effects of EPO on H_2O_2-induced expression of inflammatory cytokines. $N=6$ each from the groups. $*p<0.05$ vs. untreated, $^{\#}p<0.05$ vs. EPO alone.

that we also found that Stat3, Stat5 and Akt , but not ERK, were all significantly activated in hearts with 10-week-old MIs, and that the level of activation was further increased by EPO (Fig. 5).

3.5. Direct effects of EPO on cardiac cells and their molecular mechanisms (in vitro experiments)

The direct effects of EPO on cardiac cells were investigated using cultured neonatal cardiomyocytes and cardiac fibroblasts. Oxidative stress has been reported to play a critical role for progression of heart failure [23], and we here treated cardiac cells with H_2O_2. In response to 100 μmol/l H_2O_2, cardiac fibroblasts produced substantial amounts of inflammatory cytokines (IL-1β, IL-6, TNF-α, and TGF-β1), an effect that was significantly attenuated by pretreatment with EPO (Fig. 6A). In cardiomyocytes exposed to H_2O_2, intense 8-OHdG immunostaining was observed mainly in the perinuclear regions of cells, suggesting oxidative damage was primary to the mitochondrial DNA (Fig. 7A, upper panel). Approximately 60% of the cells were affected, but the incidence of 8-OHdG-positivity was dose-dependently suppressed by pretreatment with EPO (Fig. 7A, lower panel). According to the MTT assay, no significant cell death was noted at 1 and 6 h after H_2O_2 addition in either cardiomyocytes or fibroblasts (data not shown).

To investigate the molecular signaling involved in the anti-inflammatory and anti-oxidative effects of EPO on cardiac cells exposed to H_2O_2, parthenolide, a specific inhibitor of Stat signaling, or wortmannin, an inhibitor of PI3K/Akt signaling, was added simultaneously with EPO to the cardiac cells prior to treatment with H_2O_2. The concentrations of parthenolide and wortmannin used here were found to inhibit activation of Stat and Akt in cardiac fibroblasts and cardiomyocytes, respectively (Figs. 6B, upper panel and

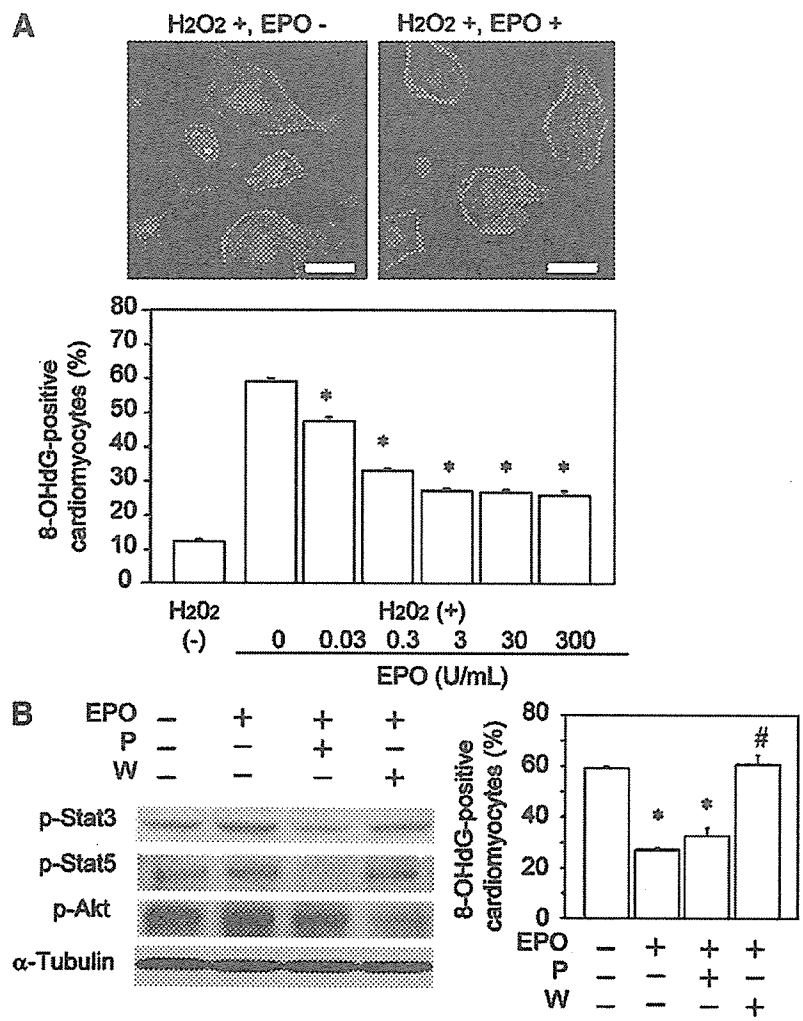

Fig. 7. (A) Dose-dependent effect of EPO on H_2O_2-induced 8-OHdG production (oxidative damage) in cultured cardiomyocytes; top panels, confocal microphotographs showing actin (red), 8-OHdG (green) and nuclei (blue). Scale bars, 10 μm. (B) Wortmannin (W) but not parthenolide (P) inhibits the effects of EPO on H_2O_2-induced 8-OHdG production (oxidative damage). $N=6$ each from the groups. $^*p<0.05$ vs. untreated, $^\#p<0.05$ vs. EPO alone.

Table 1
Functionality, fibrosis, cytokine expression and oxidative damage in hearts with 14-week-old MIs (Protocol-2)

	No treatment ($n=10$)	EPO treatment ($n=10$)	p value
LV geometry and function			
LVDd (mm)	5.7±0.16	5.1±0.10	0.0101
LVEF (%)	38±1.4	49±1.2	<0.0001
SBP (mm Hg)	77.4±2.8	83.6±3.2	0.2290
Heart Rate (beats/min)	553.8±6.4	551.3±17.9	0.9323
LVEDP (mm Hg)	7.2±1.9	2.9±0.38	0.0151
$+dp/dt$ (mm Hg/s)	4120±287	5416±318	0.0302
$-dp/dt$ (mm Hg/s)	−3926±372	−4874±243	0.0547
Fibrosis in noninfarcted area			
Sirius red-positive area (%)	6.3±0.38	2.8±0.35	0.0001
Inflammatory cells in noninfarcted area			
$CD45^+$ cells (/HPF))	5.4±0.82	2.6±0.57	0.0230
$F4/80^+$ cells (/HPF)	2.1±0.50	0.7±0.15	0.0228
Myocardial cytokine levels			
IL-1β (pg/mg)	25.1±3.94	13.3±1.45	0.0468
IL-6 (pg/mg)	13.4±2.82	5.7±0.44	0.0469
TNF-α (pg/mg)	59.9±6.43	25.7±1.64	0.0067
TGF-β1 (relative value)	100±0	69.5±2.60	<0.0001
Oxidative damage in cardiomyocytes			
8-OHdG^+ cells (%)	6.4±0.39	3.1±0.15	0.0002

SBP, systolic blood pressure. See text for other abbreviations.

7B, left panel). Parthenolide also completely suppressed the inhibitory effect of EPO on inflammatory cytokine expression in cardiac fibroblasts, while wortmannin diminished the protective effect of EPO against oxidative damage in cardiomyocytes (Figs. 6B, lower panels and 7B, right panel). Collectively then, these findings suggest that the anti-inflammatory and anti-oxidant effects of EPO are respectively mediated by the Jak/Stat and PI3K/Akt pathways.

3.6. The beneficial effect of EPO persists even after discontinuing treatment in failing hearts (In vivo experiment: Protocol-2)

Mice with 6-week-old MIs were treated with EPO for 4 weeks in the same manner as Protocol-1, after which treatment was stopped for 4 weeks prior to examination. Notably, even after 4 weeks without treatment (14 weeks post-MI), we found significantly better cardiac function, lower inflammatory cytokine levels and less oxidative damage in EPO-treated hearts than in untreated ones (Table 1).

4. Discussion

Van der Meer et al. recently described the ability of EPO treatment to improve cardiac function in post-MI heart failure in rats, even when the treatment was started during the chronic stage of MI (3 weeks post-MI) [8]. We have confirmed this finding using a murine model (6 weeks post-MI) in the present study, and also showed that the beneficial effect persists long after (4 weeks) discontinuing the treatment. Although rapid recanalization of the occluded coronary artery is presently the best clinical approach to the treatment of acute MI, unfortunately most patients miss the chance for that therapy because, to be effective, it must be performed within a few hours after the onset of MI [24]. Patients who escape death during the acute stage of a large MI are at high risk of developing heart failure during the chronic stage [25], so that patients with post-MI heart failure account for nearly half of the candidates for cardiac transplantation [26]. Thus, development of effective therapies for post-MI heart failure that are efficacious even when begun during the chronic stage of MI would be highly desirable, which highlights the clinical importance of the present study as well as the study by van der Meer et al. [8].

It is now clear that EPO possesses direct beneficial cardiovascular effects [5]; however, the mechanisms underlying the effects on chronic heart failure are not yet fully understood. In their study, van der Meer et al. found that EPO increased angiogenesis and reversed the modulation of the myosin heavy chain phenotype in failing rat hearts [8]. In the present study, we not only confirmed that EPO increased capillary density, but also found that EPO reduces inflammation, cytokine production, fibrosis and oxidative damage in the failing myocardium. Several studies have shown that in both animals and human with failing hearts, levels of inflammatory cytokines (e.g., IL-1β, IL-6 and TNF-α) are increased in plasma [9,10] and circulating leukocytes [27], as well as in the myocardium itself [11,28,29]. Our observation that levels of IL-1β, IL-6 and TNF-α were elevated in the failing myocardium of mice 10 and 14 weeks post-MI is consistent with those earlier findings. Importantly, expression of these inflammatory cytokines is reportedly directly related to the degree of heart failure and inversely related to survival [11,30]. Moreover, the results of animal studies and some clinical pilot trials have suggested that suppression of inflammatory cytokines may improve cardiac performance [31,32]. Though speculative, it is therefore conceivable that reduction of inflammatory cytokines may be one of the mechanisms involved in the beneficial effect of EPO on failing hearts.

The present study also revealed the anti-oxidant effect of EPO on failing myocardium; EPO significantly reduced the numbers of cardiomyocytes positive for 8-OHdG, a commonly used marker of oxidative DNA damage [20]. Along with inflammatory cytokines, the overproduction of reactive oxygen species is also thought to be involved in myocardial remodeling [12]. Apparently, inflammatory cytokines can induce oxidative stress – e.g., TNF-α can directly induce mitochondrial reactive oxygen species production in cardiomyocytes, causing damage to mitochondrial DNA [33] – while oxidative stress can increase levels of inflammatory cytokines, leading to the development of a vicious cycle in failing hearts [34,35]. It is therefore noteworthy that our *in vitro* study confirmed that oxidative stress caused by H_2O_2

induced both oxidative damage in cardiomyocytes and cytokine production in cardiac fibroblasts.

We also observed that EPO treatment reduced the elevated TGF-β1 expression otherwise seen in the failing myocardium. Induction of TGF-β1 expression, as well as supplementation with exogenous TGF-β1, can protect cardiomyocytes against ischemia/reperfusion injury [36], but this beneficial effect appears only during the early phase of the cytokine's expression. Sustained expression, by contrast, leads to LV remodeling and failure following MI [37]. In addition, TGF-β1 is a major stimulator of tissue fibrosis, influencing fibroblast proliferation and extracellular matrix production, collagen and fibronectin in particular, while reducing degradation of these components [38]. The EPO-induced attenuation of the myocardial levels of TGF-β1 seen in the present study may be responsible for the observed reduction in fibrosis.

We also found that expression of EPOR is upregulated in failing hearts, possibly as a compensatory mechanism. Among downstream targets of EPOR signaling are Stat3, Stat5 and Akt, all of which showed significant activation in the hearts of EPO-treated mice. Moreover, our *in vitro* experiments showed that inhibiting Jak/Stat signaling with parthenolide completely blocked the EPO-induced reduction of inflammatory cytokine production in cardiac fibroblasts, while inhibiting PI3K/Akt signaling with wortmannin blunted EPO-induced attenuation of oxidative damage in cardiomyocytes. These findings suggest that the anti-inflammatory effect of EPO is elicited via the Jak/Stat pathway, while its anti-oxidative effect is via the PI3K/Akt pathway. Consistent with that idea, downregulation of TNF-α, IL-1 and IL-6 by IL-10 is known to be mediated by activation of Stat1 and Stat3 [39]. In addition, mice with cardiomyocyte-restricted deletion of Stat3 are susceptible to inflammation-induced heart damage and show a dramatic increase in cardiac fibrosis [40]. On the other hand, cardiotrophin-1 is known to protect cardiomyocytes from oxidative stress-induced cell death via gp130 and the PI3K/Akt and ERK intracellular cascades [41]. It is also noteworthy that in heart failure, excessive inflammatory cytokine production can reduce EPO secretion from the kidney, interfere with EPO activity in bone marrow, and reduce the iron supply to the bone marrow [42].

Finally, we recently showed that EPO improves cardiac function in a murine model of doxorubicin (DOX)-induced non-ischemic cardiomyopathy [43]. In that model, EPO-induced molecular signaling downstream of EPOR differed substantially from that seen in the present MI model. ERK activation was suppressed in DOX-induced cardiomyopathy and was restored to a significant degree by EPO, which had no effect on Stat or Akt activation. Thus, EPO's downstream signaling via EPOR apparently varies in different models of heart failure.

In summary, EPO treatment during post-MI heart failure improves cardiac function by reducing expression of inflammatory cytokines and oxidative damage, at least in part through activation of the Jak/Stat and PI3K/Akt pathways.

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Impact of atherosclerosis-related gene polymorphisms on mortality and recurrent events after myocardial infarction

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Abstract

Although previous epidemiologic studies have suggested an association between the onset of myocardial infarction (MI) and some genetic variations, the impact of these variants on recurrent cardiovascular events after MI has not been fully elucidated. We genotyped 87 polymorphisms of 73 atherosclerosis-related genes in consecutive acute MI patients registered in the Osaka Acute Coronary Insufficiency Study and compared the incidence of death and major adverse cardiac events (MACE) among the polymorphisms of each gene. After initial screening in 507 patients, we selected nine polymorphisms for screening in all 1586 patients. Multivariate Cox regression analysis revealed that G allele carriers at the position 252 of the lymphotoxin alpha (LTA) gene were independently associated with an increased risk of death (hazard ratio [HR]: 2.46; 95% CI: 1.24–4.86). In conclusion, a 252G allele of LTA is associated with an increased risk of death after AMI and may be a useful genetic predictor.

Keywords: Myocardial infarction; Polymorphism; Lymphotoxin alpha; Prognosis

1. Introduction

Myocardial infarction (MI) has become one of the leading causes of death around the world. Previous epidemiologic studies have identified certain risk factors related to the onset and prognosis of MI, such as diabetes mellitus, hypertension, hyperlipidemia and smoking. Today, attention is being focused on genetic susceptibility to MI and identification of some genomic markers may provide additional information to the standard risk factor profile as well as some insights into the underlying pathology. We have reported an association between the functional variant of lymphotoxin alpha [1] or galectin-2 [2] and susceptibility to MI in a case-control study as well as others [3,4]. Although these polymorphisms may be useful genomic markers for distinguishing high-risk subjects from an entire cohort, it does not necessarily mean that these polymorphisms have the same utility to predict recurrent events after MI because there may be different mechanisms for the initial and recurrent cardiovascular events. Accordingly, to determine genetic predictors by performing a prospective observational study on a cohort of MI survivors may provide useful information for the genetic identification of high-risk patients after MI, leading to an improved prognosis.

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In this study, we therefore evaluated the influence of 73 candidate genes, which were selected on the basis of an overview of vascular biology, inflammation, and coagulation and fibrinolysis, as well as lipid and glucose metabolism and other metabolic factors, on recurrent events after MI.

2. Methods

2.1. Patients

Among 3788 consecutive Japanese patients with acute myocardial infarction who were registered in the Osaka Acute Coronary Insufficiency Study (OACIS) from April 1998 to April 2004, 1586 survivors who gave written informed consent for data collection, blood sampling and genotyping were included in this study. Details of the Osaka Acute Coronary Insufficiency Study have been reported [5,6]. Briefly, all patients were prospectively registered immediately after the diagnosis of acute MI, based on their symptoms, electrocardiographic findings and release of cardiac enzymes. The study protocol complied with the Guidelines for Genome/Genetic Research issued by the Japanese government and was approved by the institutional ethical committee.

2.2. Selection of polymorphisms

Using public databases such as PubMed, we selected 87 polymorphisms of 73 candidate genes that have been reported to be potentially associated with atherosclerosis, vascular inflammation, coagulation and fibrinolysis and standard risk factors such as diabetes mellitus, hypertension and hyperlipidemia (Table 1).

2.3. Genotyping of polymorphisms

Venous blood was collected from each patient into tubes containing 50 nmol/L of EDTA and genomic DNA was extracted with a kit (Qiagen Hilden, Germany). The genotypes of the 87 polymorphisms were determined with a fluorescence- or colorimetry-based allele-specific DNA primer probe assay (Toyobo Gene Analysis, Tsuruga, Japan). The regions showing polymorphism were amplified by the polymerase chain reaction (PCR) with primers previously described elsewhere [1,7–15]. The reaction mixture (25 μl) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/L of each deoxynucleoside triphosphate, 1–4 mmol/L of magnesium chloride and 1 U of DNA polymerase (rTaq or KIOplus, Toyobo) in the corresponding DNA polymerase buffer. The amplification protocol comprised initial denaturation, 35–45 cycles of denaturation at 95 °C for 30 s, annealing at 55–65 °C for 30 s and extension at 72 °C for 30 s, followed by final extension at 72 °C for 2 min.

To detect the genotype by means of fluorescence, amplified DNA samples were incubated with streptavidin-conjugated magnetic beads in 96-well plates at room temperature. The plates were placed on a magnetic stand and the supernatants were harvested and transferred to the wells of a 96-well plate containing 10 mmol/L of sodium hydroxide. Then, fluorescence was assessed at excitation and emission wavelengths of 485 and 538 nm, respectively, for fluorescein isothiocyanate, or 584 and 612 nm, respectively, for Texas red. To determine the genotype by colorimetry, amplified DNA samples were denatured with 0.3 mmol/L of sodium hydroxide and subjected to hybridization at 37 °C for 30 min in hybridization buffer containing 30–45% formamide with each of two allele-specific capture probes fixed to the bottoms of the wells of a 96-well plate. After thorough washing of the wells, alkaline phosphatase-conjugated streptavidin was added to each well and the plate was incubated at 37 °C for 15 min with agitation. The wells were again washed and after the addition of a solution containing 0.8 mmol/L of 2-(4-iodophemyl)-3-(4-nitrophnyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (monosodium salt) and 0.4 mmol/L of 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt, the absorbance of the samples was assessed at a wavelength of 450 nm.

The accuracy of genotyping with this method was confirmed by restriction fragment length polymorphism analysis or by twice performing direct DNA sequencing of the PCR products of 50 randomly selected DNA samples. In each instance, the genotype determined by the allele-specific DNA primer probe assay system was identical to that determined by the other methods.

2.4. Data collection, follow-up and association studies

Research cardiologists and trained research nurses recorded data concerning sociodemographic variables, medical history, therapeutic procedures and clinical events during the patient's hospital stay. Information was obtained from the hospital medical records and by direct interview with the patients, their families and the treating physicians. After written informed consent to enter the OACIS was obtained from each patient, all in-hospital data were transmitted to the data collection center for processing and analysis. For patients who were discharged alive, follow-up clinical data concerning subsequent cardiac events were obtained at 3, 6 and 12 months after the onset of AMI and annually thereafter. The data were obtained from the research outpatient clinic or, in a few instances, by verbal or written contact with the patient's physician, the patient, or family members. The incidence of death, the incidence of death plus non-fatal MI and the incidence of major cardiac events (MACE) were compared among the different polymorphisms. MACE was defined as death from any cause, myocardial infarction, unstable angina and revascularization (including target vessel revascularization, percutaneous coronary intervention for vessels other than the primary target vessel and coronary artery bypass grafting).

To evaluate the association between the various polymorphisms and the outcome after MI, we first performed a screening study using 87 polymorphisms of 73 candidate

Table 1
The 87 polymorphisms examined in the screening study

Gene	Polymorphism	Gene	Polymorphism
Adenosine monophosphate deaminase 1	C34T	Interleukin-13	G4166A (Argll0Gln)
Adiponectin	G276T	Interleukin-18	C−607A
Adiponetcin	C383T (Argll2Cys)	Interleukin-18	G−137C
Alpha estrogen receptor	T397C	Interleukin-4 receptor alpha	A398G (Ile50Val)
Angiotensin II receptor type I	A1166C	Interleukin-6	G−174C
Angiotensinogen	T704C (Met235Thr)	Interleukin-6	C−634G
Apolipoprotein E	T3932C (Cysll2Arg)	Leptin	C−1887A
Apolipoprotein E	C4070T (Argl58Cys)	Lymphotoxin alpha	A252G
ATP-binding cassette transporter ABCC6	C3421T (Argl141 stop)	Lymphotoxin alpha	C804A (Thr26Asn)
Atrial natriuretic peptide	C708T	Matrix Gla Protein	G−7A
Beta2 adrenergic receptor	A46G (Argl6Gly)	Metalloproteinase-12 (macrophage elastase)	A−82G
Beta2 adrenergic receptor	C79G (Gln27Glu)	Metalloproteinase-7 (matrilysin) promoter	A−181G
Beta2 adrenergic receptor	C491T (Thrl64Ile)	Metalloproteinase-7 (matrilysin) promoter	C−153T
Beta3 adrenergic receptor	T190C (Trp64Arg)	Metalloproteinase-9 (gelatinase B)	C−1562T
CC chemokine receptor	G190A (Val64Ile)	Methylenetetrahydrofolate reductase	C677T (Ala222Val)
CD18	C1323T	Microsomal triglyceride transfer protein	G−493T
Cholesterol ester transfer protein	G1200A (Arg451Gln)	Monocyte chemoattractant protein 1	G−2518A
Coagulation factor V	G1691A (Arg506Gln)	Myeloperoxydase	G−463A
Coagulation factor XII	C46T	Neuropeptide Y	T1128C (Leu7Pro)
C-reactive protein	G1059C	p22phox	C242T (His72Tyr)
Dopamine D2 receptor	C1097G (Ser311Cys)	Paraoxonase	A220T (Met55Leu)
Early growth response protein-1	C−151T	Paraoxonase	A632G (Glnl92Arg)
Endothelial nitric oxide synthase	T−786C	Peroxisome-proliferator—activated receptor-alpha	C256G (Leul62Val)
Endotheline-1	G5665T (Lysl98Asn)	Peroxisome-proliferator—activated receptor-gamma	C34G (Prol2Ala)
E-selectin	A561C (Serl28Arg)	Plasminogen-activator inhibitor type I	4G−668/5G
E-selectin	G98T	Prothrombin	G20210A
Flactalkine receptor	G84635A (Val249Ile)	P-selectin	A76666C (Thr715Pro)
Ghrelin	C247A (Leu72Met)	Receptor for advanced glycation end products	G557A (Gly82Ser)
Glutamate-cysteine ligase modifier subunit	C−588T	Receptor for advanced glycation end products	T−429C
Glycogen synthase	A1426G (Met416Val)	Resistin	ATG repeat
Glycoprotein Ia	A1648G (Lys505Glu)	Scavenger receptor BI	G4A (Gly2Ser)
Glycoprotein Ia	C807T	Scavenger receptor BI	G403A (Vall35Ile)
Glycoprotein Ia	G873A	Serotonin receptor 2A	T102C
Glycoprotein IIIa	C1565T (Leu33Pro)	Soluble epoxide hydrolase	G860A
Glycoprotein VI	T13254C (Ser219Pro)	Soluble epoxide hydrolase	Arg402–403ins in exonl3
Heme oxigenase-1	GT repeats in promoter	Thrombomodulin	G−33A
Hepatic lipase	C−480T	Thrombopoietin	A5713G
Human platelet antigen-2	C1018T (Thrl45Met)	Thrombospondin 1	A2210G (Asn700Ser)
Intercellular adhesion molecule-1	G1462A (Glu469Lys)	Thrombospondin 4	G1186C (Ala387Pro)
Interleukin-1 alpha	C−889T	Toll-Like Receptor 2	C2029T (Arg677Trp)
Interleukin-1 receptor antagonist	Tandem repeat in intron2	Transforming growth factor beta 1	T29C (Leul0Pro)
Interleukin-10	G−1082A	Tumor necrosis factor alpha	G−308A
Interleukin-10	T−819C	Vascular endothelial growth factor	C−634G
		von Willebrand factor	G−1051A

Minus signs indicate the number of nucleosides upstream from the transcription-initiation site. For non-synonymous polymorphisms, the resulting amino acid change is shown in parentheses.

genes in 507 patients who were randomly selected from the total study population. This revealed nine polymorphisms that were related to recurrent events. Then, a large-scale study was performed to assess the association between these nine polymorphisms and recurrent events after MI in all 1586 patients.

2.5. *Statistical analysis*

Discrete variables were expressed as counts or percentages and were compared with the χ^2-test. Continuous variables were expressed as the mean ± S.D. and compared by the unpaired two-sided *t*-test. In the screening study, we used the χ^2-test with a cut-off *p*-value of less than 0.1 to avoid false negative associations. In the large-scale study, polymorphisms selected in the screening study were included as covariables as well as age, sex, body mass index, coronary risk factors (including diabetes mellitus, hypertension, hyperlipidemia, smoking and history of prior MI), anteroseptal MI and reperfusion therapy in Cox's proportional hazards regression model. Survival curves were constructed by Kaplan–Meier method and differences in the event-free survival rate were compared among the different genotypes using the log-rank test. Association were considered signif-

Table 2
Patient characteristics

Age (years)	64.0 ± 11.0
Male sex (%)	1225/1586 (77.2)
BMI (kg/m^2)	23.4 ± 4.0
Diabates mellitus (%)	544/1550 (35.1)
Hypertension (%)	811/1546 (52.5)
Hyperlipidemia (%)	718/1529 (47.0)
Smoking (%)	1048/1581 (66.3)
Past history of MI (%)	176/1533 (11.5)
Preangina (%)	364/1540 (23.6)
Anteroseptal MI (%)	766/1586 (48.3)
Killip class > II (%)	191/1524 (12.5)
Reperfusion therapy within 24 h (%)	1382/1582 (87.4)
PCI (%)	1362/1582 (86.1)
PTCR (%)	159/1582 (10.1)
CABG (%)	34/1555 (2.2)
Peak value of creatinine kinase (IU/l)	2628 ± 2371
Medication at discharge	
Aspirin (%)	1452/1586 (91.6)
Angiotensin-converting enzyme inhibitor (%)	1019/1566 (65.1)
Angiotensin-receptor blocker (%)	150/1560 (9.6)
Beta blocker (%)	615/1563 (39.3)
Calcium blocker (%)	375/1562 (24.0)
Statin (%)	384/1586 (24.2)

icant at a p-value of less than 0.05. All statistical analyses were performed using SPSS software (SPSS, Inc.).

3. Results

The baseline characteristics of the patients are listed in Table 2. Long-term follow-up was completed in 1585 patients (99.9%), while contact was lost with one patient. During the mean follow-up period of 831 days, there were 76 deaths, 158 deaths or MIs and 522 MACE.

The screening study in 507 patients identified nine polymorphisms for further study on the basis of a univariate χ^2-test with a p-value of less than 0.1. These were C−480T polymorphism of the hepatic lipase gene, A398G polymorphism of the interleukin-4 receptor alpha gene, G−137C polymorphism of the interleukin-18 receptor antagonist gene, A252G polymorphism of the lymphotoxin alpha gene, 4G/5G polymorphism of the platelet activator inhibitor-I gene, G860A polymorphism of the soluble epoxide hydrolase gene, T29C polymorphism of the transforming growth factor beta gene, A5713G polymorphism of the thrombopoietin gene and G−1051A polymorphism of the von Willebrand factor gene (Table 3).

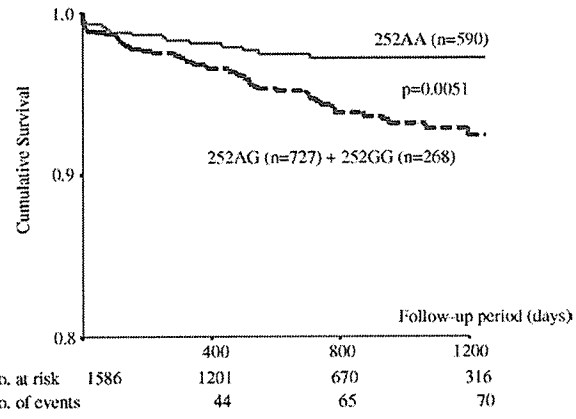

Fig. 1. Cumulative survival rate during follow-up according to the LTA. Solid line indicates LTA-252AA genotype and dashed line indicates LTA-252AG + GG genotype, respectively.

In the entire study in 1586 patients, multivariate Cox regression analysis revealed that A252G polymorphism in the LTA gene was significantly associated with an increased risk of death (Table 4). The hazard ratio for the occurrence of death in LTA G allele carriers (AG or GG genotypes) versus non-carriers (AA genotype) was 2.46 (95% CI: 1.24–4.86). Fig. 1 shows Kaplan–Meier survival curves for patients with each genotype. There was no association between each of nine polymorphisms and MACE.

4. Discussion

In this prospective observational study, we investigated the prognostic significance of 87 polymorphisms of 73 genes that have been suggested to be related to the pathophysiology of MI, and found that an LTA polymorphism were associated

Table 3
The nine polymorphisms selected by the screening study

Gene	Polymorphism	Genetic model	Event	Event rate	p-Value
Hepatic lipase	C−480T	CC + CT vs. TT	MACE	158/382 vs. 30/123	0.0002
Interleukin-4 receptor alpha	A398G (Ile50Val)	AA + AG vs. GG	Death + MI	31/314 vs. 29/192	0.0773
Interleukin-18	G−137C	CC + CG vs. GG	Death	10/116 vs. 18/389	0.0990
Lymphotoxin alpha	A252G	AA vs. AG + GG	Death	6/183 vs. 22/323	0.0949
Plasminogen activator inhibitor-1	4G/5G	4G/4G + 4G/5G vs. 5G/5G	Death	21/436 vs. 7/70	0.0900
Soluble epoxide hydrolase	G860A	AA + AG vs. GG	MACE	63/194 vs. 126/312	0.0737
Transforming growth factor-beta 1	T29C (Leu10Pro)	CC vs. CT + TT	Death	4/146 vs. 24/360	0.0800
Thrombopoietin	A5713G	AA vs. AG + GG	Death	12/107 vs. 16/399	0.0038
Von Willebrand factor	G−1051A	AA vs. AG + GG	Death	10/101 vs. 18/405	0.0319

Minus signs indicate the number of nucleosides upstream from the transcription-initiation site. For non-synonymous polymorphisms, the resulting amino acid change is shown in parentheses. MACE is determined as a combination of death, MI, unstable angina and revascularization.

Table 4
Multivariate Cox regression analysis of association between nine polymorphisms and mortality after myocardial infarction in the entire study

Gene	Polymorphism	Model	Unadjusted HR[a] 95% CI	p-Value	Adjusted[b] HR 95% CI	p-Value
Hepatic lipase	C−480T	CC+CT vs. TT	1.06 (0.61–1.85)	0.829	1.36 (0.72–2.58)	0.342
Interleukin-4 receptor alpha	A398G (Ile50Val)	AA+AG vs. GG	0.76 (0.46–1.28)	0.306	0.69 (0.37–1.28)	0.237
Interleukin-18	G−137C	CC+CG vs. GG	0.88 (0.51–1.53)	0.654	0.75 (0.39–1.44)	0.385
Lymphotoxin alpha	A252G	AA vs. AG+GG	2.33 (1.29–4.20)	0.005	2.46 (1.24–4.86)	0.010
Plasminogen activator inhibitor-1	4G/5G	4G/4G+4G/5G vs. 5G/5G	0.85 (0.45–1.63)	0.628	1.37 (0.67–2.81)	0.387
Soluble epoxide hydrolase	G860A	AA+AG vs. GG	1.13 (0.68–1.88)	0.636	1.22 (0.67–2.24)	0.516
Transforming growth factor-beta 1	T29C (Leu10Pro)	CC vs. CT+TT	1.13 (0.66–1.94)	0.662	0.77 (0.42–1.42)	0.405
Thrombopoietin	A5713G	AA vs. AG+GG	0.99 (0.55–1.79)	0.981	0.86 (0.44–1.68)	0.664
Von Willebrand factor	G−1051A	AA vs. AG+GG	0.73 (0.42–1.28)	0.266	0.78 (0.40–1.53)	0.475

[a] Hazard ratio of latter genotypes (e.g. TT in hepatic lipase) compared with former ones (e.g. CC+CT in hepatic lipase) are indicated.
[b] Age, sex, body mass index, diabetes mellitus, hypertension, hyperlipidemia, current smoker, past history of MI, anteroseptal MI, Killip class ≥ II and reperfusion therapy are included as covariables.

with an increased risk of death. Although several genes have already been shown to be associated with the onset of MI, few polymorphisms have been reported to show an association with the prognosis of MI. Accordingly, our data might provide some insights for research concerning the genetic risk of recurrent events after MI.

Lymphotoxin alpha (LTA, formerly named TNF-β) is a member of the TNF family that plays a critical role in inflammation and is located within the human leukocyte antigen class III gene cluster on human chromosome 6p21. Many studies have focused on TNF-α as a key cytokine involved in heart failure and atherosclerosis [16,17]. LTA is structurally similar to TNF-α and also has an important role in the inflammatory response by inducing monocyte migration, as well as by promotion of lymphocyte activation and proliferation [18,19]. Although this cytokine has been widely investigated to clarify its association with systemic inflammation, the role of LTA in the pathology of coronary heart disease has not been fully elucidated. Schreyer et al. reported a reduction of atherosclerosis lesion in LTA knockout mice, but not in TNF-α knockout mice, suggesting that LTA may be more important in the proatherogenic response [20]. In the LTA gene, there are some genetic variants with functional significance. An A–G substitution at nucleotide position 252 (A252G) is reported to be associated with increased transcriptional activities [1] or elevated C-reactive protein [21]. Moreover, the 252G allele links to an amino-acid coding polymorphism (804A) which increases mRNA expression of VCAM1 and selectin E [1]. Furthermore, Ozaki et al. [1] and others [22,23] found some variants of the LTA gene were associated with the onset of myocardial infarction or coronary heart disease. Our finding that the LTA polymorphism is associated with recurrent cardiovascular events confirms the important role of this cytokine, as is shown in these previous reports and suggests the need for adjunctive treatment for patients with the polymorphism. The mechanism of the LTA polymorphisms for increased cardiovascular events is still unclear. Several environmental predictors of the prognosis of MI, such as high age, diabetes mellitus, hypertension, time to revascularization, severity of MI (i.e. Killip class or left ventricular function) and therapeutic interventions, have been established on the basis of clinical data. In the present study, Cox multivariate analysis revealed that the 252G allele of LTA was a predictor of death independent from these known predictors. Detailed in vivo and in vitro studies of the mechanisms by which the genetic polymorphism influences cardiovascular events will be needed to solve this issue and clarify the position of this polymorphism within the constellation of the established predictors.

There were some limitations to our study. One limitation was the possibility of survivor bias. Patients dying before or soon after admission were excluded from our study, so it is possible that they had a particular polymorphism and their exclusion biased our results. However, the frequencies of the LTA polymorphism in the study population were in Hardy–Weinberg equilibrium, so it is unlikely that acute mortality is dominated by specific polymorphisms of LTA. Another limitation is ethnic differences. However, the frequency of the 252G allele of LTA in this study population was similar to that in Iwanaga et al. [22] and Keso et al. [24] studies, so the influence of ethnic differences should be minimal.

In conclusion, A252G polymorphism of the LTA gene was significantly associated with an increased risk of death after MI and may be one of the most important genetic determinants that have been detected so far. To confirm our findings, further large-scale epidemiologic studies of various ethnic groups are needed, as well as experimental studies to clarify the precise mechanisms involved. Determination of the genes related to an increased risk of recurrent events after MI could be of clinical significance to identify patients with a high genetic risk, and thus provide better treatment after MI.

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Appendix A

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Myocarditis and Heart Failure Associated With Hepatitis C Virus Infection

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ABSTRACT

Background: The aim of study is to determine the prevalence of hepatitis C virus (HCV) infection and myocardial injury among patients enrolled in the Myocarditis Treatment Trial. HCV infection has recently been noted in patients with cardiomyopathies and myocarditis. However, prevalence of HCV infection in myocarditis and heart failure remains to be clarified.

Methods and Results: Patients with heart failure up to 2 years in duration without a distinct cause were enrolled in the trial between 1986 and 1990. Frozen blood samples were available from 1355 among 2233 patients enrolled and examined for presence of anti-HCV antibodies, circulating cardiac troponins I and T, and N-terminal pro-brain natriuretic peptide (NT-proBNP). Anti-HCV antibodies were identified in 59 of 1355 patients (4.4%). This higher prevalence of HCV infection than that observed in the general US population (1.8%), varied widely (0–15%) among the different medical centers and regions. The concentrations of circulating cardiac troponin (cTn) I were elevated in 17 of 56 patients (30%), and cTnT was detectable in 28 of 59 patients (48%) with HCV antibodies, suggesting the persistence of ongoing myocardial injury. The concentrations of NT-proBNP were elevated in 42 of 42 patients (100%) with HCV antibodies, (10,000 ± 5860 pg/mL), a mean value significantly greater than in 1276 patients without HCV antibody (2508 ± 160 pg/mL, $P < .0001$).

Conclusion: Anti-HCV antibodies were identifiable in sera stored for 13 to 17 years and were more prevalent in patients with myocarditis and HF than in the general population. In regions where its prevalence is high, HCV infection may be an important cause of myocarditis and HF. NT-proBNP is a more sensitive marker of myocardial injury than cardiac troponins in patients with heart failure from HCV myocarditis.

Key Words: Myocarditis, Heart failure, Hepatitis C virus, Troponin, proBNP.

Hepatitis C virus (HCV) has infected an estimated 170 × 10^6 individuals worldwide and, in the next few years, the number of annual deaths from HCV-related liver disease and cancer in the US may exceed the number of deaths caused by the human immunodeficiency virus.[1] A screening test developed in 1990 has nearly eliminated the spread of HCV through blood transfusions in industrial countries, and the sharing of contaminated needles is now by far the most common mode of infection. As a result, the US Centers for Disease Control and Prevention estimates that new infections have decreased from approximately 230,000/year in the 1980s to fewer than 36,000 in the US in 1996. However, because most individuals infected in earlier decades are alive, it is estimated that 1.8% of the US population harbors the virus.[2] As these patients become older, HCV-related liver disease, now accounting for 8000 to 10,000 deaths/year in the US, and the single most common indication for liver transplants, is likely to increase.[1]

HCV has been associated with several extrahepatic manifestations, among which the best characterized are mixed cryoglobulinemia, itself associated with a risk of developing B-cell non-Hodgkin's lymphoma, and glomerulonephritis, probably related to inflammatory disease of the small

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