Anti–Monocyte Chemoattractant Protein-1 Gene Therapy Attenuates Left Ventricular Remodeling and Failure After Experimental Myocardial Infarction

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Background—Increased expression of monocyte chemoattractant protein-1 (MCP-1) has recently been described in clinical and experimental failing heart. However, its pathophysiological significance in heart failure remains obscure. We thus determined whether MCP-1 is increased in post–myocardial infarction (MI) hearts and its blockade can attenuate the development of left ventricular (LV) remodeling and failure.

Methods and Results—Anterior MI was produced in mice by ligating the left coronary artery. After 4 weeks, MI mice exerted LV dilatation and contractile dysfunction in association with myocyte hypertrophy and interstitial fibrosis of noninfarcted LV. MCP-1 mRNA levels were increased by 40-fold in noninfarcted LV 1 day after ligation, which persisted until 28 days. To block the MCP-1 signals, an N-terminal deletion mutant of the human MCP-1 gene was transfected into the limb skeletal muscle 3 days before and 14 days after ligation. This method improved the survival rate of mice with MI at 4 weeks (61% versus 87%, \( P < 0.05 \)) as well as attenuated LV cavity dilatation and contractile dysfunction, interstitial fibrosis, recruitment of macrophages, and myocardial gene expression of tumor necrosis factor-\( \alpha \) and transforming growth factor-\( \beta \) compared with the nontreated MI mice despite the comparable infarct size calculated as percent LV circumference.

Conclusions—The activation of MCP-1 expression contributes to the LV remodeling and failure after MI. An anti–MCP-1 gene therapy can be a useful novel strategy for preventing post-MI heart failure. (Circulation. 2003;108:2134-2140.)

Key Words: heart failure • remodeling • inflammation

The changes in left ventricular (LV) geometry, such as cavity dilatation associated with myocyte hypertrophy and interstitial fibrosis, referred to as remodeling, contribute to the development of depressed cardiac function in heart failure. Recent studies have demonstrated that inflammatory responses may cause myocardial damage and fibrosis, leading to progressive impairment of cardiac function. Specifically, monocyte chemoattractant protein (MCP)-1, which belongs to the C-C chemokine superfamily, is increased in both experimental and clinical heart failure. In addition, transgenic cardiac overexpression of MCP-1 results in myocarditis and subsequent development of heart failure. MCP-1 can be induced in numerous cell types, including vascular endothelial cells, smooth muscle cells, monocytes/macrophages, and cardiac myocytes in response to various stimuli, such as tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) and angiotensin II (Ang II), both of which play an important role in the pathogenesis of heart failure. Enhanced myocardial MCP-1 levels induce the infiltration and activation of inflammatory cells, such as monocytes/macrophages and lymphocytes. In addition, it can also promote the induction of other cytokines, matrix metalloproteinase (MMPs), and transforming growth factor-\( \beta \) (TGF-\( \beta \)) through an autocrine/paracrine mechanism, possibly representing a vicious cycle operative in the development of myocardial remodeling. Based on these findings, we hypothesized that the increased myocardial levels of MCP-1 might play an important role in the pathogenesis of cardiac remodeling and its inhibition could attenuate the development of heart failure. Despite recent advances in recognizing a role of MCP-1, no previous studies have established its pathogenetic significance in heart failure.

Therefore, the purpose of this study was to determine whether the blocking of MCP-1 signaling can attenuate the progressive LV dysfunction in a murine model of post–myocardial infarction (MI) heart failure by using a gene therapeutic strategy to transfect an N-terminal deletion mutant of the human MCP-1 gene into skeletal muscles.

Methods

Anti–MCP-1 Gene Therapy

The study was approved by our Institutional Animal Research Committee and conformed to the animal care guidelines of the...
American Physiological Society. To block MCP-1 signals in vivo, we used an N-terminal deletion mutant of MCP-1, 7ND, cDNA constructed by recombinant polymerase chain reaction with a wild-type human MCP-1 cDNA (a generous gift from Dr. Yoshimura, National Cancer Institute, Bethesda, Md) as template and inserted into the BamHI (5′) and NotI (3′) sites of the pcDNA expression vector plasmid. This method was based on our previous experiments, in which 7ND could bind to MCP-1 (CCR2) receptor and block MCP-1–mediated signals in vivo.11 Briefly, anesthetized mice were injected in the bilateral tibial muscles with either empty or 7ND plasmid (100 μg) in PBS using a 27-gauge needle fitted with a plastic collar limiting muscle penetration to approximately 5 mm. To improve the gene transfection efficiency, the myotoxic agent bupivacaine (0.25% wt/vol, 100 μL) was injected into the muscles 3 days before the transfection.12 Transfected 7ND can be secreted as its protein into the circulating blood, as evidenced by the elevation of plasma 7ND concentration from <20 to 140 to 220 pg/mL after 14 days. The circulating 7ND binds to the MCP-1 receptor on target cells in remote organs, thereby effectively blocking MCP-1 signaling in an intact animal.

Creation of MI
We created MI in male CD-1 mice (Kyudo Co Ltd, Saga, Japan), 5 to 8 weeks old and 25 to 35 g body weight, by ligation of the left coronary artery.10 Empty plasmid or 7ND was transfected 3 days before and 14 days after ligation.

Experimental Protocol 1: 28-Day Post-MI Study

Survival
The survival analysis was performed in the sham (n=20), sham+7ND (n=20), MI (n=48), and MI+7ND (n=31) mice. During the study period of 4 weeks, cages were inspected daily for deceased animals. All deceased mice were examined for the presence of MI as well as pleural effusion, serous fluid within the chest wall cavity, and cardiac rupture, diagnosed by the presence of blood clot within the pericardial sac.

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. A 2D 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 mid upper left anterior chest wall. The transducer was then gently moved cephalad or caudal and angled until desirable images were obtained. After it was ensured that the imaging was on axis (based on roundness of the LV cavity), 2D targeted M-mode tracings were recorded at a paper speed of 50 mm/sec. Then, a 1.4F micromanometer-tipped catheter (Millar) was inserted into the right carotid artery and advanced into the left ventricle to measure LV pressures.10 One subset of 2 investigators (T.S. and M.I.), who were not informed of the experimental groups, performed in vivo LV function studies.

Myocardial Pathology
Infarct size was determined by the methods described in rats13 and also in mice.14 The left ventricles were cut from apex to base into 3 transverse sections. Five-micrometer sections were cut and stained with Masson’s trichrome. Infarct length was measured along the endocardial and epicardial surfaces from each of the LV sections, and the values from all specimens were summed. Total LV circumference was calculated as the sum of endocardial and epicardial segment lengths from all LV sections. Infarct size (in percent) was calculated as total infarct circumference divided by total LV circumference. In our preliminary study, we confirmed excellent reliability of infarct size measurements, in which a morphometric methodology similar to that used in this study was used. The intraobserver and interobserver variabilities between 2 measurements divided by these means, expressed as a percentage, were less than 5%. Therefore, our technique could be considered to allow reliable assessment of infarct size in mice. Myocyte cross-sectional area and collagen volume fraction were determined.10

Experimental Protocol 2: Time-Dependent Studies of Immunohistochemistry, Cytokines, and MMPs
The following studies were performed in a separate group of additional mice treated identically to experimental protocol 1.

Immunohistochemistry
LV sections were immunostained with a rat anti-mouse monocye/macrophage antibody (MOMA-2, Serotec Inc). The slides were washed and incubated with biotinylated, affinity-purified goat anti-rat IgG. After avidin-biotin amplification, the slides were incubated with 3’,3’-diaminobenzidine and counterstained with hematoxylin.

To quantify the number of MOMA-2–positive monocytes/macrophages, each section (5 per heart) was scanned at ×40 magnification. The number of MOMA-2–positive cells in each section was counted, and the average number of the positive cells per square centimeter was calculated for each animal. The myocardial tissues with MI were carefully dissected into 2 parts, 1 consisting of the infarcted LV with the per-infarct rim (a 0.5- to 1-mm rim of normal-appearing tissue) and the remaining noninfarcted LV.

Cytokine Genes
Total RNA was isolated from noninfarcted LV, and ribonuclease protection assay (PharMingen) was performed with 5 μg of total RNA to determine the expression level of genes, including MCP-1, TNF-α, TGF-β, and interleukin-1β. Each value was normalized to that of GAPDH in each template set as an internal control.

MMPs
Crude protein extracts (10 μg) were subjected to a 7.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. MMP-9 and MMP-13 protein levels were detected using monoclonal antibodies against recombinant mouse MMP-9 (both activated and latent forms; R&D Systems) and human MMP-13 (Santa Cruz), respectively.

Experimental Protocol 3: MCP-1 Expression In Vitro
We performed another experiment that assessed MCP-1 gene expression in cultured neonatal rat cardiac myocytes and fibroblasts exposed to TNF-α and Ang II. Primary cultures of cardiac myocytes and fibroblasts were prepared from the ventricles of neonatal Wistar rats. Cultured cells were incubated in the presence or absence of TNF-α or Ang II. After 24 hours of incubation at 37°C, the cells were solubilized in 500 μL of 0.5% SDS, 1 mmol/L dithiothreitol, and 50 mmol/L Tris-HCl, pH 7.5, and the cell lysates were collected and stored at –80°C until the time of analysis. Total RNA was extracted from each sample by the acid guani- dinium thiocyanate-phenol-chloroform method, denatured with formaldehyde and formamide, fractionated by electrophoresis on formaldehyde-agarose gels, transferred to nylon membranes (Hybond N+), and immobilized by UV irradiation. The membranes were hybridized overnight with rat MCP-1 cDNA or mouse GAPDH cDNA and labeled with [32P]dCTP by random priming.

Statistical Analysis
Data are expressed as mean±SEM. Survival analysis was performed by the Kaplan-Meier method, and between-group difference in survival was tested by the log-rank test. Between-group comparison of means was performed by 1-way ANOVA, followed by t tests. The Bonferroni’s correction was done for multiple comparisons of means.

Results
Survival
7ND transfection in MI mice (n=31) was associated with an increased survival during 4 weeks compared with nontrans-
fected MI mice (n=48) (Figure 1). The power of survival analysis was calculated to be 0.62, and the hazard ratio was 0.29. All mice that died were confirmed to have MI on postmortem examination. Death was most likely related to progressive heart failure or arrhythmia. Two 7ND-treated MI (6%) and 7 MI (15%) mice died from LV rupture (P/NS).

LV Function and Structure
Serial echocardiographic measurements were performed at 3 days before surgery, baseline, and 3 days, 14 days, and 28 days after surgery in another group of randomly grouped sham (n=12), sham 7ND (n=12), MI (n=12), and MI+7ND (n=12) mice. Figure 2 demonstrates that LV chamber size increased in both MI and MI+7ND and that the extent of increase was the same between groups after 3 days, indicating the comparable infarct size calculated as percent LV (% LV) circumference between groups. However, 7ND transfection significantly attenuated progressive LV dilatation and dysfunction thereafter caused by MI (Figures 2 and 3 and Table). In comparison with sham, MI animals showed a significant increase in the thickness of noninfarcted region, which was attenuated by 7ND.

There was no significant difference in heart rate and mean aortic blood pressure among 3 groups (Table). LV end-diastolic pressure was increased in MI, which was attenuated in 7ND-treated MI mice. 7ND partially normalized LV dP/dt_max, which was significantly reduced in MI mice.

Body weight was similar among 3 groups. Right ventricular weight/body weight was increased in the MI, and 7ND attenuated this increase. Coinciding with an increased LV end-diastolic pressure, lung weight/body weight was increased in the MI group, which was also attenuated by 7ND. The prevalence of pleural effusion was significantly lower in 7ND-transfected MI mice.

Infarct Size Calculated as % LV Circumference
Infarct size calculated as % LV circumference 28 days after ligation was comparable (49±3% versus 54±6%; P/NS) between MI (n=27) and 7ND-transfected MI (n=30) mice. In a separate group of additional animals, it was also comparable (53±3% versus 49±2%; P/NS) between MI (n=7) and 7ND-transfected MI (n=7) mice 3 days after ligation.

Myocardial Pathology
The transverse LV sections obtained from the MI mouse revealed an anteroapical wall of infarct. Consistent with echocardiographic data, 7ND-transfected MI mice had significantly smaller LV diameters compared with MI (Figure 4 and Table). Photomicrographs of LV sections showed an increased myocyte cross-sectional area in MI, which was significantly attenuated by 7ND. These results are concordant with LV wall thickness data obtained from echocardiography. Interstitial fibrosis was increased in MI (Figure 4E), which was inhibited by 7ND (Figure 4F). Morphometric quantification of collagen volume fraction confirmed these findings (Table).

Myocardial Immunohistochemistry
The immunoreactive staining for macrophages was barely detectable in the LV obtained from sham. In contrast, in day 3 post-MI animals, macrophage infiltration was clearly increased in both infarcted and noninfarcted LV (Figure 5A). This increase persisted during 28 days after MI. As expected, 7ND transfection significantly reduced the infiltration of macrophages (Figure 5A). T lymphocytes (CD4 and CD8), to which MCP-1 can be also chemotactic, were detected in the MI mice, which were not altered by 7ND (data not shown). Similarly, the infiltration of neutrophils was not affected by 7ND.
MCP-1 mRNA levels were significantly higher in MI than sham-operated animals (Figure 6). There was a peak elevation in MCP-1 levels on days 1 and 3, and its expression declined but still remained upregulated for an extended duration up to 28 days. Interestingly, 7ND attenuated the increase in MCP-1 gene expression in MI. Moreover, MI induced TNF-α and TGF-β1 (Figure 6), which was also attenuated by 7ND, suggesting that their induction occurs through MCP-1-dependent pathways. Similarly, interleukin-1β was increased by 2-fold at day 1 of MI, which was also inhibited by 7ND.

**Induction of MCP-1 by TNF-α and Ang II In Vitro**

TNF-α (10 ng/mL, 6 hours) exposure caused a 35-fold increase ($n=6$, $P<0.01$) in MCP-1 mRNA levels in cardiac fibroblasts. Similarly, it also increased MCP-1 in myocytes (17-fold increase; $n=4$, $P<0.01$). In addition, Ang II (100 nmol/L, 6 hours) increased MCP-1 in fibroblasts by 3.3-fold ($n=6$, $P<0.01$). However, Ang II (10 to 100 nmol/L, 6 to 24 hours) did not induce any significant changes in MCP-1 from myocytes.

**Discussion**

This is the first report to provide evidence of increased myocardial expression of MCP-1 and the beneficial effects of
MCP-1 signaling inhibition on the development of heart failure. We demonstrated that 7ND improved survival and attenuated LV dilatation and contractile dysfunction compared with the vehicle-treated MI mice. These effects were associated with a decrease in interstitial fibrosis as well as macrophage infiltration and myocardial TNF-α and TGF-β gene expression. Thus, our observations suggest that an anti-MCP-1 strategy may be of therapeutic benefit against the evolution of post-MI failure.

Previous studies have shown that MCP-1 is markedly upregulated in the ischemic myocardium and is responsible for the recruitment of mononuclear cells into the injured myocardium. Monocyte-derived macrophages and mast cells may produce cytokines and growth factors necessary for fibroblast proliferation and neovascularization, leading to effective repair and scar formation. Thus, in the setting of acute MI or ischemia reperfusion injury, the expression of MCP-1 is likely beneficial. In the present study, post-MI hearts were also associated with an upregulation of MCP-1 gene expression (Figure 6). In failing hearts, MCP-1 has been shown to be increased in humans as well as in animal models. Our data suggest that sustained MCP-1 expression can lead to sustained cytokine expression and inflammatory responses that lead to inadvertent myocardial damage. Thus, the beneficial effects of MCP-1 may be lost when its expression is sustained, thereby leading to sustained inflammatory responses that contribute to myocardial remodeling and failure.

The beneficial effects of 7ND transfection were not attributable to its MI size-sparing effect, because the infarct size calculated as % LV circumference was comparable between MI and MI+7ND mice at 28 days as well as 3 days of MI. Furthermore, its effects might not be attributable to those on hemodynamics, because blood pressure and heart rate were not altered (Table).
Transfection of 7ND reduced a number of macrophages infiltrated into the post-MI hearts (Figure 5). There are several reports showing the infiltration of lymphocytes and monocytes/macrophages into the failing myocardium. Therefore, by mediating the recruitment of macrophages into myocardial tissue, MCP-1 may play an important role in the pathogenesis of heart failure. In addition, recent studies have suggested that MCP-1 may exert other biological activities than recruiting monocytes/macrophages, because the expression of MCP-1 does not always correlate with the extent of their infiltration. Therefore, the effects of MCP-1 shown in this study might not be attributable solely to its chemoattractant properties on inflammatory cells. In fact, 7ND reduced the expression of TNF-α in the post-MI hearts (Figure 6). TNF-α has profound and wide-ranging effects that can initiate a cascade of myocyte hypertrophy and apoptosis. Therefore, a proposed mechanism of 7ND for reverse LV remodeling is related to the attenuation of this cytokine after MI. In addition, MCP-1 can directly stimulate collagen production via upregulation of TGF-β expression in cardiac fibroblasts. Therefore, the inhibition of interstitial fibrosis in MI by 7ND (Figure 4) might be attributable to the attenuation of TGF-β (Figure 6). However, we cannot exclude the possibility that the longer-term inhibition of MCP-1 signaling may enhance LV remodeling and even cause myocardial rupture. We thus apparently need additional studies to clarify this crucial issue. Furthermore, 7ND was associated with the decrease in MCP-1 itself, which might be attributable to the inhibition of infiltrating macrophages (Figure 5A), one of the major cellular sources of MCP-1 in the myocardium. It was also associated with the attenuation of MMP-9 activation (Figure 5B), which might be involved in the inhibition of myocardial fibrosis and the resultant LV failure. Moreover, cytokines including TNF-α might evoke a secondary MCP-1 production from other types of cells, including cardiac myocytes and fibroblasts, and establish a positive loop, amplifying and sustaining the proinflammatory response. Thus, there is an intimate link between MCP-1 and cytokines, MMPs, and growth factors in the hearts through an autocrine/paracrine mechanism, possibly representing a vicious cycle implicated in myocardial failure. Therefore, an inhibition of MCP-1 signaling may attenuate the proinflammatory response and thus the development of myocardial remodeling.

The mechanism responsible for the upregulation of MCP-1 in MI has not been delineated in the present study. Our in vitro studies demonstrated that TNF-α can directly induce MCP-1 expression in both cardiac myocytes and fibroblasts, in agreement with the previous studies. In addition, Ang II could also induce MCP-1 gene expression in cardiac fibroblasts, as has been shown in vascular tissues. Even though these in vitro results could not be simply equated with in vivo animals, they indicate that TNF-α and Ang II, both well-known neurohumoral factors activated in the setting of heart failure, can directly induce MCP-1 expression.

There are several methodological issues to be acknowledged in this study. First, even though in vivo assessment of LV function with echocardiography is feasible and reproducible in the mouse, it still might be difficult to interpret the indices in dilated LV. The validation study has shown that the intraobserver and interobserver variabilities of our echocardiographic measurements for LV cavity dimensions were small and measurements made in the same animals on separate days were highly reproducible. Therefore, our technique could be considered to allow noninvasive assessment of LV structure even in mice with large MI. Importantly, even though the differences between MI and MI + 7ND mice were small, they were considered to be meaningful. Second, the heart rate values in the present study (430 to 460 bpm) were lower than those (600 bpm) measured in conscious mice. Therefore, it should be taken into account that LV size and function results might be greatly influenced by the differences in anesthetic regimens and the experimental conditions, such as heart rate.

The present study suggests that MCP-1 expression contributes to LV remodeling and failure after MI, potentially through increased cardiac fibrosis via enhanced TGF-β and MMP-9 expression. An anti-MCP-1 gene therapy might be a useful strategy for preventing post-MI failure though the definitive mechanisms by which it exerts beneficial effects and needs additional investigation.

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