Cardiac Overexpression of Monocyte Chemoattractant Protein-1 in Transgenic Mice Prevents Cardiac Dysfunction and Remodeling After Myocardial Infarction

Hajime Morimoto, Masafumi Takahashi, Atsushi Izawa, Hirohiko Ise, Minoru Hongo, Pappachan E. Kolattukudy, Uichi Ikeda

Abstract—Myocardial infarction (MI) is accompanied by inflammatory responses that lead to the recruitment of leukocytes and subsequent myocardial damage, healing, and scar formation. Because monocyte chemoattractant protein-1 (MCP-1) (also known as CCL2) regulates mononuclear inflammatory responses, we investigated the effect of cardiac MCP-1 overexpression on left ventricular (LV) dysfunction and remodeling in a murine MI model. Transgenic mice expressing the mouse JE-MCP-1 gene under the control of the α-cardiac myosin heavy chain promoter (MHC/MCP-1 mice) were used for this purpose. MHC/MCP-1 mice had reduced infarct area and scar formation and improved LV dysfunction after MI. These mice also showed induction of macrophage infiltration and neovascularization; however, few bone marrow–derived endothelial cells were detected in MHC/MCP-1 mice whose bone marrow was replaced with that of Tie2/LacZ transgenic mice. Flow cytometry analysis showed no increase in endothelial progenitor cells (CD34+/Flk1− cells) in MHC/MCP-1 mice. Marked myocardial interleukin (IL)-6 secretion, STAT3 activation, and LV hypertrophy were observed after MI in MHC/MCP-1 mice. Furthermore, cardiac myofibroblasts accumulated after MI in MHC/MCP-1 mice. In vitro experiments revealed that a combination of IL-6 with MCP-1 synergistically stimulated and sustained STAT3 activation in cardiomyocytes. MCP-1, IL-6, and hypoxia directly promoted the differentiation of cardiac fibroblasts into myofibroblasts. Our results suggest that cardiac overexpression of MCP-1 induced macrophage infiltration, neovascularization, myocardial IL-6 secretion, and accumulation of cardiac myofibroblasts, thereby resulting in the prevention of LV dysfunction and remodeling after MI. They also provide a new insight into the role of cardiac MCP-1 in the pathophysiology of MI. (Circ Res. 2006;99:891-899.)

Key Words: cytokines ■ heart failure ■ hypertrophy ■ inflammation ■ myocardial infarction

Myocardial infarction (MI) is accompanied by inflammatory responses that lead to the recruitment of leukocytes and subsequent myocardial damage, healing, and scar formation. Recruitment and activation of monocytes/macrophages in the infarcted myocardium have been shown to contribute importantly to the processes that occur after MI. The activated macrophages lead to the release of cytokines and proteinases, which can induce further inflammation and left ventricular (LV) remodeling. Meanwhile, recent evidence indicates that some endothelial progenitor cells (EPCs) are derived from monocytic lineage cells and participate in neovascularization in ischemic tissues. Moreover, monocytic-derived EPCs secrete a large amount of angiogenic factors such as the vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), thereby suggesting that monocytes/macrophages could improve LV dysfunction and remodeling after MI.

Chemokines are a family of potent chemoattractive cytokines that regulate locomotion and trafficking of leukocytes in basal and inflammatory processes; however, it has been recently observed that chemokines are expressed by nonhematopoietic cells such as endothelial cells, smooth muscle cells, and cardiomyocytes, and their function extends far beyond leukocyte activity. Monocyte chemoattractant protein-1 (MCP-1) (also known as CCL2) is a major chemokine that induces the recruitment and activation of monocytes, T cells, and NK cells, and it has been implicated in diseases characterized by monocyte-rich infiltrates. MCP-1 has been shown to be upregulated in experimental MI models and promotes mononuclear cell recruitment into the infarcted heart. Anti–MCP-1 gene therapy improves survival and attenuates LV dilatation and dysfunction in a murine MI model. Furthermore, targeted deletion of its receptor CCR2 in mice also improved LV dilatation and dysfunction after
MI, suggesting a deleterious role for MCP-1 in postinfarct LV dysfunction and remodeling. Hence, MCP-1 inhibition is presently regarded to be a target for therapeutic intervention against MI. However, angiogenic and cardioprotective effects of MCP-1 have been reported. These studies suggest diverse effects of MCP-1 on myocardial damage and remodeling after MI. Because MCP-1 is produced in the infarcted heart and the effect of MCP-1 is regulated by its topical concentration, we hypothesized that MCP-1 that is topically produced in the heart plays a critical role in the pathophysiology of MI. In the present study, we investigated the effect of cardiac MCP-1 overexpression on LV dysfunction and remodeling in a murine MI model. Transgenic mice that express the mouse JE-MCP-1 gene under the control of the cardiac myosin heavy chain promoter (MHC/MCP-1 mice) were used for this purpose. The findings obtained from this study provide a new insight into the role of cardiac MCP-1 in the pathophysiology of MI.

**Materials and Methods**

MHC/MCP-1 mice (background: FVB) were generated as previously described. FVB/N mice were purchased from Clea Japan Inc (Tokyo, Japan) and used as the age-matched wild-type controls for MHC/MCP-1 mice. At 12 weeks of age, no differences in the cardiac function and hypertrophy between wild-type and MHC/MCP-1 mice were observed (Figure I of the online data supplement, available at http://circres.ahajournals.org). FVB transgenic mice that express β-galactosidase under the control of the Tie-2 promoter (Tie2/LacZ mice) were purchased from The Jackson Laboratory (Bar Harbor, Me). Mice aged 8 to 12 weeks (n=191) were used in this study. They were fed a standard diet and water and were maintained on a 12-hour light and dark cycle. All experiments in this study were performed in accordance with the Shinshu University Guide for Laboratory Animals, which conforms to NIH Guidelines.

Cell culture, MI protocols, histology, immunohistochemistry, X-gal staining, bone marrow transplantation, flow cytometry analysis, serum cytokine levels, echocardiography, real-time RT-PCR analysis, and statistical analysis are described in the online data supplement.

**Results**

**Infarct Area, Scar Formation, and LV Function**

To investigate the role of cardiac overexpression of MCP-1 in MI, infarct area and scar formation were assessed at 14 days after MI. As shown in Figure 1A and 1B, the infarct area was significantly decreased in MHC/MCP-1 mice compared with wild-type mice (P<0.05). Masson’s trichrome staining showed that scar formation was also significantly reduced in MHC/MCP-1 mice compared with wild-type mice (P<0.01) (Figure 1C and 1D). We also assessed the acute infarct size at 24 hours after MI and found that there was no significant difference between wild-type and MHC/MCP-1 mice (27.4%±2.3% versus 23.3%±3.8%, P=0.381, n=6).

We next assessed LV function at baseline, 48 hours, 7 days, 14 days, 21 days, and 28 days after MI by using echocardiography. Table 1 shows that both wild-type and MHC/MCP-1 mice had similar LV dimension and function under baseline conditions. In wild-type mice, a marked decrease in percentage of fractional shortening (FS) was observed at 48 hours after MI, and this decrease was sustained for 28 days. In contrast, percentage of FS was maintained after MI in MHC/MCP-1 mice (14 days: 34.1%±1.0% [wild-type] versus 42.6%±0.7% [MHC/MCP-1]; P<0.001). Furthermore, in MHC/MCP-1 mice, the diastolic and systolic LV wall thicknesses were significantly increased after MI compared with that at baseline (P<0.01 to P<0.001).

**Macrophage Infiltration and Capillary Formation**

Because macrophage infiltration in the heart is a prominent feature of MHC/MCP-1 mice, immunohistochemical analysis of the macrophage marker F4/80 was performed. In baseline conditions, the number of infiltrated macrophages in the heart of MHC/MCP-1 mice was slightly increased compared with that of wild-type mice (Figure 2B). The number of infiltrated macrophages was increased in the border (infarct) area of the heart after MI in wild-type mice, and this macrophage infiltration was...
further increased in MHC/MCP-1 mice (P<0.05, Figure 2A and 2B). To assess the capillary density in the border area of the heart, immunohistochemical analysis of the endothelial cell marker CD31 was also performed. The capillary density determined by CD31 expression was significantly increased in MHC/MCP-1 mice compared with that in wild-type mice (P<0.01, Figure 2C and 2D), thereby suggesting that MHC/MCP-1 mice promote macrophage infiltration and neovascularization in the border area in MI.

**Contribution of Bone Marrow–Derived Cells**

To determine the contribution of bone marrow–derived EPCs to the neovascularization in MI in MHC/MCP-1 mice, we used bone marrow–transplanted mice whose bone marrow was replaced with that of Tie2/LacZ mice. In wild-type mice whose bone marrow was replaced with that of Tie2/LacZ mice (Tie2/LacZ→wild-type), no LacZ-positive cells were detected in the heart. In contrast, few LacZ-positive cells were observed in the border area of the heart in MHC/MCP-1 mice whose bone marrow was replaced with that of Tie2/LacZ mice (Tie2/LacZ→MHC/MCP-1) (Figure 3A).

To further investigate the contribution of EPCs, we checked whether EPCs were mobilized from the bone marrow to the peripheral circulation after MI in wild-type and MHC/MCP-1 mice. Predictably, flow cytometry analysis showed that the percentage of Mac-1^+Gr-1^- cells (monocyte marker) was increased at 7 days after MI in wild-type mice, and the increase after MI in MHC/MCP-1 mice was significantly greater than that in wild-type mice (Figure 3B). The percentage of CD34^+ cells (hematopoietic and endothelial marker) was increased only at 14 days after MI in wild-type mice, and in MHC/MCP-1 mice, the CD34^+ cell percentage was significantly greater than that in wild-type mice at baseline, 7 days, and 14 days after MI (Figure 3C). However, in MHC/MCP-1 mice, the percentage of Flk-1^- cells (endothelial marker) was not increased (Figure 3D) and the percentage of CD34^-Flk-1^- double-positive cells (EPC marker) was significantly less than that of wild-type mice (Figure 3E). These results indicate that the number of peripheral monocytes, not EPCs, was highly increased after MI in MHC/MCP-1 mice.

Because lethal irradiation might affect LV function, we measured LV function after MI in bone marrow–transplanted mice by using echocardiography. Similar to the observation with wild-type and MHC/MCP-1 mice, we confirmed that in comparison with Tie2/LacZ→wild-type mice, Tie2/LacZ→MHC/MCP-1 mice showed a significant improvement in LV dysfunction after MI (Figure 3F). These findings

### TABLE 1. Echocardiographic Findings After MI in Wild-Type and MHC/MCP-1 Mice

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>48 hours</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
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<tr>
<td><strong>Wild-type</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>n</td>
<td>30</td>
<td>8</td>
<td>24</td>
<td>23</td>
<td>15</td>
<td>15</td>
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<tr>
<td>BW, g</td>
<td>29.3±0.5</td>
<td>25.9±1.2</td>
<td>28.0±0.5</td>
<td>29.4±0.5</td>
<td>30.1±0.5</td>
<td>31.1±0.6</td>
</tr>
<tr>
<td>IVSd, mm</td>
<td>0.78±0.02</td>
<td>0.74±0.04</td>
<td>0.74±0.03</td>
<td>0.78±0.03</td>
<td>0.78±0.03</td>
<td>0.77±0.03</td>
</tr>
<tr>
<td>LVDd, mm</td>
<td>4.10±0.06</td>
<td>4.22±0.13</td>
<td>4.52±0.10*</td>
<td>4.54±0.11*</td>
<td>4.69±0.07†</td>
<td>4.71±0.06†</td>
</tr>
<tr>
<td>LVPWd, mm</td>
<td>0.81±0.01</td>
<td>0.84±0.09</td>
<td>0.83±0.02</td>
<td>0.85±0.03</td>
<td>0.81±0.02</td>
<td>0.85±0.05</td>
</tr>
<tr>
<td>IVSs, mm</td>
<td>1.33±0.02</td>
<td>1.17±0.08</td>
<td>1.27±0.04</td>
<td>1.22±0.04</td>
<td>1.23±0.05</td>
<td>1.15±0.03*</td>
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<tr>
<td>LVDs, mm</td>
<td>2.24±0.03</td>
<td>2.77±0.12*</td>
<td>3.00±0.09†</td>
<td>3.00±0.10†</td>
<td>3.20±0.08†</td>
<td>3.23±0.06†</td>
</tr>
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<td>LVPWs, mm</td>
<td>1.47±0.02</td>
<td>1.23±0.09†</td>
<td>1.30±0.02†</td>
<td>1.26±0.03†</td>
<td>1.23±0.03†</td>
<td>1.24±0.03†</td>
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<tr>
<td>FS, %</td>
<td>46.3±0.4</td>
<td>34.4±2.1†</td>
<td>33.0±0.8†</td>
<td>31.4±1.0†</td>
<td>31.7±0.7†</td>
<td>31.6±0.7†</td>
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<tr>
<td>FS, % of baseline</td>
<td>100</td>
<td>76.2±4.4†</td>
<td>75.2±1.9†</td>
<td>76.1±2.6†</td>
<td>71.5±1.7†</td>
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<td><strong>MHC/MCP-1</strong></td>
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<td>n</td>
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<td>8</td>
<td>23</td>
<td>23</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>BW, g</td>
<td>28.4±0.6</td>
<td>27.0±0.6</td>
<td>27.7±0.5</td>
<td>28.7±0.5</td>
<td>30.5±0.4</td>
<td>31.0±0.5*</td>
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<tr>
<td>IVSd, mm</td>
<td>0.81±0.02</td>
<td>0.84±0.05</td>
<td>0.97±0.03§</td>
<td>0.98±0.02§</td>
<td>0.96±0.04†§</td>
<td>0.96±0.03§</td>
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<tr>
<td>LVDd, mm</td>
<td>3.94±0.06</td>
<td>3.84±0.08</td>
<td>4.17±0.06†</td>
<td>4.37±0.07†</td>
<td>4.49±0.09†</td>
<td>4.33±0.10†</td>
</tr>
<tr>
<td>LVPWd, mm</td>
<td>0.85±0.02</td>
<td>1.01±0.07</td>
<td>1.02±0.04§</td>
<td>1.06±0.02§</td>
<td>1.02±0.04§</td>
<td>1.06±0.03††</td>
</tr>
<tr>
<td>IVSs, mm</td>
<td>1.40±0.01</td>
<td>1.30±0.08</td>
<td>1.48±0.04§</td>
<td>1.49±0.02§</td>
<td>1.44±0.06§</td>
<td>1.36±0.03§</td>
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<tr>
<td>LVDs, mm</td>
<td>2.12±0.04</td>
<td>2.35±0.09§</td>
<td>2.39±0.04§</td>
<td>2.50±0.06†§</td>
<td>2.71±0.07§</td>
<td>2.63±0.08§</td>
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<tr>
<td>LVPWs, mm</td>
<td>1.45±0.02</td>
<td>1.44±0.07</td>
<td>1.49±0.03§</td>
<td>1.54±0.03§</td>
<td>1.45±0.02§</td>
<td>1.46±0.03§</td>
</tr>
<tr>
<td>FS, %</td>
<td>46.3±0.5</td>
<td>39.0±1.4†</td>
<td>42.6±0.7†§</td>
<td>42.6±0.7†§</td>
<td>39.6±0.8†§</td>
<td>39.5±0.6†§</td>
</tr>
<tr>
<td>FS, % of baseline</td>
<td>100</td>
<td>85.1±2.9†</td>
<td>92.2±1.3†§</td>
<td>90.1±1.5†§</td>
<td>88.4±2.0†§</td>
<td>88.2±1.6†§</td>
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</tbody>
</table>

Data represent the mean±SEM. BW indicates body weight; IVSd, interventricular septal diastolic thickness; LVDd, LV diastolic dimension; LVPWd, LV posterior wall diastolic thickness; IVSs, interventricular septal systolic thickness; LVDs, LV systolic dimension; LVPWs, LV posterior wall systolic thickness. *P<0.05, †P<0.01 vs wild-type; ‡P<0.001 vs wild-type, §P<0.05 vs wild-type.
suggest a small contribution of bone marrow–derived EPCs to neovascularization after MI in MHC/MCP-1 mice.

Involvement of Inflammatory Cytokines
To investigate whether inflammatory cytokines are involved in the reduction of infarct area and scar formation after MI in MHC/MCP-1 mice, we determined the serum levels of MCP-1, interleukin (IL)-6, IL-10, IL-12p70, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α. As expected, the serum level of MCP-1 was strikingly increased in MHC/MCP-1 mice under baseline conditions, but no increase was detected in the levels of other cytokines (Table 2). At 14 days after MI, the serum levels of MCP-1 and IL-12p70 were increased in wild-type as well as MHC/MCP-1 mice (MCP-1, \( P<0.01 \); IL-12p70, \( P<0.01 \)). There was no difference between wild-type and MHC/MCP-1 mice in terms of the increased MCP-1 levels after MI. Notably, the serum IL-6 level was markedly increased after MI in MHC/MCP-1 mice when compared with that in wild-type mice (\( P<0.05 \)).

To explore the cellular sources of IL-6 in MHC/MCP-1 mice, real-time RT-PCR analysis was performed to detect IL-6 and TNF-α expression in the infarct heart and peripheral blood cells in the early phase after MI. IL-6 mRNA expression in the heart of wild-type mice was increased at 6 hours after MI (Figure 4A). In MHC/MCP-1 mice, IL-6 mRNA expression in the heart was also clearly increased at 6 hours after MI; however, no increased IL-6 mRNA expression was detected in peripheral blood cells isolated from both wild-type and MHC/MCP-1 mice. Moreover, the level of IL-6 mRNA expression in the heart of MHC/MCP-1 mice significantly increased at 24 hours, compared with that in wild-type mice. There was no significant increase in TNF-α mRNA expression in the heart and peripheral blood of wild-type and MHC/MCP-1 mice (supplemental Figure II). Immunohistochemical staining also showed that the expression of MCP-1 and IL-6 proteins was increased in the infarct heart of MHC/MCP-1 mice (Figure 4B). These findings suggest that not only MCP-1 but also IL-6 produced by the infarct heart might be involved in the beneficial effects that were observed in MHC/MCP-1 mice.

Cardiac Hypertrophy and STAT3 Activation
Because IL-6 has been reported to induce cardiac hypertrophy and exert a cardioprotective effect through the STAT3 signaling pathway,16 we tested whether cardiac hypertrophy and STAT3 activation were induced in MHC/MCP-1 mice. Representative histological findings of cross-sectional cardiomyocytes are shown in Figure 5A and 5B. There was no difference between wild-type and MHC/MCP-1 mice in terms of the cross-sectional diameters of cardiomyocytes at baseline. However, the cross-sectional diameters of cardiomyocytes at 14 days after MI in MHC/MCP-1 mice were significantly greater than those of wild-type mice (\( P<0.001 \)).
Furthermore, activated STAT3 (phosphorylated form) was visualized in the nuclei of the infarct heart in MHC/MCP-1 mice but not in those of wild-type mice (Figure 5C).

To confirm the activation of STAT3 by IL-6 and investigate the effect of MCP-1 on STAT3 activation in cardiomyocytes, we used mouse neonatal cultured cardiomyocytes in vitro and tested for STAT3 activation by Western blotting. Treatment with IL-6 clearly activated STAT3 within 5 minutes, peaked at 30 minutes, and then declined to the basal level (Figure 5D), whereas treatment with MCP-1 showed only slight STAT3 activation. It is noteworthy that combined treatment of IL-6 with MCP-1 synergistically activated STAT3, and this activation was sustained at least during the 120 minutes observation period. To assess cellular hypertrophy, the 2D cell area was quantified in cardiomyocytes treated with IL-6. As shown in Figure 5E, treatment with IL-6 for 48 hours increased the size of cardiomyocytes in vitro. Furthermore, we measured the heart weights and body weights and calculated the ratio of heart weights to body weights. The ratio of heart weights to body weights after MI in MHC/MCP-1 mice was significantly increased compared with that in wild-type mice (Figure 5G). These results suggest that IL-6 induced cardiac hypertrophy through STAT3 activation after MI in MHC/MCP-1 mice.

**Figure 3.** Contribution of bone marrow–derived EPCs. A, Heart sections were obtained from bone marrow–transplanted mice (Tie2/LacZ–wild-type and Tie2/LacZ–MHC/MCP-1) at 14 days after MI and stained with hematoxylin/eosin (left and middle) and X-gal (right). The arrow indicates a LacZ-positive cell. B through E, Blood samples were collected from the same wild-type and MHC/MCP-1 mice at baseline, 7 days, and 14 days after MI. The percentage of Mac-1/H11001/Gr-1/H11002 cells (monocyte marker), CD34/H11001 cells (hematopoietic and endothelial marker), Flk-1/H11001 cells (endothelial marker), and CD34/H11001/Flk-1/H11001 cells (EPC marker) was assessed by flow cytometry. Results are expressed as means±SEM (n=4 to 5). *P<0.05 vs baseline; †P<0.001 vs wild-type. F, LV function was assessed by echocardiography at baseline, 7 days, and 14 days after MI in bone marrow–transplanted mice (Tie2/LacZ–wild-type, n=10; Tie2/LacZ–MHC/MCP-1, n=5). The bar graph shows percentage of FS. Results are expressed as means±SEM.

**Table 2.** Serum Cytokine Levels at Baseline and Fourteen Days After MI in Wild-Type and MHC/MCP-1 Mice

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Baseline (pg/mL)</th>
<th>Day 14 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-Type</td>
<td>MHC/MCP-1</td>
</tr>
<tr>
<td>MCP-1</td>
<td>42.9±0.8</td>
<td>5170.2±763.4§</td>
</tr>
<tr>
<td>IL-6</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>IL-10</td>
<td>&lt;20</td>
<td>&lt;20</td>
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<tr>
<td>IL-12p70</td>
<td>&lt;20</td>
<td>24.9±18.0</td>
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<tr>
<td>IFN-γ</td>
<td>&lt;20</td>
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</tr>
<tr>
<td>TNF-α</td>
<td>&lt;20</td>
<td>&lt;20</td>
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</tbody>
</table>

Data represent the mean±SEM (n=4–5). *P<0.05, †P<0.01 vs baseline; ‡P<0.05, §P<0.01 vs wild type.
**Accumulation of Cardiac Myofibroblasts**

Cardiac fibroblasts have been shown to differentiate into myofibroblasts during the process of myocardial repair and remodeling after MI.\(^{10,18}\) Because myofibroblasts are characterized by the presence of α-smooth muscle actin (α-SMA), immunohistochemical analysis of α-SMA was performed. The number of α-SMA–positive myofibroblasts was notably increased at 14 days after MI in MHC/MCP-1 mice compared with that in wild-type mice (Figure 6A and 6B).

To explore the factors responsible for the differentiation of fibroblasts into myofibroblasts, we used murine neonatal cardiac fibroblasts in vitro. Treatment with MCP-1 remarkably increased the differentiation of fibroblasts into myofibroblasts under the normoxic condition (Figure 6C). Similarly, treatment with IL-6 alone or combined treatment of MCP-1 with IL-6 also increased its differentiation. Furthermore, hypoxic condition increased its differentiation, and its differentiation was further enhanced in the presence of MCP-1.

**Discussion**

The major findings of this study are as follows. (1) Cardiac overexpression of MCP-1 reduced infarct area and scar formation and improved LV dysfunction and remodeling after MI.\(^{10,18}\) (2) Cardiac overexpression of MCP-1 also induced macrophage infiltration and capillary formation in the border area of MI. However, a small contribution of bone marrow–derived EPCs to the neovascularization was observed. (3) Cardiac overexpression of MCP-1 induced marked myocardial IL-6 secretion, STAT3 activation, and LV hypertrophy in the MI heart. (4) Combined treatment of IL-6 with MCP-1 synergistically stimulated and sustained STAT3 activation in cardiomyocytes in vitro. (5) Cardiac overexpression of MCP-1 increased the accumulation of cardiac myofibroblasts, and an in vitro study demonstrated that MCP-1 and hypoxia synergistically induced the differentiation of cardiac fibroblasts into myofibroblasts. These findings suggest that cardiac overexpression of MCP-1 induced macrophage infiltration, neovascularization, cardiac IL-6 secretion, and accumulation of cardiac myofibroblasts, thereby resulting in the prevention of LV dysfunction and remodeling after MI.

The beneficial role of MCP-1 in MI is controversial. Inhibition of MCP-1 or its signaling has been shown to attenuate LV damage and remodeling after MI.\(^{6,7}\) Conversely, MCP-1 has direct angiogenic effects, and human endothelial cells express CCR2.\(^{8}\) In addition, a recent study demonstrated that MCP-1 has a cardioprotective effect against hypoxia in cardiomyocytes in vitro.\(^{9}\) Dewald et al\(^{10}\) recently demonstrated that MCP-1 gene disruption led to decreased and delayed macrophage infiltration in the healing infarct and delayed replacement of injured cardiomyocytes with granulation tissue. They also showed that MCP-1 deficiency had decreased the expression of inflammatory cytokines such as TNF-α, IL-1β, IL-10, and transforming growth factor (TGF)-β and had diminished myofibroblast accumulation, thereby suggesting the beneficial role of MCP-1 in myocardial healing after MI. The reason for the discrepancy in the role of MCP-1 in MI remains unclear. We postulate that the topical MCP-1 concentration may be important because the chemotactic function of MCP-1 depends on the MCP-1 concentration gradient.\(^{11,12}\) Therefore, we used MHC/MCP-1 mice that topically overexpress MCP-1 in hearts and demonstrated that these mice exhibited a reduction in the infarct area and scar formation and prevention of LV dysfunction and remodeling after MI. Martire et al\(^{19}\) recently reported that cardiac overexpression of MCP-1 could prevent myocardial damage against short-term ischemia/reperfusion injury; their observations supported our data. Taken together, these findings suggest...
the beneficial effects of topical cardiac MCP-1 production in the heart after MI.

We demonstrated that macrophage infiltration and capillary formation were significantly increased after MI in MHC/MCP-1 mice. In the noninfarct area, the similar number of macrophages in MHC/MCP-1 mice was observed, compared with that of wild-type mice. A recent study reported that Mac-1– but not Mac-3–positive cells were increased in the heart of 2-month-old MHC/MCP-1 mice.20 In this regard, Mac-1 is expressed on both granulocytes and monocytes/macrophages, whereas Mac-3 is specifically expressed by activated macrophages. Similarly, F4/80 antibody used in this study specifically recognizes activated macrophages, suggesting that the number of activated macrophages in the noninfarct area was similar between wild-type and MHC/MCP-1 mice. We also showed the similar acute infarct sizes after MI between wild-type and MHC/MCP-1 mice; this suggests that neovascularization rather than cytoprotective effects might be responsible for the beneficial effects of MCP-1. Recent evidence indicates that MCP-1 promotes neovascularization in ischemic tissues through several possible mechanisms. First, MCP-1 directly stimulates VEGF induction by monocytes/macrophages21 and endothelial cells,22 leading to neovascularization. Second, recent evidence indicates that monocytes/macrophages produce matrix metalloproteinase–dependent tunnels and promote neovascularization.23 In fact, Moldovan et al24 demonstrated that this phenomenon was observed in the heart of MHC/MCP-1 mice, suggesting that
this mechanism might contribute to neovascularization in this study. Third, bone marrow–derived monocytic lineage cells are suggested to function as EPCs and participate in neovascularization in the ischemic tissues.2–4 Flow cytometric analysis showed that there was no increase in the EPCs (CD34+/H11001/Flk-1/H11001 cells) in peripheral circulation after MI in MHC/MCP-1 mice. In this regard, Harraz et al3 reported that CD34-negative angioblasts are a subset of CD14-positive monocytic cells and that these monocytes have the potential to transdifferentiate into endothelial cells, thereby suggesting that monocytic cell–derived EPCs might have other surface markers. We detected few bone marrow–derived endothelial cells after MI in MHC/MCP-1 mice. Interestingly, EPCs have shown to induce the proliferation of the neighboring resident endothelial cells.25 Thus, further investigations are required to elucidate the role of EPCs in the heart of MHC/MCP-1 mice after MI.

IL-6 is a pleiotropic cytokine with varying effects on cells of the immune system and other tissues, including cardiomyocytes. Interestingly, we found that a substantial amount of IL-6 was secreted after MI in MHC/MCP-1 mice, although there was no significant increase at baseline. The latter findings were supported by Kolattukudy et al13 who reported that cardiac overexpression of MCP-1 did not induce the production of proinflammatory or morphogenic cytokines, including IL-6, up to 100 days of age. In the present study, cardiomyocytes were defined as the main cellular source of IL-6 production after MI, and IL-6 promoted the cellular hypertrophy of cardiomyocytes.26 Additionally, cardiomyocytes respond to hypoxic conditions to augment the production of IL-6.27 Although the role of IL-6 in cardiac tissues has not been fully understood, it is now recognized that the cytokines of the IL-6 family might prevent heart failure through antiapoptotic and hypertrophic effects on cardiomyocytes. Furthermore, recent investigations indicate that the STAT3-mediated signaling pathway is responsible for these beneficial effects.16 Indeed, Negoro et al28 demonstrated that MI activates the JAK/STAT pathway mainly in the border area of MI and that pharmacological inhibition of this pathway results in the deterioration of myocardial viability. Interestingly, combined treatment of IL-6 with MCP-1 synergistically stimulated and sustained STAT3 activation in cardiomyocytes, thereby suggesting the presence of crosstalk signaling pathways between IL-6 and MCP-1. Taken together, these findings suggest that IL-6 contributes to the prevention of LV damage and subsequent remodeling after MI in MHC/MCP-1 mice.

The present study demonstrated that accumulation of cardiac myofibroblasts was increased in MHC/MCP-1 mice after MI. Previous studies demonstrated that proliferation of myofibroblasts is observed during the process of MI repair,18 and these cells disappear by an apoptotic mechanism in association with necrotic tissue during fibrotic scar formation,20 thereby suggesting a role for myofibroblasts in scar formation and remodeling after MI. Consistent with our findings, Dewald et al10 recently reported that MCP-1 deficiency diminished myofibroblast accumulation in a murine MI model. Our in vitro results also suggest that MCP-1 directly stimulated the differentiation of fibroblasts into myofibroblasts, and this differentiation was enhanced by hypoxic conditions. Taken together, these findings suggest a critical role for MCP-1 in cardiac scar formation and remodeling after MI.

In conclusion, we clearly demonstrated that cardiac overexpression of MCP-1 induced macrophage infiltration, neovascularization, cardiac IL-6 secretion, and accumulation of cardiac myofibroblasts, thereby resulting in the prevention of LV dysfunction and remodeling after MI. Although the MCP-1 levels in MHC/MCP-1 mice are not physiological, the current results may provide insights into gene-based drug delivery of MCP-1. Furthermore, several previous studies
suggest the inhibition of MCP-1 as a potential target for therapeutic intervention\(^6\); however, our study indicates that further investigations are necessary to elucidate the precise role of MCP-1 in ischemic heart diseases before its clinical application.

**Acknowledgments**

We thank Junko Nakayama, Tomoko Hamaji, and Kazuko Misawa for excellent technical assistance.

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**Disclosures**

None.

**References**

Cardiac Overexpression of Monocyte Chemoattractant Protein-1 in Transgenic Mice Prevents Cardiac Dysfunction and Remodeling After Myocardial Infarction

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Materials and Methods

Cell culture and reagents

Murine neonatal cardiomyocytes and cardiac fibroblasts were prepared from the ventricles of 1-day-old mice with minor modifications of the protocol used for rat neonatal cardiomyocyte isolation. Briefly, after dissociation with 0.25% trypsin (Invitrogen, Carlsbad, CA) followed by 0.8 mg/mL collagenase (Wako Pure Chemical Industries, Inc., Osaka, Japan), the cells were washed and resuspended in Dulbecco’s modified Eagle’s medium (DMEM: Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS: Hyclone, Logan, UT). For isolation of cardiac fibroblasts, the cells were plated onto culture dishes for 1 hour, during which time cardiac fibroblasts readily attached to the bottom of the culture dishes. The non-attached cells (cardiomyocytes) were removed and plated onto other culture dishes. The isolated cardiomyocytes and cardiac fibroblasts were grown in 10% FBS-containing DMEM. Primary cardiomyocytes and cardiac fibroblasts were used in the experiments. To expose the cells to hypoxia, they were placed in Aneropac (Mitsubishi Gas Chemical, Tokyo, Japan) for 24 hours.

Murine recombinant MCP-1 and interleukin (IL)-6 were purchased from Pepro-Tech Inc. (Rocky Hill, NJ). The remaining reagents were obtained from Sigma unless otherwise specified.

MI protocols

After anesthetizing mice with isoflurane, intubation was performed. The mice were ventilated with a rodent ventilator (Minivent type 845, Harvard Apparatus). A left thoracotomy was performed in the 4th or 5th intercostal space. An 8-0 nylon suture was placed directly underneath the left auricle in the interventricular groove. Successful coronary occlusion was verified by observing the development of a pale color in the distal myocardium after the ligation. The lungs were re-expanded using positive
pressure at end-expiration, and the thoracotomy and skin incision were closed with 3-0 silk. Extubation was performed when spontaneous respiration resumed.

**Histology and immunohistochemistry**

Mice were euthanized after irrigation with saline (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan), and their blood was completely washed out. The hearts were embedded in OCT compound (Tissue-Tek, Sakura Finetechinal Co., Ltd., Tokyo, Japan) and frozen on dry ice. The hearts were sectioned transversely from the apex to the site of the ligature underneath the left atrium. Tissue sections (10-µm thick) were cut on a cryostat (CM-1900, Leica Microsystems GmbH, Wetzlar, Germany) and examined histologically. A total of 10 sections were selected from each heart to perform morphometrical assessments of the LV myocardium and infarct size. The sections were stained with hematoxylin-eosin (HE) and Masson’s trichrome (MT). Measurements were performed using Scion Image software (beta 4.03, Scion Corporation, MD). The infarct size was calculated as a percentage of total LV area. The extent of fibrosis was measured in 4 sections from each heart and the value was expressed as the ratio of the MT stained area to the total LV free wall. All measurements were conducted in a double-blind manner by 2 independent researchers.

For immunohistochemical analysis, the heart sections were incubated with primary antibodies against mouse CD31 (clone MEC13.3, BD Bioscience, San Jose, CA), F4/80 (clone A3-1, RDI, Flanders, NJ), α-smooth muscle actin (α-SMA: clone 1A4, Sigma), MCP-1 (ab7202: Abcam plc, Cambridge, UK), IL-6 (Santa Cruz Biotechnology, CA), and phospho-signal transducer and activator of transcription 3 (phospho-STAT3 [Tyr705], Cell Signaling Technology Inc., Beverly, MA) followed by biotin-conjugated secondary antibodies. The sections were washed and treated with avidin peroxidase (ABC kit, Vector Laboratories, Burlingame, CA). The stain was developed using the DAB substrate kit (Vector Laboratories). The sections were then
counterstained with hematoxylin. To specifically localize mouse primary monoclonal antibodies on mouse tissues, mouse on mouse (M.O.M.) basic kits (Vector Laboratories) were used. For immunofluorescence staining, Cy3-labeled mouse anti-mouse IgG was used as the secondary antibody. For nucleic acid staining, the cells were incubated with 4', 6-diamidino-2-phenylindole (DAPI: Wako Pure Chemical Industries, Ltd.). No signals were detected when irrelevant IgG was used instead of the primary antibody as the negative control. All measurements were conducted in a double-blind manner by 2 independent researchers.

**X-gal staining**

For detection of LacZ reporter activity, arterial sections were fixed in acetone, stained using X-gal Substrate Set (HistoMark, KPL, Gaithersburg, MD) according to the manufacturer’s instructions, and carefully examined under a microscope for the presence of blue staining.²

**Bone marrow transplantation**

Bone marrow transplantation was performed as described previously.³ Whole bone marrow cells from Tie2/LacZ mice were harvested by flushing the femurs with phosphate-buffered saline (PBS). Red blood cells were lysed with ACK buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.2) at 4°C for 20 min. The cells were washed 3 times with PBS and resuspended in 0.5 mL PBS. Recipient mice (wild-type and MHC/MCP-1 mice, 8-week-old) were lethally irradiated with a total dose of 9 Gy (MBR-155R2, Hitachi, Japan) and injected with bone marrow cells through the tail vein. To verify the reconstitution of bone marrow after transplantation by this protocol, we used GFP mice as the donors. Flow cytometry analysis revealed that at 6 weeks after bone marrow transplantation, peripheral blood cells consisted of more than 95% GFP-positive cells.
Flow cytometry analysis

Blood samples were collected from the mice at baseline, 7 days, and 14 days after MI. Circulating cells were identified using a nucleated cell fraction. The nucleated cells were double-labeled as follows. The PerCP-conjugated anti-CD11b (Mac-1) antibody (clone M1/70, BD Biosciences), FITC-conjugated anti-Ly-6G (Gr-1) antibody (clone 1A8), FITC-conjugated anti-CD34 monoclonal antibody (clone RAM34, BD Biosciences) and PE-conjugated anti-Flk-1 antibody (VEGFR2/KDR, clone Avas12a1, BD Biosciences) were used. The cells were examined by flow cytometry (FACSCalibur, Becton Dickinson) and analyzed using CellQUEST software ver.3.3 (Becton Dickinson).

Echocardiography

Transthoracic echocardiography was performed at the indicated time after MI using a Vivid Five echocardiography system (GE Yokogawa Medical Systems, Tokyo, Japan) as described previously.4 Ketamine (50 mg/kg) and xylazine (10 mg/kg) were administered intraperitoneally for mild sedation. Two-dimensional targeted M-mode echocardiograms were obtained along the short axis of the LV at the level of the papillary muscles, and at least 3 consecutive beats were recorded. Left ventricular end-systolic diameter (LVESD) and left ventricular end-diastolic diameter (LVEDD) were defined as the phases in which the smallest and largest area of LV, respectively, were obtained. Percentage fractional shortening (%FS) was calculated using the standard formula: %FS = [(LVEDD – LVESD)/LVEDD] x 100. All measurements were conducted in a double-blind manner by 2 independent researchers.

Real-time RT-PCR analysis

Total cellular RNA was prepared using ISOGEN (Nippon Gene Co., Ltd.,
Toyama, Japan) or RNA-Bee (Tel-Test, Inc., Friendswood, TX), according to the manufacturer’s instructions. Real-time RT-PCR analysis was performed by using the ABI Prism 7000 system (Applied Biosystems, Inc.) to detect the mRNA expression of IL-6, tumor necrosis factor-α (TNF-α), and β-actin. The following primers (oligonucleotide sequences are given in parentheses in the order of antisense and sense primer) were used. IL-6 (5’-ACAACCACGGCCTCCTACTT-3’ and 5’-CACGATTTCCCAGAGAACATGTG-3’), TNF-α, (5’-CCCCAAAGGGATGAGAAGTTC-3’ and 5’-GCTTTGTCACCTCGAATTTTGAGAA-3’), and β-actin (5’-CCTGAGCGCAAGTACTCTGTGT-3’ and 5’-GCTGAGCGCAAGTACTCTGTGT-3’). The expression levels of each target gene were normalized by subtracting the corresponding β-actin threshold cycle (C_T) values; this was done by using the \(\Delta\Delta C_T\) comparative method.

**Serum levels of inflammatory cytokines**

The serum levels of MCP-1, IL-6, IL-10, IL-12p70, interferon-γ (IFN-γ), and TNF-α were assessed using the CBA Mouse Inflammation Kit (BD Biosciences) according to the manufacturer’s instructions.

**Western blot analysis**

The expression levels of total STAT3 and the phosphorylated form of STAT3 were analyzed by western blotting.\(^5\) Briefly, the cells were washed with ice-cold PBS and then lysed in RIPA buffer (25 mM Tris-HCl, pH 7.5; 2.5 mM EDTA; 10 mM sodium pyrophosphate; 50 mM NaF; 137 mM NaCl; 1% Triton X-100; 10% glycerol; 1% deoxycholic acid; 0.1% SDS; fresh 0.1 mM phenylmethyl sulfonyl fluoride (PMSF); 100 µM Na_3VO_4; and a protease inhibitor cocktail). Cell lysates were prepared by scraping, sonication, and centrifugation. Cellular protein concentrations were
determined by the DC protein assay (Bio-Rad). Cell lysates were subjected to SDS-PAGE under reducing conditions, and the protein bands were then transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk for 2 hours at room temperature, and then incubated with STAT3 or the phospho-STAT3 antibody (Cell Signaling Technology Inc.) for 1 hour at room temperature. This was followed by incubation with the secondary antibody conjugated to horseradish peroxidase for 1 hour. Immunoreactive bands were visualized by an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK).

**Statistical analysis**

Data are shown as means ± SEM. Multiple group comparison was performed by one-way analysis of variance (ANOVA) followed by the Scheffe’s F-test procedure for comparison of means. Comparison between two groups was analyzed by the two-tailed t-test following the F-test. Values of $P<0.05$ were considered statistically significant.

**References**


**Supplemental figure legend**

**Supplemental figure 1**

(A) Echocardiography was performed in wild-type and MHC/MCP-1 mice at 12 weeks of age. (B) Body weights (BW) and heart weights (HW) were measured and the ratio of H.W./B.W. was calculated in wild-type and MHC/MCP-1 mice at 12 weeks of age. There were no differences in these parameters between wild-type and MHC/MCP-1 mice.

**Supplemental figure 2**

Total RNA was extracted from the heart and peripheral blood (PB) in wild-type and MHC/MCP-1 mice at baseline, 6 hours, and 24 hours after MI and analyzed for TNF-α mRNA expression by real-time RT-PCR. Results are expressed as means ± SEM (n=5).
## Supplemental figure 1

### A

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### B

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Supplemental figure 2

The graph shows the relative TNF-α mRNA expression in Wild-type and MHC/MCP-1 conditions at baseline, 6h, and 24h in both heart and PB samples.