Critical Role of Monocyte Chemoattractant Protein-1/CC Chemokine Ligand 2 in the Pathogenesis of Ischemic Cardiomyopathy

Nikolaos G. Frangogiannis, MD; Oliver Dewald, MD; Ying Xia, MD; Guofeng Ren, PhD; Sandra Haudek, PhD; Thorsten Leucker; Daniela Kraemer; George Taffet, MD; Barrett J. Rollins, MD, PhD; Mark L. Entman, MD

Background—Cardiac interstitial fibrosis plays an important role in the pathogenesis of ischemic cardiomyopathy, contributing to systolic and diastolic dysfunction. We have recently developed a mouse model of fibrotic noninfarctive cardiomyopathy due to brief repetitive myocardial ischemia and reperfusion. In this model, fibrotic changes are preceded by marked and selective induction of the CC chemokine monocyte chemoattractant protein-1 (MCP-1). We hypothesized that MCP-1 may mediate fibrotic remodeling through recruitment of mononuclear cells and direct effects on fibroblasts.

Methods and Results—Wild-type (WT) and MCP-1-null mice underwent daily 15-minute coronary occlusions followed by reperfusion. Additional WT animals received intraperitoneal injections of a neutralizing anti-MCP-1 antibody after the end of each ischemic episode. Hearts were examined echocardiographically and processed for histological and RNA studies. WT mice undergoing repetitive brief myocardial ischemia and reperfusion protocols exhibited macrophage infiltration after 3 to 5 days and marked interstitial fibrosis in the ischemic area after 7 days, accompanied by ventricular dysfunction. MCP-1-null mice had markedly diminished interstitial fibrosis, lower macrophage infiltration, and attenuated ventricular dysfunction compared with WT animals. MCP-1 neutralization also inhibited interstitial fibrosis, decreasing left ventricular dysfunction and regional hypocontractility. Cardiac myofibroblasts isolated from WT but not from MCP-1-null animals undergoing repetitive myocardial ischemia and reperfusion demonstrated enhanced proliferative capacity. However, MCP-1 stimulation did not induce cardiac myofibroblast proliferation and did not alter expression of fibrosis-associated genes.

Conclusions—Defective MCP-1 signaling inhibits the development of ischemic fibrotic cardiomyopathy in mice. The profibrotic actions of MCP-1 are associated with decreased macrophage recruitment and may not involve direct effects on cardiac fibroblasts. (Circulation. 2007;115:584-592.)

Key Words: inflammation ■ ischemia ■ reperfusion ■ immunology ■ pathology ■ fibrosis ■ cardiomyopathy

Monocyte chemoattractant protein-1 (MCP-1)/CC chemokine ligand 2, a member of the CC chemokine family, is upregulated in inflammatory and fibrotic processes and plays a key role in the development of pulmonary, renal, and skin fibrosis. The profibrotic actions of MCP-1 are thought to reflect its role in mononuclear cell recruitment and activation. Macrophages create a fibrotic milieu by releasing a wide variety of cytokines and growth factors. In addition, recent evidence suggests that MCP-1 may directly modulate fibroblast phenotype and gene expression, stimulating extracellular matrix production and metabolism.

Clinical Perspective p 592

We have recently demonstrated that reversibly dysfunctional myocardial segments from patients with ischemic cardiomyopathy exhibit an active inflammatory reaction, associated with MCP-1 upregulation and leukocyte infiltration. Inflammation ultimately leads to fibrous tissue deposition in the cardiac interstitium. Established interstitial fibrosis and extensive collagen deposition are associated with a decreased likelihood of functional recovery after surgical revascularization of the myocardium. We postulated that the development of myocardial fibrosis in patients with chronic ischemic heart disease results from activation of inflammatory pathways due to brief episodes of ischemia that do not induce cardiomyocyte death.

We have previously demonstrated that a single brief coronary occlusion followed by reperfusion resulted in reactive oxygen-dependent CC chemokine upregulation in both...
mouse\textsuperscript{9} and canine\textsuperscript{10} models, without inducing any permanent structural changes in the myocardium. Daily brief episodes of coronary occlusion and reperfusion induced a persistent reactive oxygen–mediated upregulation of MCP-1 mRNA in the myocardium (in the absence of significant cytokine induction), however, followed by myofibroblast accumulation and extensive interstitial fibrosis.\textsuperscript{11} Cardiac fibrosis regressed on discontinuation of the ischemic protocol and showed features resembling the pathological characteristics of human reversible ischemic cardiomyopathy associated with myocardial hibernation.\textsuperscript{8}

The present study examines whether MCP-1 induction plays an important role in the pathogenesis of fibrotic cardiomyopathy. We studied the effects of defective MCP-1 signaling in the development of interstitial fibrosis and dysfunction due to brief repetitive myocardial ischemia/reperfusion (I/R). Both MCP-1 gene disruption and inhibition with a neutralizing antibody protected the myocardium from the development of interstitial fibrosis and attenuated left ventricular dysfunction. In vitro studies indicated that these effects may not be due to direct actions of MCP-1 on cardiac myofibroblasts. The present findings suggest that MCP-1 plays a critical role in the development of interstitial fibrosis in ischemic noninfarctive cardiomyopathy.

### Methods

#### Animal Surgery and Repetitive I/R Protocol

Wild-type (WT) C57/BL6 mice (obtained from Charles River Laboratories, Wilmington, Mass) and MCP-1\textsuperscript{-/-} mice were used for experiments. MCP-1\textsuperscript{-/-} animals were genotyped with established polymerase chain reaction protocols. Female and male WT and MCP-1-null mice, 8 to 12 weeks of age, were anesthetized by an intraperitoneal injection of sodium pentobarbital (10 mg/g body weight) for the initial surgery. We used an established closed-chest mouse model of brief repetitive I/R to minimize the effects of surgery-related inflammation.\textsuperscript{11} Daily 15-minute coronary occlusions followed by reperfusion were performed for 3 to 7 consecutive days. An echocardiographic examination was performed 5 hours after the last ischemic episode, and the heart was immediately excised, fixed in zinc-formalin, and embedded in paraffin for histological studies, snap-frozen, and stored at −80°C for RNA isolation or enzymatically digested for fibroblast isolation. Sham animals were prepared in identical fashion without undergoing repetitive I/R protocols. Mice used for histology underwent 5 and 7 days of repetitive I/R, and for RNA extraction, 3 days of repetitive I/R (8 animals per group). Animals used for fibroblast isolation underwent 5 days of repetitive I/R.

We examined the effects of MCP-1 antibody inhibition on development of fibrotic cardiomyopathy using intraperitoneal injection of 20 μg of a goat anti-mouse MCP-1 neutralizing antibody (R&D Systems, Minneapolis, Minn), or goat IgG, at the end of each experiment. MCP-1\textsuperscript{-/-} mice were stimulated with recombinant mouse MCP-1 (10 to 100 ng/mL) for 4 to 16 hours. At the end of the experiment, RNA was extracted from the fibroblasts, and a ribonuclease protection assay (RiboQuant; Pharmingen) was performed with a commercially available peroxidase kit (Vector Laboratories, Burlingame, Calif) and developed with diaminobenzidine plus nickel (Vector) as described previously.\textsuperscript{12} The Mouse on Mouse kit (Vector) was used for α-SMA immunohistochemistry. Unmasking with trypsin was used for MCP-1 staining, whereas optimal identification of CD31-positive cells required unmasking and tyramide amplification with the TSA kit (Perkin Elmer, Boston Mass) as described previously.\textsuperscript{12} Quantitative assessment of macrophage density was performed by counting the number of F4/80-positive cells in the ischemic (left anterior descending coronary artery territory) and control area (posterior wall). Histochemical staining for collagen was performed with picrosirius red, and collagen content was quantitatively assessed with ImageQuaNT software.\textsuperscript{13}

#### RNA Extraction and Ribonuclease Protection Assay

RNA from the excised hearts was extracted as described previously.\textsuperscript{13} The mRNA expression levels of the cytokines tumor necrosis factor-α, interleukin (IL)-6, and IL-10; the growth factors transforming growth factor (TGF)-β1, TGF-β2, and TGF-β3; and the matrix-cellular protein osteopontin (OPN) were determined with a ribonuclease protection assay (RiboQuant; Pharmingen).\textsuperscript{11} After phosphorimaging of the gels, the signals were quantified with ImageQuaNT software and normalized to the housekeeping gene L32.

#### Isolation of Mouse Cardiac Fibroblasts

Mouse cardiac fibroblasts were isolated by enzymatic digestion with a collagenase buffer. Briefly, 3 hearts were isolated, dissected free of vessels and atria, transferred to 1 mL of collagenase buffer, and quickly minced into small pieces. Digestion with collagenase buffer continued until no visible tissue fragments were left. The isolated cell suspensions from each round were pelleted and washed. All cell suspensions were combined, plated on a T75 tissue-culture flask (Corning Corp, Acton, Mass) in full medium supplemented with 10% of fetal bovine serum (HyClone, Logan, Utah) and antibiotic-antimycotic solution. After overnight incubation, nonadherent cells were removed, and adherent cells were cultivated. On reaching confluence, cells were detached with trypsin/EDTA, split in a 1:2 or 1:4 ratio, and recultured. Characteristic fibroblast morphology was determined visually under a light microscope. Because the phenotype of fibroblasts can be influenced by growth conditions such as passage and cell density,\textsuperscript{14} only fibroblasts at passage 1 to 5 were used for experiments.

To study the effects of MCP-1 on fibroblast matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) mRNA expression, fibroblasts isolated from control WT hearts were stimulated with recombinant mouse MCP-1 (mMCP-1, 10 to 100 ng/mL; R&D Systems) for 4 to 16 hours. At the end of the experiment, RNA was extracted from the fibroblasts, and a ribonuclease protection assay was performed with a commercially available kit (Pharmingen). Expression of MMP-2, MMP-3, MMP-8, MMP-9, TIMP-1, TIMP-2, TIMP-3, and TIMP-4 was quantitatively assessed and normalized to L32.

#### Western Blotting

Cardiac fibroblasts isolated from control WT hearts were stimulated with recombinant mouse MCP-1 (10 to 100 ng/mL) for 4 to 16 hours. At the end of the experiment, protein was extracted from the fibroblasts, and expression of collagen type I, procollagen type III, tenasin-C, and α-SMA was studied with Western blotting.\textsuperscript{13} The following antibodies were used: rabbit anti-collagen type I (Rock-
land Immunochemicals, Inc, Gilbertsville, Pa), goat anti-procollagen III (Santa Cruz Biotechnology, Santa Cruz, Calif), rabbit antitenascin C (Chemicon), and rabbit anti-α-SMA (Abcam, Cambridge, Mass). Protein expression was quantitatively assessed after normalization for GAPDH levels (rabbit anti-GAPDH; Santa Cruz).

***Immunocytochemistry***

Pure fibroblast cultures were confirmed by immunocytochemistry with antibodies against vimentin (mesenchymal cell marker), α-SMA (both by Sigma), and collagen I (Rockland). Nonfibroblast contaminants were identified by antibodies against desmin (smooth muscle cell marker; Sigma) and CD31 (endothelial cell marker; Pharmingen). Cells were grown on 12-mm glass cover slips (BD BioCoat, BD Pharmingen), fixed in 2% paraformaldehyde, blocked, and incubated in primary antibody for 1 hour at room temperature. After they were washed with PBS, cells were incubated first with biotinylated anti-IgG reagent, then with horseradish peroxidase–conjugated avidin. Peroxidase substrate solution was added, and color development was observed under light microscopy.

***Fibroblast Proliferation Assay***

Proliferation was determined by bromodeoxyuridine incorporation with a commercially available colorimetric kit (Roche Applied Science, Indianapolis, Ind). To normalize data from different experiments, proliferation in response to serum was expressed as fold increase to cells maintained in serum-free medium. The proliferative response was compared between fibroblasts isolated from WT and MCP-1-null hearts after 5 days of repetitive I/R.

***Statistical Analysis***

Statistical analysis was performed with ANOVA followed by t test corrected for multiple comparisons (Student-Newman-Keuls) and with a statistical significance level <0.05. All data are expressed as mean±SEM.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

***Results***

MCP-1 Localization in the Ischemic Myocardium After Brief Repetitive I/R

Intense staining for MCP-1 was noted in the ischemic area after 5 days of repetitive I/R (Figure 1A and 1B). MCP-1 immunoreactivity was predominantly localized in cells with morphological characteristics of mononuclear cells. Mortality in MCP-1−/− and WT Mice Undergoing Brief Repetitive I/R

WT and MCP-1-deficient mice had similar survival rates after the initial instrumentation surgery (90.48% versus 91.77%). No WT animals and only 1 MCP-1-null mouse died during the repetitive I/R protocol.

MCP-1−/− Mice Exhibited Attenuated Left Ventricular Dysfunction After Brief Repetitive I/R

WT mice developed left ventricular dysfunction and regional hypocontractility of the ischemic anterior left ventricular wall after 7 days of repetitive I/R (Figure 1C and 1D). In contrast, MCP-1-null mice exhibited attenuated global and regional left ventricular dysfunction, showing significantly higher fractional shortening and anterior wall thickening after 7 days of repetitive I/R than WT animals (P<0.01).

MCP-1−/− Mice Showed Decreased Interstitial Fibrosis After Brief Repetitive I/R

To explore the pathological basis of the protective effects of MCP-1 deficiency on the development of cardiomyopathy, we studied the histological characteristics of WT and MCP-1−/− hearts after brief repetitive I/R. After 5 days of coronary I/R, WT mice showed increased interstitial cellularity (Figure 2A and 2C). After 7 days, the cardiac interstitium was widened, and picrosirius red staining revealed extensive deposition of collagen in the ischemic area (Figure 2D and 2F). In contrast, MCP-1−/− mice demonstrated only modest cellular infiltration after 5 days of I/R (Figure 2B) and had attenuated interstitial fibrosis in the ischemic area compared with WT mice (Figure 2E). MCP-1-null mice had markedly lower collagen content after 7 days of I/R compared with WT mice (collagen percent staining: WT, 19.4±1.02%; versus knockout, 10.09±0.42%; P<0.01; Figure 2G). In addition, myofibroblasts, identified as extravascular spindle-shaped α-SMA–expressing cells, were less abundant in the ischemic area of MCP-1−/− mice after 5 days of I/R than in WT animals (Figure 3A and 3B).
MCP-1-/- Mice Show Decreased Macrophage Infiltration in the Ischemic Area

To examine whether MCP-1 deficiency inhibits macrophage recruitment in the ischemic myocardium, we assessed macrophage density in mouse hearts using immunohistochemical staining with F4/80. Macrophages were rarely seen in control hearts in either WT or MCP-1-/- mice (Figure 3G). In WT mice, repetitive I/R resulted in significant macrophage infiltration into the ischemic area (left anterior descending coronary artery territory) after 5 days of repetitive I/R (Figure 3C and 3E). Macrophages were located around microvessels of the ischemic myocardial area (Figure 3E and 3F). MCP-1-/- mice had significantly lower macrophage density after 5 days of repetitive I/R than did the WT group (macrophage density: WT, 210.6±25.97 versus MCP-1-/-, 112±16.3 cells/mm²; P<0.01; Figure 3D and 3I).

MCP-1-/- Mice Show Decreased Expression of the Matricellular Proteins OPN and Tenascin-C After Repetitive I/R, Which Suggests Attenuated Interstitial Remodeling

Brief repetitive I/R results in marked induction of the matricellular proteins OPN (Figure 4A) and tenascin-C (Figure 4B), markers of interstitial remodeling. MCP-1-/- mice demonstrated markedly attenuated OPN mRNA induction after 3 days (Figure 4A) and significantly lower tenascin-C expression after 5 days of repetitive I/R (Figure 4C), consistent with decreased fibrotic remodeling of the ischemic myocardium.

Repetitive Brief I/R Does Not Induce Proinflammatory Cytokine Synthesis in WT and MCP-1-Null Mice; TGF-β Isoform Induction Is Comparable in WT and MCP-1-/- Animals Undergoing Brief I/R Protocols

The proinflammatory cytokines tumor necrosis factor-α, IL-1β, and IL-6 and the inhibitory cytokine IL-10 showed no significant upregulation after brief repetitive I/R in either WT or MCP-1-/- mice (cytokine:L32 ratio <0.02). TGF-β1 and TGF-β3 mRNA expression was modestly induced in both WT and MCP-1-null mice after 3 days of brief repetitive I/R. Levels of cardiac TGF-β isoform expression after brief coronary occlusion and reperfusion were comparable in WT and MCP-1-null hearts (P=NS; Figure 5).

MCP-1 Antibody Neutralization Decreases Interstitial Fibrosis and Left Ventricular Dysfunction in Mice Undergoing Brief Repetitive I/R

MCP-1 antibody neutralization protected mice undergoing repetitive I/R from the development of fibrotic cardiomyopathy. Antibody-treated mice had significantly lower collagen deposition after 7 days of reperfusion (Figure 6A) than vehicle-treated animals (collagen percent staining: vehicle-treated mice, 21.47±2.32% versus antibody-treated mice, 12.91±0.99%; P<0.01). In addition, antibody neutralization attenuated left ventricular dysfunction, which resulted in increased fractional shortening (Figure 6B) and enhanced anterior wall thickening (Figure 6C) after 7 days of brief repetitive I/R.

Fibroblasts Isolated From WT Mice Undergoing Repetitive I/R Exhibit Increased Proliferative Capacity, Which Is Abrogated in MCP-1-/- Mice

Cardiac fibroblasts isolated from control and cardiomyopathic mouse hearts were characterized at passage 1 as vimentin-positive, CD31-negative (nonendothelial) cells. Cultured cardiac fibroblasts showed expression of α-SMA but were desmin-negative, exhibiting features of phenotypic modulation to myofibroblasts. Incubation of control cardiac myofibroblasts with 5% FBS markedly increased their proliferative activity; however, MCP-1 stimulation in the absence of serum had no significant effect on myofibroblast proliferation (Figure 7A). When incubated with 5% FBS, cardiac myofibroblasts isolated from WT ischemic and reperfused hearts had significantly higher proliferative activity than myofibroblasts isolated from MCP-1-null hearts after 5 days of repetitive I/R (Figure 7B).

MCP-1 Stimulation Does Not Alter Expression of Genes Associated With Cardiac Fibrosis

To assess the effects of MCP-1 on fibroblast MMP and TIMP expression, isolated cardiac myofibroblasts from control WT mice were stimulated with mMCP-1 (10 or 100 ng/mL) for 4 to 16 hours. Western blotting experiments demonstrated that MCP-1 did not affect α-SMA, collagen type I, procollagen...
type III, or tenascin-C protein expression by cardiac myofibroblasts (Table). Ribonuclease protection assay analysis demonstrated that both unstimulated and MCP-1–stimulated cardiac myofibroblasts showed negligible MMP-8, MMP-9, and TIMP-4 mRNA expression (mRNA levels \( <2\% \) of L32; not shown). Control cardiac myofibroblasts showed significant mRNA expression of MMP-2, -3, TIMP-1, TIMP-2, and TIMP-3, which remained unchanged after stimulation with MCP-1 (Table).

**Discussion**

Development of interstitial fibrosis plays an important role in the pathogenesis of ischemic cardiomyopathy and contributes to both diastolic and systolic dysfunction by increasing both passive and active stiffness of the ventricle. Interstitial collagen deposition, in the absence of a completed infarction, is often found in dysfunctional myocardial segments from patients with ischemic cardiomyopathy and correlates in-
versely with contractile reserve.\textsuperscript{16} Although the role of the development of myocardial fibrosis in the pathogenesis of ischemic cardiomyopathy has been recognized, the pathogenic mechanisms responsible for fibrous tissue deposition in the myocardium, in the absence of cardiomyocyte death, remain unknown.\textsuperscript{17,18}

Fibrous tissue deposition in healing myocardial infarcts is critically involved in postinfarction cardiac repair, depends on activation of inflammatory pathways, and ultimately results in replacement of dead cardiomyocytes with a collagen-based scar. Although collagen deposition is an important component of the reparative mechanisms after myocardial infarction, evidence suggests that fibrogenic pathways are also triggered by brief or sublethal ischemic insults that do not result in significant cardiomyocyte loss. We have previously demonstrated that a single brief (15 minutes) coronary occlusion, followed by reperfusion, induces marked upregulation of the CC chemokine MCP-1 in the canine myocardium in a reactive oxygen–dependent manner but is not sufficient to elicit leukocyte infiltration.\textsuperscript{10} However, brief repetitive coronary occlusion/reperfusion induces persistent upregulation of MCP-1 that leads to development of fibrotic changes and left ventricular dysfunction in the absence of myocardial infarction.\textsuperscript{11} The present study establishes the critical role of MCP-1 in the development of ischemic noninfarctive cardiomyopathy. MCP-1-null mice exhibited attenuated interstitial fibrosis (Figure 2) and systolic dysfunction (Figure 1) compared with WT animals after brief repetitive I/R. MCP-1 neutralization also protected mice against the development of ischemic cardiomyopathy (Figure 6).

The most thoroughly characterized CC chemokine, MCP-1/CC chemokine ligand 2, is a potent chemoattractant for monocytes, T cells, and NK cells and has been implicated in diseases characterized by monocyte-rich infiltrates.\textsuperscript{19–21} Recent investigations have suggested that MCP-1 and its cognate receptor, CCR2, may play an important role in the development of pulmonary\textsuperscript{1,22} and renal\textsuperscript{2} fibrosis. MCP-1 overexpression in the murine heart results in marked macrophage infiltration, dilative remodeling, and fibrosis.\textsuperscript{23} MCP-1 may mediate its profibrotic effects through several distinct mechanisms. First, mononuclear cells chemotactically attracted through MCP-1/CCR2 signaling may be an important source of fibrogenic mediators, such as TGF-\beta and fibroblast growth factors. In vitro studies have demonstrated that MCP-1 enhances portal fibroblast proliferation and myofibroblast differentiation,\textsuperscript{24} upregulates collagen and TGF-\beta1 expression by rat pulmonary fibroblasts,\textsuperscript{4} and stimulates production of MMP-1 and TIMP-1 by human skin fibroblasts.\textsuperscript{25} In pulmonary fibrosis, the fibrogenic actions of MCP-1 may be mediated at least in part through inhibition of prostaglandin E2, a potent suppressor of fibroblast proliferation and activation.\textsuperscript{26} Third, MCP-1 may be an important mediator in the recruitment of fibro-
MCP-1 Stimulation Does Not Alter Expression of Proteins Associated With Fibrosis in Isolated Cardiac Fibroblasts

<table>
<thead>
<tr>
<th>Protein/Transcript</th>
<th>Control</th>
<th>4-h MCP-1 (10 ng/mL)</th>
<th>16-h MCP-1 (10 ng/mL)</th>
<th>16-h MCP-1 (100 ng/mL)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein expression (protein: GAPDH)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-SMA</td>
<td>1.14±0.12</td>
<td>1.18±0.04</td>
<td>1.15±0.08</td>
<td>1.18±0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Collagen I</td>
<td>0.70±0.11</td>
<td>0.60±0.03</td>
<td>0.58±0.03</td>
<td>0.73±0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Procollagen III</td>
<td>0.62±0.08</td>
<td>0.55±0.04</td>
<td>0.49±0.04</td>
<td>0.97±0.28</td>
<td>NS</td>
</tr>
<tr>
<td>Tenascin C</td>
<td>1.08±0.24</td>
<td>0.84±0.02</td>
<td>0.97±0.14</td>
<td>1.01±0.02</td>
<td>NS</td>
</tr>
<tr>
<td>mRNA expression (gene:L32)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>0.021±0.003</td>
<td>0.019±0.001</td>
<td>0.019±0.001</td>
<td>0.021±0.002</td>
<td>NS</td>
</tr>
<tr>
<td>MMP-3</td>
<td>0.024±0.003</td>
<td>0.022±0.001</td>
<td>0.021±0.003</td>
<td>0.018±0.001</td>
<td>NS</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>1.45±0.13</td>
<td>1.42±0.02</td>
<td>1.60±0.10</td>
<td>1.85±0.17</td>
<td>NS</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>0.71±0.05</td>
<td>0.63±0.01</td>
<td>0.81±0.05</td>
<td>0.81±0.05</td>
<td>NS</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>0.70±0.05</td>
<td>0.67±0.02</td>
<td>0.66±0.04</td>
<td>0.78±0.03</td>
<td>NS</td>
</tr>
</tbody>
</table>

Figure 7. Although cardiac myofibroblasts isolated from MCP-1-null mice undergoing repetitive I/R protocols have decreased proliferative response, MCP-1 does not stimulate proliferation of control cardiac fibroblasts. A, Cardiac fibroblasts were isolated from control WT hearts. MCP-1 stimulation, in the absence of serum, does not induce cardiac fibroblast proliferation. In contrast, serum incubation markedly increases fibroblast proliferative activity (**P<0.01). B, Cardiac fibroblasts isolated from WT mice after 5 days of repetitive I/R demonstrated an intense proliferative response after stimulation with serum. In contrast, cardiac fibroblasts isolated from MCP-1-null animals after 5 days of repetitive I/R show suppressed proliferative activity on stimulation with serum (**P<0.01 vs WT I/R). C indicates control unstimulated fibroblasts.

cytes, a circulating population of cells that share leukocyte and mesenchymal markers and are capable of myofibroblast differentiation.27 Recent investigations indicated that MCP-1/CCR2 signaling is important for recruitment of fibrocytes to the alveolar space after fibrotic injury in the lung.28

The present experiments did not support direct effects of MCP-1 on cardiac myofibroblasts as the primary mechanism responsible for fibrosis after brief repetitive I/R. MCP-1 stimulation did not modulate collagen, tenascin-C, MMP, and TIMP synthesis by isolated cardiac myofibroblasts (Table) and failed to induce fibroblast proliferation. MCP-1 gene disruption, however, significantly decreased macrophage infiltration after brief repetitive I/R (Figure 3) and resulted in suppression of OPN mRNA synthesis (Figure 4), a matricellular protein markedly induced during monocyte-to-macrophage differentiation.20 Furthermore, myofibroblasts isolated from WT hearts after 5 days of brief repetitive I/R exhibited much higher proliferative capacity than myofibroblasts derived from MCP-1-null hearts undergoing repetitive coronary occlusion and reperfusion protocols (Figure 7B). These findings suggest that the profibrotic actions of MCP-1 may depend on paracrine signals released by leukocytes recruited in the myocardium through MCP-1/CCR2 interactions. However, both WT and MCP-1-null hearts demonstrated comparable levels of TGF-β1, TGF-β2, and TGF-β3 mRNA expression (Figure 5) after repetitive I/R, which suggests that the effects of MCP-1 are not mediated through the transcriptional regulation of TGF-β isoforms.

MCP-1 appears to play an important role in regulating fibrous tissue deposition in the injured heart. We have recently demonstrated that MCP-1 critically regulates mononuclear cell recruitment and activation in healing myocardial infarcts.30 MCP-1-null mice show delayed replacement of dead cardiomyocytes with granulation tissue, suppressed cytokine expression, and diminished myofibroblast infiltration in the infarcted myocardium, which leads to attenuated left ventricular remodeling. Thus, MCP-1 is a crucial mediator for timely clearance of dead tissue from the healing infarct but also enhances the inflammatory response and stimulates fibrous tissue deposition, which leads to adverse remodeling. In contrast, brief repetitive I/R is not associated with significant cardiomyocyte loss. Thus, activation of MCP-1-induced inflammation in response to brief repetitive ischemic insults does not serve to clear dead cardiomyocytes and results in development of interstitial fibrosis, inducing cardiac dysfunction.

The present findings have important implications for our understanding of the pathogenesis of cardiomyopathy in patients with chronic ischemic heart disease. Patients with advanced ischemic cardiomyopathy exhibit extensive collagen deposition in the form of segmental replacement and diffuse interstitial fibrosis.31 Bundles of fibrillar collagen surround small clusters of cardiomyocytes,31 contributing to the genesis of ventricular dysfunction in the absence of extensive myocyte loss. Patients with severe coronary disease have frequent brief episodes of demand ischemia that do not lead to myocardial infarction but may induce reactive oxygen–mediated chemokine upregulation. Myocardial MCP-1 induction stimulates mononuclear cell recruitment, creating a
fibrogenic milieu that induces extracellular matrix deposition and regional myocardial dysfunction. We have recently demonstrated that dysfunctional myocardial segments from patients with ischemic cardiomyopathy show increased MCP-1 expression and local leukocyte infiltration. Furthermore, segments with recovery of function after surgical revascularization exhibit higher MCP-1 levels and enhanced inflammatory activity compared with segments with irreversible dysfunction. In contrast, persistently dysfunctional myocardium exhibits decreased inflammatory activity and enhanced collagen deposition. These findings suggest that the development of ischemic cardiomyopathy is a continuous process: At an early stage, brief episodes of ischemia may induce an inflammatory response that activates fibrogenic pathways. Prolonged activation of ischemia-induced inflammatory pathways may trigger inhibitory mechanisms (such as TGF-β activation) that suppress proinflammatory mediator synthesis, however, while inducing synthesis of genes associated with fibrosis.

The present findings suggest that MCP-1 may be a novel therapeutic target in patients with ischemic cardiomyopathy. Reversibility of the cardiomyopathic process may depend on timely downregulation of the inflammatory process and inhibition of the MCP-1 response. This could gradually lead to lower numbers of resident macrophages and diminished synthesis of fibrogenic substances. In contrast, segments with persistent dysfunction may have reached a “point of no return” at which long-standing hypoxia-mediated inflammation has led to induction of inhibitory mediators, suppression of MCP-1 synthesis, and extensive fibrosis. Thus, there may be a narrow window of therapeutic opportunity for the use of anti-MCP-1 strategies in ischemic cardiomyopathy. In addition, MCP-1 inhibition may be fraught with risks in patients with chronic ischemic cardiomyopathy. MCP-1 may be important in the formation of collateral vessels in the ischemic myocardium by recruiting macrophages capable of releasing angiogenic mediators and by exerting direct angiogenic actions on endothelial cells. The significance of these interactions in myocardial angiogenesis cannot be tested in the model used in the present study.

Inflammatory mechanisms play a critical role in the pathogenesis of cardiac fibrosis and dysfunction. Although activation of inflammatory pathways has a reparative function in healing infarcts, chemokine induction in response to brief reversible ischemic insults occurs in the absence of a completed infarction, has no protective effects, and results in fibrotic interstitial remodeling. Early inhibition of the chemokine response may suppress activation of fibrogenic pathways, preventing irreversible fibrosis and dysfunction.

Sources of Funding
This work was supported by National Institutes of Health grants R01 HL-76246 (Dr Frangogiannis) and P01 HL-42550 (Drs Entman and Frangogiannis) and a grant-in-aid from the American Heart Association (Dr Frangogiannis). Dr Dewald is supported by the Deutsche Forschungsgemeinschaft and BONFOR Medical School, University of Bonn, Germany.

Disclosures
Dr Rollins has received honoraria from Novartis Pharma and Merrimack Pharmaceuticals. The remaining authors report no conflicts.

References
7. Frangogiannis NG, Shimi S, Chang SM, Ren G, Dewald O, Gersch C, Shen G, Aggeli C, Reardon MJ, Letsou GV, Espada R, Ramchandani M, Entman ML, Zoghbi WA. Inhibition of the MCP-1 response. This could gradually lead to lower numbers of resident macrophages and diminished synthesis of fibrogenic substances. In contrast, segments with persistent dysfunction may have reached a “point of no return” at which long-standing hypoxia-mediated inflammation has led to induction of inhibitory mediators, suppression of MCP-1 synthesis, and extensive fibrosis. Thus, there may be a narrow window of therapeutic opportunity for the use of anti-MCP-1 strategies in ischemic cardiomyopathy. In addition, MCP-1 inhibition may be fraught with risks in patients with chronic ischemic cardiomyopathy. MCP-1 may be important in the formation of collateral vessels in the ischemic myocardium by recruiting macrophages capable of releasing angiogenic mediators and by exerting direct angiogenic actions on endothelial cells. The significance of these interactions in myocardial angiogenesis cannot be tested in the model used in the present study.

Inflammatory mechanisms play a critical role in the pathogenesis of cardiac fibrosis and dysfunction. Although activation of inflammatory pathways has a reparative function in healing infarcts, chemokine induction in response to brief reversible ischemic insults occurs in the absence of a completed infarction, has no protective effects, and results in fibrotic interstitial remodeling. Early inhibition of the chemokine response may suppress activation of fibrogenic pathways, preventing irreversible fibrosis and dysfunction.

Disclosures
Dr Rollins has received honoraria from Novartis Pharma and Merrimack Pharmaceuticals. The remaining authors report no conflicts.
Development of interstitial fibrosis plays an important role in the pathogenesis of ischemic cardiomyopathy and contributes to both diastolic and systolic dysfunction by increasing both passive and active stiffness of the ventricle. Inflammatory mechanisms may critically regulate fibrotic remodeling of the myocardium. The present study establishes the critical role of the CC chemokine monocyte chemotactrant protein-1 (MCP-1) in the development of fibrotic cardiomyopathy using a mouse model of brief repetitive myocardial ischemia and reperfusion. MCP-1-null mice exhibited decreased interstitial fibrosis and attenuated systolic dysfunction compared with wild-type animals after brief repetitive myocardial ischemia and reperfusion. MCP-1 neutralization also protected mice against the development of ischemic cardiomyopathy. Our findings have important implications for our understanding of the pathogenesis of cardiomyopathy in patients with chronic ischemic heart disease. Patients with advanced ischemic cardiomyopathy exhibit extensive collagen deposition in the form of segmental replacement and diffuse interstitial fibrosis. Patients with severe coronary disease have frequent brief episodes of demand ischemia that do not lead to myocardial infarction but may induce reactive oxygen–mediated chemokine upregulation. Myocardial MCP-1 induction stimulates mononuclear cell recruitment, which creates a fibrogenic milieu that induces extracellular matrix deposition and regional myocardial dysfunction. Our findings suggest that MCP-1 may be a novel therapeutic target in patients with ischemic cardiomyopathy. The transient induction of MCP-1 in the ischemic myocardium, however, suggests that there may be a narrow window of therapeutic opportunity for the use of anti-MCP-1 strategies in ischemic cardiomyopathy. In addition, MCP-1 inhibition may carry the risk of inhibiting collateral vessel formation in patients with chronic ischemia.
Critical Role of Monocyte Chemoattractant Protein-1/CC Chemokine Ligand 2 in the Pathogenesis of Ischemic Cardiomyopathy
Nikolaos G. Frangogiannis, Oliver Dewald, Ying Xia, Guofeng Ren, Sandra Haudek, Thorsten Leucker, Daniela Kraemer, George Taffet, Barrett J. Rollins and Mark L. Entman

Circulation. 2007;115:584-592
doi: 10.1161/CIRCULATIONAHA.106.646091
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/115/5/584

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/