Targeted Deletion of CC Chemokine Receptor 2 Attenuates Left Ventricular Remodeling after Experimental Myocardial Infarction

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A key component of cardiac remodeling after acute myocardial infarction (MI) is the inflammatory response, which modulates cardiac tissue repair. The purpose of this study was to investigate the relationship between the monocytic inflammatory response and left ventricular remodeling after MI using mice deficient in CC chemokine receptor 2 (CCR2), the primary receptor for the critical regulator of CC chemokine ligand 2. Immunohistochemical analysis revealed rapid infiltration of macrophages into infarcted tissue within 7 days in wild-type (WT) mice. However, this process was greatly impaired in CCR2-deficient (CCR2^{-/-}) mice. Echocardiography demonstrated beneficial effects of CCR2 deficiency on left ventricular remodeling at 7 and 28 days after MI. *In situ* zymography showed augmented gelatinolytic activity in WT mice within 7 days after MI, whereas gelatinolytic activity was barely detectable in CCR2^{-/-} mice. Moreover, the distribution of gelatinolytic activity in serial sections was very similar to the distribution of macrophages rather than neutrophils. Expression of matrix metalloproteinases and tumor necrosis factor- α mRNAs was up-regulated in infarcted regions from WT mice compared to CCR2^{-/-} mice at 3 days after MI. Direct inhibition of CCR2 functional pathway might contribute to the attenuation of left ventricular remodeling after MI. (*Am J Pathol* 2004, 165:439–447)

Left ventricular remodeling after acute myocardial infarction (MI) is dependent on the extent of the initial ischemic damage and the subsequent healing of the infarct and surrounding tissues.^{1,2} Excessive degradation of extracellular matrix components in infarcted regions appears to result in pathological cardiac remodeling, left ventricular dilatation, and cardiac failure. Matrix metalloprotein-

ases (MMPs) are thought to be the major physiological regulators of the extracellular matrix and have been implicated in the pathogenesis of left ventricular remodeling after MI, which can result in postinfarct ventricular failure.³ Indeed, there is increasing evidence that gelatinases (MMP-2 and MMP-9), important MMPs that also have broader substrate specificities for type IV collagen, elastin, and vitronectin, play a crucial role in the process of ventricular remodeling after MI.^{4–6}

Recent studies have also demonstrated that a key component of cardiac remodeling after MI is the inflammatory response, which can modulate left ventricular tissue repair.^{6–10} It appears that matrix degradation is focused to the area of inflammation and injury after myocardial ischemia/reperfusion, which suggests a significant role for neutrophil-derived MMP-9 activation in myocardial tissues.^{10,11} However, Ducharme and colleagues⁶ demonstrated that MMP-9-deficient mice exhibited attenuated left ventricular dilatation, reduced macrophage infiltration, and collagen accumulation after MI, which suggested a relationship between macrophage infiltration into the infarcted tissue and cardiac remodeling by MMP activation. Nonetheless, as yet there has been no direct evidence that macrophage infiltration into infarcted myocardium accelerates left ventricular remodeling through the promotion of activated MMPs.

Based on these observations, we hypothesized that macrophage infiltration is a critical step in ventricular remodeling after MI. In the present study, we used genetically modified mice deficient in CC chemokine receptor 2 (CCR2), the major receptor for CC chemokine ligand 2 [CCL2, formerly known as monocyte chemoattractant protein-1 (MCP-1)]. CCR2-deficient (CCR2^{-/-}) mice have been shown to exhibit pronounced defects in CCL2-induced leukocyte firm adhesion to microvascular endothelium and reduced monocyte extravasation.¹² The purpose of the present study was to investigate the relationship between the monocytic inflammatory response and left ven-

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tricular remodeling after experimental MI using mice deficient in CCR2 gene expression.

Materials and Methods

Animal Preparation

CCR2^{-/-} mice were generated by gene targeting as previously described,¹² and were backcrossed over seven times to the control C57BL/6J strain. Wild-type (WT) mice of the same genetic background were purchased from the Jackson Laboratory (Bar Harbor, ME). CCR2^{-/-} and WT female mice were bred at the Animal Resource Facility at the Kumamoto University under specific pathogen-free conditions. All animal procedures were approved by the Animal Research Committee at the Kumamoto University, and all procedures conformed to the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources. CCR2^{-/-} and WT mice were fed a regular chow diet and were used for experiments between 8 and 12 weeks of age. CCR2^{-/-} ($n = 138$) and WT ($n = 138$) mice that survived 6 hours after coronary ligation or sham operation were randomly divided into groups for microscopic cardiac analysis, total RNA isolation, and echocardiographic evaluation.

Left Coronary Ligation

Mice were anesthetized using pentobarbital sodium (70 mg/kg) via intraperitoneal injection. In the supine position, endotracheal intubation was performed under direct laryngoscopy, and the mice were ventilated with a small animal respirator (tidal volume, 0.3 ml; rate, 110 breaths/minute; Shinano Co., Tokyo, Japan). All surgical procedures were performed using an operating microscope (Olympus Co., Tokyo, Japan). A left thoracotomy was performed between the fourth and fifth ribs. The left anterior descending artery was visualized under the microscope after removing the pericardial sac, and an 8-0 nylon suture was placed through the myocardium into the anterolateral wall of left ventricle (LV). Significant electrocardiogram and color changes at the ischemic area were considered indicative of successful coronary occlusion. The chest cavity was then closed in layers with 5-0 silk, and the animals were gradually weaned from the respirator. The endotracheal tube was removed once spontaneous respiration resumed, and the animals were placed on a warm pad maintained at 37°C until the mice were completely awake.

Echocardiography

Serial echocardiographic measurements at baseline, 7, and 28 days after surgery were performed using a Sonos 4500 with a high-frequency transducer (12 MHz) (Philips Co.). Mice were weighed and anesthetized with 2% tribromoethanol solution (0.01 ml/g) via intraperitoneal injection. The mice were held carefully in the left hand by grasping the mouse at the skin on the back of the neck

and wrapping the tail as previously described.¹³ Good two-dimensional views of the LV were obtained for guided M-mode measurements of the LV internal diameter at end diastole (LVDD) and end systole (LVDS), as well as the interventricular septal wall thickness, and posterior wall thickness at the same points. Percent fractional shortening (%FS) were calculated by the following formulas: $\%FS = [(LVDD - LVDS)/LVDD] \times 100$. Mice were randomly numbered, and genotypes were confirmed by Southern blot analysis at the end of the echocardiographic studies as previously reported.¹²

Light Microscopy and Morphometric Analysis

At 1, 3, 7, 14, and 28 days after coronary ligation, mice were sacrificed for gross and microscopic cardiac analyses, with 6 to 10 animals studied for each time point. Heart tissues were fixed in 4% paraformaldehyde solution at 4°C for 4 hours, embedded in OCT compound (Miles, Elkhart, IN), frozen in liquid nitrogen, and cut by a cryostat into 6- μ m-thick sections. Sections were routinely stained with hematoxylin and eosin for light microscopy, and Masson's trichrome and van Gieson stains were also performed for evaluation of myocardial fibrosis. Infarct size was determined by the method previously reported.¹⁴ In brief, infarct length was measured along the endocardial and epicardial surfaces from each of the three LV sections, and values from all sections 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 times 100. Infarct area was calculated as the percentage of MI area relative to the entire left ventricular tissue area as previously reported.¹⁵ The collagen volume fraction was calculated as the ratio of the sum of the total area of interstitial fibrosis to the sum of the total connective tissue area plus the myocyte area in the entire visual field of the section, as previously reported.^{6,15}

Immunohistochemistry

Immunohistochemistry was performed according to an indirect immunoperoxidase method using the following antibodies: anti-CD68 (FA-11; Serotec, Oxford, UK); anti-granulocyte (Gr-1; Southern Biotechnology, Birmingham, AL); anti-T lymphocyte (Thy1.2; Caltag Lab., San Francisco, CA); and Ly-1: Pharmingen, San Diego, CA); and anti-B lymphocyte (B220, Pharmingen). After inhibition of endogenous peroxidase activity by the method of Isobe and colleagues,¹⁶ the sections were incubated with the monoclonal antibodies described above at 4°C overnight. The goat anti-rat Ig-conjugated peroxidase-labeled polymer amino acid (Nichirei, Tokyo, Japan) was used as the secondary antibody. After visualization with 3,3'-diaminobenzidine, sections were stained with hematoxylin for nuclear staining and were mounted with resin. As negative controls, the same procedures were performed but without the primary antibodies. For cell enumeration, numbers of positive cells in the infarcted region for each

Table 1. Body Weight (BW), Heart Weight (HW), and HW/BW in Mice at Baseline and at 28 Days after Coronary Ligation

	Day	WT MI	WT sham	CCR2 ^{-/-} MI	CCR2 ^{-/-} sham
Body weight (BW), g	0	19 ± 0.2	20 ± 0.3	19 ± 0.2	19 ± 0.2
	28	21 ± 0.2*	21 ± 0.3*	20 ± 0.2*	20 ± 0.3*
Heart Weight (HW), mg	28	129 ± 6 ^{††}	97 ± 1	112 ± 4 [†]	97 ± 3
HW/BW, mg/g	28	6.2 ± 0.3 ^{†§}	4.7 ± 0.1	5.5 ± 0.2 [†]	4.9 ± 0.2

Data are expressed as mean ± SEM. Statistical significance was determined by Student's *t*-test and analysis of variance.

**P* < 0.01 versus baseline.

[†]*P* < 0.01 versus each sham-operated group.

^{††}*P* < 0.01 versus CCR2^{-/-} MI group.

[§]*P* < 0.05 versus CCR2^{-/-} MI group.

antibody were counted and calculated as the number per 1 mm².

Detection of Gelatinolytic Activity by *in Situ* Zymography

To detect gelatinolytic activity in the infarcted heart tissues, we performed *in situ* zymography using an approach previously reported.¹⁷ Fresh specimens of infarcted myocardium were embedded without fixation in OCT compound (Miles). Serial frozen sections were made by a cryostat and mounted onto slides coated with 7% gelatin solution (Fuji Photo Film Co., Tokyo, Japan) or slides only. Gelatin films with sections were incubated for 6 hours at 37°C in a moisture chamber and stained with Biebrich Scarlet solution (Wako, Osaka, Japan). The gelatin in contact with the proteolytic areas of the sections was digested, giving zones of enzymic activity indicated by negative staining. The digested areas in the sections were compared with serial sections immunostained with monoclonal antibodies for CD68 (FA-11) or granulocytes (Gr-1). To distinguish MMP activity from other proteinase activity in the tissue, gelatin films containing the MMP inhibitor 1,10-phenanthroline (Fuji Photo Film Co.) were used, and the frozen sections were mounted as described above.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Assay

Total RNA from heart tissues at 3 days after MI was extracted by the RNeasy B method (Tel-Test, Inc., Friendswood, TX). Total RNA was reverse-transcribed into cDNA using random primers (Life Technologies, Inc., Rockville, MD). For detection of MMP-8, MMP-9, and MMP-13, tissue inhibitor of metalloproteinase-1 (TIMP-1), TIMP-4, and tumor necrosis factor- α (TNF- α) mRNA levels in heart tissue, real-time PCR was performed using an ABI PRISM 7700 sequence detection system with TaqMan Universal PCR Master Mix and Assays-on-Demand gene expression probes (Applied Biosystems, Foster City, CA). TaqMan Rodent 18S Ribosomal RNA Control Regents VIC (Applied Biosystems) was used as an endogenous control gene. A standard curve for the serial dilution of murine heart cDNA was generated. The amplification cycle consisted of 2 minutes at 50°C, 10 minutes at 95°C, 15 seconds at 95°C, and 1 minute at 60°C.

Relative quantitation values of targets were normalized according to the endogenous 18S ribosomal RNA gene control.

Statistical Analysis

Data are expressed as mean ± SEM. Paired data were compared by Student's *t*-test. Comparisons between multiple groups were performed by one-way analysis of variance followed by Fisher's protected least significant difference test. Comparisons of the time-related changes in LVDD and %FS among groups were performed by two-way analysis of variance followed by Bonferroni's multiple-comparison *t*-test. Results with *P* < 0.05 were considered statistically significant.

Results

Basic Measurements at Baseline and at 28 Days after Coronary Ligation

At baseline, mean body weights were not significantly different between the four groups, and significantly increased in both MI groups and sham-operated groups at 28 days (Table 1). Relative heart weights (heart weight/body weight) significantly increased in MI animals compared to the respective sham-operated groups (*P* < 0.01), but were greater in WT MI mice than in CCR2^{-/-} MI mice (*P* < 0.05, Table 1). The death rate 28 days after MI was 7.5% for WT mice and 8.1% for CCR2^{-/-} mice, with the most prevalent cause of death being cardiac rupture. There were no significant differences in terms of survival or cardiac rupture rates between WT and CCR2^{-/-} mice at 28 days after MI.

Infarct Size, Infarct Area, and Collagen Volume Fraction

To evaluate remodeling of the infarcted myocardium, infarct size, infarct area, and collagen volume fraction were measured 7 days after MI. As shown in Figure 1, B and C, infarct size and area were almost identical between the two MI groups (57 ± 0.8% and 39 ± 2%, respectively, for the WT group versus 53 ± 2% and 42 ± 3%, respectively, for the CCR2^{-/-} group). Collagen vol-

Figure 1. **A:** Representative examples of Masson's trichrome and van Gieson staining at 7 days after experimental MI. Stained slides demonstrate that necrotic centers of infarcted regions remain devoid of fibrosis in CCR2^{-/-} mice, with less accumulation of collagen in CCR2^{-/-} mice than in WT mice. **B** and **C:** Infarct size (**B**) and infarct area (**C**) in WT and CCR2^{-/-} mice. **D:** Collagen volume fraction from van Gieson-stained myocardium as a percentage of stained tissue in muscle areas and connective tissue in visual fields of the sections. *, $P < 0.01$ versus CCR2^{-/-} mice.

ume fraction in infarcted regions increased significantly in WT mice compared to CCR2^{-/-} mice ($75 \pm 3\%$ in WT mice versus $60 \pm 3\%$ in CCR2^{-/-} mice) (Figure 1D). In addition to the reduced collagen accumulation in infarcted regions, necrotic centers tended to remain devoid of fibrosis in CCR2^{-/-} mice, as shown in Figure 1A. Collagen volume fractions in noninfarcted regions were not significantly different between WT and CCR2^{-/-} groups (Figure 1D).

Immunohistochemical Analysis of the Constituent Cells of the Infarcted Myocardium

To identify and quantitate the constituent cells in the infarcted myocardium, cells immunostaining positive for

cell-specific monoclonal antibodies were counted. Staining for the macrophage marker FA-11 revealed the rapid infiltration of macrophages into infarcted regions in WT mice, which peaked at 7 days after MI. However, this process was greatly impaired in CCR2^{-/-} mice (Figures 2 and 3A). In contrast, the accumulation of Gr-1-positive granulocytes into infarcted regions increased, peaked at 3 days after MI, and gradually decreased in both groups (Figure 3B). However, the number of Gr-1-positive cells was higher in CCR2^{-/-} mice than in WT mice at 7 days after MI. Although some cells stained positive for Thy-1.2, Ly-1, and B220 monoclonal antibodies (T- and B-cell makers) in WT and CCR2^{-/-} mice, the numbers of these cell types were not significantly different between the two groups (Figure 3, C and D).

Figure 2. Immunohistochemical detection of FA-11-positive macrophages in infarcted regions from WT (left) and CCR2^{-/-} (right) mice at 1, 3, 7, 14, and 28 days after experimental MI. Scale bars, 100 μ m.

Echocardiographic Measurements

Serial two-dimensional and M-mode echocardiography was performed in sham-operated ($n = 6$) and MI ($n = 14$) mice in each group. There were no significant differences in LVDD and %FS between all groups at baseline. As shown in Figure 4A, marked dilatation of LVDD was observed 7 and 28 days after MI in both WT and CCR2^{-/-} mice, however, these changes were attenuated in CCR2^{-/-} mice compared with WT mice. %FS decreased at 7 and 28 days after MI in both groups, but was also attenuated in CCR2^{-/-} mice compared to WT mice at 28 days after MI (Figure 4B).

Figure 3. Differences in numbers of FA-11-positive macrophages (A), Gr-1-positive granulocytes (B), B220-positive B cells (C), and Ly-1-positive T cells (D) in infarcted regions from WT and CCR2^{-/-} mice. Data points represent the number of positive cells per 1 mm² in infarcted tissues. *, $P < 0.01$ versus CCR2^{-/-} mice. †, $P < 0.05$ versus WT mice.

Gelatinolytic Activity in Infarcted Myocardium by *In Situ* Zymography

To examine whether rapid infiltration of macrophages into the infarcted myocardium was associated with left ventricular remodeling after MI, we performed *in situ* zymography using gelatin films to detect gelatinolytic activity in the infarcted myocardium. Although no gelatinolytic activity was detected at 1 day after MI in both groups, *in situ* zymography demonstrated the presence of augmented gelatinolytic activity in WT animals 3 days after MI. No comparable gelatinolytic activity was observed in CCR2^{-/-} mice (Figure 5B). Although gelatinolytic activity in the infarcted regions peaked at 7 days after MI in both groups, CCR2^{-/-} mice had significantly less gelatinolytic activity compared to WT animals (Figure 5, A and B). The distribution pattern of gelatinolytic activity was very similar to the distribution of macrophages in the serial sections, but not to the distribution of neutrophils (Figure 5A).

Figure 4. Serial changes in LVDD (A) and %FS (B) in sham-operated ($n = 6$, each group) and MI ($n = 14$, each group) WT and CCR2^{-/-} mice. *, $P < 0.01$ versus CCR2^{-/-} MI group.

Figure 5. A: *In situ* zymography and immunohistochemistry using monoclonal antibodies for CD68 (FA-11) or granulocytes (Gr-1) in serial sections of infarcted myocardium from WT (left) and CCR2^{-/-} mice (right). Lysis of gelatin in contact with the proteolytic areas of the sections is indicated by negative staining on the slides. To distinguish MMP activity from the activity of other proteinases in the tissue, gelatin films containing 1,10-phenanthroline as an MMP inhibitor (indicated as MMP+I in the figures) are used. **B:** Morphometric analysis of the gelatinolytic area/infarct area at 1, 3, 7, 14, and 28 days after experimental MI in WT and CCR2^{-/-} mice. *, $P < 0.01$ versus CCR2^{-/-} mice. Scale bars, 500 μ m.

The activity decreased at 14 and 28 days after MI in both groups (Figure 5B). We also used gelatin films containing the MMP inhibitor 1,10-phenanthroline to distinguish MMP activity from that of other tissue proteinases. Gelatinolytic activity was almost completely inhibited on gelatin films containing the MMP inhibitor (Figure 5A).

Figure 6. Real-time RT-PCR results for MMP-8 (A), MMP-9 (B), MMP-13 (C), TIMP-1 (D), TIMP-4 (E), and TNF- α (F) mRNA levels in infarcted (I), noninfarcted (NI), and sham-operated (S) myocardium from WT and CCR2^{-/-} mice at 3 days after MI. Relative quantitation values of these mRNA levels were normalized with respect to endogenous control 18S ribosomal RNA gene expression. *, $P < 0.01$ versus the other group. †, $P < 0.05$ versus the other group.

Quantitative RT-PCR for MMP-8, MMP-9, MMP-13, TIMP-1, TIMP-4, and TNF- α mRNA Expression in Infarcted Myocardium

To evaluate the expression of MMPs, TIMPs, and the proinflammatory cytokine in infarcted or noninfarcted myocardial tissues, we quantified cardiac MMP-8, MMP-9, MMP-13, TIMP-1, TIMP-4, and TNF- α mRNA levels in WT and CCR2^{-/-} mice by two-step RT-PCR with real-time amplicon detection. As shown in Figure 6, A to C, the expressions of MMP-8, MMP-9, and MMP-13 mRNAs increased significantly in infarcted regions compared to noninfarcted or sham-operated myocardial tissues at 3 days after MI in WT mice, and were greater in infarcted regions of WT mice than CCR2^{-/-} mice. TIMP-1 mRNA expression also increased significantly in infarcted regions compared to noninfarcted or sham-operated myocardial tissues at 3 days after MI in WT mice, and were greater in infarcted regions of WT mice than CCR2^{-/-} mice (Figure 6D). On the other hand, TIMP-4 mRNA was down-regulated in infarcted regions compared to noninfarcted or sham-operated myocardial tissues at 3 days after MI in WT mice, and decreased in infarcted regions of WT mice than CCR2^{-/-} mice (Figure 6E). Moreover, a proinflammatory cytokine, TNF- α mRNA was up-regulated in infarcted regions compared to noninfarcted or sham-operated myocardial tissues at 3 days after MI in WT mice, and were greater in infarcted regions of WT mice than CCR2^{-/-} mice (Figure 6F).

Discussion

The results of our study clearly demonstrated that CCR2 deficiency had a critical impact on left ventricular remodeling after MI. We observed that macrophages rapidly infiltrated into infarcted myocardium in WT mice within 7 days after MI, and that this process was greatly impaired in CCR2^{-/-} mice. In contrast, the accumulation of Gr-1-positive granulocytes in infarcted regions was similar in both groups, although total granulocyte numbers were higher in CCR2^{-/-} mice than in WT mice at 7 days after MI. No significant differences in Thy-1.2-, Ly-1-positive T-cell numbers or B220-positive B-cell numbers were observed between the two groups. These findings suggested that the reduced infiltration of macrophages into infarcted regions in CCR2^{-/-} mice was an important contributor to the prevention of left ventricular remodeling after MI. Although CCR2 is currently the only known high-affinity CCL2 receptor, low-affinity receptors such as CCR1 and CCR5 can also lead to macrophage accumulation in lesions, but to a lesser extent.¹⁸ Besides these cloned receptors, recent reports have indicated the existence of putative new CCL2 receptor(s) on smooth muscle cells¹⁹ and hepatic stellate cells.²⁰ Thus, it is possible that other receptors may also be related to macrophage infiltration into the infarcted myocardium in this model, as macrophage infiltration into infarcted regions was not completely abolished in CCR2^{-/-} mice. Identification of all of the chemokines expressed at different stages of experimental MI would provide more insight to CCR2-independent macrophage accumulation in the infarcted tissues.

The difference in macrophage accumulation between WT and CCR2^{-/-} mice corresponded to a difference in the collagen volume fraction in the infarcted region at 7 days after MI. In the present study, collagen volume fractions in infarcted regions increased more significantly in WT mice than in CCR2^{-/-} mice at 7 days after MI. In addition to reduced collagen accumulation in infarcted regions, necrotic centers tended to remain devoid of fibrosis in CCR2^{-/-} mice. Possible mechanisms for reduced collagen accumulation in infarcted regions in CCR2^{-/-} mice might reflect the decreased infiltration of macrophages in the infarcted myocardium. Macrophages are known to play an important role in the wound-healing process through matrix degradation, neovascularization, and recruitment and proliferation of fibroblasts.²¹ Furthermore, there is evidence that macrophages express many growth factors and proinflammatory cytokines, including platelet-derived growth factor, basic fibroblast growth factor, transforming growth factor- β , interleukin-1 β , and TNF- α .²²⁻²⁵ In the present study, we also showed that TNF- α mRNA expression was up-regulated in the infarcted regions of WT mice compared to CCR2^{-/-} mice at 3 days after MI, which indicated the close relationship between augmented macrophage infiltration and the expression of inflammatory cytokines. It is possible that the macrophages infiltrating into infarcted regions might accelerate the collagen production by inducing the growth factors and proinflamma-

tory cytokines in the infarcted tissues and promote the development of tissue fibrosis in this model.

The present study demonstrated the close relationship between the macrophage infiltration and MMPs activation in infarcted myocardium. MMPs belong to the zinc-containing endopeptidase family²⁶ and are synthesized as a latent form (zymogen or pro-MMP), and are then activated by proteolytic cleavage of the amino-terminal domain or by conformational changes induced by factors such as oxidative stress.^{27,28} MMPs are involved in extracellular matrix remodeling in tissues during various physiological and pathological conditions, including embryonic development, inflammation, and cancer.²⁹ Recent studies have demonstrated that increased MMP activity may be responsible for the degradation of extracellular matrix in infarcted myocardial tissue after MI.³⁰⁻³³ Ducharme and colleagues⁶ found that MMP-9-deficient mice exhibited attenuated left ventricular dilatation, reduced macrophage infiltration and collagen accumulation after MI, which suggested a relationship between macrophage infiltration into infarcted tissues and cardiac remodeling by MMP activation. Very recently, it has been reported that N-terminal deletion mutant of the human CCL2 gene attenuated the recruitment of macrophages and LV cavity dilatation after MI in mice, which indicated that the inhibition of CCL2 signaling may contribute to prevent LV remodeling and failure after MI.³⁴ However, there has been no direct evidence that macrophage infiltration into the infarcted myocardium accelerates left ventricular remodeling through increased levels of activated MMPs. In the present study, we performed *in situ* zymography using gelatin films to detect gelatinolytic activity in infarcted myocardial tissues to determine whether the macrophages that infiltrated into the infarcted myocardium produced active MMPs. *In situ* zymography demonstrated augmented gelatinolytic activity in WT animals within 7 days after MI, whereas no comparable increase in gelatinolytic activity was observed in CCR2^{-/-} mice. Moreover, the distribution pattern of gelatinolytic activity was very similar to the pattern of macrophage distribution in serial sections, and not to the pattern of neutrophil distribution. Furthermore, the quantitative RT-PCR revealed that the expressions of MMP-8, MMP-9, and MMP-13 mRNAs increased significantly in infarcted regions of WT mice than CCR2^{-/-} mice 3 days after MI. Inflammatory cells including macrophages are known to produce these MMP subtypes in various pathological conditions.³⁴⁻³⁷ Recent studies have demonstrated that MMPs were synthesized by isolated fibroblasts and cardiac myocytes.^{38,39} Our present findings suggested that the infiltrating macrophages in infarcted regions might be the source of the active MMPs produced during the acute phase of MI, and indicated a close relationship between macrophage infiltration and left ventricular remodeling of the infarcted myocardium. TIMP-1 mRNA expression was greater in infarcted regions of WT mice than CCR2^{-/-} mice 3 days after MI, which indicated the compensatory increase of inhibitor expression because of the presence of activated MMPs. On the other hand, TIMP-4 mRNA was down-regulated in infarcted regions of WT mice than CCR2^{-/-} mice. Four

TIMPs have been characterized, namely, TIMP-1, TIMP-2, TIMP-3, and TIMP-4, which share a high-sequence homology but have unique characteristics.^{32,40,41} TIMP-4 is expressed predominantly and constitutively in the heart compared with other organs, suggesting that TIMP-4 plays an important homeostatic role in the normal myocardium.⁴¹ Our results suggested that an increased imbalance in MMPs/TIMPs system as well as elevated MMP activity existed in infarcted regions in WT mice compared with CCR2^{-/-} mice. A proinflammatory cytokine, TNF- α mRNA was greater in infarcted regions of WT mice than CCR2^{-/-} mice 3 days after MI. TNF- α is known to activate MMPs, including collagenase type 1, stromelysin-1, and gelatinase A and B,^{42,43} indicating that its local production may have an important effect on the myocardial remodeling process. It is possible that TNF- α overexpression in infarcted regions may contribute to the progression of left ventricular remodeling after MI. In the present study, we demonstrated the up-regulation of MMP-8, MMP-9, and MMP-13 in the infarcted myocardium at acute phase using our model. Some experimental data have suggested that MMP-2 and MMP-9 play crucial roles in both the acute and chronic phases of the myocardial response to ischemia.⁴⁻⁶ Furthermore, Heymans and colleagues⁵ have demonstrated that urokinase-type plasminogen activator (u-PA), which belongs to the plasminogen system that includes the MMPs cascade, plays a pivotal role in cardiac rupture and ventricular remodeling after MI. With regard to the presence of the other proteinases in the infarcted tissue, we performed *in situ* zymography using gelatin films that contained an MMP inhibitor to distinguish the MMP activity from other proteinase activities. Gelatinolytic activity was almost completely inhibited on the gelatin films that contained the MMP inhibitor, which indicated that the dominant gelatinolytic activity in infarcted tissues was because of MMPs.

In the present study, we found no significant differences in survival rate and cardiac rupture rate between WT and CCR2^{-/-} mice at 28 days after MI. The reason for this may involve the finding that the survival rate at 28 days after MI was relatively high in both groups despite infarct size and area being comparable to previous studies.^{5,15,44,45}

In summary, we found that inhibition of macrophage infiltration via the CCL2/CCR2 pathway attenuated left ventricular remodeling after experimental MI. In infarcted regions of WT mice, the gelatinolytic activity was augmented because of the increased imbalance in MMPs/TIMPs system (up-regulation of MMPs and loss of TIMP-4), whereas the activity was attenuated in CCR2^{-/-} mice. Our present study indicated a novel mechanism to improve cardiac function and left ventricular remodeling after MI. Direct inhibition of CCR2 functional pathway might contribute to the attenuation of left ventricular remodeling after MI.

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References

- Pfeffer JM, Pfeffer MA, Fletcher PJ, Braunwald E: Progressive ventricular remodeling in rat with myocardial infarction. *Am J Physiol* 1991, 260:H1406-H1414
- Rumberger JA: Ventricular dilatation and remodeling after myocardial infarction. *Mayo Clin Proc* 1994, 69:664-674
- Creemers EE, Cleutjens JP, Smits JF, Daemen MJ: Matrix metalloproteinase inhibition after myocardial infarction: a new approach to prevent heart failure? *Circ Res* 2001, 89:201-210
- Peterson JT, Li H, Dillon L, Bryant JW: Evolution of matrix metalloproteinase and tissue inhibitor expression during heart failure progression in the infarcted rat. *Cardiovasc Res* 2000, 46:307-315
- Heymans S, Luttun A, Nuyens D, Theilmeier G, Creemers E, Moons L, Dyspersin GD, Cleutjens JP, Shipley M, Angellilo A, Levi M, Nube O, Baker A, Keshet E, Lupu F, Herbert JM, Smits JF, Shapiro SD, Baes M, Borgers M, Collen D, Daemen MJ, Carmeliet P: Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. *Nat Med* 1999, 5:1135-1142
- Ducharme A, Frantz S, Aikawa M, Rabkin E, Lindsey M, Rohde LE, Smith CW: Targeted deletion of matrix metalloproteinase-9 attenuates left ventricular enlargement and collagen accumulation after experimental myocardial infarction. *J Clin Invest* 2000, 106:55-62
- Hawkins HK, Entman ML, Zhu JY, Youker KA, Berens K, Dore M, Smith CW: Acute inflammatory reaction after myocardial ischemic injury and reperfusion. Development and use of a neutrophil-specific antibody. *Am J Pathol* 1996, 148:1957-1969
- Matsumura K, Jeremy RW, Schaper J, Becker LC: Progression of myocardial necrosis during reperfusion of ischemic myocardium. *Circulation* 1998, 97:795-804
- Dreyer WJ, Michael LH, West MS, Smith CW, Rothlein R, Rossen RD, Anderson DC, Entman ML: Neutrophil accumulation in ischemic canine myocardium. Insights into time course, distribution, and mechanism of localization during early reperfusion. *Circulation* 1991, 84:400-411
- Borregaard N, Cowland JB: Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* 1997, 89:3503-3521
- Lindsey M, Wedin K, Brown MD, Keller C, Evans AJ, Smolen J, Burns AR, Rossen RD, Michael L, Entman M: Matrix-dependent mechanism of neutrophil-mediated release and activation of matrix metalloproteinase 9 in myocardial ischemia/reperfusion. *Circulation* 2001, 103:2181-2187
- Kuziel WA, Morgan SJ, Dawson TC, Griffin S, Smithies O, Ley K, Maeda N: Severe reduction in leucocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proc Natl Acad Sci USA* 1997, 94:12053-12058
- Takuma S, Suehiro K, Cardinale C, Hozumi T, Yano H, Shimizu J, Mullis-Jansson S, Sciacca R, Wang J, Burkoff D, Di Tullio MR, Homma S: Anesthetic inhibition in ischemic and nonischemic murine heart: comparison with conscious echocardiographic approach. *Am J Physiol* 2001, 280:H2364-H2370
- Patten RD, Aronovitz MJ, Deras-Mejia L, Pandian NG, Hanak GG, Smith JJ, Mendelsohn ME, Konstam MA: Ventricular remodeling in a mouse model of myocardial infarction. *Am J Physiol* 1998, 274:H1812-H1820
- Ichihara S, Senbonmatsu T, Price JR E, Ichiki T, Gaffney FA, Inagami T: Targeted deletion of angiotensin II type 2 receptor caused cardiac rupture after acute myocardial infarction. *Circulation* 2002, 106:2244-2249
- Isobe Y, Nakane PK, Brown WR: Studies on translocation of immunoglobulins across intestinal epithelium. I. Improvements in the peroxidase-labeled antibody method for application to study of human intestinal mucosa. *Acta Histochem Cytochem* 1977, 10:161-171
- Nakada M, Nakamura H, Ikeda E, Fujimoto N, Yamashita J, Sato H, Seiki M, Okada Y: Expression and tissue localization of membrane-type 1, 2, and 3 matrix metalloproteinases in human astrocytic tumors. *Am J Pathol* 1999, 154:417-428
- Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, Power CA: International Union of Pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* 2000, 152:145-176
- Schecter AD, Rollins BJ, Zhang YJ, Charo IF, Fallon JT, Rossikhina M,

- Giesen PLA, Nemerson Y, Taubman MB: Tissue factor is induced by monocyte chemoattractant protein-1 in human aortic smooth muscle and THP-1 cells. *J Biol Chem* 1997, 272:28568–28573
20. Marra F, Romanelli RG, Giannini C, Failli P, Pastacaldi S, Arrighi MC, Pinzani M, Laffi G, Montalto P, Gentilini P: Monocyte chemotactic protein-1 as a chemoattractant for human hepatic stellate cells. *Hepatology* 1999, 29:140–148
21. Singer AJ, Clark RA: Cutaneous wound healing. *N Engl J Med* 1999, 341:738–746
22. Rappolee DA, Mark D, Banda MJ, Werb Z: Wound macrophages express TGF- α and other growth factors in vivo: analysis by mRNA phenotyping. *Science* 1988, 241:708–712
23. Assoian RK, Fleurdelys BE, Stevenson HC, Miller PJ, Madtes DK, Raines EW, Ross R, Sporn MB: Expression and secretion of type beta transforming growth factor by activated human macrophages. *Proc Natl Acad Sci USA* 1987, 84:6020–6024
24. Leibovich SJ, Polverini PJ, Shepard HM, Wiseman DM, Shively V, Nuseir N: Macrophage-induced angiogenesis is mediated by tumour necrosis factor- α . *Nature* 1987, 329:630–632
25. Leibovich SJ, Ross R: The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. *Am J Pathol* 1975, 78:71–100
26. Hooper NM: Families of zinc metalloproteinases. *FEBS Lett* 1994, 354:1–6
27. Birkedal-Hansen H: Proteolytic remodeling of extracellular matrix. *Curr Opin Cell Biol* 1995, 7:728–735
28. Okamoto T, Akaike T, Nagano T, Miyajima S, Suga M, Ando M, Ichimori K, Maeda H: Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: a novel mechanism for procollagenase activation involving nitric oxide. *Arch Biochem Biophys* 1997, 342:261–274
29. Woessner Jr JF: Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J* 1991, 5:2145–2154
30. Takahashi S, Barry AC, Factor SM: Collagen degradation in ischemic rat hearts. *Biochem J* 1990, 265:233–241
31. Cheung PY, Sawicki G, Wozniak M, Wang W, Radomski MW, Schulz R: Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. *Circulation* 2000, 101:1833–1839
32. Schulze CJ, Wang W, Suarez-Pinzon WL, Sawicka J, Sawicki G, Schulz R: Imbalance between tissue inhibitor of metalloproteinase-4 and matrix metalloproteinases during acute myocardial ischemia-reperfusion injury. *Circulation* 2003, 107:2487–2492
33. Wang W, Schulze CJ, Suarez-Pinzon WL, Dyck JR, Sawicki G, Schulz R: Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. *Circulation* 2002, 106:1543–1549
34. Dollery CM, Owen CA, Sukhova GK, Krettek A, Shapiro SD, Libby P: Neutrophil elastase in human atherosclerotic plaques: production by macrophages. *Circulation* 2003, 107:2829–2836
35. Ohashi K, Nemoto T, Nakamura K, Nemori R: Increased expression of matrix metalloproteinase 7 and 9 and membrane type 1-matrix metalloproteinase in esophageal squamous cell carcinomas. *Cancer* 2000, 88:2201–2209
36. Vos CM, Gartner S, Ransohoff RM, McArthur JC, Wahl L, Sjulson L, Hunter E, Conant K: Matrix metalloproteinase-9 release from monocytes increases as a function of differentiation: implications for neuroinflammation and neurodegeneration. *J Neuroimmunol* 2000, 109: 221–227
37. Kiili M, Cox SW, Chen HW, Wahlgren J, Maisi P, Eley BM, Salo T, Sorsa T: Collagenase-2 (MMP-8) and collagenase-3 (MMP-13) in adult periodontitis: molecular forms and levels in gingival crevicular fluid and immunolocalisation in gingival tissue. *J Clin Periodontol* 2002, 29:224–232
38. Xie Z, Singh M, Siwik DA, Joyner WL, Singh K: Osteopontin inhibits interleukin-1 β -stimulated increases in matrix metalloproteinase activity in adult rat cardiac fibroblasts. Role of protein kinase C- ζ . *J Biol Chem* 2003, 278:48546–48552
39. Coker ML, Jolly JR, Joffs C, Etoh T, Holder JR, Bond BR, Spinale FG: Matrix metalloproteinase expression and activity in isolated myocytes after neurohormonal stimulation. *Am J Physiol* 2001, 281:H543–H551
40. Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP: Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur J Cell Biol* 1997, 74:111–122
41. Greene J, Wang M, Liu YE, Raymond LA, Rosen C, Shi YE: Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. *J Biol Chem* 1996, 271:30375–30380
42. Gottschall PE, Yu X: Cytokines regulate gelatinase A and B (matrix metalloproteinase 2 and 9) activity in cultured rat astrocytes. *J Neurochem* 1995, 64:1513–1520
43. Galis ZS, Muszynski M, Sukhova GK, Simon-Morrissey E, Unemori EN, Lark MW, Amento E, Libby P: Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. *Circ Res* 1994, 75:181–189
44. Hayashidani S, Tsutsui H, Shiomi T, Ikeuchi M, Matsusaka H, Suematsu N, Wen J, Egashira K, Takeshita A: Anti-monocyte chemoattractant protein-1 gene therapy attenuates left ventricular remodeling and failure after experimental myocardial infarction. *Circulation* 2003, 108:2134–2140
45. Hayashidani S, Tsutsui H, Shiomi T, Suematsu N, Kinugawa S, Ide T, Wen J, Takeshita A: Fluvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, attenuates left ventricular remodeling and failure after experimental myocardial infarction. *Circulation* 2002, 105: 868–873