Genetic Modification of Mesenchymal Stem Cells
Overexpressing CCR1 Increases Cell Viability, Migration, Engraftment, and Capillary Density in the Injured Myocardium

Jing Huang, Zhiping Zhang, Jian Guo, Aiguo Ni, Arjun Deb, Lunan Zhang, Maria Mirotsou, Richard E. Pratt, Victor J. Dzau

Rationale: Although mesenchymal stem cell (MSC) transplantation has been shown to promote cardiac repair in acute myocardial injury in vivo, its overall restorative capacity appears to be restricted mainly because of poor cell viability and low engraftment in the ischemic myocardium. Specific chemokines are upregulated in the infarcted myocardium. However the expression levels of the corresponding chemokine receptors (eg, CCR1, CXCR2) in MSCs are very low. We hypothesized that this discordance may account for the poor MSC engraftment and survival.

Objective: To determine whether overexpression of CCR1 or CXCR2 chemokine receptors in MSCs augments their cell survival, migration and engraftment after injection in the infarcted myocardium.

Methods and Results: Overexpression of CCR1, but not CXCR2, dramatically increased chemokine-induced murine MSC migration and protected MSC from apoptosis in vitro. Moreover, when MSCs were injected intramyocardially one hour after coronary artery ligation, CCR1-MSCs accumulated in the infarcted myocardium at significantly higher levels than control-MSCs or CXCR2-MSCs 3 days postmyocardial infarction (MI). CCR1-MSC–injected hearts exhibited a significant reduction in infarct size, reduced cardiomyocytes apoptosis and increased capillary density in injured myocardium 3 days after MI. Furthermore, intramyocardial injection of CCR1-MSCs prevented cardiac remodeling and restored cardiac function 4 weeks after MI.

Conclusions: Our results demonstrate the in vitro and in vivo salutary effects of genetic modification of stem cells. Specifically, overexpression of chemokine receptor enhances the migration, survival and engraftment of MSCs, and may provide a new therapeutic strategy for the injured myocardium. (Circ Res. 2010;106:1753-1762.)

Key Words: mesenchymal stem cells ■ engraftment ■ chemokine receptor ■ myocardial repair

Over the past few years adult stromal mesenchymal stem cells have been extensively investigated for their potential in developing cell based therapies for the treatment of cardiac injury and/or other regenerative diseases. Numerous studies have shown that transplantation of those cells in postinfarct mice decreased mortality, reduced infarct size and improved cardiac function. More recently, preliminary data from a clinical study of MSCs in 69 postinfarct patients also demonstrated improved left ventricular function. Despite the progress, many barriers for translating the promise of stem cell therapy into practice still exist. Homing, engraftment and survival of the transplanted cells in the ischemic area still pose major problems, with most of the cells being lost within hours of the transplantation. Moreover, the mechanisms determining these processes are still not well understood.

Chemokine induction is one of the prominent features in the postischemic heart associated with neutrophil infiltration and potential angiogenic effects. Importantly, various chemokine/chemokine receptor axes are essential and potent regulators of chemotactic activities for a wide range of cell types such as monocytes and stem cells. We and others have demonstrated that many chemokines, including chemokine (c-c motif) ligand 7 (CCL7), chemokine (c-x-c motif) ligand 1 (CXCL1), chemokine (c-x-c motif) ligand 2 (CXCL2), and others were significantly upregulated in the heart following myocardial infarction (MI) and might be implicated in regulating engraftment and homing of MSCs to infarcted myocardium. Although functionally active receptors for those chemokines have been identified in MSCs, their respective expression levels are relatively low.

In this study, we have hypothesized that genetic modifications of the MSCs to enhance levels of specific chemokine receptors can improve the engraftment and survival of the
cells to injured myocardium. Specifically, we asked whether MSCs overexpressing chemokine (c-c motif) receptor 1 (CCR1) which is the receptor for CCL7, or chemokine (c-x-c motif) receptor 2 (CXCR2) which is the receptor for both CXCL1 and CXCL2, exhibited improved engraftment and myocardial salvage after acute MI.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org and includes detailed information regarding the following: RT-PCR and real-time RT-PCR of chemokine and chemokine receptors, isolation and culture of MSCs, retroviral construct design, retroviral packaging and producer cell lines, transduction of MSCs, Western blotting, transmigration assay, ELISA for vascular endothelial growth factor (VEGF), human umbilical vein endothelial cell (HUVEC) culture and tube formation assay on matrix gels, 5-bromo-2'-deoxyuridine (BrdUrd) incorporation assay, caspase 3/7 assay and TUNEL assay, small interfering (si)RNA transfection, intramyocardial MSC delivery, tracking of the green fluorescent protein (GFP)/cells injected in mice, area at risk (AAR) and infarct size determination, capillary and arteriolar density assessment, assessment of the collagen deposition and fibrosis, histochemical and immunofluorescent examination, echocardiographic study, cytokine arrays, and statistical analysis.

Results

Chemokine Gene Expression Was Upregulated in the Infarcted Tissue

We have shown previously that specific chemokines are upregulated in the injured murine myocardium.6,10 To determine the role of the local chemokine milieu in MSC transplantation after MI, we first confirmed the expression levels of a set of known chemokines in the border zone of the injured area by RT-PCR and real time RT PCR (Figure 1A and 1B). The results confirmed that mRNA levels of CXCL1, CXCL2 (both ligands for CXCR2), and CCL7 (a ligand for CCR1) were markedly upregulated after MI. The highest levels of expression were observed at 24 hours, although increased levels could still be detected at 7 days after MI. The mRNA levels of CCL5, another ligand for CCR1, were not upregulated significantly. To investigate a possible role for a chemokine/chemokine receptor axis in MSC fate, we then evaluated the gene expression of the corresponding chemokine receptors, namely CCR1, CXCR2, and CXCR4, in MSCs. Because chemokine receptors are robustly expressed in different bone marrow leukocyte subtypes,11 we isolated mRNA from whole bone marrow cells (wBMCs) as positive control. As expected CCR1, CXCR2 and CXCR4 were all expressed in wBMCs. CXCR4 was detected in MSCs, albeit at reduced levels compared to the wBMCs. Interestingly, very low expression of the CCR1 and CXCR2 genes was found in the MSCs under the conditions tested (Figure 1C).

CCR1 Overexpression Increased MSC Chemotaxis In Vitro

To test the potential of an effect of the receptors in chemokine-induced MSC migration, murine MSCs were retrovirally transfected with GFP, CCR1-GFP, or CXCR2-GFP constructs (Figure 2A), and sorted by flow cytometry for GFP expression. Cells with robust green fluorescence signal were separated and used in this study (Figure 2B). Western blot analysis, performed in parallel, confirmed the overexpression (3 to 4 X) of the chemokine receptors on targeted cells (Figure 2C). The functionality of the engineered MSCs was then assessed using a migration assay.
Transwell assay. In detail, control GFP-MSCs, CCR1-MSCs, or CXCR2-MSCs were stimulated with 1 μg/mL of chemokines known to correspond to the receptors overexpressed on these cells (CCL5 for receptor CCR1 and interleukin-8 for receptor CXCR2). As shown in Figure 2D, overexpression of CCR1 significantly increased chemokine-induced MSC chemokinesis (≈90% increase, P<0.05). In contrast, MSCs overexpressing CXCR2 did not demonstrate an increased response to interleukin-8–mediated chemotaxis.

**CCR1 Overexpression Protects MSCs From Serum Deprivation–Induced Apoptosis In Vitro**

To further study the biology of the genetically modified cells, we first examined the rate of cell proliferation by performing 5-bromo-2′-deoxyuridine (BrdUrd) incorporation assays. As shown in Figure 3A, neither CCR1 nor CXCR2 overexpression affected the proliferation of MSCs compared with control MSCs. However, when serum deprivation was used as in vitro model to mimic the stress conditions found in the injured heart, the CCR1, but not the CXCR2, overexpressing MSCs did show enhanced levels of survival. Characteristically, after 12 hours of serum withdrawal, the activity of proapoptotic caspase 3/7 of control MSCs exceeded that of CCR1-MSCs by 1.5-fold. Overexpression of CXCR2 did not affect caspase 3/7 activity (Figure 3B). Follow-up analysis by TUNEL staining showed that the percentage of TUNEL-positive cells was significantly reduced in CCR1-overexpressing MSCs as compared with control cells. CXCR2 overexpression did not render significant survival advantage to MSCs (Figure 3C and 3D).

We have previously demonstrated that MSCs secrete paracrine factors which potentially exert proangiogenic and cardioprotective effects in the injured myocardium. To examine whether the VEGF levels were affected in the CCR1 or CXCR2 overexpressing cells, we first performed ELISA to measure the VEGF levels in the culture medium of MSCs. After 12 hours of serum deprivation, the culture supernatants of CCR1-MSCs...
contained more VEGF compared with CXCR2-MSCs and control MSCs supernatants (Online Figure I, A). Moreover, when culture supernatants from CCR1-MSCs was added in HUVECs, significantly increased levels of tube formation was observed as compared to HUVECs treated with control MSCs culture supernatants (Online Figure I, B). However, addition of CXCR2-MSCs supernatants did not enhance tube formation in HUVECs. Interestingly, when the VEGF values in the culture supernatants of CCR1-MSCs, CXCR2-MSCs and control MSCs were normalized to the number of viable cells (TUNEL-negative cells), the VEGF values were similar (Online Figure I, C), suggesting that the higher VEGF level in culture supernatant of CCR1-MSCs is attributable to the resistance of these cells to serum deprivation-induced apoptosis, rather than an increase in secretion per cell.

siRNA-Mediated Downregulation of Endogenous Ligands Expression Attenuated the Protective Effects of CCR1 Overexpression Against Serum Deprivation–Induced Apoptosis in MSCs

To characterize the role of endogenous CCR1 ligands such as CCL5 and CCL7 in protecting CCR1-MSCs from apoptosis, we down-modulated the expression of those genes in CCR1-MSCs using siRNA. After 72 hours transfection with either CCL5 or CCL7 siRNA, CCL5 mRNA content in CCR1-MSCs decreased 98% and CCL7 mRNA content decreased 46%, respectively, compared to negative control siRNA treated cells. siRNA treatment itself did not cause nonspecific downregulation of gene expression, as demonstrated by the β-actin control (Figure 4A and 4B). The effects of CCL5 and CCL7 gene silencing in CCR1-MSCs survival were then evaluated (Figure 4C). Following CCL7 siRNA transfection, 75% ± 21.1% of CCR1-MSCs underwent apoptosis versus 38.3% ± 16.6% in the control siRNA transfected cells (~2-fold change). We also observed a trend for enhanced apoptosis in CCL5 siRNA transfected CCR1-MSCs. These data indicated that endogenous ligands of CCR1, especially CCL7, are critical for the high apoptosis resistance of CCR1-MSCs.

Enhanced Accumulation and Survival of Transplanted CCR1-Overexpressing MSCs in Injured Murine Myocardial Tissue Three Days After MI

To examine the in vivo effects of chemokine receptor overexpression in MSCs, MI was produced in mice by permanent ligation of the left anterior descending coronary artery. Mice

Figure 3. CCR1 overexpression enhanced MSC cells survival by protection from apoptosis. A, BrdUrd incorporation assays were performed and the percentage of BrdUrd positive MSCs vs total MSCs was calculated. B, Caspase 3/7 luminescence assay for MSCs after 12 hour serum withdrawal. RLU indicates relative light units. C, Representative fluorescence images of TUNEL (red), DAPI (blue), and GFP (green) signal in MSCs after 12 hour serum deprivation. Colocalization (purple nuclei) of TUNEL and DAPI can be observed in some cells. D, Quantitative analysis of images represented in C. The percentage of TUNEL-positive MSCs vs total MSCs is shown. Data represent means±SD; n=3 to 4. *P<0.05 vs control MSCs under serum deprivation.
were randomly assigned to receive intramyocardial injection of a mixture of $0.75 \times 10^5$ LacZ− MSCs added to $3 \times 10^5$ MSCs which were retrovirally transduced with GFP, CCR1-GFP, or CXCR2-GFP. The LacZ− MSCs were injected to account for any technical variability with the cell delivery (see Material and Methods for details). Three days after MI, GFP+ cells could be detected in the infarcted ventricular myocardium (Figure 5A). In sections from mouse hearts injected with the mixture of GFP-MSC and LacZ-MSC, the ratio of GFP+/LacZ− ranged from 3:1 to 5:1 (Online Figure II), consistent with the 4:1 ratio of the injected cells ($3 \times 10^5$ GFP-MSCs, $0.75 \times 10^5$ LacZ− MSCs). In hearts injected with the mixture of CXCR2-GFP-MSCs and LacZ MSCs, the ratio of GFP+/LacZ− was greater in the first few sections but, in the remainder of the sections, was very similar to that observed in hearts injected with mixture of GFP-MSC and LacZ-MSC, suggesting the overexpression of CXCR2 had little or no effect of migration into the infarct zone (Online Figure II, bottom). In contrast, in mouse hearts injected with mixture of CCR1-MSC and LacZ-MSC (Online Figure II, top), the ratio of GFP+/LacZ− was higher in all sections compared to the ratio observed in hearts injected with mixture of GFP-MSC and LacZ-MSC, suggesting that expression of CCR1 enhanced MSC migration into the infarct zone. Calculation of the area under the curve for the 3 groups supported this observation (Figure 5B).

When TUNEL staining was performed on these heart sections, a significantly greater number of viable GFP+ cells were observed in the myocardium of the CCR1-MSC as compared to control GFP MSC group (Figure 5C and 5D). We also observed lower percentage of TUNEL and GFP-double-positive cells in CCR1-GFP MSC group as compared with CXCR2-MSC group and control-GFP MSC group, though it did not reach statistical significance (Figure 5E).

**Intramyocardial Injection of CCR1-Overexpressing MSCs Reduced the Infarction Size and Increased Cardiomyocytes Survival and Capillary Density Three Days After MI**

To evaluate the functional significance of the overexpression of CCR1 or CXCR2 on MSCs, we measured the infarct area and AAR by Evans Blue dye perfusion and triphenyltetrazolium chloride staining in heart sections of treated mice 3 days after MI. As anticipated, injection of control MSCs caused a minor but significant decrease in infarct. Importantly, mice injected with MSCs overexpressing CCR1, but not CXCR2, exhibited a significantly reduced infarct/AAR ratio compared with mice injected with control MSCs or CXCR2-MSCs as evidenced by Mason’s trichrome staining (Online Figure III).

A reduction in cardiomyocyte cell death was also evident when we examined for apoptosis. TUNEL and α-sarcomeric actin-double-positive cells were counted and the percentage of TUNEL-positive cardiomyocytes/total nuclei was calculated and
normalized to control MSCs group. The data demonstrated that the percentage of apoptotic cardiomyocytes was lower in CCR1-MSC group as compared with CXCR2-MSC group and control-MSC group (Figure 6C). No changes were observed in cardiac myocyte regeneration as no significant effects in proliferation, fusion or differentiation were detected by GFP, α-sarcomeric actin and/or Ki67 costaining (data not shown).

Because we have shown that MSCs secrete angiogenic factors and promote angiogenesis in vitro, we measured capillary density in the infarction adjacent border zone (Online Figure IV, A). The capillary density increased significantly in CCR1-MSC transplanted hearts as compared to control cell transplanted hearts, whereas in CXCR2-MSC injected hearts, the capillary density was not increased significantly. Modest increase of capillary density was observed in the control GFP-MSC injected group compared with PBS injected group (Figure 6D). To address whether the injected MSCs differentiated to endothelial cells, we performed GFP and lectin costaining and did not detect any double-positive cells (data not shown).

Interestingly, when percentage of infarction/AAR ratio and number of capillaries/mm² were analyzed together, there was a significant negative correlation between infarct size and capillary density (Online Figure IV, B; r = −0.90).

**Intramyocardial Injection of CCR1-Overexpressing MSCs Prevented Cardiac Remodeling and Restored Cardiac Function Four Weeks After MI**

To investigate the long-term effects of the CCR1-MSC transplantation, we measured collagen deposition and fibrosis by Masson’s trichrome staining in heart sections 4 weeks after MI. Mouse hearts injected with MSCs overexpressing CCR1 exhibited a significantly reduced fibrotic volume compared to hearts injected with PBS and control MSCs (Figure 7A and 7B).

We also measured capillary density in the infarction adjacent border zone. The capillary density increased significantly in CCR1-overexpressing MSC injected hearts as compared to PBS and GFP-MSCs injected hearts, whereas there was no significant difference between CCR1-MSCs group and Sham group (Figure 7C). Although we were able to detect TUNEL and α-sarcomeric actin-double-positive cells 4 weeks post MI (Online Figure V, A), the apoptotic cardiomyocyte number was very low and there was no difference among the MI groups. Additionally, double-positive Ki67 and α-sarcomeric actin cells could be detected 4 weeks post MI (Online Figure V, B) but at very low number and again there was no difference among the MI groups. Although few GFP positive cells were detectable, we were not able to detect GFP and α-sarcomeric actin or GFP and lectin-double-positive cells.

More importantly, dramatic functional changes could be detected in the CCR1-MSC–treated mice as evidenced by echocardiography studies (Table). After 4 weeks MI, both left ventricular (LV) end diastolic dimension and LV end systolic dimension were significantly reduced in the CCR1-MSC group compared to the PBS group indicating an attenuation of LV dilation. LV mass was also significantly smaller in the CCR1-MSC–treated group. Finally, similar changes were observed for the fractional shortening, which was significantly improved in the CCR1-MSCs group compared PBS control group (∼12% increase). The CCR1-MSC–treated group also showed improved levels compared to the GFP-MSC in all parameters measured.

**Discussion**

Although stem cell transplantation shows great promise as a tool for cardiac reparative and regenerative therapy, effective
engraftment and survival of the transplanted stem cells within the ischemic myocardium remain as major limitations. Incorporation of stem cells into cardiac tissue is regulated by multiple processes including cell recruitment, migration and adhesion. In addition, survival in the ischemic microenvironment is challenging for the transplanted cells because of the lack of oxygen and nutrients. A complete understanding of the mechanisms that enhance MSC migration and survival in injured tissue therefore is imperative for improving their repair capacity and therapeutic application. Yet, the predominant primary signaling pathways that orchestrate local engraftment of MSCs remain to be fully elucidated.

The ischemic myocardium expresses an array of biological mediators, in particular chemokines and cytokines. Chemokine/chemokine receptor axes are essential and potent regulators of chemotactic activities for a wide range of cell types. In particular, the stromal cell–derived factor-1/CXCR4 axis has been shown to play an important role in guiding hematopoietic stem cells into ischemic myocardium.

In the present study, we hypothesized that additional chemokine/receptor pairs, namely, CCL7/CXCR1 and CXCL1,2/CXCR2, might be involved in MSC migration to, and engraftment in injured myocardium. Our experiments confirm that specific chemokines are upregulated transiently in the murine ischemic myocardium, including CCL7, CXCL1 and CXCL2. However, the expression levels of the corresponding receptors of these chemokines, namely, CXCR1 and CXCR2, were low in MSCs. To investigate whether enhancement of the above chemotactic axis would affect the biological properties of stem cells, we overexpressed the CCR1 and CXCR2 receptors in MSCs by gene transfer.

Our data show that CCR1-overexpressing, but not CXCR2-overexpressing, MSCs exhibit higher chemotactic activity. Furthermore, CCR1-MSCs, but not CXCR2-MSCs, show increased resistance against serum deprivation-induced apoptosis in vitro. Thus, overexpressing CCR1 has an effect on increasing cell viability in addition to migration. By using siRNA-mediated gene silencing, we also demonstrated that MSC expression of CCL5 and CCL7 (both CCR1 ligands) is
important in protecting MSCs from serum withdrawal-induced apoptosis. Research into the targeting of CCR1 may lead to therapies in inflammation and immunosuppression. However, its potential positive role in stem cell biology or in cardiovascular disease has not been extensively evaluated. Interestingly, an interaction between CCR1/CCL5 has been shown recently to be involved in affecting intimal smooth muscle-like cells proliferation and migration. In addition, it has been reported that CCL5 which interacts with CCR1, can induce ERK-1, ERK-2, FAK, and STAT phosphorylation in MSCs. The activation of these signaling pathways might be responsible for the enhanced cell survival observed in CCR1-MSCs. The exact mechanism of CCR1 prosurvival effect is worthy of future investigations.

Our previous data has shown that MSCs secrete paracrine factors that can mediate cell survival, repair processes, as well as a variety of angiogenic factors such as VEGF, basic fibroblast growth factor, and hepatocyte growth factor, many of which are upregulated by hypoxia. In support of this notion, other groups have demonstrated that intramyocardial transplantation of MSCs induces myocardial angiogenesis and subsequently lead to increased cardiomyocyte survival. In this report, we further investigated the production of VEGF by CCR1-overexpressing MSCs. Under normal culture conditions, no difference was detected in the levels of VEGF secretion between CCR1-MSCs and control MSCs. After serum deprivation, VEGF secretion was increased dramatically in CCR1-MSCs compared to control MSCs. However, the increased VEGF secretion in CCR1-MSCs is attributable to the resistance of these cells to serum deprivation-induced apoptosis, rather than an increase in secretion per cell.

The enhanced biological properties of CCR1-MSCs observed in vitro were also present on injection of those cells into the murine myocardium after MI. A greater number of CCR1-MSCs accumulated within myocardium tissue and those cells showed increased migratory capacity toward the area of injury compared to the control MSC or CXCR2-MSC groups. More importantly, the infarct size of the CCR1-MSC–injected group was significantly reduced compared to the control MSC group. Additionally, a reduced number of apoptotic cardiomyocytes were observed in CCR1-MSC–injected hearts indicating protective effects for the injured myocardium. Compared to the control MSC injected hearts, the CCR1-MSC– injected hearts showed increased capillary density in the peri-infarct area, which was not observed in the

Figure 7. Intramyocardial injection of CCR1-MSCs prevented cardiac remodeling and restored cardiac function 4 weeks after MI. A, Representative Masson’s trichrome staining for heart sections 4 weeks after MI. B, Quantification of the fibrotic volume. Fibrotic volume in each heart was expressed as a percentage by calculating (sum of infarct volume from all sections/sum of LV volumes from all sections)×100. Data are presented as means±SEM; n=4 to 9. C, Capillaries were stained by lectin in infarct adjacent border zone. The number of capillaries/mm² was counted. Data represent means±SEM; n=3 to 10 mice per group. *P<0.05 vs PBS group; #P<0.05 vs GFP-MSCs group.
CXCR2-MSC injected group. We also demonstrated a strong negative correlation between infarct size and capillary density in hearts, suggesting that improved blood perfusion of the myocardium might at least partially contribute to these protective effects. Furthermore, these beneficial effects were maintained beyond the acute phase of infarction (3 days time point). Intramyocardial injection of CCR1-overexpressing MSCs further prevented cardiac remodeling and restored cardiac function 4 weeks after MI.

These observations are consistent with our previous observations that the release of biologically active mediators is significant in mediating the beneficial effects of stem cell therapy via a paracrine mechanism. A possible interpretation of the data are that the relative preservation of capillary density simply reflects preservation of border zone viability through a direct effect on existing cardiac cells rather than cell regeneration. As a result, increased capillary density reflects reduced scar formation rather than regeneration. Indeed, we were unable to detect either the transdifferentiation of the injected CCR1-MSCs into cardiomyocytes or endothelial cells or the proliferation of the cardiomyocytes. An alternative hypothesis would be that MSCs injected into ischemic hearts, via paracrine effects, may attract and activate circulating and/or resident cardiac stem cells. Higher levels of VEGF secreted because of the better survival of CCR1-MSCs in the ischemic environment may account at least partially for such effects. Indeed, VEGF has been shown to result in mobilization of bone marrow-derived endothelial progenitor cells as well as possibly contributing in the spontaneous differentiation of stem cells toward the cardiac myocyte fate. It is also worthy to note that G-CSF (granulocyte colony-stimulating factor) production in day 3 after MI hearts injected with CCR1-MSCs was significantly higher compared to hearts injected with control MSCs, although other cytokine production did not change (Online Figure VI). It has been reported that G-CSF increases the mobilization of stem cells from bone marrow into peripheral circulation resulting in myocardial protection after MI. Whether the injection of CCR1-MSCs attracts and activates cardiac stem cells as well as MSCs requires further in depth investigation.

In summary, our efforts to genetically manipulate MSCs ex vivo by enhancing the expression of CCR1 show that overexpression of CCR1 enhances MSC survival, migration, and engraftment in ischemic myocardium. Meanwhile, CCR1-MSC injection reduced cell apoptosis and infarction size, increased capillary density, prevented cardiac remodeling, and restored cardiac function (Online Figure VII). This approach has advanced our understanding of chemokine/receptor axis and stem cell biology, and may offer a new strategy for improved stem cell therapy for injured myocardium.

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Disclosures
None.

References
Novelty and Significance

What Is Known?

- Chemokine/chemokine receptor axes are essential and potent regulators of chemotactic activities for a wide range of cell types, including stem cells.
- Chemokines CCL7, CXCL1, and CXCL2 are upregulated in the heart following myocardial infarction.
- The expression levels of the corresponding receptors CCR1 and CXCR2 are low in MSCs.

What New Information Does This Article Contribute?

- Overexpression of CCR1, but not CXCR2, increased MSC migration and viability in vitro and in vivo.
- Intramyocardial injection of CCR1-MSCs protected myocardium injury, prevented cardiac remodeling, and restored cardiac function.

Despite the great promise of MSC therapy for acute myocardial injury, the effective engraftment of the transplanted stem cells into the ischemic myocardium still poses a challenge for improving their therapeutic application. Ischemic myocardium expresses high level of chemokines, including CCL7, CXCL1, and CXCL2. However, the expression levels of the corresponding receptors CCR1 and CXCR2 are low in MSCs. We overexpressed the CCR1 and CXCR2 receptors in MSCs by gene transfer and demonstrated that overexpressing CCR1, but not CXCR2, increases MSC migration and viability in vitro. When we injected CCR1-MSCs into murine myocardium after myocardial infarction (MI), a greater number of CCR1-MSCs accumulated in the injured myocardium and migrated toward the area of injury. Injection of CCR1-MSCs produced protective effects for the injured myocardium 3 days after MI and prevented cardiac remodeling and impairment of cardiac function 4 weeks after MI. Genetic manipulation of MSCs ex vivo by enhancing the expression of CCR1 advances our understanding of the chemokine/chemokine receptor axis and of stem cell biology and may offer a new strategy for improving stem cell therapy for injured myocardium.
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Materials and Methods

RT-PCR and real-time RT-PCR of chemokine and chemokine receptors
mRNA was isolated from the infarction adjacent border zone and MSCs using an RNeasy Mini Kit (Qiagen). Equal amounts of total RNA from each sample were taken for cDNA synthesis using a SuperScript III First-Strand kit (Invitrogen) according to the manufacturer’s protocol. The following primers were synthesized by Operon. CCL5, forward 5'- ATATGGCTCGGACACCACTC-3', reverse 5'-TCCTTTGAGGTGACACGCAAAACACG-3', product length 131 bp; CCL7, forward 5' GTGTCCCTGGGAAGCTGTTA-3', reverse 5' - CTTTGAGGTGAGTTTCCA-3', product length 121 bp; CXCL1, forward 5' - GCTGGGATTGCCACCTCAAAG-3', reverse 5' - AAGGGAGCTTCAAGG-3', product length 128 bp; CXCL2, forward 5' - GCCAAGGGTTGACTTCAAGGA-3', reverse 5' - TTCAGGGTCGCAAGGCAACTT-3', product length 121 bp; CCR1, forward 5' - ACTCCACTCCATGCCAAAAG-3', reverse 5' - CTAGGACATTGCCACCTTCAAGG-3', reverse 5' - CACGGATGTCTACCTGCTGA-3', reverse 5' - AAGGGAGCTTCAAGG-3', product length 128 bp; beta actin, forward 5' - GTGTATTCCCCTCCATCGTG-3', reverse 5' - GGGTCAGGATACCTCTCTCTG-3', product length 114 bp. The conditions for RT-PCR were: 5 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 56°C, 30 seconds at 72°C, followed by an extension for 5 minutes at 72°C. PCR products were resoled by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. Real-time PCR was conducted in an ABI PRISM 7700 Sequence Detector (AB Applied Biosystems). PCR reactions were performed in SYBR green PCR master mix (Qiagen). The cycling program included a 10 minute initial preincubation at 95°C followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute.

Isolation and culture of MSC
MSCs were isolated from wild-type male C57BL/6J mice (Harlan) as described previously1-3. Briefly, bone marrow was flushed from femur and tibia of mice. Nucleated cells were purified with Ficoll gradients and cultured in alpha minimal essential medium (alpha-MEM; GIBCO) supplemented with 15% FBS at 37°C with 5% CO2 for 24 hours. The unattached cells were removed by washing with PBS. The attached cells were cultured in alpha MEM until reaching 80% confluence. Then, cells that could be lifted by incubating in trypsin/EDTA within 2 minutes were collected and seeded into new plates at a density of 5,000 cells/mm². This procedure was repeated once more. In this study, cells in passage 3-4 were used and fluorescence-activated cell sorting (FACS) analysis demonstrated that these cells expressed MSC markers CD44 and CD105, but did not express hematopoietic markers CD34 and CD45. Some bone marrow flushed from femur and tibia of mice was kept as whole bone marrow cells for further RNA preparation.

Retroviral constructs design
A bicistronic expression system consisting of an internal ribosome entry site (IRES) and a GFP (Clontech) was cloned into the murine stem cell virus vector pMSCV (Clontech). cDNA for murine CCR1 and CXCR2 were purchased from Clontech and inserted in front of IRES-GFP in pMSCV under the control of the constitutively expressed promoter, a specifically designed 5’ long terminal repeat (LTR) from the murine stem cell PCMV virus. This promoter prevents transcriptional suppression and drives high-level constitutive expression of a target gene. All of the constructs were confirmed by automated DNA sequencing by Duke DNA analysis Finch LIMS.
**Retroviral packaging and producer cell lines**

RetroPack™PT67 packaging cell lines were routinely maintained in Dulbecco Modified Eagle medium (DMEM) supplemented with 10% FBS. The pMSCV-IRES-GFP, pMSCV-CCR1/IRES-GFP, and pMSCV-CXCR2/IRES-GFP constructs were transfected into packaging cells to produce high-titer retrovirus. PCDNA3.1 vector which contains neomycin resistant gene was cotransfected to allow the selection of stable retroviral producer cell lines using G418 (1.5mg/mL).

**Transduction of MSCs**

Retroviral targeting on MSCs was performed by a multiplicity of infection. An infection cocktail containing retroviral supernatant, 6 mg/mL polybrene, and 10% FBS in alpha-MEM with 2.5 x 10⁵ target MSCs were cultured in 60mm dish for 24 hours at 37°C with 5% CO₂. The medium was removed and replaced with fresh medium and incubated for a further 24 hours. We assessed successful retroviral transduction by detecting of GFP under a fluorescent microscope. Cells were then trypsinized, and sorted by flow cytometry for GFP. Supernatant of retrovirus encoding nuclear-localized lacZ was obtained from the Harvard Gene Therapy Initiative.

**Western blotting**

MSC lysates of equivalent protein were separated by SDS-PAGE gel. Protein was transferred electrophoretically to PVDF membrane (Bio-Rad). Membranes were blocked in 5% milk in PBS-T (0.1% Tween20) at room temperature for 1hour. Then membranes were probed with Rabbit anti CCR1 or Rabbit anti CXCR2 antibodies (1:1,000 diluted; Abcam) followed by a HRP conjugated goat anti-rabbit secondary antibody (1:5,000 diluted; Cell signaling Tec.). Finally, the membranes were developed using ECL advance detection kit (GE healthcare) and exposed to x-ray films. The band density was analyzed using an IPLab software.

**Transmigration assay**

The assays were carried out using transwell cell culture inserts (Corning) that had polyethylene terephthalate filters (12-µm pore size) on the bottom. MSC suspension serum free DMEM (8 x 10⁵cells/400µL) were added into the inserts. The inserts were set into 12-well plates that held 1mL of 1 µg/mL of chemokine, including recombinant murine CCL5 (Peprotech Inc.), and recombinant human IL-8 (R&D), diluted in serum free DMEM and cultured for 3 hours at 37°C with 5% CO₂. Cells that had not penetrated the filters were wiped out with cotton swabs, and cells that had migrated to the lower surface of the filters were fixed in methanol and then stained with hematoxylin. After being washed, the cell migration was observed with a light microscope at a magnification of x 100, and pictures were captured with a computer system. The number of migrated cells was counted. The mean of five individual fields in the center of the filter which exhibited the highest cell migration was obtained for each well.

**ELISA for VEGF**

The levels of VEGF of culture supernates of MSCs were determined by ELISA using a commercially available Quantikine kit. (R&D) The assay was performed according to the instruction manual provided by the manufacturer. All samples and standards were measured in duplicate.

**HUVEC culture and tube formation assay on matrix gels**
HUVECs were cultured in endothelial cell growth medium (EGM, Clonetics) supplemented with 2% fetal bovine serum (FBS), 1% penicillin/streptomycin, and EC growth supplement at 37°C and 5% CO2. Tube formation assay was performed as described previously4,5. Matrix gel (Matrigel) was purchased from Chemicon International, Inc. The gel was allowed to polymerize in a 24-well plate for 1 hour at 37°C. HUVECs were seeded at 7.5 x 10^4 per well and grown in culture supernate of mouse MSCs for 18 hours. After being washed, tube formation was observed with a light microscope at a magnification of x100, and pictures were captured. The number of tubes was counted. The number was counted in five different areas per well, and the average value was determined for each well.

**BrdU incorporation assay**

Briefly, 0.75 x 10^4 MSCs were seeded in 8 well chamber slides, grown overnight in 10% FBS and then starved in 2% FBS for 24 hours. Cells were then switched to 10% FBS for 24 hours. During the final 12 h of incubation, 10 µmol/L 5-bromo-2′-deoxyuridine (BrdU) was added into culture. The cells were fixed, permeabilized, the DNA denatured, and cells were exposed to anti-BrdU monoclonal antibody (1:1,000 diluted; Abcam) followed by an Alexa Fluor 594 conjugated goat anti-mouse secondary antibody (1:200 diluted; Molecular Probes). Fluorescence was monitored with a fluorescence microscope at a magnification of x100, and images were captured. We counted BrdU labeled cells in at least 2 separate fields, 3-4 wells/group. The percentage of proliferating cells is calculated as the number of BrdU^+ cells/number of total cells.

**Caspase 3/7 assay and TUNEL assay**

Caspase 3/caspase 7 activities were evaluated with a commercial Caspase 3/7 luminescent assay kit (Promega), following the manufacturer’s recommendations. We also performed TUNEL assay to label DNA strand breaks with an in situ TMR red detection kit (Roche). Fluorescence was monitored with a fluorescence microscope, and images were captured. To quantitate apoptosis, we counted labeled cells in at least 3 separate fields. The percentage of apoptosis is calculated as the number of TUNEL-positive cells/DAPI-labeled nuclear numbers. All experiments were repeated three times.

**siRNA transfection**

The small interfering RNA (siRNA) oligonucleotides for chemokines and scramble control were custom constructed by Ambion. The sequences of the siRNA templates were: CCL7, sense sequence CCAGAUGGGCCCAAUGCAUtt, anti-sense sequence AUGCAUUGGGCCCAAUCUGtt; CCL5, sense sequence GGGUUUCUUGAUUCACCtt, anti-sense sequence GGUCAGAAUCAAGAAACCCtc. Mouse MSCs were inoculated at a density of 1 x 10^4 cells/well in 8-well chamber slides to 70-80% confluency and transfected with 160 pg of siRNA and 1.6 µl of Lipofectamine 2000 in 100µl OPTI-MEM (GIBCO) for 24 hours. The medium was removed and replaced with fresh growth medium. The cells were harvested at 24 and 72 hours after transfection. Efficiency of knockdown by siRNA was assessed by RT PCR and real-time RT PCR. CCL5 and CCL7 mRNA levels from CCL5 or CCL7 siRNA transfected cells were compared with those from cells transfected with non-specific negative control siRNA (Ambion).

**Intramyocardial MSC delivery**

Permanent ligation of left anterior descending (LAD) coronary artery was performed in female Balb/c mice (8-10 weeks old) as described previously6. One hour after MI, MSCs were injected intramyocardially 1 mm above the ligation site. Animals were handled
according to approved protocols and animal welfare regulations of the Institutional Review Board at Duke University Medical Center.

**Tracking of the GFP+ cells injected in mice**

Quantification of green fluorescent protein (GFP) positive MSCs was performed on serial sections as described previously. The aim of this series of experiments was to determine if overexpression of specific chemokine receptors could influence migration of MSCs into the infarct zone. Since the migration of cells into the infarct zone would be influenced by the injection site (among other variables) we mixed control MSCs expressing β-galactosidase (LacZ+ MSCs) with the experimental MSCs expressing the specific chemokine receptor and GFP (CCR1-MSCs or CXCR2-MSCs) at a ratio of 1:4, thus any differences between the migration of the experimental MSCs and the control MSCs would be due to the expression of the specific chemokine receptor. In each animal, the migration of control LacZ+ MSCs and experimental GFP+ MSCs into the infarct zone was measured; changes in the ratio of GFP+/LacZ+ served as an index of changes in migration due to expression of the specific chemokine receptor.

Specifically, mice received a mixture of 0.75 x 10^5 LacZ+ MSCs and 3 x 10^5 GFP+ MSCs in 20 μl injected 1 mm above the ligation site 1 hour post infarct. Mice were sacrificed 3 days after injection. Hearts were perfused with 1x PBS and sliced transversally above the ligation suture. Tissue fixation was minimized to reduce autofluorescence. The tissue was embedded in OCT, and cut by a cryostat from the base (above the ligation) towards the apex until the ligation suture was reached. Beginning at this point, 10 μm sections were cut and collected throughout the entire lesion and divided into 18 groups of 130 μm. The first section of each of the 18 groups was analyzed.

Sections were stained using a rabbit anti β-galactosidase antibody (1:10,000; Abcam), then an Alexa Fluor 594 conjugated goat anti rabbit secondary antibody (1:200; Molecular Probes). Digital pictures for each section were taken. Using IPLab software, we measured the green fluorescent area, which corresponded to the dense cellular colonies. We calculated a ratio between the green (GFP+) area and red (LacZ+) area (GFP+/LacZ+) and the ratios were plotted as a function of migration distance from the ligation site. The area under the curve (AUC) was calculated using the trapezoidal method.

\[
AUC = \frac{1}{2} \times (\text{GFP}^+ / \text{LacZ}^+ \text{ area ratio}_1 + \text{GFP}^+ / \text{LacZ}^+ \text{ area ratio}_2) \times 0.13 + \frac{1}{2} \times (\text{GFP}^+ / \text{LacZ}^+ \text{ area ratio}_2 + \text{GFP}^+ / \text{LacZ}^+ \text{ area ratio}_3) \times 0.13 + \ldots + \frac{1}{2} \times (\text{GFP}^+ / \text{LacZ}^+ \text{ area ratio}_{17} + \text{GFP}^+ / \text{LacZ}^+ \text{ area ratio}_{18}) \times 0.13
\]

where “0.13” indicates the distance in mm between the two assayed sections.

**Area at risk and infarct size determination**

At 3 days post-MI, 0.3 ml of 1.5% Evan’s Blue dye in 1x PBS was injected retrograde into the brachiocephalic artery to delineate the in vivo area at risk (AAR). The nonischemic area, which was not at risk, was stained blue. The heart was excised and cut into four 1-mm-thick transverse slices below the ligature, parallel to the atrioventricular groove. Each slice was incubated in a 1% solution of triphenyltetrazolium chloride (TTC) at 37°C for 15 minutes to differentiate infarcted area (pale) from viable (brick red) myocardial area. The infarct area and the AAR from each section were measured by using Image J software, and the values obtained were averaged. The ratio
of infarct area/AAR were calculated and expressed as a percentage. The individuals conducting the experiment were blinded to the experimental groups.

**Capillary and arteriolar density assessment**
Hearts were analyzed 3 days and 4 weeks post-MI for capillary and arteriolar density by lectin staining as described previously. Briefly, cross sections of the hearts were incubated with 5 μg/mL Alexa Fluor 594 conjugated Griffonia Simplicifolia lectin GS-II (Molecular Probes) at 4°C overnight. After costaining with DAPI, the sections were visualized under microscope at a magnification of x 200 and photographed. Two photomicrographs were taken from the infarct border zone of each section. Six sections were selected for counting, giving a total of 12 fields per mouse. The number of capillaries was assessed using IPLab image analysis software. The capillary numbers were counted and data were estimated as capillaries/mm².

**Assessment of the collagen deposition and fibrosis**
Hearts were analyzed 3 days and 4 weeks post-MI for collagen deposition and fibrosis as previously described. Briefly, tissue sections of 10 µm were collected approximately every 130 µM from apex to base and were stained with Masson's trichrome to visualize the infarct scar. In each section, infarct scar area and the area of LV myocardium were traced manually using ImageJ 1.34 software in the digital images and measured automatically by the computer. Fibrotic and LV volumes were calculated by taking the areas and multiplying it by the section thickness. Total fibrotic and LV volumes in each heart were then calculated by the sum of fibrotic and LV volumes from all sections. Fibrotic volume in each heart was finally expressed as a percentage by calculating [sum of infarct volume from all sections/sum of LV volumes from all sections]×100. Samples from 4-9 animals were analyzed this way and the data are presented as mean ± SEM.

**Histochemical and immunofluorescent examination**
Histological cryopreserved tissue sections from mice hearts collected at 3 days and 4 weeks post-MI were used for histochemical and immunofluorescent examination. For in situ TUNEL staining, the same kit as in vitro TUNEL assay was used. The sections were visualized under microscope at a magnification of x 100 and photographed using a digital camera. To calculate the TUNEL-positive cardiomyocytes/total nuclei for tissue sections, the sections were first stained with α-sarcomeric actin antibody (mouse monoclonal first antibody, 1:500, Sigma; Qdot 605 goat F(ab')2 anti-mouse IgG conjugates (H+L) secondary antibody, 1:100, In vitrogen; visualized with Qdot 605 filter) and GFP antibody (chick first antibody, 1:1000, Abcam; Alexa Fluor 488 goat anti-chicken IgG secondary antibody, 1:400, In vitrogen), then stained with TUNEL kit and DAPI. Digital photographs were also taken under confocal microscopy at 200x magnification or fluorescent microscopy at 400x magnification. The total number of nuclei was determined by manual counting of DAPI-stained nuclei. For cardiomyocyte proliferation analysis, the sections were triple stained with α-sarcomeric actin, GFP and Ki67 antibodies (Ki67 antibody: rabbit polyclonal first antibody, 1:200, Lab Vision; Alexa Fluor 594 goat anti-rabbit IgG secondary antibody, 1:400, In vitrogen). The nuclei were also stained with DAPI.

**Echocardiographic study**
Serial transthoracic echocardiography was performed on conscious mice from all groups with an HDI 5000 echocardiograph (Philips) and a Vevo 770 high-resolution imaging system (VisualSonics) pre-, 2 and 4 weeks post-MI as previously described. LV end
diastolic and end systolic dimensions (LVEDD, LVESD, respectively), LV mass (LVm), fractional shortening (FS) were quantified. Percentage of FS reduction after 4 weeks of MI was expressed as (FS of pre-MI – FS of 4 weeks post-MI)/FS of pre-MI X 100%.

**Cytokine arrays**
For cytokine analysis, we performed mouse cytokine antibody array (RayBiotech, Inc.) following the manufacturer’s instructions. Briefly, the array membranes were incubated with blocking buffer followed by diluted mouse heart tissue lysates (500ug/ml) at 4°C overnight. Then, the membranes were washed, incubated with biotin-conjugated Abs at 4°C overnight. After washing, HRP-conjugated strepavidin were added to the membranes, and incubated at room temperature for 2 hours. Finally, the membranes were incubated in detection buffer and exposed to x-ray films. The spot density was analyzed using an IPLab software.

**Statistical analysis**
Student’s t test was used for two-group comparison. Analysis of variance (ANOVA) followed by an unpaired Student t test with Bonferroni’s correction was used for multiple group comparisons, with significance accepted at the $P < 0.05$. 


Online Supplement Figures and Legends.

Online Figure I. Culture supernatants from CCR1-MSCs showed enhanced angiogenic effects A, B). (A) VEGF levels in culture supernatants of control GFP, CCR1-, or CXCR2-MSCs subjected to 12h serum deprivation as measured by ELISA. (B) HUVECs tube formation in Matrigel assay. Culture supernatants were collected from MSCs after 12h serum deprivation and added into HUVECs culture. After 48h, the number of tubes was counted. (C) VEGF levels were measured by ELISA and adjusted by the number of viable cells after serum deprivation, then normalized to control MSCs group and presented as percentage. Data represent mean ± SD; n = 3-4; *P < 0.05 compared with control MSCs.
Online Figure II. Overexpression of CCR1, but not CXCR2, enhanced the migration ability of murine MSCs into infarcted myocardium. Mice received a mixture of $0.75 \times 10^5$ LacZ+ MSCs and $3 \times 10^5$ GFP+ MSCs (control GFP-MSC, CCR1-MSCs or CXCR2-MSCs) injected 1 mm above the ligation site 1 hour post infarct. Three days post-MI sections were stained using anti β-galactosidase antibody (red). Digital pictures for each section were taken. The control GFP, CCR1-, or CXCR2- MSCs are in green; LacZ-MSCs are in red; Nuclei are in blue (DAPI staining). The green (GFP+) area and red (LacZ+) area were measured and the GFP+/LacZ+ area ratio was calculated for each section from the ligation site down to the apex. The GFP+/LacZ+ area ratio are plotted as a function of migration distance from the ligation site (see Material and Methods).
Online Figure III. Intramyocardial injection of CCR1-MSCs, but not CXCR2-MSCs, reduced collagen deposition 3d post-MI. (A) Representative images of Masson’s trichrome staining for heart sections (myocardial cells, red; collagen, blue). (B) The percentage of initial collagen deposition was measured. Data represent mean ± SEM; n = 6-8 mice per group; *P < 0.05 compared with control MSC injected mice.
Online Figure IV. Intramyocardial injection of CCR1-MSCs, but not CXCR2-MSCs enhanced capillary density in the infarcted myocardium. (A) Representative images of lectin staining (red) in infarct adjacent border zone 3d post-MI. Nuclei were in blue (DAPI staining). (B) The infarction size was significantly negative correlated with capillary density. The percentage of infarction/AAR ratio and number of capillaries/mm² were analyzed together and the correlation coefficient was calculated (r = -0.90).
Online Figure V. Representative image of immunofluorescence staining for heart sections 4 weeks post MI. (A) TUNEL-positive cardiomyocytes. TUNEL-positive: red; cardiomyocyte: α-sarcomeric actin, strong red; GFP: negative, green; nuclei: DAPI, blue. (B) Proliferating cardiomyocyte. Ki67 positive: red; cardiomyocyte: α-sarcomeric actin, strong red; GFP: negative, green; nuclei: DAPI, blue.
Online Figure VI. Cytokine production in 3d post-MI mouse hearts injected with MSCs. Tissue lysates of hearts were analyzed for cytokine production using a mouse cytokine antibody array. (A) X-ray films for membranes incubated with tissue lysates from either control MSC injected heart or CCR1-MSC injected heart. Arrows point to blots for granulocyte colony-stimulating factor (G-CSF). (B) Densitometric analysis for the intensity of spots. The fold is relative to normalized values of control MSC injected heart. Expression level of G-CSF, which has been shown to increase the mobilization of stem cells from BM into peripheral circulation and protect myocardium after MI\textsuperscript{9,10}, was enhanced dramatically in CCR1-MSC injected heart.
Online Figure VII. A schematic diagram of the suggested mechanisms through which CCR1 overexpressing MSCs confer protection to the ischemic myocardium.

References


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