Essential Role of ICAM-1/CD18 in Mediating EPC Recruitment, Angiogenesis, and Repair to the Infarcted Myocardium

Yaojiong Wu, James E. Ip, Jing Huang, Lunan Zhang, Kenichi Matsushita, Choong-Chin Liew, Richard E. Pratt, Victor J. Dzau

Abstract—Bone marrow–derived endothelial progenitor cells (EPCs) have the ability to migrate to ischemic organs. However, the signals that mediate trafficking and recruitment of these cells are not well understood. Using a functional genomics strategy, we determined the genes that were upregulated in the ischemic myocardium and might be involved in EPC recruitment. Among them, CD18 and its ligand ICAM-1 are particularly intriguing because CD18 and its heterodimer binding chains CD11a and CD11b were correspondingly expressed in ex vivo–expanded EPCs isolated from rat and murine bone marrows. To further verify the functional role of CD18 in mediating EPC recruitment and repair to the infarcted myocardium, we used neutralizing antibody to block CD18. Blockade of CD18 in EPCs significantly inhibited their attachment capacity in vitro and reduced their recruitment to the ischemic myocardium in vivo by 95%. Moreover, mice receiving EPCs that were treated with control isotype IgG exhibited significantly increased capillary density in the infarct border zone, reduced cardiac dilatation, ventricular wall thinning, and fibrosis when compared with myocardial infarction mice receiving PBS and CD18 blockade reversed the EPC-mediated improvements to the infarcted heart. Thus, our results suggest an essential role of CD18 in mediating EPC recruitment and the subsequent functional effects on the infarcted heart. (Circ Res. 2006;99:315-322.)

Key Words: CD18 ■ EPC ■ recruitment ■ myocardial infarction ■ heart repair

Previous studies have suggested that bone marrow–derived endothelial progenitor cells (EPCs) could migrate to the foci of ischemia and promote repair of the injured organs. In animal models of myocardial infarction (MI), injection of ex vivo–expanded EPCs significantly improved blood flow and cardiac function and reduced left ventricular (LV) scarring. Similarly, infusion of ex vivo–expanded EPCs improved the neovascularization in hind limb ischemia models. Moreover, pilot trials using ex vivo–expanded EPCs isolated from rat and murine bone marrows. To further verify the functional role of CD18 in mediating EPC recruitment and repair to the infarcted myocardium, we used neutralizing antibody to block CD18. Blockade of CD18 in EPCs significantly inhibited their attachment capacity in vitro and reduced their recruitment to the ischemic myocardium in vivo by 95%. Moreover, mice receiving EPCs that were treated with control isotype IgG exhibited significantly increased capillary density in the infarct border zone, reduced cardiac dilatation, ventricular wall thinning, and fibrosis when compared with myocardial infarction mice receiving PBS and CD18 blockade reversed the EPC-mediated improvements to the infarcted heart. Thus, our results suggest an essential role of CD18 in mediating EPC recruitment and the subsequent functional effects on the infarcted heart.

In this study, we developed a functional genomics strategy to identify the mediators of bone marrow–derived stem cell recruitment to the infarcted myocardium. We defined the term recruitment hereby that includes chemotraction, adhesion, migration, retention, and accumulation. We generated expression profiles of MI heart and identified 16 chemokines, cytokines, and adhesion molecules that were significantly upregulated in myocardial ischemic injury whose complementary receptors were also expressed in EPCs. Accordingly, we identified ligand and receptor pairs potentially involved in EPC recruitment to the ischemic myocardium, which include ICAM-1 (ischemic myocardium)/CD18 (integrin β2, EPC), SDF-1 (ischemic myocardium)/CXCR4 (EPC), fibronectin-1 and VCAM-1 (ischemic myocardium/integrin α4 (EPC), and selectin (ischemic myocardium)/selectin ligand (EPC). We further examined the functional involvement of ICAM-1/CD18 in EPC recruitment and repair of the infarcted myocardium. We found that CD18 and its heterodimer binding chains CD11a and CD11b were highly expressed in expanded EPCs but declined with successive passage. Blockade of CD18 in EPCs by neutralizing antibody significantly reduced EPC recruitment to the ischemic myocardium, attenuated neovascularization, and worsened pathological remodeling.

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Materials and Methods

Isolation and Characterization of EPCs
We used EPCs derived from rat bone marrow because of the low yield of EPCs from mice. Athymic nude mice were used as receipts to avoid potential immunorejection to the transplanted rat EPCs. EPCs were isolated from the bone marrow of femurs and tibias of SD rats (male, 150 to 175g, Harlan) and BalbC mice (male, 5 to 7 weeks old, Harlan). Single bone marrow–nucleated cells were isolated by subsequent purification over Ficoll gradients. EPCs were isolated by cell sorting of the Flk1 and CD34 double-positive population11–13 and cultured in endothelial cell basal medium-2 (Clonetics) with supplementation as previously described.14 Confirmation of endothelial-cell lineage was performed as previously described15,15 in early passage cells. Briefly, FACS and indirect immunostaining were performed using antibodies directed against Flk-1, Tie-2, CD34, c-kit (Santa Cruz), VE-cadherin, CD31 (BD Pharmingen), and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate–acetylated low-density lipoprotein (DiI-acLDL). The cells were also analyzed on FACS for CD18, CD11a, and CD11b using fluorescein isothiocyanate (FITC)– or phycoerythrin (PE)-conjugated antibodies (BD Pharmingen). A mouse endothelial cell line, bEnd3 (American Type Culture Collection), was used as control for endothelial lineage marker expression.

Transplantation of Ex Vivo–Expanded EPCs
Rat EPCs collected after 7 days in culture were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes) as described previously.3,11,16 Trypan blue exclusion analysis of DiI-labeled EPCs (DiI-EPCs) at 24 and 72 hours showed no increase in cell death (data not shown). Immediately before injection, 0.5 ×10⁶ EPCs were incubated with anti-CD18 monoclonal antibody (mAb) (clone WT.3, IgG1, c; BD Pharmingen) or a control IgG isotype (a mouse IgG1, nonrelevant control antibody) at a concentration of 20 μg/mL for 30 minutes on ice. The cells were pelleted and resuspended in PBS before injection. To induce MI, athymic nude mice (female, 8 to 10 weeks old; Harlan) underwent permanent ligation of LAD coronary artery. One hour after MI, mice received a LV intracavity injection of 0.5 ×10⁶ Dil-EPCs pretreated with anti-CD18 EPCs-CD18 mAb group (DiI-labeled rat EPCs treated with blocking monoclonal antibody against CD18), control IgG (EPCs-IgG group [DiI-labeled rat EPCs treated with control isotype IgG]), or equal volume of PBS (PBS group). The needle was inserted at the apex away from the injected area. Extreme care was taken to avoid introduction of EPCs directly into the myocardium. MI hearts receiving a LV intracavity injection of equal volume of PBS were used as control. Sham animals underwent open chest surgery without coronary artery ligation and received LV cavity injection of the same amount of EPC-IgG. The mice were euthanized on day 3 and day 14.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results

Expression Profiles of Chemokines and Adhesion Molecules in the Ischemic Myocardium and Complementary Analysis of Their Receptors in EPCs
We developed a functional genomics strategy to identify the mediators of bone marrow–derived EPC recruitment to the infarcted myocardium. Our approach is based on the hypothesis that specific mobilizing and chemoattractant molecules released by the ischemic myocardium interact specifically with corresponding receptors on EPCs to induce mobilization, and that adhesion receptors in the ischemic myocardium are upregulated, activated, and bind to specific counter-receptors on the surface of the EPCs to enlist migration and engraftment. Accordingly, we generated expression profiles of MI heart after 8 and 24 hours by Affymetrix microarray analysis. Because our goal was to identify cytokines and adhesion receptors involved in trafficking and recruitment of EPCs into ischemic myocardium, we focused on a subset of 461 probes (of >22 000 probes on this array) related to cell adhesion, chemokines, cytokines, and chemotaxis, and 46 genes were found significantly upregulated. We further narrowed our focus on the 17 upregulated genes whose receptors might be expressed in EPCs and confirmed their expression by real-time PCR, which indicated that 16 of them had dramatically increased expression after MI and 15 of them had increased expression at both time points: 8 and 24 hours post-MI, including SDF1, E-selectin, ICAM-1, and VCAM-1 (Table 1). Examination of the expression of the receptors of these upregulated chemokines and adhesion molecules in EPCs after 7 days in culture by real-time PCR analysis indicated that all of them were expressed, including CXCR4, E-selectin ligand, CD18, and integrin α4 (Figure 1). We hypothesized that these ligand/receptor pairs were potentially involved in EPC recruitment and repair to the infarcted myocardium.

EPCs Express CD18 That Declines With Successive Ex Vivo Expansion
The involvement of SDF1/CXCR4 and selectin/selectin ligand in EPC recruitment to injured tissues was reported recently.16–18 In this study, we focused on the examination of the role of ICAM-1 (upregulated in ischemic myocardium) and CD18 (in EPCs) pair in mediating EPC recruitment to the

TABLE 1. Chemokines and Adhesion Molecules Upregulated in the Ischemic Myocardium

| Chemokine (C-C motif) ligand 2 | Ccl2 | 551 | 36.6 |
| Chemokine (C-C motif) ligand 6 | Ccl6 | 22.6 | 18 |
| Chemokine (C-C motif) ligand 7 | Ccl7 | 125 | 32.6 |
| Chemokine (C-C motif) ligand 9 | Ccl9 | 3.8 | 1.8 |
| Chemokine (C-X-C motif) ligand 1 | Cxcl1 | 1300 | 18.9 |
| Chemokine (C-X-C motif) ligand 2 | Cxcl2 | 2141 | 68.8 |
| Fibronectin 1 | Fn1 | 3.7 | 31 |
| Intercellular adhesion molecule 1 | Icam1 | 6.9 | 0.6 |
| Interleukin 6 | Il6 | 768 | 40 |
| Selectin, endothelial cell | Sele | 26.8 | 4.9 |
| Transforming growth factor, β1 | Tgfb1 | 2.3 | 0.3* |
| Transforming growth factor, β2 | Tgfb2 | 2.2 | 2.4 |
| Thrombospondin 1 | Tlbs1 | 167.7 | 10.7 |
| Tenascin C | Tnc | 98 | 178 |
| Vascular cell adhesion molecule 1 | Vcam1 | 1.0 | 0.6 |
| Vascular endothelial growth factor A | Vegfa | 0.2* | 0.2* |
| Stromal cell derived factor-1 | Sdf1 | 0.7 | 0.7 |

Real-time PCR showing fold increases (average of three analyses) of cytokines and adhesion molecules in MI vs sham hearts after 8 and 24 hours (P<0.05 except *P>0.05).
infarcted heart. Fluorescence-activated cell sorting (FACS) analysis indicated that the ex vivo–expanded EPCs expressed endothelial markers (Figure 2), and 95% of the cells express CD18, CD11a, and CD11b on the cell surface (Figure 2), but the positive populations declined with successive passage (Table 2). Similar results were obtained with cultured EPCs derived from the bone marrow of Balb/C mice (data not shown).

**CD18 Blockade Reduces EPC and Leukocyte Adhesion to HUVECs**

As the ligand of CD18, ICAM-1 mRNA expression was confirmed upregulated 8 and 24 hours after MI in the ischemic myocardium by real-time PCR analysis (Figure 3A, \( P < 0.01 \)). Immunohistochemistry analysis of the myocardium 24 hours after sham operation or 8, 24, and 72 hours after MI using an anti–ICAM-1 mAb with counterstaining of von Willebrand factor (vWF) for endothelial cells or sarcomeric \( \alpha \)-actin for myocytes indicated that in the myocardium of sham-operated animals, ICAM-1 was mainly colocalized to endothelial cells of larger blood vessels; however, in the MI myocardium, especially in the 8- and 24-hour MI myocardium, ICAM-1 expression extended to endothelial cells of capillaries and, to a lesser extent, myocytes. Upregulation of ICAM-1 sustained in the 72-hour MI myocardium in the infarct border zone (Figure 3B). Previous studies have indicated that CD18 plays a key role in leukocyte adhesion to activated endothelial cells and extravasation to the inflammatory zones through interaction with ICAM-1.19,20 We tested the blocking ability of CD18 blocking mAb (clone WT.3) by examining whether it could block CD18 and ICAM-1 binding. Rat leukocytes, which express CD18 on the surface, were preincubated with WT.3 or isotype IgG followed by incubation with FITC conjugated ICAM-1. ICAM-1 binding to leukocyte was determined by FACS analysis. The result indicated that 10 \( \mu \)g/mL WT.3 sufficiently blocked FITC-labeled ICAM-1 binding to rat leukocytes (Figure 3C). To examine the functional involvement of CD18 in mediating EPC recruitment, we first examined whether CD18 was involved in EPC adhesion. We seeded EPCs on HUVEC monolayers in the presence of anti-CD18 mAb WT.3 or isotype IgG, and leukocytes were used as a control. FACS analysis indicated that under our condition of culture, 70% of the HUVECs expressed ICAM-1 on the cell surface (Figure 3D). The presence of anti-CD18 mAb significantly reduced EPC (Figure 3E through 3G) and leukocyte adhesion to HUVEC monolayers (Figure 3H through 3J) (\( P < 0.0001 \)).

**CD18 Blockade Reduces EPC Recruitment to the Infarcted Myocardium**

Three days after LV intracavity injection, EPCs-IgG were found principally in the areas of infarcted ventricular myocardium. In contrast, EPCs-CD18 mAb were barely found in the infarcted heart sections (Figure 4A). Quantification of Dil-labeled EPCs after whole heart digestion 3 days after injection (Figure 4B) indicated a 33-fold greater number of EPCs in the MI hearts compared with those in the sham hearts (\( n = 5, P < 0.001 \)). Treatment of EPCs with anti-CD18 antibody before injection reduced EPCs in the MI hearts by 95% (\( n = 5, P < 0.001 \)). To examine the specificity of EPC migration to the MI heart, we also quantified the Dil-labeled EPCs in the spleen (Figure 4B). In the sham-operated mice, there were 4.7-fold more EPCs in the spleens than in the hearts (\( P < 0.01 \)). In contrast, in the MI mice, 15-fold more EPCs...
were in the hearts than in the spleens (P<0.001). MI led to a reduction of EPCs found in the spleen (P<0.001); CD18 blockade attenuated this reduction. When we examined the frozen heart sections 2 weeks after administration EPCs-IgG under fluorescence microscope, we found a considerable number of DiI-EPCs localized to the infarct border zone (Figure 4C). The infarct was indicated by Masson’s trichrome staining in successive sections of the heart (data not shown). In contrast, EPCs-CD18 mAb were barely detected in the infarcted hearts at 2 weeks (Figure 4D). To examine if macrophages in the lesion uptake dead DiI-EPCs and contribute to DiI-positive cells in the myocardium, we conducted immunostaining using a monoclonal antibody against CD68, which was detected with a FITC-conjugated secondary antibody. We observed CD68-positive cells and DiI-EPCs but barely detected double stained cells (Figure I in the online data supplement), indicating that the contribution of macrophage to DiI positive cells is minor.

<table>
<thead>
<tr>
<th>EPC Expansion Passages and Surface Receptor Populations</th>
<th>CD34</th>
<th>Flk1</th>
<th>VE-cadherin</th>
<th>CD31</th>
<th>CD18</th>
<th>CD11a</th>
<th>CD11b</th>
<th>CXCR4</th>
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<tr>
<td>EPC 7 days</td>
<td>97±1.6</td>
<td>99±1.2</td>
<td>96±1.8</td>
<td>76±2.3</td>
<td>96±1.8</td>
<td>96±2.1</td>
<td>95±1.7</td>
<td>96±2.3</td>
</tr>
<tr>
<td>EPCp1</td>
<td>N/A</td>
<td>97±2.4</td>
<td>95±2.2</td>
<td>87±3.1*</td>
<td>69±2.8*</td>
<td>69±3.4*</td>
<td>82±3.7*</td>
<td>78±4.1*</td>
</tr>
<tr>
<td>EPCp3</td>
<td>N/A</td>
<td>99±1.9</td>
<td>98±1.7</td>
<td>91±5.9*</td>
<td>29±5.3*</td>
<td>27±5.5*</td>
<td>27±4.8*</td>
<td>38±4.4*</td>
</tr>
<tr>
<td>bEnd3</td>
<td>93</td>
<td>84</td>
<td>98</td>
<td>56</td>
<td>3</td>
<td>18</td>
<td>18</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Values are expressed as percentage. FACS analysis indicated the positive percentages of ex vivo–expanded EPCs of 7 days in culture, passage 1 and passage 3 (average of 3 experiments, *P<0.01). bEnd3 cells were used as control for endothelial lineage markers.
Previous studies have shown that exogenous EPCs promote neovascularization. To investigate the effect of EPC transplantation on vasculature in infarcted myocardium and to assess the influence of CD18 blockade on EPCs, we examined the myocardial vasculature in the infarct border zone 2 weeks after exogenous EPC administration. Capillary density was assessed morphometrically after histochemical staining with Bandeiraea simplicifolia lectin I (isolectin B4). As shown in Figure 5, capillary density was significantly higher in mice receiving EPCs treated with isotype IgG than with CD18 mAb (2282 ± 204 versus 1282 ± 197, n=5, P<0.0001).

CD18 Blockade Abolishes Exogenous EPC-Mediated Protection of the Infarcted Heart

In the previous studies, EPC transplantation was shown to reduce infarct size and improve heart function. To examine the impact of CD18 blockade on exogenous EPC-mediated myocardial protection, we examined the heart morphology 2 weeks after MI. In mice receiving EPCs-IgG, 4 of 7 hearts appeared normal in size, but in mice receiving EPCs-CD18 mAb, 7 of 8 hearts were apparently enlarged and dilated, which appeared similar to the MI hearts receiving vehicle PBS injection (Figure 6A). Masson’s trichrome staining showed significantly reduced collagen deposition in the infarcted hearts of mice receiving EPCs-IgG than those of mice receiving EPCs-CD18 mAb (Figure 6B and 6C, P<0.05), which exhibited a similar amount of fibrosis than in the MI hearts receiving vehicle PBS injection (P<0.05). Consistent with this, measurement of the left ventricles indicated that MI mice receiving EPCs-IgG had significantly reduced LV dilatation (Figure 6D and 6E, P<0.05) and increased LV wall thickness (Figure 6D and 6E, P<0.005) than MI mice receiving vehicle PBS injection. In contrast, MI mice receiving EPCs-CD18 mAb exhibited similarly increased LVD and reduced LV wall thickness than MI mice receiving vehicle PBS injection (Figure 6D and 6E, P>0.05).

Discussion

Previous studies have suggested that bone marrow–derived EPCs could migrate to the foci of ischemia and promote
repair of the injured organs.\textsuperscript{1} Injection of ex vivo–expanded EPCs has exhibited improvement in blood flow, cardiac function, infarct size, and neovascularization of the infarcted heart.\textsuperscript{2–4} EPCs derived from cord blood were found within tumor microvessels, extravasated into the interstitium, and incorporated into neovessels, suggesting that EPCs possess capacities of trafficking, migration, and engraftment.\textsuperscript{22}

However, the signals that mediate trafficking and recruitment of these cells to injured myocardium are not well understood. Using a functional genomics approach coupled with real-time PCR analysis, we identified ligand/receptor pairs potently involved in mediating EPC recruitment and engraftment to the ischemic myocardium, which include ICAM-1 (ischemic myocardium)/CD18 (EPC), SDF-1 (ischemic myocardium)/CXCR4 (EPC), fibronectin-1 and VCAM-1 (ischemic myocardium)/integrin α4 (EPC), and selectin (ischemic myocardium)/selectin ligand (EPC). Of these, SDF1/CXCR4 and selectin/selectin ligand have been reported recently to be involved in EPC recruitment process,\textsuperscript{16–18} thereby validating our functional genomics strategy for the identification of mediators in EPC recruitment to the infarcted myocardium.

In this study, we show an essential role of CD18/ICAM-1 in EPC recruitment to the ischemic myocardium. Our real-time PCR analysis indicates that the expression of ICAM-1 in the ischemic myocardium, which include ICAM-1 (ischemic myocardium)/CD18 (EPC), SDF-1 (ischemic myocardium)/CXCR4 (EPC), fibronectin-1 and VCAM-1 (ischemic myocardium)/integrin α4 (EPC), and selectin (ischemic myocardium)/selectin ligand (EPC). Of these, SDF1/CXCR4 and selectin/selectin ligand have been reported recently to be involved in EPC recruitment process,\textsuperscript{16–18} thereby validating our functional genomics strategy for the identification of mediators in EPC recruitment to the infarcted myocardium.

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Expression of ICAM-1 in cardiomyocyte has been reported previously and is considered as a mechanism for adhesion of leukocytes to myocytes after ischemic injuries.\textsuperscript{23,24} The expression of CD18 and its heterodimer binding chains CD11a and CD11b, the receptor of ICAM-1, were detected in EPCs. Further, using FACS analysis, we confirmed the expression of the receptors on the surface in \textasciitilde 95% of ex vivo–expanded EPCs derived from both rat and mouse bone marrow. Blockade of CD18 with a neutralizing antibody significantly reduced ICAM-1 binding to leukocyte and inhibited EPC and leukocyte adhesion to HUVECs. We found very limited DiI-EPCs in the hearts of sham-operated mice, which were several-fold lower than that in the spleens. After acute MI, however, we found a 33-fold increase of the EPCs recruited to the heart, which was 15-fold higher than the amount in the spleen. Histologic analysis indicated that the EPCs were recruited into the ischemic myocardium and retained in the infarct border zone. This result is consistent with a previous observation, in which the radioactively labeled EPCs were injected, and radioactivity was mainly localized in the liver and spleen of the sham-operated rats, whereas the radioactivity of the infarcted heart was higher than that of the sham heart.\textsuperscript{8} Normally, ICAM-1, along with CXCR4, is differentially expressed in the endothelia of different organs. ICAM-1 and CXCR4 are constitutively expressed on the cell surface of the endothelia in the bone marrow and spleen, which contributes to the homing of the circulating progenitor cells to these organs.\textsuperscript{25–28} CD18 blockade significantly reduces recruitment of EPCs to the infarcted hearts by more than 90%, supporting an essential role of CD18 in mediating EPC recruitment and retention in the ischemic myocardium. However, our conclusion is limited by the lack of a negative control with another
cell surface binding antibody (IgG). Thus, our results could not rule out the possibility that the anti-CD18 IgG-coated EPC complex might be removed nonspecifically from the circulation by the reticuloendothelial system, thereby reducing their numbers in the heart. Nevertheless, our result is consistent with a recent finding in which Sca-1+/Lin- hematopoietic progenitor cells from CD18-deficient mice were found less capable of migration to sites of hind limb ischemia.59 It has been known that CD18 is crucial for leukocyte firm adhesion to the activated endothelial cells and subsequent extravasation.19 CD18-deficient mice exhibit severe defects in leukocyte recruitment, adhesion, and extravasation in response to inflammatory stimuli.20,30 Loss of the CD18 ligand ICAM-1 also causes defect in lymphocyte adhesion and lymphoid tumor cell metastasis.31 On the other hand, stem cell recruitment is complex and involves the interaction of several ligand receptor pairs. Previous studies have suggested that selectin/selectin ligand and SDF1/CXCR4 might be involved in EPC recruitment.16–18 However, overexpression of SDF-1 in the normal heart did not enhance the recruitment of bone marrow–derived lineage negative cells,17 strongly suggesting involvement of multiple factors in the event.

We found that MI mice receiving CD18-blocked EPCs exhibited as severe cardiac enlargement, LV dilatation, wall thinning, and fibrosis as those receiving no EPC treatment and much more severe than those receiving EPCs treated with IgG, suggesting that CD18 blockade abolished exogenous EPC-mediated myocardial protection and/or repair. Interestingly, a recent study suggested that preactivation of CD18 on EPCs by activating antibodies augmented the EPC-induced neovascularization in a murine model of hindlimb ischemia.29 Taken together, these findings suggest a therapeutic potential in increasing recruitment of bone marrow–derived stem cells to injured tissues.

Previous studies have suggested that 3 mechanisms may be involved in EPC-mediated myocardial protection and repair after acute MI: reendothelialization of the denuded blood vessels, neovascularization, and paracrine effect.32 In this study, we confirmed the incorporation of the exogenous EPCs into the endogenous capillaries, as has been observed previously.1,11 Moreover, we found that mice receiving EPCs with CD18 blockade had significantly reduced capillary density in the infarct border zones of the myocardium than the mice receiving EPCs without CD18 blockade. Previous studies have shown that cultured EPCs release growth factors, such as vascular endothelial growth factor, hepatocyte growth factor, granulocyte colony–stimulating factor, granulocyte-macrophage colony–stimulating factor, and platelet-derived growth factor-B,33 that could exert protective effect on myocardial cells. Indeed, many of these growth factors have been known to promote cell proliferation, enhance cell survival, and facilitate cardiac repair after acute MI.

Different preparations of EPCs have shown varied degrees of recruitment to the ischemic tissues in the previous studies.8–10,35 One important determinant may be the level of expression of the key receptors for trafficking, migration, and adhesion on the expanded EPCs, such as CD18 and its heterodimer binding chains. In this study, we found that the CD18 positive EPCs declined with successive expansion passages, and mature endothelial cells do not express CD18 and its heterodimer-binding chains CD11a and CD11b. We speculate that this phenomenon might explain the previous reports that infusion of mature endothelial cells, such as HUVEC, gastroepiploic artery endothelial cells, and mouse saphenous vein endothelial cells, did not show benefits in improving tissue ischemia.2,4,36

In this study, we identified 16 ligands in the ischemic myocardium with complementary receptor expression in EPCs. Our data suggest an essential role of ICAM1/CD18 in EPC recruitment and repair of the infarcted myocardium. These results support the validity of our functional genomics approach in the identification of the mediators in EPC recruitment and repair of the infarcted heart. However, given the complexity of EPC recruitment and repair, we will need to study more receptor–ligand interactions individually and in combination to fully understand the cellular and molecular mechanisms that govern these events.

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Disclosures
None.

References


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Materials and Methods

Expression Profiling of Myocardium of Acute Ischemic Injury and Complementary Expression Analysis of EPCs

BalbC mice (female, 8-10 weeks old, Harlan) underwent left anterior descending (LAD) coronary artery ligation as previously described.\textsuperscript{1} Eight hours and twenty four hours after MI, total RNA was extracted from ischemic myocardium of the infarct border zone (n = 3 at each time point). Corresponding regions from sham-operated littermates were used as controls (n = 3 per time point). Total RNA was used for Affymetrix microarray analysis. The upregulated chemokines and adhesion molecules with potential complementary receptor expression in EPCs were further verified by Real Time PCR. The expression of complementary receptors of the chemokine and adhesion molecules in EPCs was detected by Real Time PCR of the EPCs which were derived from rat bone marrow and were cultured for 7 days. The primer sequences can be found as Supplementary Table 1 on line. The animals were used with approval of the Harvard Medical Area Standing Committee on Animals.

Cell Adhesion Assay and CD18 Blockade

The azide-free WT.3 mAb (BD Pharmingen) is an IgG1 mAb that reacts with rat CD18. The antibody neutralized CD18 function \textit{in vitro} and \textit{in vivo} in numerous studies.\textsuperscript{2,3} The saturating concentration of the antibody in rat EPCs (10 \(\mu\)g/ml) was determined by FACS
analysis as previously described. The inhibitory effect of WT.3 on CD18 binding to ICAM-1 was tested. Rat ICAM-1 (R&D Systems) or control IgG was FITC conjugated using ProtOn Fluorescein Labeling Kit (Vector Laboratories) following the manufacture’s instruction. Rat peripheral leucocytes isolated by density gradient sedimentation in Ficoll were incubated with IgG or WT.3 at a concentration of 10 μg/ml in HBSS containing 0.5% BSA for 20 minutes at room temperature. The cells were then incubated with FITC-conjugated rat ICAM-1 at a concentration of 10 μg/ml for 30 minutes at 37°C. After washes, the samples were subjected to FACS analysis. Cells without staining and cells stained with FITC-conjugated IgG were used as negative controls. Leukocyte and EPC adhesion to human umbilical vein endothelial cells (HUVEC) monolayers was examined as previously described. HUVECs (Clonetics) were cultured in EBM endothelial basal medium with supplementation (Clonetics), and passage 3 or 4 cells were used for the study. The expression of ICAM-1 in HUVECs was detected by FACS analysis using PE-conjugated anti-human ICAM-1 mAb (BD Pharmingen). Rat peripheral leucocytes were suspended at a density of 1x10^6 in M199 plus 0.1% BSA. Rat EPCs after 7 days of culture were labeled with fluorescent carbocyanine 1,1’-dioctadecyl-1 to 3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI) dye (Molecular Probes) as previously described and suspended at a density of 0.5 x10^6 EMB-2 (Clonetics) plus 0.1% BSA. After incubation with WT.3 mAb at concentrations of 5 and 10 μg/ml, or isotype IgG (10 μg/ml) for 20 minutes at room temperature, the cells were added to confluent monolayers of HUVECs in 48-well plates, and incubated for 1.5 hour for the leucocytes and 45 min for the EPCs. The cultures were washed twice with HBSS for removal of non-adherent cells and the adherent cells were
fixed with 2% PFA. The total number of cells per high-powered field was counted after photograph. Two fields per well were photographed and 6 replicate wells were used for each condition.

**Quantification of Dil-labeled EPCs in the Heart and Spleen**

Three days after LV cavity injection of Dil-EPCs, the spleen and the heart were harvested. The organs were weighed, cut into small pieces, and underwent 3 sequential digestions (digestion buffer: 0.002% glucose, 0.1% collagenase, and 0.5% trypsin in PBS) in a 37°C shaking water bath for 15 minutes each. Enzymatic activity was neutralized with IMDM containing 10% FBS immediately after completion of each digestion. The digestions were pooled and the cells were pelleted by centrifugation. The Dil-positive cells were counted under fluorescence microscope.

**Histological Assessment of Vasculature and Fibrosis**

Two weeks after MI, the mouse hearts were perfused with PBS, arrested in diastole with KCl, and fixed with 4% PFA. Three transverse slices of the heart, one above and two below the ligation (base-, mid, apex portion), were embedded in OCT for histology. Tissue sections from the mid-portion of the heart were used for immunohistochemistry. For ICAM-1 detection, heart sections were incubated with a mAb against mouse ICAM-1 (eBiosciences) and detected with FITC-conjugated secondary antibody. The sections were double stained with a mAb against sarcomeric alpha actin (Sigma) for myocyte or an antibody against vWF (Santa Cruz) for endothelial cells and the signal was detected with a Cy3-conjugated secondary antibody. Nuclei were stained with Hoeschst.
Capillary density was assessed morphometrically by examination of 2 randomly selected fields per section (6 μm in thickness) in the peri-infarct areas in 10 sections of left ventricular myocardium subserved by the occluded left anterior descending coronary artery after histochemical staining with Bandeiraea simplicifolia lectin I (isolectin B4) (Vector Laboratories).\(^9\)^\(^{,}\)\(^{10}\) Briefly, tissue sections were incubated with biotinilated isolectin B4 at 4°C overnight, treated with alkaline phosphatase and developed in Vector Red (Vector Laboratories). The individual who performed the quantification was blinded to the treatment groups. To examine potential contribution of macrophages to DiI positive cells, heart sections were immunostained with a monoclonal antibody against CD68 (Santa Cruz) and detected with a FITC conjugated secondary antibody.

The mid-section was used to measure LV wall thickness and chamber diameter as described previously.\(^{11}\) Two sections from each of the three heart slices were subjected to Masson's Trichrome staining for fibrosis. The areas of blue staining were quantified morphometrically and expressed as a ratio of the entire left ventricular wall area.

**Statistics**

All values were expressed as mean ± SD. Student’s paired t test was performed for comparison of data between the IgG treated and CD18 mAb treated samples, and one-way ANOVA test was used for multiple group comparisons. A probability (\(P\)) value < 0.05 was considered significant.
Supplementary Table 1. Real Time PCR primers (from 5’ to 3’)

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Abbreviations: SDF-1, stromal cell derived factor-1; CC, chemokine (C-C motif); CXC, chemokine (C-X-C motif); VEGF, vascular endothelial growth factor; IL, Interleukin; VCAM, Vascular Cell Adhesion Molecule; ICAM, Intercellular Cell Adhesion Molecule; TGF, Transforming Growth Factor; TGFBRII, TGFB receptor; Thbs1, thrombospondin1; SelL, E-selectin ligand.

Supplementary Figure 1. Immunostaining of macrophages

Figure 1. Immunostaining of macrophages. Heart sections of 2 week MI myocardium received Dil-labeled EPCs pre-treated with IgG were immunostained with a monoclonal antibody against CD68 and detected with a FITC conjugated secondary antibody (green). Dil-positive cells are indicated in red. Nuclei were stained with Hoechst (blue).
Reference List


