Stromal Cell–Derived Factor-1α Plays a Critical Role in Stem Cell Recruitment to the Heart After Myocardial Infarction but Is Not Sufficient to Induce Homing in the Absence of Injury

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Background—After myocardial infarction (MI), bone marrow–derived cells (BMDCs) are found within the myocardium. The mechanisms determining BMDC recruitment to the heart remain unclear. We investigated the role of stromal cell–derived factor-1α (SDF-1) in this process.

Methods and Results—MI produced in mice by coronary ligation induced SDF-1 mRNA and protein expression in the infarct and border zone and decreased serum SDF-1 levels. By quantitative polymerase chain reaction, 48 hours after intravenous infusion of donor-lineage BMDCs, there were 80.5±15.6% more BDMCs in infarcted hearts compared with sham-operated controls ($P<0.01$). Administration of AMD3100, which specifically blocks binding of SDF-1 to its endogenous receptor CXCR4, diminished BMDC recruitment after MI by 64.2±5.5% ($P<0.05$), strongly suggesting a requirement for SDF-1 in BMDC recruitment to the infarcted heart. Forced expression of SDF-1 in the heart by adenoviral gene delivery 48 hours after MI doubled BMDC recruitment over MI alone ($P<0.001$) but did not enhance recruitment in the absence of MI, suggesting that SDF-1 can augment, but is not singularly sufficient for, BDMC recruitment to the heart. Gene expression analysis after MI revealed increased levels of several genes in addition to SDF-1, including those for vascular endothelial growth factor, matrix metalloproteinase-9, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1, which might act in concert with SDF-1 to recruit BMDCs to the injured heart.

Conclusion—SDF-1/CXCR4 interactions play a crucial role in the recruitment of BMDCs to the heart after MI and can further increase homing in the presence, but not in the absence, of injury. **(*Circulation*. 2004;110:3300-3305.)**

Key Words: cell adhesion molecules ■ cells ■ growth factors ■ myocardial infarction

Peripheral and bone marrow–derived stem cells (BMDCs) are a promising therapy for the treatment of ischemic cardiomyopathy. Although the differentiation capacity of BMDCs directly injected into the heart after myocardial infarction (MI) is controversial, they result in improved ventricular function,[1–3] which may be due to production of cytokines that potentially restore heart function and vascularization.[4] Early experience with autologous unfractionated bone marrow (BM) via the endocardial and intracoronary routes has been promising with respect to improvement in ischemic parameters.[5,6] The invasive nature of BM harvest and subsequent cardiac delivery, however, has limited the clinical utility of cell therapies. An alternative approach to stem cell (SC) therapy is to manipulate the natural factors responsible for homing to sites of injury.

Myocardial ischemia mobilizes and potentially homes BMDCs. Circulating endothelial progenitor cells increase after cardiac ischemia or vascular injury.[7,8] After MI, BMDCs are detected in the peri-infarct region as endothelial cells and rarely, myocytes.[3,9] In addition, SCs delivered intravenously within 24 hours of MI engraft in the heart and have a similar differentiation capacity.[10,11] The observation that the SCs localize to the peri-infarct region suggests the presence of local chemotactic factors such as stromal cell–derived factor-1α (SDF-1).

SDF-1 and its receptor CXCR4 are required for BMDC homing to the BM.[12] SDF-1 is also involved in stress-induced recruitment of SCs to the liver and neointima.[13,14] The role of SDF-1 in coronary artery disease is less clear. Although SDF-1 is expressed in atherosclerotic plaques, circulating levels of the chemokine are decreased in unstable coronary syndromes.[15,16] SDF-1 is upregulated in the heart early after MI, and exogenous expression late after MI increases vascular density and improves ventricular function when granulo-

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cyte colony stimulating factor is coadministered, which may result in adverse side effects such as in-stent restenosis.[17,18] In ischemic hindlimbs, exogenous SDF-1 did not promote limb salvage in the absence of delivered endothelial precursor cells but did decrease endothelial cell apoptosis.[19]

To clarify the role of SDF-1 in SC recruitment to the myocardium, we quantified the accumulation of systemically delivered BMDCs with overexpression of SDF-1 in the presence and absence of myocardial injury. Using the chemokine receptor CXCR4 antagonist AMD3100, we assessed the effect of blocking the SDF-1/CXCR4 interaction on the homing of delivered SCs. In addition, we examined the expression of factors potentially involved in the homing and retention of stem cells in the myocardium, including SC factor (SCF), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), vascular endothelial growth factor (VEGF), and matrix metalloproteinase-9 (MMP-9).

Methods

Construction of Adenoviral Vectors

Murine SDF-1α was released from the expression vector pORF5 (InvivoGen) and cloned into pACCMV.pLpASR−. Replication-deficient, recombinant adenovirus (AdV)-5 vectors were generated as previously described.[20] An AdV clone containing full-length SDF-1 (AdV.SDF) was confirmed by polymerase chain reaction (PCR). The control AdV was similarly constructed to contain a red fluorescent gene in the same shuttle vector (AdV.CONT). The AdV.SDF titer was 1.51×10^{11} plaque-forming units (PFU)/mL, and the titer for AdV.CONT was 2.3×10^{11} PFU/mL.

Animal Studies

Mice were cared for in accordance with National Institutes of Health guidelines, and all procedures were approved by the Yale University Animal Care and Use Committee.

Murine MI and Viral Transduction

Female, 8-week-old SCID mice (NOD-CB17-Prkdc scid/j, Jackson Laboratory, Bar Harbor, Me) were used for experiments involving cell transplantation (n=28), and CD-1 mice (Charles River, Wilmington, Mass) were used for gene and protein expression experiments (n=46). Mice were anesthetized with xylazine (5 mg/kg IP, Boehringer Ingelheim) and ketamine hydrochloride (100 mg/kg IP, Fort Dodge Animal Health). The mice were intubated and ventilated (Harvard Apparatus), and the heart was exposed through a lateral thoracotomy. With the aid of a surgical microscope (Leica MZ95), a 7-0 silk suture was placed blindly to occlude the left anterior descending coronary artery to produce an MI. Before chest closure, infarction was confirmed by observation of a demarcation of injury with blanching of the myocardium. Sham surgery involved the same cardiac exposure without placement of the coronary suture. For AdV delivery, 1×10^9 PFU of AdV.SDF-1 or AdV.CONT diluted in 15 μL of phosphate-buffered saline (PBS), or PBS alone, was injected into the left ventricular wall alone or immediately after MI into the peri-infarct zone.

The CD-1 hearts were harvested 48 hours after AdV transduction and at 48, 72, and 96 hours and 7 days after MI. The atria and right ventricular free wall were removed, and the entire left ventricle was frozen in LN_2 for isolation of protein and RNA. In 1 subset of the CD-1 hearts harvested 48 hours after MI, only the infarct and peri-infarct regions of the left ventricle were harvested for RNA to determine whether local and global left ventricular gene expression differed.

BM Isolation and Lineage Depletion

Donor BMCs were harvested from 8- to 12-week-old, knock-in MLC-2v cre mice (±) as previously described.[21] These mice are heterozygous for green fluorescent protein (GFP) cDNA, thereby allowing identification of donor cells by PCR on genomic DNA. Lineage-negative (Lin−) BM was obtained by depletion with the use of magnetic columns (Miltenyi Biotec Inc) and biotinylated, lineage-specific antibodies (anti-CD4, -CD8, -CD11, -B220, -Gr1, and -Ter119; Pharmingen). This method results in <2% Lin+ cells confirmed by flow cytometry.

SCID mice underwent intravenous delivery of 3×10^5 Lin− BMCs in the internal jugular vein in all experiments 48 hours after MI and/or AdV transduction. Seventy-two hours after SC delivery, heart was harvested, the atria and right ventricular free wall were removed, and the entire left ventricle was frozen in LN_2 for subsequent DNA isolation to quantify homing as described later.

In Vivo Inhibition of CXCR4

To block SDF-1–induced homing of delivered cells after MI, we used the bicyclam AMD3100 (Sigma), a potent and specific antagonist of the chemokine receptor CXCR4.[22] Before intravenous delivery, Lin− BMCs were incubated for 30 minutes at 37°C with 5 μg/mL AMD3100. The cells were washed in PBS and then immediately delivered intravenously.

Flow Cytometric Analysis

Fluorescence-activated cell sorting (FACS) detection of CXCR4 expression was performed on Lin− BM stem cells immediately after isolation with the use of phycoerythrin-conjugated anti-CXCR4 (PharMingen) and assayed on a FACS system (FACscan, Becton Dickinson).

Migration Assay

To investigate cell migration in response to SDF-1 and CXCR4 inhibition with AMD3100, a modified Boyden chamber assay was performed with a 96-well, microchemotaxis chamber (NeuroProbe) with 5-μm pores. Murine SDF-1 (PharMingen) diluted to 100 ng/mL or medium alone was placed in the lower half of the chemotaxis chamber. Lin− BMCs were incubated for 30 minutes at 37±C with or without 5 μg/mL AMD3100. The cells were resuspended in Iscove's modified Dulbecco's medium with or without SDF-1 100 ng/mL and placed on the upper compartment. After incubation at 37°C for 2 hours and subsequent dilution in a known concentration of fluorescent beads (Spherotech), the number of migrated cells was measured by flow cytometry.

PCR for Quantification of Homing

Genomic DNA was isolated from left ventricular myocardial tissue with the Easy-DNA kit (Invitrogen). In brief, the frozen tissue was pulverized, resuspended in the DNA isolation solutions, and incubated overnight at 60°C with 100 μg of protein degrader. After DNA precipitation, samples were treated with 40 μg/mL RNase for 30 minutes at 37°C. GFP primers were as follows: forward, 5′-GATGGCCCTGTCCTTTTACCA-3′ and reverse, 5′-TTTCGTTGGGATCTTTCGAAA-3′), and that for a fluorogenic GFP probe was 5′ HEX ACAACCATTACCTGTCCACA-CAATCTGCC BHQ-1 3′, Biosearch Technologies). The DNA Engine Opticon 2 Continuous Fluorescence Detection System was used (MJ Research, Inc). PCR began with a 1-minute, 94°C denaturation step followed by 40 cycles at 94°C for 30 seconds, 58°C for 1 minute, and 72°C for 1 minute.

ELISA for Murine SDF-1α and VEGF

Quantitative immunoassays were used for both SDF-1 and VEGF, according to the manufacturer's protocol (R&D Systems). Blood was obtained by cardiac aspiration and serum was isolated after clotting. Tissue extracts from heart and liver were prepared by homogenization and lysis with 25 mmol/L Tris, 1% Triton X-100, 0.5 mmol/L EDTA, 150 mmol/L NaCl, 10 mmol/L NaF, and a protease inhibitor cocktail (Roche Mannheim). Recombinant murine SDF-1 and

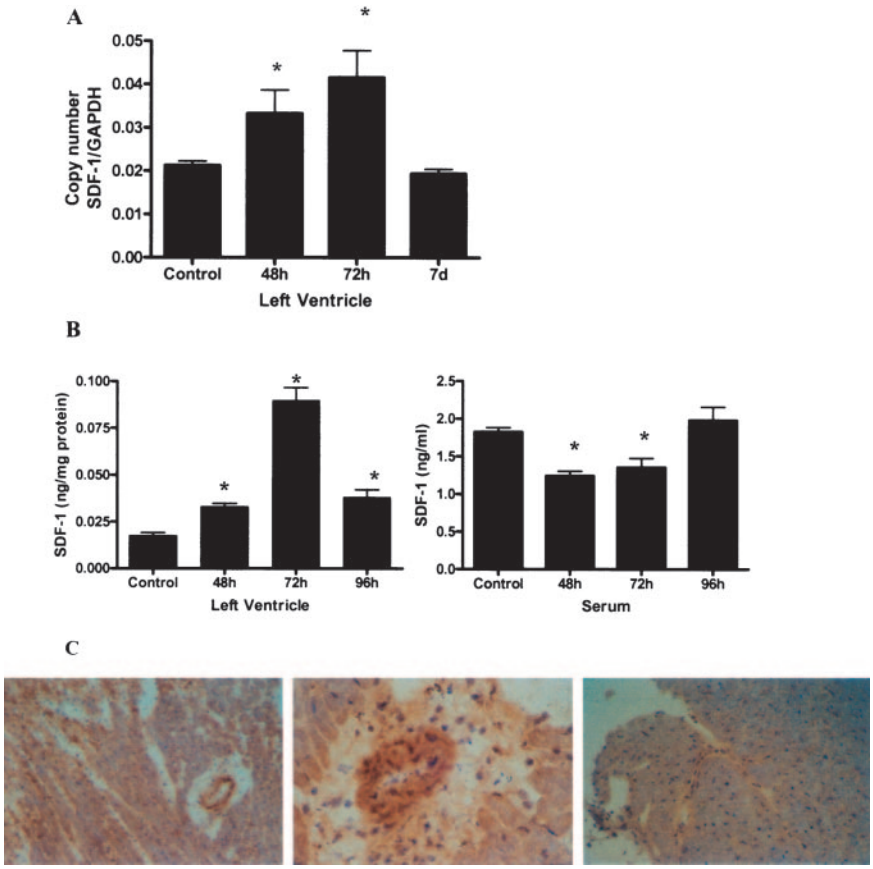

Figure 1. SDF-1 upregulation in infarcted myocardium. A, Relative mRNA level of SDF-1 in infarcted animals vs sham over time shows 95.7±29.5% increase in SDF-1 copy number at 72 hours (*$P<0.05$) that normalized by 7 days. B, ELISA for SDF-1 shows 410.6±57.9% increase in left ventricle (*$P<0.01$) and 25.9±6.8% decrease in serum (*$P<0.05$) at 72 hours. C, Immunohistochemical staining of SDF-1 (brown) in peri-infarct myocytes and blood vessels (left) and higher-magnification view of border-zone vessel (middle) but not in remote areas of myocardium (right). Abbreviations are as defined in text.

murine VEGF supplied with the kits were used to generate standard curves. Total protein was quantified by the Bradford assay.

Immunohistochemistry for Detection of SDF-1 Expression

Immunohistochemistry for SDF-1 was performed on myocardial tissue harvested 48 hours after infarction and frozen in OCT compound. Frozen 8-μm sections were fixed in acetone. Endogenous peroxidase and biotin were blocked (Vector Laboratories), and rabbit anti-mouse SDF-1α (RDI, diluted 1:100 in 1% blocking serum) was added for 30 minutes. Rabbit anti-mouse IgG (H+L) served as a negative control (Jackson Immunoresearch). Biotinylated goat anti-rabbit IgG was applied for 30 minutes, and the slides were developed with avidin:biotinylated enzyme complex and diaminobenzidine, according to the manufacturer's instructions (Vector Laboratories), counterstained with hematoxylin, and mounted in mounting medium (Vectamount).

Gene Expression Analysis

Myocardial RNA was stabilized, homogenized, and initially extracted with RNA STAT-60 (Tel-test, Inc). The RNA was further purified over RNA columns, and DNA was removed by on-column DNase digestion with an RNase-free DNase set (Qiagen). cDNA was generated with random primers. Quantitative reverse transcription (RT)–PCR was carried out with SYBR Green JumpStart Taq Ready Mix (Sigma) on 10 ng of cDNA template and normalized for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PCR products were confirmed by examination of agarose gels. The following oligonucleotides were used as primers: for SDF-1 forward, 5′-A C C A G T C A G C C T G A G C T A C C-3′ and reverse, 5′-C A C T T T A A T T T C G G G T C A A T G C-3′; for CXCR-4 forward, 5′-T C A G T C A G G G G G A T G A C A G G-3′ and reverse, 5′-T G G C C C T T G G A G T G T G A C A G C-3′; for VEGFA forward, 5′-A A G G A G A G C A G A A G T C C C A T G A-3′ and reverse, 5′-C A C A G G A C G G C T T G A A G A T G T-3′; for SCF forward, 5′-C T G C G G G A A T C C T G T G A C T G-3′ and reverse, 5′-C C A G A A G A G T A G T C A A G C T G A G-3′; for VCAM forward, 5′- T C T C T C A G G A A A T G C C A C C C-3′ and reverse, 5′-C A C A G C C A A T A G C A G C A C A C-3′; for ICAM forward, 5′-G G C A C C C A G C A G A A G T T G T T-3′ and reverse, 5′-C C T C A G T C A C C T C T A C C A A G-3′; for MMP-9 forward, 5′-A G A A G C A G T C T C T A C G G C C G-3′ and reverse, 5′-T G A T G G T C C C A C T T G A G G C C-3′; and for GAPDH forward, 5′-A C A G C A A C T C C C A C T C T T C C-3′ and reverse, 5′-G C C T C T C T T G C T C A G T G T C C-3′.

Gene expression data are expressed as the change in percentage or copy number over baseline. For quantification of SDF-1 copy number, a standard curve was generated with the SDF plasmid for AdV production (9145 bp) and the formula $C=G/(MX/A)$, where C=copy number, M=number of base pairs, X=682 g/mol, A=Avogadro's constant (6×10^{23}), and G=DNA concentration.

Data and Statistical Analysis

Gene expression data were verified by performing experiments in triplicate. ELISA, FACS, and chemotaxis studies were performed in duplicate. Results are presented as mean±SEM. Statistical significance was evaluated with an unpaired Student's t test for comparison between 2 groups or by ANOVA for multiple comparisons. A value of $P<0.05$ was considered significant.

Results

MI Increases Expression of SDF-1 in the Infarct Border Zone

We determined the temporal expression of SDF-1 mRNA in the left ventricle after MI by quantitative RT-PCR. SDF-1 increased by 56.7±12.7% and 95.7±29.5% at 48 and 72 hours, respectively, after MI ($P<0.05$) and returned to baseline by 7 days (Figure 1A). ELISAs showed a significant

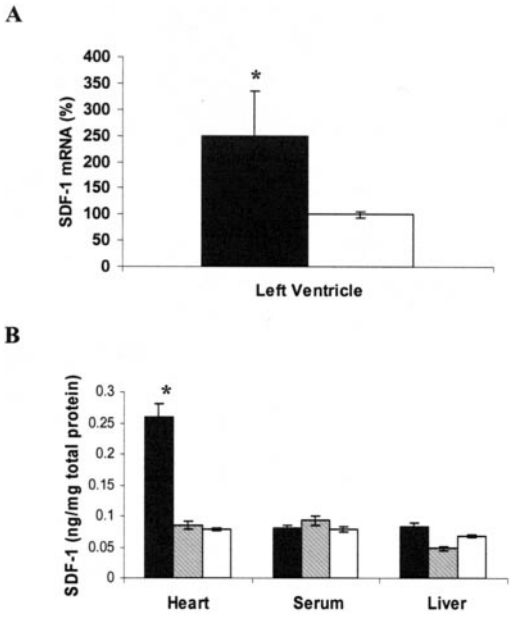

Figure 2. A, Effect of AdV.SDF transduction on cardiac muscle (black) compared with AdV.CONT (white) mRNA SDF-1 expression. *$P<0.01$ for cardiac muscle. B, ELISA for SDF-1 tissue and serum levels 48 hours after AdV.SDF (black), AdV.CONT (gray), or PBS direct myocardial injection (white); *$P<0.01$ vs AdV.CONT and PBS. Abbreviations are as defined in text.

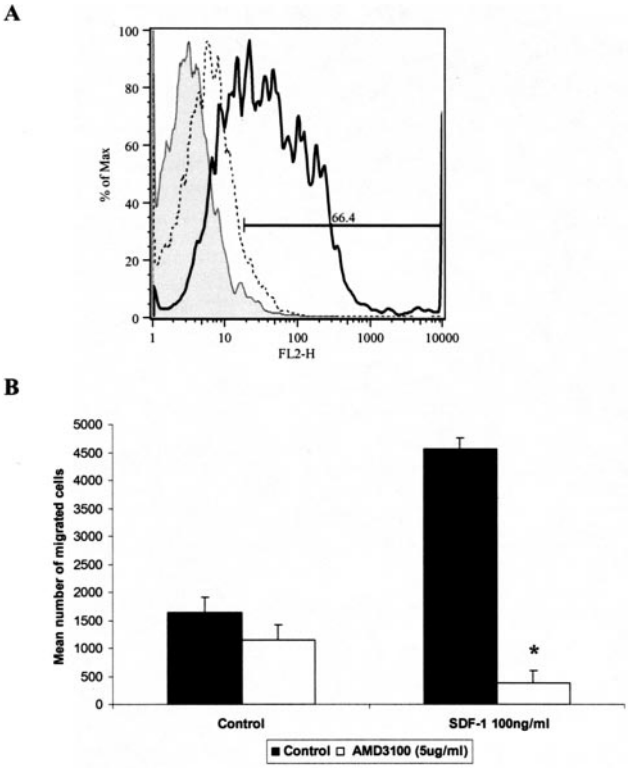

Figure 3. A, Immunofluorescence flow cytometry (semilog plot) of Lin− murine BM cells for CXCR4 (solid line), negative IgG isotype control (dashed line), and unstained cells (filled). Results are shown as fluorescence histograms with relative cell number and fluorescence intensity (FL2-H). BMCs were positive by 65.5±2.7% for CXCR4. B, Chemotaxis of BMCs toward 100 ng/mL SDF in absence and presence of CXCR4 receptor inhibitor AMD3100. Chemotaxis is represented as mean number of cells migrated into bottom chamber. AMD3100 inhibited 91.5±2.2% of SDF-1–induced migration (*$P<0.01$). Abbreviations are as defined in text.

increase in the left ventricular SDF-1 protein level after MI, which peaked at 410.6±57.9% at 72 hours ($P<0.01$) and remained >2-fold elevated over baseline at 96 hours ($P<0.05$). Conversely, the serum level of SDF-1 as measured by ELISA was significantly decreased at 48 hours (31.9±3.4%, $P<0.05$) and 72 hours (25.9±6.8%, $P<0.05$) but returned to baseline by 96 hours (Figure 1B). By immunohistochemistry, both cardiomyocytes and blood vessels in the infarct and border zones expressed SDF-1, in contrast to the remote areas of myocardium (Figure 1C).

AdV-Mediated SDF-1 Expression

Transduction of the myocardium with AdV.SDF resulted in a 2.5-fold increase of SDF-1 mRNA compared with AdV-.CONT ($P<0.01$, n=8; Figure 2). The level of SDF-1 protein expression by ELISA after direct myocardial injection of AdV.SDF was significantly increased in cardiac tissue, but there was no increase in SDF-1 levels in the liver or serum, consistent with targeted delivery to the myocardium.

BMSC CXCR4 Expression and Migration

FACS analysis of Lin− BMCs showed high surface expression of CXCR4 compared with an isotype control, 65.5±2.7% (Figure 3A). The ability to block SDF-1–induced chemotaxis was assessed with the CXCR4 inhibitor AMD3100. Incubation with AMD3100 (5 μg/mL) inhibited migration by 91.5±2.2% compared with control cells ($P<0.01$, Figure 3B).

SDF-1 Mediates Injury-Induced Homing

The percentage of donor BMCs in recipient tissue was calculated by real-time PCR to quantify the copy number of GFP (donor-specific) versus GAPDH genes in extracted genomic DNA. Values were derived by using a standard curve generated from mixtures of donor and recipient DNAs. MI resulted in an 80.4±15.6% increase in the homing of Lin− cells to the left ventricle after intravenous administration ($P<0.01$ vs control; Figure 4). Blockade of the SDF-1/CXCR4 interaction with AMD3100 after MI reduced homing by 64.2±5.5% ($P<0.05$), suggesting that SDF-1 is a major required factor in injury-induced homing. Further supporting this, overexpression of SDF-1 in the peri-infarct region by Ad.SDF-1 delivery 48 hours after MI increased homing to the left ventricle an additional 2-fold over MI alone ($P<0.01$ vs infarct). In contrast, SDF-1 overexpression by AdV.SDF-1 delivery in the absence of MI had no effect (P=NS), strongly suggesting that SDF-1 is required, but not sufficient, for SC recruitment to the heart.

MI and SDF-1 Transduction Effects of Gene Expression

By quantitative RT-PCR, expression of CXCR4, VCAM-1, ICAM-1, VEGF, and MMP-9 was increased in the left ventricle after MI ($P<0.05$), with the greatest increase in MMP-9. SCF expression was unchanged (Figure 5A). Al-

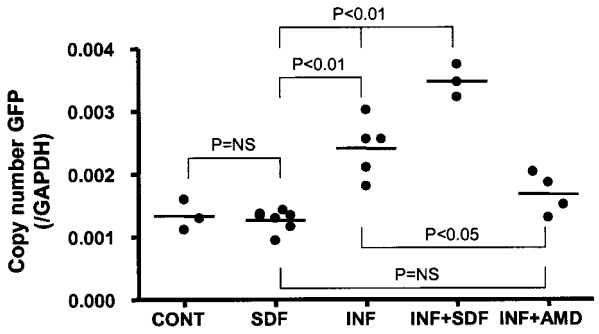

Figure 4. Detection of donor GFP+ BMCs in recipient SCID left ventricle by PCR of genomic DNA. SDF in absence of injury did not result in increase in GFP cell accumulation. Infarction increased accumulation of GFP cells, and infarction plus exogenous SDF caused further significant increase. Neutralization of CXCR4 by AMD3100 significantly inhibited homing of GFP cells. Probability values as depicted. Data summarize 2 experiments (n=21 mice). INF indicates infarction; AMD, AMD3100. All other abbreviations are as defined in text.

though VEGF levels in the entire left ventricle were increased after MI, VEGF was not increased when only the infarct and per-infarct regions were analyzed. This finding was in contrast to expression of the other genes, which were more upregulated in the immediate peri-infarct area compared with the entire left ventricle (data not shown). SDF-1 overexpression by AdV.SDF in the absence of injury resulted in an increase in both VEGF mRNA and protein (Figure 5B) ($P<0.05$ vs AdV.CONT) but did not affect the other factors.

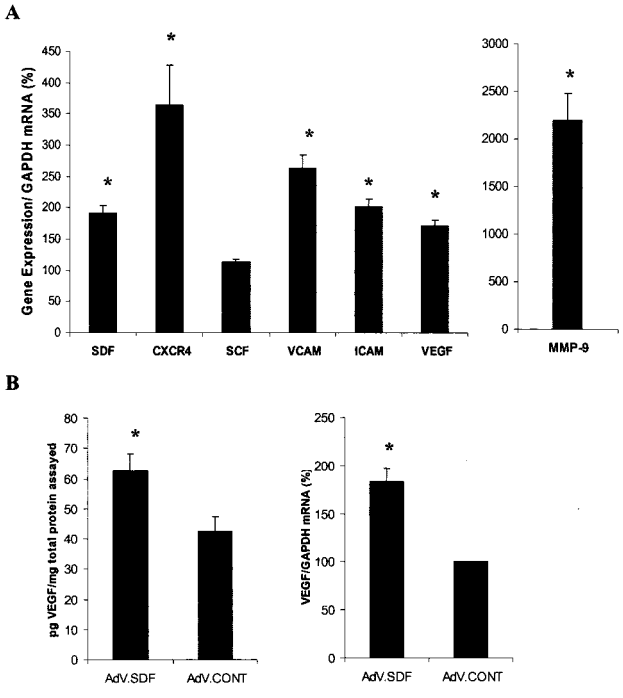

Figure 5. A, Gene expression after MI evaluated by RT-PCR. *$P<0.05$ vs sham-operated animals. PI indicates peri-infarct. B, SDF-1–induced upregulation of VEGF mRNA by RT-PCR and VEGF protein by ELISA. *$P<0.05$. All other abbreviations are as defined in text.

Discussion

Herein we establish that SDF-1 is required for post-MI recruitment of SCs to the heart and that forced overexpression of SDF-1 can augment SC homing after infarction. Although SDF-1 is required, it is not sufficient for SC homing, a finding that has significant implications concerning the potential clinical settings in which SDF-1 could be used to augment SC recruitment to the heart.

That SDF-1 is not singularly sufficient reflects the need for concomitant expression of additional factors. We contend that SC recruitment is a 2-step process that begins with binding of SCs to adhesive complexes in the vasculature around an injury zone, followed by local chemotaxis to the site of engraftment. We have corroborated SDF-1 expression in the vasculature. SDF-1 could serve an adhesive function in this context by binding CXCR4-expressing cells to the vessel wall. We have also demonstrated VCAM and ICAM upregulation after MI, and these molecules have previously been shown to be important in SC recruitment.[23–25] That SDF-1 expression alone did not increase VCAM and ICAM may explain why SDF-1 is not sufficient as a singular agent. In our view, it will be important to fully delineate the repertoire of adhesion complex alterations that occur in the setting of tissue injury to fully understand the recruitment process.

We contend that SDF-1 is of primary importance in mediating local chemotaxis of SCs. In support of this concept, we found that SDF-1 is significantly increased after MI and most highly expressed in the peri-infarct zone, the area to which BMDCs are recruited.[9] Interestingly, we also found that serum levels of SDF-1 were decreased 48 to 72 hours after MI. In patients, SDF-1 plasma levels decrease with unstable angina, consistent with our findings.[16] Other groups have reported an increase in plasma SDF-1 within 24 hours after vascular injury in mice.[14] This discrepancy in SDF-1 serum levels may relate to the type of injury or may represent an early, transient increase, as we did not evaluate SDF-1 levels before 48 hours. SDF-1 expression increases in the injured vasculature, contributing to the recruitment of circulating progenitor cells into the neointima during intimal hyperplasia.[14] In a related scenario, patients treated with G-CSF with or without peripheral blood mononuclear cell infusion develop both an improvement of ventricular function and a high rate of in-stent restenosis.[18] This could be due to recruitment of other CXCR4-expressing cell types, including mature blood cells such as lymphocytes, monocytes, megakaryocytes, and platelets.[26,27] G-CSF also upregulates expression of the CXCR4 receptor.[28]

One explanation for the decrease in SDF-1 levels in unstable angina and MI is that SDF-1 is degraded by MMPs.[28,29] Patients with acute coronary syndromes have elevated levels of MMP-2 and MMP-9.[30] We found a >20-fold increase in MMP-9 in the murine heart after MI. Matrix turnover is a prominent component of post-MI remodeling, and MMP-9–mediated matrix alterations may create a favorable tissue environment for SC migration. MMP-9 also plays documented roles in SDF-1 degradation and SC mobilization from the marrow. Furthermore, in the setting of liver injury, MMP-9 is associated with increased CXCR4 expression on CD34+ cells, and MMP inhibitors reduce homing of

these cells to the liver.[13,31] How the prominent post-MI increase in MMP-9 expression that we found affects SC recruitment to the heart remains unclear.

The purpose of our study was to determine the role of SDF-1 in BMDC recruitment to the myocardium in the setting of acute MI. We intravenously delivered Lin− BMCs from genomically distinct mice and identified donor cells by PCR. We did not observe homing in response to SDF-1 in the absence of injury, but in the presence of injury, further expression of SDF-1 resulted in a significant additional increase in donor-cell accumulation. Our work contributes to the work by Askari et al,[17] who evaluated the effects of stable expression of SDF-1 in a model of ischemic cardiomyopathy. They demonstrated that overexpression of SDF-1 in the myocardium 8 weeks after MI, when intrinsic levels of SDF-1 are comparable to those of controls, followed by administration of granulocyte colony stimulating factor, led to an increase in cell proliferation. Double staining for SC markers such as CD34 and CD117 was used as indirect evidence that the proliferating cells were of BM origin.

In summary, this is the first study to show that SDF-1 plays a major role in the accumulation of SCs in the heart after MI and can further increase homing when given by gene delivery after injury.

Acknowledgment

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