Local Delivery of Protease-Resistant Stromal Cell Derived Factor-1 for Stem Cell Recruitment After Myocardial Infarction

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Background—Local delivery of chemotactic factors represents a novel approach to tissue regeneration. However, successful chemokine protein delivery is challenged by barriers including the rapid diffusion of chemokines and cleavage of chemokines by proteases that are activated in injured tissues. Stromal cell–derived factor-1 (SDF-1) is a well-characterized chemokine for attracting stem cells and thus a strong candidate for promoting regeneration. However, SDF-1 is cleaved by exopeptidases and matrix metalloproteinase-2, generating a neurotoxin implicated in some forms of dementia.

Methods and Results—We designed a new chemokine called S-SDF-1(S4V) that is resistant to matrix metalloproteinase-2 and exopeptidase cleavage but retains chemotactic bioactivity, reducing the neurotoxic potential of native SDF-1. To deliver S-SDF-1(S4V), we expressed and purified fusion proteins to tether the chemokine to self-assembling peptides, which form nanofibers and allow local delivery. Intramyocardial delivery of S-SDF-1(S4V) after myocardial infarction recruited CXCR4+/c-Kit+ stem cells (46±7 to 119±18 cells per section) and increased capillary density (from 169±42 to 283±27 per 1 mm²). Furthermore, in a randomized, blinded study of 176 rats with myocardial infarction, nanofiber delivery of the protease-resistant S-SDF-1(S4V) improved cardiac function (ejection fraction increased from 34.0±2.5% to 50.7±3.1%), whereas native SDF-1 had no beneficial effects.

Conclusions—The combined advances of a new, protease-resistant SDF-1 and nanofiber-mediated delivery promoted recruitment of stem cells and improved cardiac function after myocardial infarction. These data demonstrate that driving chemotaxis of stem cells by local chemokine delivery is a promising new strategy for tissue regeneration. (Circulation. 2007;116:1683-1692.)

Key Words: angiogenesis ■ chemokines ■ heart failure ■ myocardial infarction ■ stem cells ■ tissue

Local delivery of chemotactic factors to drive stem cell recruitment into infarcted myocardium represents a potentially novel approach to regeneration. This concept is supported by recent reports of forced expression of chemokines by gene transfer, which can improve cardiac function after myocardial infarction (MI). However, safety issues and unpredictability in dose and timing of expression can limit gene delivery strategies. Thus, new approaches for precisely controlled delivery of chemokine proteins to drive chemotaxis of stem cells have potential for promoting tissue regeneration.

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Stromal cell–derived factor-1 (SDF-1, CXCL12) is a member of the chemokine family that plays a key role in homing of hematopoietic stem cells during embryonic development and after stem cell transplantation. However, SDF-1 is cleaved by matrix metalloproteinase (MMP)-2,5, yielding an N-terminal tetrapeptide and a neurotoxic remnant of SDF-1(5-68) (Figure 1A), and MMP-2 is activated by tissue injury. SDF-1 is also cleaved by Dipeptidyl Peptidase IV (DPPIV/CDC26) (Figure 1A), a serine exopeptidase that releases Xaa-Pro dipeptides from the N-terminus of polypeptides. DPPIV is a transmembrane protein in endothelial cells and leukocytes. Thus, proteolytic activity will likely limit the effectiveness of SDF-1 in the inflammatory environment of infarcted myocardium.

It has not been shown previously that chemokines can be made resistant to local proteases and retain their bioactivity. Furthermore, because small proteins can diffuse readily through extravascular matrix, controlled delivery of chemokines may be essential for driving chemotaxis. New bioin-
Structive biomaterials such as self-assembling peptides\textsuperscript{11,12} have the potential to control delivery within tissues. These short peptides form a 3-dimensional scaffold of nanofibers (NFs) at physiological pH and osmolarity,\textsuperscript{13} and fusion proteins of self-assembling peptide sequences with chemokines could control chemokine delivery. This approach would yield a “smart-release” strategy (in which the material responds to cues in the local environment), allowing release of the chemokine in the injured tissue and creating the gradient in chemokine concentration required for stem cell recruitment.

In the present study, we present 2 advances that both proved necessary to enable chemokine protein delivery for recruitment of stem cells to injured myocardium. First, we show that designing the chemokine SDF-1 to resist proteases is essential for retaining in vivo bioactivity. Second, we show that direct tethering of the chemokine to NFs allows local delivery and bioactivity. When these 2 approaches were combined, stem cell recruitment to the myocardium was promoted and cardiac function improved after MI. These data demonstrate how bioengineered chemokine delivery can promote tissue repair.

Methods

Construction of Plasmids

The DNA sequence of mature SDF-1\textalpha was cloned from human cDNA into the pET-Sumo vector (Invitrogen, Carlsbad, Calif); the codon for an extra N-terminal serine residue was incorporated to facilitate cleavage by Sumo protease and to increase resistance to DPPIV cleavage. Because the active site of SDF-1 is at the N-terminus, fusion proteins were made by incorporating extra sequences at the C-terminus. Fusion proteins consisted of the SDF-1 sequence and the RAD16-II sequence (referred as RAD, 16 amino acids). SDF-1 was spaced from RAD by a linker, which consisted of 6 glycines (6G), a 6–amino acid sequence susceptible to MMP-2 cleavage (MCS) or a scrambled sequence (SCR) consisting of the same amino acids in random order, and a MCS sequence spaced from the RAD sequence by an extra 10 glycines (Table in the online-only Data supplement).

Mutations of MMP-2 Cleavage Site

To make SDF-1 resistant to MMP-2 cleavage, mutagenesis of specific amino acids was performed on the basis of substrate sequences of MMP-2 described by Netzel-Arnett et al.\textsuperscript{14} Polymerase chain reaction was performed with Pfx50 DNA Polymerase (Invitrogen): 18 cycles consisting of 94°C for 30 seconds, 55°C for 1 minute, and 68°C for 10 minutes. Template plasmid was digested with \textit{DpnI} (New England Biolabs, Beverly, Mass) and inserted in GC10-competent cells (PGC Scientific Corp, San Diego, Calif). All sequences were confirmed by DNA sequencing.

SDF-1 Purification and Expression

Sumo-S-SDF-1 fusion proteins were expressed in Rosetta DE3 \textit{Escherichia coli} (Novagen, Madison, Wis) and grown to an optical density of 1.5 (600 nm) at 37°C. Cells were induced with 0.25 mmol/L isopropyl\textsubscript{16}β-D-thiogalactoside for 4 hours and harvested by centrifugation. S-SDF-1 was purified by a 4-step procedure; all steps were performed at 21°C. Cells from a 4-L growth were lysed in lysis buffer (6 mol/L guanidine, 20 mmol/L phosphate, pH 7.8, 500 mmol/L NaCl) and homogenized. The first purification step consisted of affinity purification by the polyhistidine tag present in Sumo-S-SDF-1 fusion proteins with Ni-NTA (Invitrogen). Ni-NTA resin was washed with wash buffer (8 mol/L urea, 30 mmol/L 2-mercaptoethanol, 1 mmol/L EDTA, 50 mmol/L Tris, pH 7.8, 500 mmol/L NaCl) and homogenized. The first purification step consisted of affinity purification by the polyhistidine tag present in Sumo-S-SDF-1 fusion proteins with Ni-NTA (Invitrogen). Ni-NTA resin was washed with wash buffer (8 mol/L urea, 500 mmol/L NaCl, 20 mmol/L phosphate, pH 6.2), and bound protein was eluted at pH 4. The second purification and refolding step was performed on a cation exchange high-performance liquid chromatography column (HiPrep 16/10 SP FF, Amersham Biosciences, Inc, Piscataway, NJ). The sample was adjusted to binding buffer (8 mol/L urea, 500 mmol/L NaCl, 20 mmol/L phosphate, pH 6.2), and bound protein was eluted at pH 4. The second purification and refolding step was performed on a cation exchange high-performance liquid chromatography column (HiPrep 16/10 SP FF, Amersham Biosciences, Inc, Piscataway, NJ). The sample was adjusted to binding buffer (8 mol/L urea, 30 mmol/L 2-mercaptoethanol, 1 mmol/L EDTA, 50 mmol/L Tris, pH 8) and loaded on the column. Refolding of Sumo-S-SDF-1 was performed on the column with a 2-hour run of refolding buffer (50 mmol/L Tris, pH 8, 75 mmol/L NaCl, 0.1 mmol/L reduced glutathione, and 0.1 mmol/L oxidized glutathione). Sumo-S-SDF-1 was eluted with a
step gradient (0.5 to 1 mol/L NaCl) and concentrated (Centricon, Millipore, Billerica, Mass). The Sumo-S-SDF-1 fusion protein was cleaved by Sumo Protease 1 (1U/50 μg protein, Lifesensors Inc, Malvern, Pa) in 50 mmol/L Tris, pH 8.0, and 500 mmol/L NaCl. The sample was adjusted to 0.1% trifluoroacetic acid and loaded on a C18 reverse-phase high-performance liquid chromatography column (Delta-Pak C18, Waters, Milford, Mass) as a final purification step. The column was subjected to a linear gradient from 30% to 40% acetonitrile in 0.1% trifluoroacetic acid.

**SDF-1 Activity Test**

Activity of purified S-SDF-1 was tested by migration of Jurkat T-lymphocytic cells and compared with commercial SDF-1 (R&D Systems, Minneapolis, Minn). Jurkat cells (150,000) were plated in the upper well (Multiscreen 5-mm plates, Millipore), and protein was added in the lower well. Cells were counted in the lower well after 3 hours. All experiments were performed in triplicate.

**MMP and DPPIV/CD26 Cleavage Experiments**

S-SDF-1 or S-SDF-1(S4V) was incubated for 1 hour at 21°C with 50 ng activated MMP-2 or MMP-9 in TCNB buffer. For DPPIV/CD26 cleavage experiments, S-SDF-1, commercial SDF-1, or S-SDF-1(S4V) was incubated for 1 hour at 21°C with 50 ng DPPIV. Additional details are provided in the online-only Data Supplement.

**Radioactive Labeling of Proteins With Acetic Anhydride-Carbonyl-14C**

We incubated 500 pmol S-SDF-1(S4V), S-SDF-1(S4V)-6G-RAD, S-SDF-1(S4V)-SCR-RAD, S-SDF-1(S4V)-MCS-RAD, or S-SDF-1(S4V)-MCS10G-RAD 6 times for 10 minutes with 2.5 μCi acetic anhydride-carbonyl-14C (Sigma, St Louis, Mo) on ice. Proteins were dialyzed (3500 MWCO Slide-A-Lyzer Dialysis Cassette, Pierce, Woburn, Mass) 2 times for 2 hours against sterile PBS to remove unbound radioactivity.

**Binding Experiments of Fusion Proteins to Self-Assembling Peptides**

Self-assembling peptides (MIT Biopolymers Laboratory, Boston, Mass) were dissolved in 295 mmol/L sucrose/3 mmol/L acetic acid in water and sonicated for 10 minutes. The peptide solution was mixed with radioactive fusion proteins (30 nmol/L unless otherwise

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Figure 2. Purity and activity of different SDF-1 proteins. A, Representative reverse-phase high-performance liquid chromatography trace as the last purification step. B, Coomassie stain of different SDF-1 proteins. SDS-PAGE was run under reducing conditions. C, Activity test of S-SDF-1, S-SDF-1(S4V), and commercial SDF-1 on migration of Jurkat cells (n = 3). D, Activity test of fusion proteins compared with S-SDF-1 (n = 3). Values are mean ± SEM.
stated); 100 µL was added to a 0.4-µm-pore-size culture plate insert (Millicell-CM, Millipore, Billerica, Mass) and left overnight at 4°C to allow NFs to form. Radioactivity was measured by scintillation counting of wash fractions (combination of every 2 wells) and of NFs in inserts.

**MI and Injection of Peptide NFs**

All animal protocols were approved by the Harvard Medical School Standing Committee on Animals. MI was produced in male Sprague-Dawley rats (weight, 200 to 230 g; Charles River Laboratories, Wilmington, Mass, and Harlan, Indianapolis, Ind). Briefly, rats were anesthetized with pentobarbital (60 mg/kg) and buprenorphine (0.05 mg/kg), and after tracheal intubation, hearts were exposed via left thoracotomy. After pericardiectomy, the left coronary artery was ligated by suturing with 6-0 prolene at the location 2 mm below the left atrial appendix. For the sham operation, suturing was performed without ligature. Peptide NFs (peptide sequence, AcN-NIRARADADARADADA-CNH2; from SynPep Corp, Dublin, Calif, and MIT Biopolymers) with or without 30 nmol/L S-SDF-1, S-SDF-1(S4V), S-SDF-1-6G-RAD, S-SDF-1-MCS-RAD, S-SDF-1(S4V)-6G-RAD, or S-SDF-1(S4V)-MCS-RAD was dissolved in 295 mmol/L sucrose and sonicated to produce a 1% solution for injection. A total of 80 µL self-assembling peptide NFs was injected into the infarcted border zone at 3 locations (equal amount for each injection) immediately after coronary artery ligation. Injections were completed within 2 minutes after coronary ligation. After injection, the chest was closed, and animals were allowed to recover on a heating pad. The animals received buprenorphine 0.05 mg/kg twice daily for 48 hours as an analgesic. A total of 200 animals underwent MI surgery. Of the 200, 20 died during surgery or within the first 24 hours as an analgesic. A total of 80

**Hemodynamic Measurement**

At 28 days after surgery, rats were anesthetized with pentobarbital, and hemodynamics were measured with 2F pressure-volume sensing catheters (Millar Instruments, Houston, Tex). The catheter was inserted into the right carotid artery and arterial pressure was measured. The catheter was advanced to the left ventricle for pressure-volume measurements. After stabilization, baseline left ventricular pressure-volume loops were recorded. At the end of each catheterization, 50 µL of 25% saline was injected into the right atrium through a polyethylene catheter inserted into the left jugular vein to determine the volume attributed to parallel conductance of the surrounding tissue. The volume calibration and the hemodynamic data were analyzed with commercial software (PVAN3.2, Millar Instruments).

**Immunofluorescence Microscopy**

Myocardial sections were deparaffinized, rehydrated, and pretreated with boiling 10 mmol/L sodium citrate (pH 7.2) for 30 minutes, followed by incubation with antibodies against α-smooth muscle actin (Sigma), isolectin (Molecular Probes, Carlsbad, Calif; Invitrogen), e-Kit, Fk-1, CD34 (Santa-Cruz Biotechnology, Santa Cruz, Calif), and CXCR4 (Abcam, Cambridge, Mass) at 21°C for 2 hours and then Alexa Fluor–conjugated secondary antibodies (Molecular Probes, Invitrogen). After counterstaining with DAPI (0.1 µg/mL), sections were mounted and observed under fluorescence microscopy. All quantitative analyses were performed by an observer unaware of treatment group.

**Detection of Sumo-S-SDF-1 by Immunofluorescence Staining**

In an experiment separate from the 200-rat experiment above, MI was produced in 32 male rats as described. In this experiment, 80 µL NFs alone, with 5 µmol/L Sumo-S-SDF-1-6G-RAD or with Sumo-S-SDF-1(S4V)-6G-RAD, and Sumo-S-SDF-1(S4V)-6G-RAD alone was injected in the infarcted area. Hearts were harvested after 5 minutes (day 0) and at 1, 3, or 7 days and fixed with 4% paraformaldehyde. Immunofluorescence staining for cardiac sarcolemic actin (Sigma) and for Sumo-fragment (Abcam) was performed. Microscope settings and acquisition times were identical for all slides; the images for Sumo staining were not altered digitally.

**Statistical Analysis**

Normal distribution was tested with the Kolmogorov-Smirnov test. If normally distributed, a 2-tailed t test was performed for 2 independent samples, and a 1-way ANOVA was performed for >2 independent samples, followed by Dunnett multiple comparison. A Kruskal-Wallis test was performed if data were not normally distributed. MMP cleavage experiments of linker sequences in fusion proteins, SDF-1 ELISA, and Western analysis are detailed in the online-only Data Supplement.

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

**Results**

S-SDF-1(S4V) Is Resistant to DPPIV, MMP-2, and MMP-9 Cleavage

Recombinant SDF-1 was expressed in E coli and purified in 4 steps: affinity purification with Ni-agarose; cation exchange chromatography, including oxidative refolding; cleavage of an N-terminal Sumo fragment by Sumo protease; and finally reverse-phase chromatography purification. An additional N-terminal serine residue was incorporated to allow cleavage by Sumo protease and to protect against the exopeptidase DPPIV/CD26, which recognizes the XaaPro sequence (All SDF-1 proteins with the extra serine were thus called S-SDF-1; Figure 1B). Purity of S-SDF-1 was >95% by reverse-phase chromatography and by Coomassie staining (Figure 2A and 2B). The recombinant S-SDF-1 was as active as a commercial preparation of SDF-1 on migration of Jurkat T-lymphocytes (Figure 2C).

SDF-1, which binds to its cognate CXCR4 receptor, is cleaved by MMP-2 to a truncated, neurotoxic remnant that activates the CXCR3 receptor. Because MMP-2 is activated in injured tissues like infarcted myocardium, 4 different mutations were constructed in the MMP-2 cleavage site of S-SDF-1. S-SDF-1(L5W) and S-SDF-1(L5E) showed minimal activity on T-cell migration (Figure 1A) in the online-only Data Supplement), whereas S-SDF-1(S4V) and S-SDF-1(L5P) showed bioactivity comparable to S-SDF-1. Because S-SDF-1(L5P) was difficult to purify, S-SDF-1(S4V) was selected for further experiments. As shown by the concentration-response curve of T-cell migration (Figure 2C), S-SDF-1(S4V) showed a rightward shift in bioactivity compared with S-SDF-1; S-SDF-1 and S-SDF-1(S4V) bioactivities were completely blocked by AMD3100, a specific CXCR4 inhibitor. Incubation of S-SDF-1 with MMP-2 resulted in the predicted truncated protein (Figure 1C) that was unable to activate T cells (Figure 1D), consistent with previous studies demonstrating that MMP-2 truncates SDF-1 at its first 4 amino acids (Figure 1A). The mutated S-SDF-1(S4V) was not cleaved (Figure 1C) and retained its bioactivity after incubation with MMP-2 (Figure 1D). These findings were confirmed by incubation of Sumo-S-
SDF-1 and Sumo-S-SDF-1(S4V) (the protein obtained after the cation exchange refolding step but before the Sumo-protease cleavage step) with different doses of MMP-2 and MMP-9 (Figure 1b of the online-only Data Supplement); Sumo-S-SDF-1 was cleaved by MMP-2 in a dose-dependent manner, whereas Sumo-S-SDF-1(S4V) was not. Sumo-S-SDF-1 was partially cleaved by MMP-9 after 4 hours at the highest concentration tested and more significantly cleaved after 48 hours by MMP-9, indicating slower cleavage of S-SDF-1 by MMP-9 compared with MMP-2. Sumo-S-SDF-1(S4V) was not cleaved by MMP-9. Thus, the S-SDF-1(S4V) variant of SDF-1 was bioactive but resistant to cleavage by the exopeptidase DPPIV/CD26, MMP-2, and MMP-9.

S-SDF-1(S4V)-RAD Fusion Proteins Are Bioactive and Incorporate Into Self-Assembling Peptide NFs

After the protease-resistant variant of SDF-1 was designed, fusion proteins containing S-SDF-1 or S-SDF-1(S4V) and the self-assembling peptide RAD16-II sequence (RARADARADADA) were designed (Table in the online-only Data Supplement) and purified (Figure 3A). Four different linkers were included between the chemokine and the self-assembling peptide: a 6-amino acid linker susceptible to MMP-2 cleavage (MMP-2 cleavage site [MCS]), the same linker with an extra 10 glycines (MCS10G), a scrambled (SCR) linker containing the same 6 amino acids as the MCS linker but in random order, and a linker consisting of only 6G. Purity of all proteins was >95% as evaluated by Coomassie stain (Figure 2B). C-terminal modification of S-SDF-1 did not affect its bioactivity (Figure 2D), as anticipated because the active site of SDF-1 is located near the N-terminus.

To demonstrate incorporation of the fusion proteins into the self-assembling peptide NFs (Figure 3A), we labeled S-SDF-1(S4V) and S-SDF-1(S4V)-RAD fusion proteins by acetylation of lysine residues with acetic anhydride-carbonyl-14C. Labeled S-SDF-1(S4V) proteins (30 nmol/L) were mixed with self-assembling peptide NFs. Nanofibers were washed 16 times for 15 minutes with 300 µL PBS, and every 2 wash fractions were combined and measured with scintillation counting. C, Radioactivity remaining in NFs after 16 or 32 washes was measured with scintillation counting and normalized to radioactivity of starting concentration of S-SDF-1(S4V). D, The 30-nmol/L initial concentration of S-SDF-1(S4V)-6G-RAD in 1% NFs. Graph shows radioactivity remaining in NFs. Zero days represents NFs after 16 washes; other samples were washed twice daily with 300 µL PBS until harvest. E, Different S-SDF-1-RAD fusion proteins were incubated with MMP-2 or MMP-9. Coomassie staining of SDS-PAGE. All experiments n=4 or more. Values are mean±SEM. *P<0.05; **P<0.001.
S-SDF-1(S4V)-RAD Improves Cardiac Function After MI

MI is a common cause of heart failure, and recruitment of stem cells by SDF-1 could improve cardiac function.\(^1\) We measured background SDF-1 protein concentration in infarcted cardiac tissue by ELISA (Figure IIIa of the online-only Data Supplement) following experimental MI in rats. SDF-1 concentration decreased from 2.4±0.3 nmol/mL at baseline to 1.0±0.1 nmol/mL after 24 hours (\(P<0.001\); \(n=6\)) and remained at this level for at least 7 days. Sensitivity of the ELISA was similar for full-length S-SDF-1 and cleaved SDF-1(5-68) (Figure IIIb). The decline in SDF-1 after infarction suggests that controlled delivery of SDF-1 signaling could improve cardiac function.

To establish that self-assembling peptides can deliver chemokines in vivo, immunofluorescence staining for the Sumo fragment was performed. NFs alone, NFs with Sumo-S-SDF-1-6G-RAD, NFs with Sumo-S-SDF-1(S4V)-6G-RAD, or Sumo-S-SDF-1(S4V)-6G-RAD alone was injected after MI. Sumo-S-SDF-1(S4V)-6G-RAD without NFs was easily detectable immediately after injection but was undetectable at 1 day and beyond (Figure 4). In contrast, Sumo-S-SDF-1(S4V)-6G-RAD with NFs was detectable at 1, 3, and 7 days, indicating that chemokine delivery with NFs was successful in vivo. In a separate experiment, we treated rats after MI with the same 4 groups and used homogenized tissue at the same 4 time points for Western analysis. The Sumo tag could be readily identified immediately after injection in the 3 groups in which protein was injected (Figure IV of the online-only Data Supplement). However, after 1 day, the Sumo tag could be detected only in the Sumo-S-SDF-1(S4V)-6G-RAD group. The detection limit of the Western blot with this Sumo-specific antibody was \(\approx 100\) nmol/L, which corresponds to 300 pmol/3 mL lysis buffer or 37.5% of the total amount of injected protein (800 pmol). This indicates that after 1 day, >37.5% of the Sumo-S-SDF-1(S4V)-6G-RAD was still present, but remaining protein was below the threshold after 3 days. The Sumo tag of Sumo-S-SDF-1-6G-RAD could not be detected after 1 day, which we speculate could be due to cleavage by MMP-2.

The effects of the chemokines on MI were examined in 9 groups in a randomized and blinded study of 176 rats surviving surgery: sham, MI, MI+SDF-1, MI+SDF-1(5-68), MI+SDF-1(5-68)+SDF-1, MI+SDF-1-6G-RAD, MI+SDF-1-6G-RAD+MCS-RAD, MI+SDF-1-6G-RAD+MCS-RAD, MI+SDF-1-6G-RAD+MCS-RAD, MI+SDF-1-6G-RAD+MCS-RAD, MI+SDF-1-6G-RAD+MCS-RAD. All rats underwent left ventricular catheterization for hemodynamic measurements at 28 days after surgery. Both MI+NS-SDF-1(5-68)+MCS-RAD (\(n=18\); \(P=0.002\)) and MI+NS-SDF-1(5-68)+MCS-RAD (\(n=16\); \(P=0.004\)) significantly increased ejection fraction compared with the MI-only group (\(n=21\)) (\(P=0.004\); the Table). A significant improvement in end-systolic volume occurred in the MI+NS-SDF-1(5-68)+MCS-RAD group (\(P=0.036\)) and the MI+NS-SDF-1(5-68)+MCS-RAD group (\(P=0.004\)). Other treatment groups were not significantly different from the MI-only group. These data demonstrate that the MMP-resistant S-SDF-1(5-68), but not S-SDF-1 alone, improves cardiac function. Furthermore, controlled delivery by NPs was essential, whereas release by MMP cleavage was not essential for benefit.
S-SDF-1(S4V)-RAD Increases Capillary Density and Recruitment of CXCR4⁺ Cells After MI

Isolectin and α-smooth muscle actin immunofluorescence stainings were performed on hearts harvested 28 days after MI (Figure 5A). Capillary density increased from 169±42 per 1 mm² in the MI only group to 269±33 per 1 mm² in the MI+NF/S-SDF-1(S4V)-6G-RAD group (nonsignificant, *P=0.067) and 283±27 per 1 mm² in the MI+NF/S-SDF-

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<td>9010±862</td>
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HR indicates heart rate; MAP, mean arterial pressure; LVESV, left ventricular end-systolic volume; LVEDV, left ventricular end-diastolic volume; LVESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure; SV, stroke volume; EF, ejection fraction; CI, cardiac index; and SWI, stroke work index. Values are mean±SEM.

*P<0.05 vs MI; †P<0.005 vs MI.
(S4V)-MCS-RAD group (P=0.035) (Figure 5B). No significant differences were observed in arteriolar density (Figure 5C). CXCR4 cells were found in the infarct and border zone but were rare in sham-operated hearts (not shown). The density of CXCR4 cells increased from 46±7 cells per section in MI only to 132±35 cells per section in the MI+NF/S-SDF-1(S4V)-6G-RAD group (P=0.002) and to 119±18 cells per section in the MI+NF/S-SDF-1(S4V)-MCS-RAD group (P=0.008) (Figure 6A). Of the CXCR4 cells, 40.4±4.1% were positive for CD34 (Figure 6B). All observed CXCR4 cells also were positive for c-Kit (Figure 6C), and 72.1±5.5% were positive for Flk-1/VEGF-R2 (Figure 6D).

**Discussion**

Driving chemotaxis of stem cells and progenitor cells to infarcted myocardium could promote tissue regeneration. In the present study, we describe a protease-resistant SDF-1, S-SDF-1(S4V), which is resistant to MMP-2 and DPPIV/CD26 cleavage. S-SDF-1(S4V) improved cardiac function after MI when it was tethered to self-assembling peptides for controlled delivery. The improvement in cardiac function was accompanied by greater capillary density and recruitment of CXCR4/c-Kit/Flk-1 cells.

Proteases regulate the activity of many chemokines. Some chemokines like CXCL7 or CXCL8 have increased activity after N-terminal cleavage, whereas others like CXCL10 or CXCL12/SDF-1 decrease activity after N-terminal cleavage. MMP-2 cleaves SDF-1 at the N-terminus, resulting in a tetrapeptide and a neurotoxic SDF-1(5-67) remnant. A physiological role for this cleavage is unknown, but inactivation of SDF-1 by MMPs in the bone marrow could allow mobilization of hematopoietic stem cells from the bone marrow.

MMP-2 upregulation after MI is well documented and plays a role in extracellular matrix turnover and remodeling after MI. MMP-2 cleavage of SDF-1 may be 1 reason for the inefficiency of SDF-1 protein injection after MI as published by Abbott et al, whereas continuous production of SDF-1 in situ by gene therapy as used by Askari et al improves cardiac function. Because all the S-SDF-1 isoforms produced in our laboratory were resistant to DPPIV, we cannot definitively show that degradation of SDF-1 by DPPIV plays a major role after MI. However, none of the S-SDF-1 proteins with an extra serine at the N-terminus were degradable by DPPIV, and this DPPIV resistance may have enhanced in vivo homing.

Our experiments with S-SDF-1(S4V)-MCS-RAD showed that although the MCS linker sequence is completely cleaved by MMP-2 when the protein is in solution, only one fourth of the chemokine is actually released when the same fusion protein is incorporated into NFs. Both reduced accessibility to the relatively large 72-kDa MMP-2 enzyme, and trapping of the chemokine in the 3-dimensional network of the NFs might account for this finding. In fact, our in vivo studies suggested that “smart release” of chemokines by MMPs may not be necessary.

S-SDF-1(S4V)-RAD fusion proteins improved cardiac function and capillary density after MI. This finding, combined with the increase in CXCR4/c-Kit/Flk-1 cells seen in the same treatment groups, argues for an endothelial progenitor cell–mediated benefit. These results are consistent with previous findings that show that SDF-1 is a potent chemoattractant for endothelial progenitor cells. How-
ever, it is unlikely that the increase in capillary density alone is sufficient to explain the improvement in ejection fraction. An additional effect of SDF-1 on cardiomyocyte hypertrophy or contractility might play a role. Furthermore, some studies have shown differentiation of endothelial progenitor cells into cardiomyocytes. Our study also raises the hypothesis that SDF-1 attracts cardiac progenitor cells that have cardiomyocyte differentiation potential. The chemotactic factors that attract cardiac progenitor cells remain to be identified, but myocardial delivery of chemotaxis could have a role in recruiting resident cardiomyocyte precursors.

In summary, designing SDF-1 to be protease resistant, together with delivery by self-assembling peptides, improved cardiac function after MI. This benefit may arise from increased vascularization by attraction of endothelial progenitor cells to the heart. This protease-resistant SDF-1 may be useful for preventing heart failure but also for repairing other injured tissues in which proteases are active. Finally, this study demonstrates the unique potential of driving chemotaxis by bioengineered chemokine delivery.

Sources of Funding
Dr Segers was supported by a PhD fellowship of the Research Foundation–Flanders and by a Belgian American Educational Foundation research fellowship. Dr Tokunou was supported by a fellowship of the Banyu Life Science Foundation International. This work was supported by National Institutes of Health grant EB003805.

Disclosures
None.

References

Figure 6. S-SDF-1(S4V)-RAD increases CXCR4+ cells after MI. A, CXCR4+ cells per section. n=7 or more. Values are mean±SEM. B, CD34-CXCR4 double stain of infarcted myocardium. Arrow shows CXCR4+/CD34+ cell, arrowhead, CXCR4+/CD34- cell. C, CXCR4-c-Kit double stain of infarcted myocardium. D, Flk-1/VEGF-R2-CXCR4 double stain of infarcted myocardium. Scale bar is 20 μm.
CLINICAL PERSPECTIVE

The dominant cause of congestive heart failure is death of cardiomyocytes resulting from myocardial infarction. New strategies are needed that increase vascularization and regeneration of infarcted myocardium. Local delivery of chemokines that attract multipotent stem cells to the injured tissue represents a novel strategy to promote regeneration, and stromal cell–derived factor-1 (SDF-1) is a recognized chemotactrant for hematopoietic stem cells and endothelial progenitor cells. However, local delivery of proteins to the myocardium is challenged by rapid diffusion from the site of injection, and SDF-1 is proteolytic degraded by matrix metalloproteinases. In the present study, we altered SDF-1 to create a new protease-resistant chemokine SDF-1(S4V). Furthermore, we prevented diffusion from the site of injection by generation of fusion proteins of SDF-1 proteins with the sequence of self-assembling peptides (called RAD). We evaluated the effects of modified SDF-1 proteins using a randomized, blinded experimental design in 176 rats that incorporated catheterization at 1 month after myocardial infarction and local delivery of the proteins. We found that local delivery of protease-resistant SDF-1(S4V) incorporated in self-assembling peptides as a fusion protein with self-assembling peptides improved systolic function at 1 month after myocardial infarction and increased capillary density. These results indicate that local myocardial delivery of SDF-1(S4V)-RAD fusion proteins may be therapeutically effective, especially if this strategy can be implemented in the catheterization laboratory after myocardial infarction.
Local Delivery of Protease-Resistant Stromal Cell Derived Factor-1 for Stem Cell Recruitment After Myocardial Infarction


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Supporting Text and Figures

Local Delivery of Protease Resistant Stromal Cell Derived Factor-1 for Stem Cell Recruitment after Myocardial Infarction

Segers, Protease resistant SDF-1 for stem cell recruitment.

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SUPPORTING METHODS

MMP and DPPIV/CD26 cleavage experiments. Human recombinant MMP-2 (R&D systems) was activated for 1 h and MMP-9 was activated for 24 h with 4-aminophenylmercuric acetate (APMA, 1 mM) at 37°C. 4 μg of S-SDF-1 or S-SDF-1(S4V) were incubated for 1 h at 21°C with 50 ng of activated MMP-2 or MMP-9 in 40 μl TCNB buffer (50 mM Tris pH 7.5, 10 mM CaCl₂, 150 mM NaCl, 0.05% Brij-35). Sample loading buffer (Invitrogen) and 2-mercaptoethanol (0.5M final concentration) were added, samples were boiled for 5 min and run on a 12% Bis-Tris gel. Positive controls consisted of same activation of MMP-2 and MMP-9 ran on a gelatin Bis-Tris gel. To visualize cleavage in a different way, 4 μg of Sumo-S-SDF-1 or Sumo-S-SDF-1(S4V) was incubated for 4 h or 48 h with different concentrations of activated MMP-2 and MMP-9. Proteins used for this experiment were obtained after cation exchange chromatography, but were not cleaved by Sumo-protease and not purified by RPC-HPLC.

For DPPIV/CD26 cleavage experiments, 2.5 μg of S-SDF-1, commercial SDF-1 (R&D Systems) or S-SDF-1(S4V) were incubated for 1 h at 21°C with 50 ng of DPPIV (R&D Systems) Tris buffer (25 mM Tris pH 8). Sample loading buffer and 2-mercaptoethanol were added, samples were boiled for 5 min and run on a 12% Bis-Tris gel.

MMP cleavage experiments of linker sequences in fusion proteins. Cleavage of linkers was tested in soluble phase (TCNB buffer) as described for the cleavage site in native SDF-1. Samples were incubated for 1 or 4 hours at 21°C. To demonstrate release of S-SDF-1(S4V) after incorporation of fusion proteins in the nanofibers, S-SDF-1(S4V)-MCS-RAD or S-SDF-1(S4V)-MCS10G-RAD were incorporated in the nanofibers as described earlier and the unbound fraction was washed away with 16 washes in TCNB. 50 ng of MMP-2 was added to the insert and incubated for 1 or 24 h at 21°C. Inserts were washed 16 times with phosphate buffered saline to wash away the cleaved fraction of S-SDF-1(S4V) and remaining protein was counted by scintillation counting of the nanofibers. Control samples were treated in the same manner, without addition of MMP-2. For N-terminal protein sequencing, S-SDF-1-SCR-RAD was cleaved by MMP-2 in TCNB as described earlier. Fragments were separated by SDS-PAGE and blotted on a PVDF membrane. The smaller fragment was studied for 10 Edman cycles on an ABI 494 Protein Sequencer.

SDF-1 ELISA after myocardial infarction. MI was produced in 24 male rats as described. Infarcted zone was harvested after 5 min (day 0), 1d, 3 d, and 7 d. Tissue was homogenized in RIPA buffer (PBS, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 1mM Sodium orthovanadate, 0.5mM PMSF, Protease inhibitor cocktail (1/1000 of Sigma, P2714, 10ml/ bottle stock)). SDF-1 protein concentrations were determined by a commercially available ELISA kit (R&D Systems). Sensitivity of the ELISA for different concentrations of S-SDF-1 and SDF-1(5-68) was measured in a separate in vitro experiment (n=4).

Western analysis. In a separate experiment, MI was produced in 32 male rats as described in the manuscript methods. 80 μl of nanofibers alone, with 10 μM Sumo-S-SDF-1-6G-RAD or with Sumo-S-SDF-1(S4V)-6G-RAD, and Sumo-S-SDF-1(S4V)-6G-RAD alone were injected in the infarcted area. Infarcted zones were harvested after 5 min (day 0), 1d, 3d, or 7d and homogenized in 3ml RIPA lysis buffer (PBS, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS, 1mM PMSF, 1mM Sodium Orthovanadate, protease inhibitor cocktail). Sample loading buffer (Invitrogen) and 2-mercaptoethanol (0.5M final concentration) were added, samples were boiled for 5 min and
20µl sample per lane was loaded on a 10% Bis-Tris gel. Proteins were transferred to a PVDF membrane (Perkin Elmer), stained with Sumo-specific antibody (Abcam) and with an anti-rabbit-HRP conjugated secondary antibody (Bio-Rad). Proteins were visualized with Western Lightning Chemiluminescence Reagent (Perkin Elmer).
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Supporting Figure 1. (a) Four different mutations of S-SDF-1 were tested for activity on Jurkat cell migration. 100nM was added to the lower well of a Boyden chamber. After 3 h, cells that had migrated to lower wells were counted (n=3). (b) 4µg of Sumo-S-SDF-1 or Sumo-S-SDF-1(S4V) was incubated with different doses of MMP-2 or MMP-9 for 4 h or 48 h at 21°C. Expected fragment size for Sumo = 13 kDa, expected fragment size for S-SDF-1 8kDa. Coomassie staining of SDS-PAGE, reducing conditions.
Supporting Figure 2. Lysine residues in different proteins were radioactively labeled with acetic anhydride-carbonyl-C14. Labeled S-SDF-1(S4V) proteins (30nM) were mixed with self-assembling peptide nanofibers. Nanofibers were washed 16 times 15 min with PBS. (a) Graph shows protein remaining in nanofibers with different starting concentrations of S-SDF-1(S4V). (b) Graph shows protein concentration remaining in different densities of nanofibers after 16 washes. (c) 4µg of the different S-SDF-1(S4V)-RAD fusion proteins were incubated with 50ng MMP-2 or MMP-9 for 1 h at 21°C. Coomassie staining of SDS-PAGE, reducing conditions. (d) 30nM of S-SDF-1(S4V)-MCS-RAD was incorporated in nanofibers which were washed 16 times to wash away unbound protein. Nanofibers were incubated with 50ng MMP-2 for 1 h and gels were washed another 16 times to wash away cleaved and released S-SDF-1(S4V). Graph represents protein that remains incorporated in nanofibers. All experiments n=4 or more. Mean±SEM.
Supporting Figure 3. (a) SDF-1 ELISA of infarcted rat myocardium at different time points after infarction (n=6). SDF-1 concentration is expressed as moles per unit of tissue volume. (b) Concentration response curve for S-SDF-1 and MMP-2 cleaved SDF-1(5-68). The sensitivity of the ELISA (measured by optical density) was the same for full length S-SDF-1 and cleaved SDF-1(5-68). Mean±SEM. *=P<0.001.
Supporting Figure 4. Western blot with Sumo-specific antibody at different time points after MI combined with injection of nanofibers and proteins. Only Sumo-S-SDF-1(S4V)-6G-RAD injected together with nanofibers was detectable after 1d. Representative images of 2 independent experiments.