Stromal Cell-Derived Factor-1 Retention and Cardioprotection for Ischemic Myocardium

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Background—Stromal cell-derived factor-1 (SDF-1) is a chemoattractant of stem/progenitor cells, and several studies have shown that SDF-1 may improve ventricular function after infarction. SDF-1 is cleaved by proteases including matrix metalloproteinase-2 (MMP-2) and CD26/dipeptidylpeptidase-4 (DPP-4), which are activated in injured tissues.

Methods and Results—We investigated the biodistribution and functional roles of SDF-1 in experimental ischemia/reperfusion injury in rats. Radiolabeled SDF-1 given by intracoronary injection was selectively concentrated in ischemic myocardium. The enhanced uptake of SDF-1 in ischemic myocardium was not mediated by its receptor, CXCR4. Mass spectrometry and Western analyses showed that SDF-1 was cleaved by DPP-4 in plasma and myocardium, whereas a bioengineered MMP-2/DPP-4–resistant form of SDF-1, SSDF-1(S4V), was highly stable. A single dose of SSDF-1(S4V) exhibited greater potency for cardioprotection than wild-type SDF-1. SSDF-1(S4V) improved cardiac function in rats even after a 3-hour ischemic period.

Conclusions—These results show that a single dose of protease-resistant SSDF-1(S4V) after myocardial infarction leads to dramatic improvement in angiogenesis and ventricular function even 3 hours after the onset of ischemia, revealing a simple, clinically feasible approach to prevention of heart failure. (Circ Heart Fail. 2011;4:509-518.)

Key Words: myocardium • ischemia • cardioprotection • angiogenesis • reperfusion • heart failure

Patients with myocardial infarction (MI) benefit from early myocardial reperfusion, which is the most effective strategy to improve clinical outcome.1 Despite the success of reperfusion, mortality and morbidity after MI remain substantial, with 5% to 6% of patients having a subsequent cardiovascular event in the next 30 days.1 Therefore, novel cardioprotective strategies are required to improve clinical outcomes in patients with ischemic heart disease.2–4

Clinical Perspective on p 518

Stromal cell-derived factor-1 (SDF-1, also known as CXCL12) is a chemokine that binds to specific G-protein–coupled 7-transmembrane receptors presented on the plasma membranes of target cells.5 SDF-1 binds to at least 1 chemokine receptor, CXCR4, and SDF-1 and CXCR4 deficient mice display similar lethal phenotypes.5,7 SDF-1 secreted by bone marrow during embryogenesis is critical for the colonization of marrow by fetal liver-derived hematopoietic stem/progenitor cells.8 Furthermore, during adult life, SDF-1 has a pivotal role in the retention and homing of hematopoietic stem/progenitor cells into the bone marrow microenvironment through the interaction with CXCR4.9 Recent studies from multiple laboratories have demonstrated that SDF-1 treatment after MI recruits hematopoietic stem cells from the circulation or bone marrow and exert beneficial effects on ventricular function, probably through inhibition of cardiomyocyte apoptosis and the promotion of angiogenesis.10–14 SDF-1 is susceptible to cleavage and inactivation by several proteases, including matrix metalloproteinase-2 (MMP-2), which is activated by tissue injury,15,16 and CD26/dipeptidylpeptidase-4 (DPP-4).17 Rendering SDF-1 resistant to proteases may improve cardioprotection after MI, and administration of a DPP-4 inhibitor may also be beneficial after infarction, possibly through prevention of SDF-1 cleavage.18,19

In the present study, we investigated the biodistribution of therapeutic SDF-1 for myocardial ischemia/reperfusion (I/R) injury; because SDF-1 has been reported to selectively home to ischemic myocardium,20 we speculated that CXCR4-mediated uptake of SDF-1 could lead to uptake and reprocessing of this chemokine.9 Surprisingly, we found that CXCR4 did not mediate selective retention of SDF-1 by ischemic myocardium. We also found that this ischemic homing is not necessary for the beneficial effects of SDF-1 because a nonhoming protease-resistant form of SDF-1 pre-
served cardiac function after I/R-injury. Our results suggest that a single dose of a bioengineered variant of SDF-1 administered at the end of reperfusion with a simple intracoronary injection may represent a new clinical approach to prevent postinfarction ventricular dysfunction.

Methods
A detailed description of the following methods is provided in the online-only Data Supplement: protein expression and purification, radiolabeling and biotin-labeling of SDF-1, Western analysis, protein identification by liquid chromatography–mass spectrometry, and immunohistochemistry and infarct size measurements.

Rat I/R Experiments
All animal protocols were approved by an institutional animal care and use committee and performed in an AAALAC-approved facility. All procedures were performed in a blinded and randomized manner, and the code of the studies was broken only after acquisition of all data. I/R injury was produced in male Sprague-Dawley rats (weight,

Table. Biodistribution of SDF-1 in I/R-Injured Rats at 10 Minutes, 30 Minutes, 4 Hours, and 24 Hours After Administration

<table>
<thead>
<tr>
<th>Organ Tissue</th>
<th>Circulation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 Minutes (n=9)</td>
</tr>
<tr>
<td>LV, ischemic region</td>
<td>1.30±1.29</td>
</tr>
<tr>
<td>LV, nonischemic region</td>
<td>0.26±0.15*</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>0.28±0.24‡</td>
</tr>
<tr>
<td>Lung</td>
<td>0.37±0.37†</td>
</tr>
<tr>
<td>Liver</td>
<td>0.46±0.29*</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.20±0.20‡</td>
</tr>
<tr>
<td>Eye</td>
<td>0.22±0.13‡</td>
</tr>
<tr>
<td>Brain</td>
<td>0.038±0.059†</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>0.21±0.18‡</td>
</tr>
</tbody>
</table>

Data are percentage of injected dose of SDF-1 per gram of organ tissue, expressed as mean±SD.

*P<0.05, †P<0.01, ‡P<0.001 compared with LV, ischemic region.
200 to 230g; Charles River Laboratories, Wilmington, MA, and Harlan, Indianapolis, IN). Rats were anesthetized with pentobarbital (60 mg/kg) or 2% to 3% isoflurane and buprenorphine (0.05 mg/kg), and, after tracheal intubation, hearts were exposed through a left thoracotomy. After pericardiotomy, the left anterior descending coronary artery (LAD) was ligated by suturing with 6-0 Prolene at a location 2 mm below the left atrial appendage. Reperfusion was induced by releasing the suture after 30, 45, 75, or 90 minutes. For the sham operation, suturing was performed without ligation. Proteins were dissolved in phosphate buffered saline (PBS) and injected in the left ventricular (LV) cavity while clamping the aorta (no longer than 10 seconds) to ensure that proteins were delivered in the coronary arteries. For the 3-hour ischemia experiments, chests were closed after ligation of the LAD and the animals were weaned from the ventilator. Chests were reopened after 3 hours, sutures were released from the LAD, and the proteins were injected as described. After injection, the chest was closed and animals were allowed to recover on a heating pad. The animals received buprenorphine 0.05 mg/kg every 6 to 12 hours for 48 to 72 hours as an analgesic.

**Biodistribution of Systemically Injected SDF-1**

The ³⁵Clabeled SDF-1 variants were injected into rats after I/R injury (ischemia for 30 minutes followed by reperfusion for 10 minutes). Ten minutes to 24 hours after protein injection, blood containing unbound SDF-1 was washed out with 90 mL of PBS, and organs including the ischemic region of the LV, nonischemic region of the LV, right ventricle, lung, liver, spleen, kidney, eye, brain and skeletal muscle were harvested. A dose of AMD3100 (1 mg/kg) that has been shown to be active in vivo²¹–²³ was given by intraperitoneal injection. Because its half-life is 2 to 3 hours,²¹ AMD3100 was injected 30 minutes before initiation of I/R injury. Tissues were weighed and processed to measure radioactivity. Tissues were placed in glass scintillation vials with 2 mL of Soluene-350 (PerkinElmer, Waltham, MA), incubated at 50°C for 3 hours, and decolorized with 30% hydrogen peroxide. Hionic-Fluor (Perkin Elmer) was added, and the scintillation vials with 2 mL of Soluen-350 (PerkinElmer, Waltham, MA), incubated at 50°C for 3 hours, and decolorized with 30% hydrogen peroxide. Hionic-Fluor (Perkin Elmer) was added, and the volume calibration and the hemodynamic data of at least 5 pressure-volume loops before each of the 3 saline injections. Relative volume units were normalized with fresh heparinized blood in calibrated cuvettes provided by the manufacturer.

**Statistical Analysis**

Unless defined otherwise, data are expressed as mean±SEM. To compare SDF-1 concentrations between different tissues, a logarithmic transformation of the concentrations in the different tissues was performed to obtain homoscedasticity. A linear mixed-effects model with compound symmetry covariance structure was estimated; post hoc comparisons were performed with a Bonferroni adjustment. To compare means of cardiac function and histological date, means were compared by means of 1-way ANOVA; post hoc comparisons were performed with a Bonferroni adjustment. Probability values were normalized with fresh heparinized blood in calibrated cuvettes provided by the manufacturer.

Figure 2. Selective retention of SDF-1 in the ischemic region of the LV is not mediated by CXCR4. A, In the SDF-1 group (n=9), rats were injected with SDF-1 (0.01 mg/kg); in the SDF-1+AMD3100 group, rats were administered 1 mg/kg body weight of AMD3100, a selective antagonist of CXCR4, IP before SDF-1 injection (n=9); in the SDF-1+SSDF-1 group, rats were injected with SDF-1 (S4V) (n=7). AMD3100 did not decrease the concentration of SDF-1 in the ischemic region of the LV. Deletion of the CXCR4 receptor-binding domain of SDF-1 (SDF-1(S4V)) diminished (not significantly) the selective retention in the ischemic region of the LV. B, Rats injected with SDF-1, which is a DPP-4–resistant form of SDF-1, showed a trend toward lower retention in the ischemic LV compared with SDF-1 (n=4, not significant). Diprotin A, an inhibitor of DPP-4, dramatically decreased the selective retention of SDF-1 in the ischemic LV (n=3, no significant differences). In the SDF-1+SSDF-1 group (n=4), more SSDF-1(S4V), which is a DPP-4 and MMP-2–resistant form of SDF-1, homed to the ischemic LV, nonischemic LV, and right ventricle (RV) compared with SDF-1.

**Results**

**SDF-1 Is Retained Selectively by Ischemic Myocardium After Intracoronary Injection**

It has been reported previously that SDF-1 can improve cardiac function after MI; however, the biodistribution of SDF-1 after intracoronary injection is unknown. To analyze the biodistribution of SDF-1 after I/R injury, the LAD was occluded in vivo for 30 minutes followed by 10 minutes of reperfusion (Figure 1A), and radiolabeled SDF-1 (0.01 mg/kg) was given by intraventricular injection while the ascending aorta was clamped. Ten minutes after SDF-1 injection, unbound SDF-1 was washed out with PBS perfusion, after which organs were harvested. SDF-1 concentrated in the ischemic region of LV compared with nonischemic LV, and the
concentration of SDF-1 in ischemic LV was significantly higher than in other organs (P<0.001 compared with all organs except liver and lung (P<0.05 compared with liver and P<0.01 compared with lung; Figure 1B and Table). Almost 2% of the injected SDF-1 dose could be detected in the heart. Furthermore, we performed a time-course study that showed that SDF-1 could be detected in the ischemic region up to 24 hours after injection (Table), whereas levels in brain and eyes were close to the detection limit of the assay.

To localize SDF-1 in the myocardium, biotinylated SDF-1 (4 μg) was injected after I/R injury; to confirm the hypoxic insult, some rats were injected with Hypoxyprobe-1 (60 mg/kg, IP) 3 minutes before ischemia. Hypoxyprobe-1 is selectively taken up by cells with an oxygen concentration <10 mm Hg. Histological analysis showed that SDF-1 was concentrated in the ischemic LV primarily in cardiomyocytes (Figure 1C through 1F), although both cardiomyocytes and capillary endothelium positively stained with the Hypoxyprobe-1-antibody (Figure 1G and 1H). These results indicate that SDF-1 given by intracoronary injection selectively concentrates in ischemic cardiomyocytes.

**Ischemic Retention of SDF-1 Is Not Mediated by CXCR4**

The SDF-1–CXCR4 axis plays a critical role in the development of heart and blood vessels, in the regulation of angiogenesis, and in the development of tumors. A recent study showed that SDF-1 is selectively retained in the ischemic myocardium, which may have implications for the development of new therapies for myocardial infarction. However, the mechanism by which SDF-1 is selectively retained in the ischemic myocardium remains unclear. In this study, we investigated the mechanism of SDF-1 retention in the ischemic myocardium using in vivo and in vitro assays. We found that SDF-1 is retained in the ischemic myocardium through a mechanism independent of CXCR4.

**Figure 3.** SSDF-1(S4V) is protease-resistant. A, Liquid chromatography–mass spectrometry identified intact and cleaved forms of SDF-1 and SSDF-1(S4V); SDF-1 or SSDF-1(S4V) was incubated in rat plasma for 0, 60, and 240 minutes, and most of SDF-1 was detected as the N-terminal cleaved form, SDF-1(3-68), at 240 minutes, whereas SSDF-1(S4V) was detected as intact during the 240-minute observation. B, Western analysis shows that injected SDF-1 was observed as a smaller band compared with recombinant SDF-1 in ischemic heart tissue: Lanes 1, 3, 4, and 6, recombinant SDF-1 (lanes 1 and 3, 7 ng; lanes 4 and 6, 3 ng); lanes 2 and 5, tissue homogenate of ischemic LV of a rat injected with SDF-1; and lane 7, tissue homogenate of ischemic LV of a rat injected with PBS. Coomassie staining shows that the loading amount of tissue on each well is comparable; the bands shown on the gel are endogenous proteins in the tissue between 20 and 30 kDa in size.
of motility and differentiation in hematopoietic stem cells, and in the pathophysiology of MI. Because expression levels of CXCR4 have been shown to be increased in ischemic myocardium, we hypothesized that the selective retention of SDF-1 in ischemic myocardium was mediated by binding to CXCR4. To test this hypothesis, we used a selective CXCR4 antagonist, AMD3100. AMD3100 injected before I/R injury did not inhibit the retention of SDF-1 in ischemic myocardium (Figure 2A). To determine if the N-terminus of SDF-1—which binds to CXCR4—is required for ischemic homing, we expressed and purified SDF-1(5-68); SDF-1(5-68) is an inactive form of SDF-1 that lacks the N-terminal CXCR4 receptor-binding region but binds to CXCR3. This truncated form of SDF-1 showed a trend toward diminished retention in ischemic myocardium compared with SDF-1 (Figure 2B). SSDF-1(S4V), which is resistant to both DPP-4 and MMP-2, significantly increased EF and significantly decreased end-systolic volume (ESV) and end-diastolic volume (EDV) compared with the PBS-only group. Diprotin A, a tripeptide specific inhibitor of DPP-4, decreased the retention of native SDF-1 in the ischemic myocardium to levels of retention comparable to SSDF-1 (Figure 2B). DPP-4 concentration, measured with ELISA, was comparable in nonischemic and ischemic myocardium, with a small but significant decrease after longer periods of ischemia (7 days). DPP-4 molar concentration was between 10 to 50 times lower than the amount of SDF-1 retained in ischemic or nonischemic myocardium (online-only Data Supplement Figure 1), suggesting that DPP-4 is not serving as a binding reservoir for SDF-1. Taken together, these data show that selective retention of SDF-1 by ischemic myocardium is not mediated by CXCR4 or CXCR3.
SSDF-1(S4V) Is More Stable than SDF-1 in Rat Plasma and in Ischemic Myocardial Tissue

Because DPP-4 cleaves SDF-1 after the proline residue at position 2 to an inactive SDF-1(3-68), we tested by Western analysis whether SDF-1 is cleaved by DPP-4 in ischemic myocardium. A specific antibody to SDF-1 (MAB350) was used to detect SDF-1(3-68).17 Ischemic LV tissue was homogenized and fractionated into cytosol and membrane-associated fractions. This Western analysis showed SDF-1 in the membrane-associated fractions, but with a smaller size than recombinant SDF-1, indicating proteolytic cleavage (Figure 3B, 2 independent experiments). No specific antibodies with sufficient sensitivity were available to detect SSDF-1(S4V) in the tissues by Western analysis. To further characterize SDF-1 cleavage in rat plasma, qualitative liquid chromatography–mass spectrometry was performed (Figure 3A). SDF-1 incubated at 37°C for 10 to 240 minutes in rat plasma revealed an average molecular mass of 7730 Da, which is 225 Da lower than that of SDF-1 (7955 Da) and is consistent with SDF-1(3-68), reflecting the loss of the first 2 residues. SSDF-1(S4V), however, retained its full length of 69 amino acids (8054 Da) during the 240-minute observation period. To confirm that SSDF-1(S4V) is retained in cardiac tissue after longer periods of ischemia, we performed an experiment in which radiolabeled SSDF-1(S4V) was injected at 90 minutes instead of 30 minutes (online-only Data Supplement Figure 2). No significant difference in retention was observed at these 2 time periods.

Resistance of SDF-1 to MMP-2 Is Important for Functional Improvement

To investigate whether DPP-4 or MMP-2 resistance is more important for functional improvement after MI, 3 different variants of SDF-1 were purified and then tested in a rat I/R injury model: native SDF-1, DPP-4–resistant SSDF-1, and DPP-4/MMP-2–resistant SSDF-1(S4V). After 75 minutes of ischemia, proteins were injected and cardiac function was measured 2 weeks after MI with pressure-volume catheters. Representative pressure-volume loops are shown in online-only Data Supplement Figure 3. Native SDF-1, the variant with the greatest retention in ischemic myocardium, did not significantly increase ejection fraction (EF). SSDF-1, which is resistant to DPP-4 but not to MMP-2 and has a lower retention in ischemic tissue than SDF-1, did not increase EF compared with PBS only. However, SSDF-1(S4V) increased EF significantly from 49.8±2.6% in the PBS-only group to 54.2±3.2% (P<0.01, Figure 4). SSDF-1(S4V) also significantly decreased end-systolic volume and end-diastolic vol-
ume compared with PBS only. These results indicate that although SSDF-1(S4V) is not selectively concentrated in ischemic myocardium compared with native SDF-1, its resistance to MMP-2 provides an outcome advantage after I/R injury in rat hearts.

In a separate and longer-duration experiment, we compared functional effects of different doses of SSDF-1(S4V) 4 weeks after 45-minute ischemia (Figure 5). This experiment confirmed the efficacy of SSDF-1(S4V) for improving cardiac function after I/R injury at a longer time period (4 weeks) after the infarct, with doses as low as 0.001 mg/kg SSDF-1(S4V) to 1 mg/kg. To limit the number of experimental animals, no sham group was included in this experiment. In our laboratory, sham-operated animals consistently have EFs between 60% and 65%. We then used the most effective dose of SSDF-1(S4V) after a 3-hour ischemia period, which might be more clinically relevant. SSDF-1(S4V) increased EF significantly from 44.1±3.1% in the PBS-only group to 54.0±3.0% (P<0.05, Figure 6). SSDF-1(S4V) also significantly decreased end-systolic volume and end-diastolic volume compared with PBS only. Furthermore, mortality after I/R injury was higher in the PBS group (11 of 24 animals) compared with the SSDF-1(S4V) group (6 of 23), but this difference was not statistically significant.

**Discussion**

The SDF-1–CXCR4 axis plays a critical role in heart development, and SDF-1 has cardioprotective effects after MI. Although the primary receptor for SDF-1 is CXCR4, a G-protein–coupled receptor, several studies question the role of CXCR4 in ventricular remodeling after MI in the adult organism. The use of SDF-1 as a therapeutic to treat MI is challenged by its sensitivity to proteases, including DPP-4 and MMP-2. In the present study, we show that SDF-1 is selectively retained by ischemic myocardium after intracoronary injection compared with nonischemic myocardium. Surprisingly, the retention by ischemic myocardium is not mediated by CXCR4—the primary receptor for SDF-1. The molar concentration of DPP-4 was 10 to 50 times lower than the amount of SDF-1 retained in cardiac tissue. This result suggests that
the mechanism of retention of SDF-1 is not a one-to-one binding to DPP-4 but rather a processing of SDF-1 by DPP-4 and retention of the products of this reaction. Mass spectrometry enabled us to identify intact and cleaved SDF-1 when incubated in rat plasma and showed that SDF-1 is rapidly cleaved in plasma compared with SSDF-1(S4V).

We engineered SSDF-1(S4V), an SDF-1 variant that is resistant to DPP-4 by addition of a serine at the N-terminus and resistant to MMP-2 by mutating the serine at position 4 to a valine. In the present study, we show that protection against proteases is important for restoration of cardiac function after MI. SSDF-1(S4V) induced a larger improvement in cardiac function than did native SDF-1. Furthermore, SSDF-1(S4V) increased capillary growth in the infarcted myocardium with a single injection by a clinically relevant delivery approach but did not decrease infarct size or apoptosis rate. Although angiogenesis is plausibly the major mechanism of the SDF-1(S4V)–induced improvement of cardiac function, it is unlikely that it is the only mechanism. Proteins that improve cardiac function after MI may do so by more than one mechanism. In addition to a direct action on migration of progenitor cells, another possible explanation for the positive effects of SSDF-1(S4V) on cardiac function is that SSDF-1(S4V) might act as a competitive antagonist of MMP-2 or DPP-4. Inhibition of both proteases might lead to decreased cleavage of endogenous SDF-1 and enhanced homing of endothelial progenitor cells. This possibility is supported by experiments showing that DPP-4 inhibitors improve cardiac function after MI by increased homing of CXCR4+ cells. In another study, we found that c-kit+ bone marrow cells can stimulate endogenous cardiogenic precursor cells, whereas SSDF-1(S4V) did not. Thus, data to date most consistently support angiogenesis as the mechanism of benefit, but other mechanisms remain plausible.

In a previous study, protease-resistant SSDF-1(S4V) was delivered by injection into the myocardium, using a hydrogel at the onset of ischemia, which makes translation to clinical practice more difficult. In the present study, SSDF-1(S4V)
was delivered after 45 minutes, 75 minutes, and 3 hours of ischemia by intravenous injection while the ascending aorta was clamped, mimicking intracoronary injection in humans. This method has potential for rapid translation into clinical care, in which protease-resistant SDF-1 could be injected in the coronary artery after coronary reperfusion, even hours after the onset of ischemia.

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Disclosures
Dr Lee is a founder of and consultant for Provascolon. Dr Segers is a founder of and consultant for Provascolon.

References
The current standard of care for myocardial infarction (MI) includes early reperfusion to limit cardiomyocyte death and subsequent use of β-blockers and other medications to prevent cardiac remodeling and reduce future events. Despite the success of current therapies, new treatments for MI are needed because many patients with MI still have development of heart failure. We explored the possibility that protease-resistant stromal cell-derived factor-1 (SDF-1) could represent a new treatment to prevent heart failure after MI. SDF-1 is a small protein that can attract endothelial progenitor cells and increase blood vessel formation. However, SDF-1 is rapidly cleaved and inactivated by proteases that are present in the inflammatory environment of infarcted myocardium. In the present study, we show in multiple randomized and blinded studies in a rodent model that protease-resistant SDF-1 improves cardiac function after MI when injected in a manner similar to intracoronary delivery. Protease-resistant SDF-1 improved cardiac function even when injection was delayed up to 3 hours after onset of ischemia. Thus, a variant of SDF-1 rendered resistant to protease cleavage could be a new therapeutic for MI, particularly because it could be injected into the coronary artery immediately after reperfusion.
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Supplemental Material

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Supplemental Methods

Protein expression and purification

Plasmids were constructed as described previously. The DNA sequence of mature SDF-1α was cloned into the pET-Sumo vector (Invitrogen, Carlsbad, CA); sequences coding for an additional serine at the N-terminus were incorporated to increase resistance to DPP-4 cleavage (called SSDF-1). Mutagenesis of the serine at position 4 to a valine was performed as described previously (called SSDF-1(S4V)). All sequences were confirmed by DNA sequencing. Sumo-SSDF-1 fusion proteins were expressed in BL21 E. coli and grown to an optical density of 1.5 (at 600nm) at 37°C. Cells were induced with 0.25mM isopropyl β-D-thiogalactoside overnight at 21°C. SSDF-1 was purified by a 3-step procedure; all steps were performed at 21°C. Cells from a 6 liter growth were lysed by sonication in lysis buffer (PBS, 10µg/ml DNase, 1mM PMSF, and 100µg/ml lysozyme). Inclusion bodies were isolated by centrifugation and washed twice (PBS, 0.5% Triton-X100). The second purification and refolding step was performed on a cation exchange HPLC column (HiPrep-16/10SP-FF, Amersham). The inclusion bodies were dissolved in binding buffer (8M Urea, 30mM 2-mercaptoethanol, 1mM EDTA, and 50mM Tris pH 8.0) and loaded on the column. Refolding of Sumo-SSDF-1 was performed on the column with a 0-100% gradient of refolding buffer (50mM Tris pH8, 75mM NaCl, 0.1mM reduced Glutathione and 0.1mM oxidized Glutathione). Sumo-SSDF-1 was eluted with a gradient of 0.1-1M NaCl. The Sumo-SSDF-1 fusion protein was cleaved by Sumo Protease (1U/50µg protein, LifeSensors Inc., Malvern, PA). The sample was adjusted to 0.1% trifluoroacetic acid (TFA) and loaded on a C18 Reversed Phase HPLC column (XTerra-Prep-MS-5µm-7.8x150mm, Waters) as a final purification step. The column was subjected to a linear gradient from 30 to 40% acetonitrile in 0.1% TFA. Activity of purified SDF-1 variants was confirmed by migration of Jurkat cells in a ChemoTx chemotaxis system (5µm pore, Neuro Probe).

Radiolabeling and biotin-labeling of SDF-1

SDF-1 and variants were labeled with 14C by acetylation with acetic anhydride [1-14C] (American Radiolabeled Chemicals, St. Louis, MO) as described previously. Proteins were dissolved in PBS or 0.3M sodium phosphate buffer, pH 7.4, with 75µCi of acetic anhydride [1-14C] added in Dioxane (Sigma-Aldrich, St. Louis, MO) to reach a concentration of 0.5µCi/µl, and then incubated at room temperature for 30min. Unlabeled acetic anhydride [1-14C] was removed by dialysis against PBS. Specific activities and protein concentrations were measured by light absorption at 280nm. For histological analysis, SDF-1 and SSDF-1(S4V) were labeled with biotin by incubation with NHS-biotin (Thermo-Fisher) as described in the manufacturer
Western analysis

Heart tissues from rats injected with SDF-1 (20µg) following I/R-injury were excised, snap frozen and homogenized on ice in 5% w/v 50mM HEPES buffer (pH 7.5, supplemented with 250mM sucrose, protease inhibitor cocktail, 0.6mM PMSF, 1mM sodium orthovanadate, and 1mM EDTA). After 30min incubation on ice, the homogenate was centrifuged and the pellet was suspended in RIPA buffer (PBS, 0.1% SDS, 1% Nonidet P-40, 0.6mM PMSF, 0.5% sodium deoxycholate, 1mM sodium orthovanadate, and protease inhibitor cocktail). Western analysis was performed with monoclonal antibody to SDF-1 (MAB350, R&D Systems, Minneapolis, MN).

Protein identification by mass spectrometry (LC/MS/MS)

Intact and truncated forms of SDF-1 and SSDF-1(S4V) were analyzed by LC/MS as previously described. SDF-1 or SSDF-1(S4V) (10µg) were incubated in 10µL of rat plasma (stored with EDTA, diluted 1/10 in PBS) for 0, 10, 30, 60, and 240min at 37°C. The activity of proteases/enzymes in plasma was terminated with formic acid (2.5µL of 2% formic acid to 100 µL reaction mixture). Samples were diluted 100 fold with water and underwent protein profile analysis with liquid chromatography coupled to mass spectrometry (LC/MS/MS). Samples (10µl) were injected onto a C8 Vydac column (50 x 2.10mm, Grace, Deerfield, IL) equipped with a CTC Analytics HTS Pal Autosampler (CTC Analytics, Switzerland) and an Agilent binary LC pump (Agilent Technologies, Santa Clara, CA). Solvent A was water with 0.1% formic acid, and mobile phase B was 90% acetonitrile with 0.1% formic acid. Samples were eluted with a linear gradient of solvent B (0 to 80% over 30min). Eluted samples were applied to MDS Sciex API QStar Pulsar quadruple time-of-flight (Q-TOF) mass spectrometer (Applied Biosystems, Foster City, CA). Data was acquired in full scan TOF MS mode (m/z, 200–2000) with positive electrospray ionization (ESI). Intact and fragmented molecules (cleaved by DPP-4, MMP-2, Elastase, Cathepsin G, and Carboxypeptidase M) of SDF-1 and SSDDF-1(S4V) were analyzed by high-resolution QSTAR LC-MS.

Immunohistochemistry and infarct size measurements

Organ tissues were fixed in 4% paraformaldehyde, and paraffin-embedded sections were deparaffinized and rehydrated before staining. Tissue sections of rats injected with biotin-SDF-1 were stained with HRP-conjugated streptavidin (DAB Peroxidase Substrate Kit, Vector Laboratories, Burlingame, CA) and tissue sections of rats injected with Pimonidazole
Hydrochloride (Hypoxyprobe™-1, Hypoxyprobe, Inc., Burlington, MA) were stained with MAB-1 (Hypoxyprobe™-1)\textsuperscript{5, 6} and counterstained with hematoxylin (Sigma). Capillaries and arterioles were stained with GS-IB4 antibody (Invitrogen) and smooth muscle actin antibody respectively, and nuclei were stained with DAPI. Apoptotic nuclei were stained using the ApoTag Fluorescein In Situ Apopotsis Detection Kit (Millipore) per manufacturer's instructions. Myocytes were stained with antibodies specific to Troponin-T (Abcam). Infarct size was measured in mid-papillary sections by the blue area stained with Trichrome Masson. The surface area of blue scar tissue was normalized to the total surface of the sections.
Supplementary Figure 1. DPP-4 concentration in non-ischemic and ischemic myocardium. DPP-4 was measured with ELISA in non-ischemic cardiac tissue and ischemic cardiac tissue at different time points after ligation of the coronary artery (n=6 for all groups).
Supplementary Figure 2

Supplementary Figure 2. Retention of SSDF-1(S4V) after different ischemia times. The amount of SSDF-1(S4V) retained in cardiac tissue was similar after 30 or 90 minutes of ischemia.
Supplementary Figure 3. Representative pressure-volume loops of the study in Figure 4. Representative PV-loops from one animal in each group of Figure 4 are presented.
Supplementary Figure 4. Protease-resistant SSDF-1(S4V) does not decrease infarct size. A, Infarct size was quantified by measuring collagen scar tissue relative to total surface area, representative samples of panel B. B, no significant differences in infarct size were observed after administration of different doses of SSDF-1(S4V).
**Supplementary Figure 5**

**A**, apoptotic nuclei were quantified in the infarct border zone 3 days after I/R-injury (n=4).

**B**, representative images of panel A, red= Troponin T, green= apoptotic nuclei (arrows), blue= DAPI.

**Supplementary Figure 5.** Protease-resistant SSDF-1(S4V) does not influence apoptosis rate. A, apoptotic nuclei were quantified in the infarct border zone 3 days after I/R-injury (n=4). B, representative images of panel A, red= Troponin T, green= apoptotic nuclei (arrows), blue= DAPI.
References


