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A critical role of Src family kinase in SDF-1/CXCR4-mediated bone-marrow progenitor cell recruitment to the ischemic heart

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Abstract

The G protein-coupled receptor CXCR4 and its ligand stromal-cell derived factor 1 (SDF-1) play a crucial role in directing progenitor cell (PC) homing to ischemic tissue. The Src family protein kinases (SFK) can be activated by, and serve as effectors of, G proteins. In this study we sought to determine whether SFK play a role in SDF-1/CXCR4-mediated PC homing. First, we investigated whether SDF-1/CXCR4 signaling activates SFK. Bone-marrow mononuclear cells (BM MNCs) were isolated from WT and BM-specific CXCR4-KO mice and treated with SDF-1 and/or CXCR4 antagonist AMD3100. SDF-1 treatment rapidly induced phosphorylation (activation) of hematopoietic Src (i.e., Lyn, Fgr, and Hck) in WT cells but not in AMD3100-treated cells or CXCR4-KO cells. Then, we investigated whether SFK are involved in SDF-1/CXCR4-mediated PC chemotaxis. In a combined chemotaxis and endothelial-progenitor-cell (EPC) colony assay, Src inhibitor SU6656 dose-dependently inhibited the SDF-1-induced migration of colony-forming EPCs. Next, we investigated whether SFK play a role in SDF-1/CXCR4-mediated BM PC homing to the ischemic heart. BM MNCs from CXCR4^{BAC}:eGFP reporter mice were i.v. injected into WT

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and SDF-1_{BAC}:SDF1-RFP transgenic mice following surgically-induced myocardial infarction (MI). eGFP⁺ MNCs and eGFP⁺c-kit⁺ PCs that were recruited in the infarct border zone in SDF-1_{BAC}:SDF1-RFP recipients were significantly more than that in WT recipients. Treatments of mice with SU6656 significantly reduced eGFP⁺ and eGFP⁺c-kit⁺ cell recruitment in both WT and SDF-1_{BAC}:RFP recipients and abrogated the difference between the two groups. Remarkably, PCs isolated from BM-specific C-terminal Src kinase (CSK)-KO (Src activated) mice were recruited more efficiently than PCs from WT PCs in the WT recipients. In conclusion, SFK are activated by SDF-1/CXCR4 signaling and play an essential role in SDF-1/CXCR4-mediated BM PC chemotactic response and ischemic cardiac recruitment.

Keywords

Src; CXCR4; SDF-1; Bone marrow progenitor cells; Stem cell homing; Ischemic tissue repair

1. Introduction

EPCs from the BM are part of the natural response to cardiac ischemic injury. Following MI, EPCs are mobilized from the BM and recruited to the site of injury, where they promote repair by inducing de novo vasculogenesis and secreting beneficial paracrine factors [1]. This endogenous repair mechanism has been employed over the last 15 years in cell-based therapies aimed to improve long-time outcomes post-MI. However only modest efficacy has been achieved; very few of the mobilized or transplanted PCs are retained in the ischemic myocardium and, consequently, poor cell recruitment is one of the primary barriers to the effectiveness of cell therapy [2].

We and others have shown that the recruitment and retention of PCs in ischemic tissue are regulated by interactions between stromal cell-derived factor-1 (SDF-1 or called CXC chemokine ligand 12 [CXCL12]) and its receptor CXC chemokine receptor 4 (CXCR4) [3,4]. CXCR4 is expressed by BM MNCs, and SDF-1 is expressed by stromal cells; both SDF-1 and CXCR4 are upregulated by hypoxia, and SDF-1–CXCR4 interaction regulates several steps of PC recruitment, including chemotaxis, transendothelial migration, and adhesion to mature endothelial cells and integrin [5]. The SDF-1/CXCR4-mediated BM PC recruitment to the ischemic heart has been shown to contribute to the vessel growth and improved myocardial function in pre-clinical models [6]. Although numerous studies demonstrate that SDF-1/CXCR4 signaling plays a crucial role in the recruitment of PCs to ischemic tissue, and the SDF-1/CXCR4 axis has been identified as a potential target for improving cell therapy, little is known about the downstream signaling mechanisms that are induced by this pathway.

The Src family protein kinases (SFK) are a class of non-receptor tyrosine kinases that relay signals downstream of cell-surface receptors to regulate diverse cellular responses including cell growth, differentiation as well as cell adhesion and movement [7]. The overall amino acid sequences of SFK, with the exception of the N-terminal ~50 residues, are highly conserved among the family members. The structural similarities allow for common mechanisms of regulation: the phosphorylation of the conserved tyrosine residues (Tyr-416 and Tyr-527). Although the molecular mechanisms of SFK activation may vary depending

upon cell types and extracellular cues, SFK are normally present in their inactive form, in which Tyr-527 is phosphorylated. By contrast, SFK are activated by the phosphorylation of Tyr-416, suggesting that these two tyrosine phosphorylation sites are involved in a reciprocal regulatory mechanism [7]. The non-receptor tyrosine kinase CSK acts as an indispensable negative regulator of SFK by specifically phosphorylating the negative regulatory site (Tyr-527) of SFK, thereby suppressing their activity and function; genetic deletion of CSK leads to spontaneous SFK activation [8]. Notably, recent studies highlight the important role of SFK in mediating chemotaxis [9]. Reports from another laboratory and ours indicate that SDF-1/CXCR4 signaling relies on SFK to activate PI3-kinase in T cells [10] and c-kit receptor tyrosine kinase in BM PCs [11]. However, whether SFK play a role in SDF-1/CXCR4-mediated BM PC chemotactic response is currently not known.

In the present study, we demonstrate that in BM PCs, SFK are activated by SDF-1/CXCR4 signaling and play an essential role in SDF-1-mediated PC migration and recruitment to the ischemic myocardium. These results warrant further investigation to modulate SFK activity in PCs for enhancing the effectiveness of cell therapy for ischemic heart disease.

2. Materials and methods

2.1. Mice

CXCR4^{fl/fl}, CXCR4_{BAC}::eGFP^{Tg} (CXCR4 reporter) and SDF1_{BAC}::SDF1-RFP^{Tg} (SDF-RFP-fusion transgenic) mice were provided by Dr. Richard J. Miller (Northwestern University, Chicago) [12]. CXCR4_{BAC}::eGFP^{Tg} mice carry a bacterial artificial chromosome (BAC) coding for eGFP expression from the CXCR4 genomic regulatory sequence (i.e., green eGFP fluorescence signals CXCR4 expression). SDF1_{BAC}::SDF1-RFP transgenic mice carry a BAC coding for the expression of an SDF-1-RFP fusion protein from the SDF-1 genomic regulatory sequence (i.e., red RFP fluorescence signals expression of the SDF-1-RFP fusion protein, and the total level of SDF-1 expression is doubled). Mx1-cre;CXCR4^{fl/fl} were generated as we described, and CXCR4 gene deletion was induced with 3 intraperitoneal (i.p.) injections of 250 µg poly(I)-poly(C) (GE Healthcare) administered at 2-day intervals [11]. CSK^{fl/fl} mice were provided by Dr. Alexander Tarakhovsky (The Rockefeller University, New York) and crossed with Tie2-cre mice (Jax lab) to generate Tie2-cre;CSK^{+/fl} (referred as Tie2-cre;CSK^{+/+} mice because the floxed allele is deleted in BM PCs, i.e., Lin⁻ and c-kit⁺ BM MNCs). Genotypes were determined via PCR with tail DNA by using primer sequences reported previously [11,13]. For all the experiments in this report, 8–10 week-old male mice were used. The animal breeding, maintenance, and surgical procedures were approved by the Institutional Animal Care and Use Committee of Northwestern University and performed in the University's Center for Comparative Medicine. For acute systemic inhibition of SFK, SU6656 was i.p. administered at a dose of 6 mg/kg based on the published reports [14] and on our dose–response studies indicating a 90% reduction in the level of Sam68 (SFK substrate) phosphorylation in BM MNCs 4 h after drug administration.

2.2. Isolation of BM MNCs and c-kit⁺ PCs

BM MNCs were isolated by density centrifugation, and Lin⁻c-kit⁺ PCs were obtained by FACS sorting as described [11].

2.3. Quantitative real-time RT-PCR (qRT-PCR)

qRT-PCR analyses of CXCR4 expression were performed as we described previously [11]. Relative gene expression was normalized to GAPDH mRNA levels.

2.4. Western blotting

Western blotting was performed with anti-phospho-Src (Tyr-416, cell signaling), anti-Lyn, and anti-Hck (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) primary antibodies and with relevant secondary antibodies (Santa Cruz Biotechnology, Inc.) via standard techniques [11]. Phospho-Src band intensities were determined densitometrically with Image J software, and normalized to Lyn or Hck intensity.

2.5. Chemotaxis assay

Chemotaxis assay was performed with a Coster Transwell (6.5 mm diameter, 5 μ m pore) as described [15]. Briefly, 10⁶ BM MNCs were seeded in the upper chamber containing AMD3100 (100 ng/mL), SU6656 (2 μ M), or vehicle. Serial concentrations of SDF-1 or vehicle (PBS) were added to the lower chamber. Following 4 h incubation at 37 °C, cells that migrated into the lower chamber were collected in 50 μ L of buffer and counted manually using a hemocytometer or directly cultured for EPC colony formation assay (below).

2.6. EPC colony formation assay

Following chemotaxis assay, the migrated MNCs were subjected to EPC colony formation assay by 11 days of continuous culture as we described previously [16].

2.7. Surgical induction of myocardial infarction (MI)

MI was induced by permanently ligating the middle of the left anterior descending (LAD) coronary artery as we described previously [4,16].

2.8. Fluorescent microscopy

Histological assessments of heart tissues were performed as we described previously [4,16]. Briefly, hearts were isolated, fixed in 4% paraformaldehyde for 4 h, incubated overnight in 30% sucrose, embedded in OCT compound (Sakura Finetek U.S.A., Inc., Torrance, CA), and snapfrozen in liquid nitrogen; then, serial cryosections were cut from 1 mm below the suture used to ligate the LAD to the apex (three consecutive sections per 1 mm) to enable quantitative histological analyses at each level. Fluorescent microscopic analyses of transplanted cells were performed as described previously [4,16]. All surgical procedures and histological analyses were performed by investigators who were blind to mouse genotypes and treatments.

2.9. Statistical analysis

Data are reported as mean $\pm$ SEM. Two-tail unpaired Student's *t* test was used for comparisons between 2 means. One-way ANOVA was used for comparisons between 3 or more means. Statistical significance was assigned if $P < 0.05$.

3. Results

3.1. SDF-1/CXCR4 signaling induces SFK phosphorylation (activation) in BM MNCs

We firstly isolated BM MNCs from BM-specific CXCR4 knockout mice (i.e., Mx1-cre⁺;CXCR4^{fl/fl} mice, derived from Mx1-cre⁺;CXCR4^{fl/fl} mice treated with poly (I)-(C) to induce CXCR4 knockout in the BM MNCs) and control Mx1-cre⁺;CXCR4^{+/+} mice. Quantitative RT-PCR confirmed that CXCR4 is expressed abundantly in the control cells but largely absent in the Mx1-cre⁺;CXCR4^{fl/fl} cells (Fig. 1A). Treatment with SDF-1 significantly increased phosphorylation of Src in the control BM MNCs, but not in Mx1-cre⁺;CXCR4^{fl/fl} BM MNCs (Fig. 1B). Notably, when CXCR4 antagonist AMD3100 was added to the control cells, SDF-1 no longer induced Src phosphorylation (Fig. 1C). Collectively, these data suggest that SDF-1 activates SFK through interacting with CXCR4.

3.2. Inhibition of SFK impairs SDF-1-induced migration of BM MNCs and EPCs

We then investigated whether SFK play a role in SDF-1/CXCR4-mediated chemotaxis. WT BM MNCs were subjected to Transwell migration assays for counting the migrated cells; and in separate experiments, the migrated cells were further cultured in a special medium for 11 days for quantifying EPCs with the capability of forming colonies. SDF-1 dose-dependently induced the migration of BM MNCs (Fig. 1D) and colony-forming EPCs (Fig. 1E). However, when SFK inhibitor SU6656 or CXCR4 antagonist AMD3100 was added to the cells, SDF-1 was no longer able to induce BM MNC and EPC migration (Figs. 1D and E). These results suggest that SDF-1 induced PC migration is dependent on SFK activity.

3.3. SFK are essential for SDF-1-mediated recruitment of BM PCs to the ischemic myocardium

Next we investigated whether SFK contribute to SDF-1/CXCR4-mediated BM PC recruitment in the ischemic cardiac tissue. BM MNCs were isolated from CXCR4^{BAC}::eGFP^{Tg} reporter mice, and i.v.-injected into SDF1^{BAC}::SDF1-RFP^{Tg} (with doubled SDF1 expression) or WT mice after surgically-induced MI; Twenty-four hours after cell injection, eGFP⁺ cells and eGFP⁺c-kit⁺ PCs were significantly more frequent in the infarct border zone (ischemic area) of SDF1^{BAC}::SDF1-RFP^{Tg} mice than in WT mice, which indicates that SDF-1 overexpression enhances the recruitment of circulating BM PCs (Figs. 2B and C). SU6656 treatments significantly reduced the amount of CXCR4^{BAC}::eGFP⁺ cells and CXCR4^{BAC}::eGFP⁺c-kit⁺ cells in both WT and SDF-1^{BAC}::SDF1-RFP recipients and abrogate the difference between the two groups (Figs. 2B and C). Thus, SFK activity is required for efficient recruitment of BM PCs from the circulation to the ischemic region, and recruitment cannot be enhanced by SDF-1 overexpression when SFK activity is inhibited.

Finally, we tested whether augmenting SFK activity in BM PCs can improve their recruitment. For these studies, we have generated Tie2-cre;CSK^{+/-} (i.e., Tie2-cre;CSK^{+/-}) mice, in which the floxed CSK allele is deleted in BM PCs via cre expression under the control of stem cell promoter Tie2. The BM Lin⁻c-kit⁺ PCs from Tie2-cre;CSK^{+/-} mice displayed a significantly higher level of phospho-Src than the control PCs from Tie2-cre;CSK^{+/+} mice (Fig. 2D). We then labeled Tie2-cre;CSK^{+/-} and Tie2-cre;CSK^{+/+} PCs with fluorescent DiI, and i.v.-injected into WT mice with surgically-induced MI. The CSK^{+/-} PCs were recruited in the ischemic heart significantly more efficiently than CSK^{+/+} PCs (Fig. 2E).

4. Discussion

Although numerous experiments support SDF-1/CXCR4 axis as a therapeutic target for enhancing PC recruitment, recent clinical studies have revealed that in patients with coronary artery disease and related conditions such as aging and diabetes, the SDF-1/CXCR4 signaling is impaired [17]. Our results identify SFK as a critical downstream effector of SDF-1/CXCR4 signaling that play an essential role in BM PC chemotactic response and ischemic cardiac recruitment. Therefore, targeted activation of SFK in BM PCs may benefit this population of patients who are candidates for angiogenic cell therapy.

Our observations indicate that SDF-1/CXCR4 induces SFK activation. CXCR4 is a G-protein coupled receptor, and two families of G-protein α subunits (G α s and G α i) activate SFK (c-Src and Hck) by binding directly to the catalytic domain and increasing substrate access to the active site [18]. CXCR4-dependent stimulation of SFK (Lyn) has also been associated with the activation of PI3K [10]. However, SFK display considerable redundancy both in their activation pathways and in the downstream effectors that mediate their biological function [7], so whether the G-proteins, PI3K, or other upstream components link the SDF-1/CXCR4 axis to SFK activation and BM PC retention, has yet to be determined.

There are nine known SFK, as well as multiple isoforms of the same SFK, are expressed in any given tissue. SU6656 inhibits all SFK [19], and the available antibodies are not specific for the phosphorylated forms of individual family members, so we used antibodies against activated SFK that are found predominantly in the hematopoietic system (i.e., Lyn, Hck, and Fgr). Nevertheless, the results reported here cannot identify which specific type of SFK is activated by SDF-1/CXCR4 signaling. We have begun a series of experiments with mice deficient in one or more SFK to characterize the roles of the individual family members.

Notably, our data indicate that systemic inhibition of SFK reduces the recruitment of not only c-kit⁺ BM PCs but also other BM cell populations (Figs. 2B and C). Because certain cell populations within the total BM cells may be detrimental to vascular repair [20], it is important that Src activity be upregulated specifically in the PCs for any potential therapeutic development.

In conclusion, the results presented here demonstrate that SDF-1/CXCR4 signaling recruits PCs to ischemic heart through activation of Src. Because BM PCs have an essential role in tissue repair, the mechanism identified by our studies could be an important component of

the physiological response to ischemic injury and may also have the potential to be modulated for enhancing the effectiveness of cell therapy.

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Abbreviations

BAC	bacterial artificial chromosome
BM	bone marrow
CSK	C-terminal Src kinase
CXCR4	CXC chemokine receptor 4
MI	myocardial infarction
PC	progenitor cell
RFP	red fluorescence protein
SDF-1	stromal-cell derived factor 1
SFK	Src family protein kinases

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Fig. 1.

SFK are activated by SDF-1/CXCR4 signaling and mediate SDF-1-induced migration of BM MNCs and EPCs. (A) qRT-PCR analyses of CXCR4 expression in BM MNCs isolated from BM-specific CXCR4 knockout (Mx1-cre;CXCR4^{-/-} or CXCR4^{-/-}) mice and Mx1-cre;CXCR4^{f/f} (Control) littermates. (B) CXCR4^{-/-} and Control BM MNCs were treated with SDF-1 (200 ng/mL) for 10 min, then subjected to western blotting analyses for phosphorylated hematopoietic Src (i.e., Lyn, Fgr, and Hck) and total Lyn proteins (*left panel*), quantified densitometrically, normalized to the levels of total Lyn, and expressed relative to the level in Control cells with vehicle treatment (-) (*right panel*). n = 6 per treatment. N.S., not significant. (C) WT BM MNCs were treated with SDF-1 and/or AMD3100 (100 ng/mL) for 10 min, and then levels of phospho-Src and total Lyn were analyzed by western blotting (*left panel*) and quantified (*right panel*). n = 5 per treatment. (D-E) Chemotaxis assays were performed in a Transwell with upper chamber containing 10⁶ WT BM MNCs with or without AMD3100 (100 ng/mL) or SFK inhibitor SU6656 (2 μM) and lower chamber containing serial concentrations of SDF-1; then the migrated cells were counted (D) or directly cultured with additional growth factors for EPC colony assays (E). (D) Quantification of the migrated BM MNCs per well. (E) Quantification of EPC colonies formed from the migrated EPCs after an 11-day culture.

Fig. 2.

SFK play an essential role in SDF-1-induced recruitment of BM PCs to the ischemic myocardium. (A) Experimental procedures evaluating the homing capacity of i.v. injected CXCR4_{BAC}::eGFP reporter BM MNCs to the ischemic myocardium of WT and SDF-1 transgenic (SDF-1_{BAC}::SDF-1/RFP^{Tg}) mice with or without systemic Src inhibition. (B–C) Quantification of the numbers of eGFP⁺ MNCs (B) and eGFP⁺c-kit⁺ PCs (C) in the ischemic border zone in histological sections. HPF, high power field. (D) Representative western blotting of phospho-Src in BM Lin⁻c-kit⁺ PCs isolated from Tie2-cre;CSK^{+/+} and Tie2-cre;CSK^{fl/fl} mice. (E) BM Lin⁻c-kit⁺ PCs from BM MNCs of Tie2-cre;CSK^{+/+} and

Tie2-cre;CSK^{+/+} mice were labeled with fluorescent DiI, then i.v.-injected to the WT mice with surgically-induced MI by following the procedures described in (A); 24 h later, the PCs recruited in the ischemic border zone were quantified histologically. HPF, high-powered field.

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