Heparin Disrupts the CXCR4/SDF-1 Axis and Impairs the Functional Capacity of Bone Marrow–Derived Mononuclear Cells Used for Cardiovascular Repair

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Rationale: Cell therapy is a promising option for the treatment of acute or chronic myocardial ischemia. The intracoronary infusion of cells imposes the potential risk of cell clotting, which may be prevented by the addition of anticoagulants. However, a comprehensive analysis of the effects of anticoagulants on the function of the cells is missing.

Objective: Here, we investigated the effects of heparin and the thrombin inhibitor bivalirudin on bone marrow–derived mononuclear cell (BMC) functional activity and homing capacity.

Methods and Results: Heparin, but not bivalirudin profoundly and dose-dependently inhibited basal and stromal cell–derived factor 1 (SDF-1)–induced BMC migration. Incubation of BMCs with 20 U/mL heparin for 30 minutes abrogated SDF-1–induced BMC invasion (16±8% of control; P<0.01), whereas no effects on apoptosis or colony formation were observed (80±33% and 100±44% of control, respectively). Pretreatment of BMCs with heparin significantly reduced the homing of the injected cells in a mouse ear-wound model (69±10% of control; P<0.05). In contrast, bivalirudin did not inhibit in vivo homing of BMCs. Mechanistically, heparin binds to both, the chemoattractant SDF-1 and its receptor, chemokine receptor 4 (CXCR4), blocking CXCR4 internalization as well as SDF-1/CXCR4 signaling after SDF-1 stimulation.

Conclusions: Heparin blocks SDF-1/CXCR4 signaling by binding to the ligand as well as the receptor, thereby interfering with migration and homing of BMCs. In contrast, the thrombin inhibitor bivalirudin did not interfere with BMC homing or SDF-1/CXCR4 signaling. These findings suggest that bivalirudin but not heparin might be recommended as an anticoagulant for intracoronary infusion of BMCs for cell therapy after cardiac ischemia. (Circ Res. 2012;111:854-862.)

Key Words: cell therapy ■ bone marrow cells ■ myocardial infarction ■ heparin ■ bivalirudin ■ cell migration ■ cell transplantation

Postinfarction heart failure remains a challenge in modern cardiology despite optimal treatment with early reperfusion and medical therapy.1,2 Cell therapy with autologous or allogeneic bone marrow–derived or tissue-resident stem/progenitor cells, eg, from adipose or cardiac tissue, has emerged as a promising treatment option for patients with acute or chronic ischemia and heart failure.3 Cell therapy with proangiogenic cells, such as bone marrow–derived mononuclear cells (BMCs), increased left ventricular ejection fraction and prevented left ventricular remodeling in some but not all clinical trials.3–8 Specifically, when intracoronary delivery of cells was used for cell therapy in patients with acute myocardial infarction, the therapeutic benefit of cell therapy was found to critically depend on the appropriate homing of the injected cells. Homing in acute ischemia is mainly guided by the chemokine SDF-1 (stromal cell–derived factor-1, also known as CXCL12) and its receptor, chemokine receptor 4 (CXCR4).9 SDF-1 is released in response to hypoxia and recruits CXCR4-expressing proangiogenic cells to sites of ischemia.10–13 Blocking the CXCR4 receptor with neutraliz-
ing antibodies or injection of CXCR4-negative BMCs leads to a significantly reduced recovery of neovascularization in mice after hindlimb ischemia.\textsuperscript{12,14} The capacity of the injected cells to migrate ex vivo in response to SDF-1 additionally predicted the neovascularization improvement and infarct size reduction in vivo.\textsuperscript{15,16}

**Methods**

**Isolation of BMCs**

Bone marrow aspirates were obtained from healthy volunteers without any evidence of coronary artery disease in their history or physical examination. The Ethics Review Board of the Hospital of the Johann Wolfgang Goethe University in Frankfurt, Germany, approved the protocol, and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each volunteer. For several experiments, BMCs were obtained from patients with ischemic cardiomyopathy undergoing intracoronary application of proangiogenic cells, which might be caused by cell clotting.\textsuperscript{20–22} Therefore, several groups added anticoagulant glycosaminoglycans such as heparin to the injected cells. Heparin sulfate is well known for its high binding activity to proteins and receptors and was previously shown to directly bind to the chematoattractant SDF-1 through a typical consensus sequence for heparin recognition.\textsuperscript{23,24} Clinically relevant concentrations of heparin negatively affected SDF-1–induced chemotaxis and inhibited CXCR4 receptor ligation in tumor cell lines.\textsuperscript{24–26} Moreover, treatment of mice with heparin significantly reduced the number of metastases by blocking CXCR4-dependent invasion of tumor cells into the bloodstream.\textsuperscript{25} However, the influence of heparin and other anticoagulants on the migration and homing function of cells used for cardiac cell therapy is unknown.

Therefore, we performed a detailed analysis of the effects of heparin on BMC homing functions in vitro and in vivo. In addition, we tested other anticoagulants, such as the direct thrombin inhibitor bivalirudin, which is a short, synthetic peptide without many of the limitations of heparin, such as its unspecific binding ability.\textsuperscript{27,28}

We show that heparin, when added in clinically relevant concentrations, significantly reduces BMC functionality by interacting with the CXCR4/SDF-1 axis and thereby abrogates the positive effects of this chemokine. In contrast, bivalirudin does not affect BMC functionality in vivo and in vitro.

**Fluorescence-Activated Cell Sorter Analysis and Immunostaining**

For internalization experiments, BMCs were preincubated with heparin (Ratiopharm) for 2 hours at 37°C. Next, cells were stained with a CXCR4 antibody (clone 1D9; BD) for 60 minutes at 4°C. Afterwards, BMCs were incubated at 37°C with SDF-1 (100 ng/mL; R&D Systems) to stimulate internalization. To detect internalized CXCR4, the surface-bound antibodies were stripped off with an acidic buffer (50 mmol/L glycine plus 100 mmol/L NaCl, pH 2.5) for 2.5 minutes, followed by extensive washing. Then, cells were analyzed by fluorescence-activated cell sorting (FACS; Becton Dickinson, FACS Canto II). For immunostaining, the CXCR4 antibody (1D9; BD) was labeled with Alexa Fluor 555 (Invitrogen), the cell membrane was stained with wheat germ agglutinin labeled with Alexa Fluor 488, and cell nuclei were stained with Hoechst 33342 (AnaSpec Inc). Confocal microscopy analysis was performed with a Zeiss LSM 510 Meta microscope.

**Assessment of Invasion Capacity of BMCs**

A total of $1 \times 10^6$ BMCs were resuspended in 250 μL of X-VIVO 15 medium and placed in the upper chamber of a modified Boyden chamber filled with Matrigel (BioCoat invasion assay, 8-μm pore size; Becton Dickinson). The chamber was then placed in a 24-well culture dish that contained 500 μL of X-VIVO 15 medium. For some experiments, 100 ng/mL SDF-1 was added to the lower chamber. After 24 hours of incubation at 37°C, transmigrated cells were counted. Invasion assays were run in duplicate.

**Ear-Wound Model**

Fifteen female SHK-1 mice, weighing 20 to 30 g and aged 8 to 12 weeks, were obtained from Charles River Laboratories (Sulzfeld, Germany). The animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine hydrochloride (10 mg/kg body weight). After disinfection of the ears, the animals were placed on a specially designed acrylic glass platform with the ears stretched out by application of 3 permanent loops (9-0, nylon). A standardized circular wound (2.25 mm in diameter, 1.25 mm in depth) was made on the dorsal side of the ears with a specially designed punch. The wounds were positioned between the ears’ anterior and medial principal neurovascular bundles. After the punch incision was performed, a full-thickness layer of skin was dissected down to the underlying cartilage layer by use of a precise microsurgical technique. BMCs were preincubated for 2 hours under different conditions (control, heparin, bivalirudin), then stained with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) and injected into the tail vein in 250 μL of PBS. The mice were placed under a microscope, and the periwound area was monitored for homing BMCs for 10 minutes. Homed cells were counted by 2 blinded investigators.
Frozen sections were cut with a cryotome with 10 μm thickness. BMCs were counted by a blinded investigator. Sections were analyzed under a fluorescence microscope, and homed BMCs were stimulated with SDF-1 for 2.5 minutes (100 ng/mL). The phosphatidylinositol 3-kinase inhibitor Ly294002 was used as a negative control for Akt phosphorylation. Cells were lysed with the Akt-ELISA lysis buffer, and the ELISA was performed with commercially available Akt1 ELISA kits (Cell Signaling Technology) that detect total Akt1 and phosphorylated Akt1 (phospho-Akt1; Ser473). Absorbance was measured with a plate reader (Synergy HT; Bio-Tek Instruments). Data are shown as a ratio of phospho-Akt to total Akt.

Heparin-Binding ELISA

Heparin (5000 U/mL) or BSA (1 μg/mL) was incubated on a 96-well immunoplate (Nunc) for 3 hours at 37°C in coating buffer (50 mmol/L Na₂CO₃, pH 9.6). After the coating buffer was removed, the wells were incubated with a CXCR4–glutathione S-transferase (GST) fusion protein (1 μg/mL; Abnova) overnight at 4°C followed by an anti-GST horseradish peroxidase (HRP) antibody (Abcam). After 3 washing steps, HRP was detected by TMB solution (Pierce), and absorbance was measured with a plate reader (Synergy HT; Bio-Tek Instruments).

CXCXR4-Binding ELISA

CXCXR4-GST protein (1 μg/mL; Abnova) was incubated on a 96-well immunoplate (Nunc) for 18 hours at 4°C in coating buffer.
Figure 1. Heparin impairs BMC migration and homing in vitro. BMCs were incubated with heparin (20 U/mL) or bivalirudin (15 μg/mL) for 2 hours, stained, and injected into the tail vein of female SHK-1 mice with an ear wound. Homed cells were detected by in vivo fluorescence microscopy after injection and counted by two blinded investigators (n=5 mice in each group, data are shown as mean±SEM). Statistical comparisons were made by the 2-sided t test or the nonparametric Mann-Whitney U test. Statistical significance was assumed at a value of \( P<0.05 \).

Results

Heparin Impairs Migration and Homing of BMCs

BMCs were incubated with heparin or the thrombin inhibitor bivalirudin in increasing, pharmacologically relevant concentrations, and invasion was measured in a modified Boyden chamber. As shown in Figure 1, heparin profoundly and dose-dependently inhibited SDF-1–induced BMC invasion (Figure 1A), whereas the thrombin inhibitor bivalirudin did not affect BMC invasion. In addition, the basal invasion capacity of the cells was significantly inhibited after incubation with heparin (Figure 1B). Similar effects were detected when BMCs isolated from patients with chronic ischemic heart disease were used for the experiments (Figure 1C). Interestingly, the addition of 20 U/mL heparin to BMCs for just 30 minutes followed by extensive washing before addition to the Boyden chamber also significantly reduced the SDF-1–induced BMC invasion capacity (Figure 1D). To exclude apoptotic effects, annexin/7-AAD was examined by FACS analysis. There was no significant difference in apoptosis and necrosis between BMCs preincubated with heparin or bivalirudin and untreated control cells (Figure 1F). Likewise, the colony-forming activity was not affected by heparin or bivalirudin (Figure 1E).

To address whether heparin affects BMC homing in vivo, BMCs were pretreated with heparin for 2 hours, washed with PBS, fluorescently labeled, and intravenously injected in mice with an ear wound, which allowed for intravital assessment of BMC homing. Preincubation of BMCs with heparin significantly reduced homing capacity compared with control cells (Figure 2). Importantly, bivalirudin did not inhibit homing of BMCs in vivo (Figure 2). Furthermore, heparin reduced in vivo homing in infarcted mouse hearts to a similar extent (49.6±27.4% of control). Taken together, these data demonstrate that heparin but not bivalirudin impairs the SDF-1–induced invasion of BMCs in vitro, and preincubation with heparin significantly reduces homing of the injected cells in vivo.

Heparin Alters CXCR4 Internalization

The SDF-1/CXCR4 axis is essential for invasion and for in vivo engraftment of BMCs. Therefore, CXCR4 surface expression was measured by FACS analysis. Interestingly, heparin significantly increased mean CXCR4 surface expression on BMCs after 2 hours and after 24 hours (Figure 3). On binding to SDF-1, the CXCR4 receptor is internalized to activate the intracellular signaling pathways, whereas the absence of a ligand leads to an increase in CXCR4 surface expression. To address the kinetics of CXCR4 receptor cycling, we performed internalization experiments as shown.
in Figure 4A. Indeed, the surface CXCR4 signal was significantly reduced after SDF-1 stimulation in control BMCs, whereas heparin abolished this effect (Figure 4). Consistently, the intracellular CXCR4 signal significantly increased after SDF-1 stimulation in control but not heparin-treated BMCs. In contrast, bivalirudin did not alter CXCR4 expression or internalization (data not shown). These findings were confirmed by immunohistochemistry, as shown in Figure 4C. Together, these results demonstrate that heparin prevents CXCR4 internalization after SDF-1 stimulation.

Heparin Inhibits SDF-1 Signaling

To verify our results and to demonstrate that the defective CXCR4 internalization caused by heparin interferes with intracellular CXCR4 signaling, we measured the activation of Akt, a known downstream signal of the CXCR4 receptor.30,31 Indeed, heparin significantly reduced SDF-1–induced Akt phosphorylation to 57±10% of untreated cells (Figure 5), whereas bivalirudin did not affect Akt phosphorylation (Figure 5), which indicates a disruption of the CXCR4/SDF-1 signaling axis by heparin but not by bivalirudin.

CXCR4/SDF-1 Interaction

Having demonstrated that heparin inhibits CXCR4 internalization and signaling, we addressed the underlying mechanisms. As heparin is well known for its binding activities to both growth factors and receptors,23,24,28,32–34 we performed binding studies with recombinant SDF-1 and CXCR4 in vitro. First, we examined whether the CXCR4 receptor binds directly to heparin. Plates were coated with heparin or BSA (as control) before the addition of recombinant GST-CXCR4 fusion protein. After being fixed with 4% formaldehyde, BMCs were dried on slides, stained with wheat germ agglutinin/Alexa Fluor 488 (WGA), and covered with a Hoechst mounting medium. Representative pictures are shown. Scale bar=5 μm.

Figure 4. Heparin prevents chemokine receptor 4 (CXCR4) internalization. A, Bone marrow–derived mononuclear cells (BMCs) were incubated at 37°C with or without heparin, followed by incubation with an anti-CXCR4 antibody at 4°C. Then, some of the cells were stimulated with Stromal cell–derived factor-1 (SDF-1) at 37°C, and the surface antibodies were stripped with an acidic buffer as indicated. B, BMCs were treated as mentioned in panel A. Then, cells were analyzed by fluorescence-activated cell sorting. Total, intracellular, and surface CXCR4 fluorescence is shown. C, BMCs were incubated at 37°C with or without heparin, followed by incubation with an anti-CXCR4 antibody at 4°C (conjugated with Alexa Fluor 555). Next, some of the cells were stimulated with SDF-1 at 37°C, and in some of the cells, the surface antibodies were stripped with an acidic buffer. After being fixed with 4% formaldehyde, BMCs were dried on slides, stained with wheat germ agglutinin/Alexa Fluor 488 (WGA), and covered with a Hoechst mounting medium. Representative pictures are shown. Scale bar=5 μm.
coated plates was comparable to GST-CXCR4 coated plates, which were used as a positive control (Figure 6), which indicates the efficient binding of heparin to CXCR4.

Next, we analyzed whether heparin interferes with binding of SDF-1 to the CXCR4 receptor. We coated plates with recombinant CXCR4 and then added biotinylated recombinant SDF-1. The biotinylated SDF-1 was detected by HRP-conjugated streptavidin. As expected, SDF-1 bound to CXCR4 (Figure 7); however, when SDF-1 was preincubated with heparin, no binding to CXCR4 was detected (Figure 7). Interestingly, when heparin was added directly to the CXCR4-coated plates, SDF-1 was still able to bind to CXCR4, which indicates that the binding of heparin to the CXCR4 receptor does not directly inhibit the binding of SDF-1 to the CXCR4 receptor.

These data show that heparin binds to SDF-1 and to CXCR4 and thereby inactivates the functional activity of the SDF-1/CXCR4 axis.

The present study demonstrates that the glycosaminoglycan heparin in pharmacoologically relevant concentrations impairs the invasion and homing capacity of BMCs used for clinical cell therapy in vitro and in vivo. Specifically, heparin interferes with the SDF-1/CXCR4 axis, which is essential for migration and in vivo engraftment of intravascularly administered BMCs. The inhibition of SDF-1 responses by heparin is mediated by an inhibition of CXCR4 receptor internalization, which blocks CXCR4 downstream signaling (Figure 8). The inhibition of CXCR4 internalization might be a consequence of direct binding of heparin to SDF-1, which inhibits SDF-1–dependent CXCR4 activation. Indeed, previous biochemical studies documented a direct binding of heparin to SDF-1, which prevented the chemoattractive activity of SDF-1 in leukemia cells, consistent with our in vitro binding studies. Recently, it was shown that heparin but not bivalirudin affects the levels of circulating sFLT1 (soluble fms-like tyrosine kinase-1), placental growth factor, and vascular endothelial growth factor during percutaneous coronary intervention procedures. Moreover, we demonstrate that heparin binds not only to SDF-1 but also directly to the CXCR4 receptor. The direct binding of heparin to the CXCR4 receptor did not prevent interaction of SDF-1 and CXCR4 but likely interfered with receptor internalization. The direct binding of heparin to CXCR4 might explain why a brief preincubation of BMCs with heparin, followed by several washing steps, still led to impairment of the SDF-1 response, although heparin was not added to the migration assays. Pretreatment of BMCs with heparin also reduced the in vivo homing of injected BMCs in the ear-wound model and inhibited homing in infarcted mouse hearts. Although we cannot formally prove that this effect is mediated by the interferences with the SDF-1/CXCR4 axis, various experimental studies have demonstrated that SDF-1 and CXCR4 are essential for homing of injected proangiogenic cells and BMCs to ischemic and injured tissue. Even half-maximal inhibition of CXCR4 expression by use of CXCR4 antisense oligonucleotides resulted in severe impairment of neovascularization improvement mediated by the injected cells. Moreover, systemic injection of unfractionated heparin and low-molecular-weight heparin inhibits CXCR4-dependent migration of cancer cells and reduces the number of metastases.

Given that recent data demonstrate a direct relationship between the number of cells retained acutely and the recovery of cardiac function after ischemia in animal models, the finding of the present study that heparin interferes with the in vivo homing of BMCs might be important for the interpretation of existing clinical trial data and the design of future studies. Although meta-analysis of all studies demonstrated that BMC therapy significantly improved the left ventricular ejection fraction of patients after acute myocardial infarction, clinical trials showed variable results. In most of the clinical trials, BMCs were isolated individually by density gradient centrifugation (Ficoll); however, the details of the further handling of the cells varied among the studies. Both, the choice of buffer (ranging from plain saline to PBS to various cell culture media) and the supplements (serum, plasma) may interfere with cell functionality. Interestingly,
several studies added heparin to the BMCs in concentrations that far exceeded the minimal dose of 0.05 U/mL that inhibited the SDF-1–induced invasion capacity of BMCs in the present study (Autologous Stem Cell Transplantation in Acute Myocardial Infarction [ASTAMI] trial, 5 U/mL heparin; Multicenter Randomized Trial of Intracoronary Infusion of Autologous Mononuclear Bone Marrow Cells or Peripheral Mononuclear Blood Cells After Primary Percutaneous Coronary Intervention [HEBE] trial: 20 U/mL heparin18,19). Although the details of the protocols (such as the dosage of heparin or the addition of serum, which may partially block the heparin effects) might influence the effects on BMC function, one may speculate that the lack of effects seen in some studies might be attributed to the conditions of cell storage.

Furthermore, it is unclear whether heparin or other anticoagulants were systemically administered to the treated patients during cell administration, because this information was not given in all of the published trials. Heparin is a state-of-the art therapy for patients undergoing coronary angiography and has shown clinical benefits in patients with acute coronary syndrome.38 However, it is unclear whether systemic administration interferes with homing of administered BMCs or even with homing of endogenous circulating progenitor cells. The findings in tumor-bearing mice suggest that systemically administered heparin indeed effectively blocks CXCR4 signaling,25,26 and one may speculate that the use of heparin might have limited the effects of strategies to augment endogenous stem cell mobilization, eg, by granulocyte–colony-stimulating factor.4 Further studies are warranted to determine whether heparin may interfere with homing of applied or endogenously mobilized cells to the injured myocardium. Importantly, because the effects of heparin can be ameliorated by excessive serum in vitro (data not shown), the concentration of heparin in relation to serum should be taken into consideration.

Finally, the data of the present study demonstrate that an alternative anticoagulant, bivalirudin, does not interfere with SDF-1/CXCR4 signaling and does not inhibit homing of injected BMCs. Bivalirudin is approved as an anticoagulant for percutaneous interventions and was shown to be superior compared to heparin in invasively treated patients with acute coronary syndrome.
myocardial infarction. Therefore, one might consider using bivalirudin instead of heparin for cell storage and potentially also for systemic anticoagulant treatment of patients undergoing intracoronary cell therapy.

In summary, heparin but not bivalirudin impairs the functionality of BMCs in vitro and in vivo by at least 2 mechanisms, including the binding of SDF-1, thereby preventing CXCR4 receptor activation, and binding to the CXCR4 receptor. Both mechanisms impair the SDF-1/CXCR4 signaling pathway (Figure 8) and thereby reduce the homing of cells for neovascularization improvement and cardiac repair after ischemia. Therefore, an alternative anti-coagulant such as the thrombin-inhibitor bivalirudin might be used for clinical cell therapy.

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Disclosures
Drs Dimmeler and Zeiher are founders and advisors of t2cure GmbH. The remaining authors report no conflicts.

References

Novelty and Significance

**What Is Known?**

- Cell therapy with bone marrow–derived mononuclear cells (BMCs) improved recovery after acute ischemia in experimental and clinical studies. Isolation and storage protocols influence the functional activity of BMCs used for cell therapy.
- The glycosaminoglycan heparin, which reduces tumor cell invasion, is routinely added to cell preparations in clinical studies to reduce clotting of the cell suspension.

**What Information Does This Article Contribute?**

- Heparin impairs the basal and SDF-1–induced migration capacity of BMCs in vitro and the homing of cells to ischemic tissue in vivo.
- Heparin binds to SDF-1 and its receptor CXCR4, thereby blocking the CXCR4–SDF-1 signaling axis crucial for cell homing and retention.

- Bivalirudin does not interfere with SDF-1/CXCR4 signaling or homing of BMCs and might be an alternative anticoagulant during cell preparation.

Data from recent meta-analyses suggest that BMC therapy improves cardiac function and survival of patients with acute myocardial infarction. However, results of individual studies have been heterogeneous, perhaps in part because varying protocols were used for cell isolation and storage. Here, we demonstrate that heparin in clinically relevant concentrations inhibits functional activity of BMCs in vitro, and pretreatment of BMCs impairs homing in vivo. Mechanistically, we show that heparin directly interacts with CXCR4 and SDF-1, which are essential for in vivo homing of BMCs. In contrast, the direct thrombin inhibitor bivalirudin does not affect BMC functionality in vivo and in vitro. Therefore, bivalirudin might be a superior alternative anticoagulant for heparin for use in cell preparations for intracoronary cell therapy.
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