Abstract. The present study was conducted to assess the effects of AMD3100 and stromal cell-derived factor 1 (SDF-1) on cellular functions and endothelial regeneration of endothelial progenitor cells (EPCs). The cell proliferation and adhesion capacity of EPCs were evaluated in vitro following treatment with AMD3100 and SDF-1 using a Cell Counting Kit-8 assay. Furthermore, the expression levels of C-X-C motif chemokine receptor 4 (CXCR4) and C-X-C motif chemokine receptor 7 (CXCR7) were detected before and after treatment with AMD3100 and SDF-1 to elucidate their possible role in regulating the cellular function of EPCs. A rat carotid artery injury model was established to assess the influences of AMD3100 and SDF-1 on endothelial regeneration. AMD3100 reduced the proliferation and adhesion capacity of EPCs to fibronectin (FN), whereas it increased the adhesion capacity of EPCs to human umbilical vein endothelial cells (HUVECs). However, SDF-1 stimulated the proliferation and cell adhesion capacity of EPCs to HUVECs and FN. Additionally, the expression levels of CXCR7 but not CXCR4 were upregulated following AMD3100 treatment, whereas the expression levels of both CXCR4 and CXCR7 were upregulated after SDF-1 treatment. In vivo results demonstrated that AMD3100 increased the number of EPCs in the peripheral blood and facilitated endothelial repair at 7 days after treatment. However, local administration of SDF-1 alone did not enhance reendothelialization 7 and 14 days after treatment. Importantly, the combination of AMD3100 with SDF-1 exhibited superior therapeutic effects compared with AMD3100 treatment alone, accelerated reendothelialization 7 days after treatment, and attenuated neointimal hyperplasia at day 7 and 14 by recruiting more EPCs to the injury site. In conclusion, AMD3100 could positively regulate the adhesion capacity of EPCs to HUVECs via elevation of the expression levels of CXCR7 but not CXCR4, whereas SDF-1 could stimulate the proliferation and adhesion capacity of EPCs to FN and HUVECs by elevating the expression levels of CXCR4 and CXCR7. AMD3100 combined with SDF-1 outperformed AMD3100 alone, promoted early reendothelialization and inhibited neointimal hyperplasia, indicating that early reendothelialization attenuated neointimal hyperplasia following endothelial injury.

Introduction

In-stent restenosis (ISR) after stent implantation remains a serious clinical challenge, and ~26.4% of patients experience ISR after implantation of stents (1). New-generation drug-eluting stents have reduced the incidence of ISR to 10% (2-4); however, local anti-proliferative therapy may interfere with vascular healing, and incomplete neointimal coverage 3-6 months after stent implantation has been identified to be associated with late stent thrombosis (5). Previous studies have demonstrated that early reendothelialization can reduce vascular neointimal hyperplasia and restenosis, indicating that endothelial regeneration is essential to prevent unfavorable vascular events (6,7). Endothelial progenitor cells (EPCs) can accelerate reendothelialization and attenuate neointimal hyperplasia (8,9). However, the concentration of circulating EPCs may be decreased in patients with risk factors for heart disease, including elevated low-density lipoprotein (LDL) cholesterol, diabetes mellitus and hypertension (10-12). The aforementioned data indicate that it is crucial for endothelial regeneration to mobilize more circulating EPCs to enhance early reendothelialization.

AMD3100, also known as plerixafor, an antagonist of C-X-C motif chemokine receptor (CXCR)4, has been proposed, instead of granulocyte colony-stimulating factor, to mobilize CD34+ hematopoietic stem or progenitor cells (HSCs) derived from bone marrow (13,14). The underlying mechanism of...
AMD3100 mobilization of progenitor cells involves interfering with the stromal cell-derived factor 1 [SDF-1, also known as C-X-C motif chemokine ligand (CXCL12)/CXCR4 signaling pathway, which is vital for the retention of EPCs in niches, and then forcing the release of circulating EPCs (15). The process of EPC homing, including mobilization, recruitment and adhesion, is regulated by key angiogenic chemokines (CXCL1, CXCL7, CXCL12 and C-C motif chemokine ligand 2) and their respective receptors (CXCR2, CXCR4 and C-C motif chemokine receptor 2). Previous studies have reported that the homing or recruitment of circulating EPCs to injury or ischemic sites by SDF-1 is an important process for executing their angiogenic and repair functions (16-18). These results indicate that AMD3100 and SDF-1 may be useful for endothelial regeneration. Therefore, the present study evaluated the effects of AMD3100 and SDF-1 on endothelial repair in a rat carotid artery injury model. Furthermore, the influence of AMD3100 and SDF-1 on the cellular function of EPCs and the expression levels of CXCR4 and CXCR7 in EPCs after treatment with AMD3100 and SDF-1 was assessed.

Materials and methods

Isolation, cultivation and identification of EPCs. A total of 20 ml fresh human umbilical cord blood was obtained from the Obstetrics Department of Shanghai Sixth People’s Hospital (Shanghai) and all participants (totally 20 patients; mean age: 24 years old) provided written informed consent. EPCs were isolated from the human umbilical cord blood by Ficoll gradient centrifugation (1,500 g) for 10 min at room temperature and cultured in endothelial basal medium (Lonza Group Ltd.) containing growth factors (hydrocortisone, 0.2 ml; human basic fibroblast growth factor-B, 2 ml; vascular endothelial growth factor, 0.5 ml; Recombinant human R3 insulin-like growth factor-1, 0.5 ml; human epidermal growth factor, 0.5 ml; ascorbic acid, 0.5 ml; and gentamicin sulfate-amphotericin, 0.5 ml). Isolation, cultivation and identification of EPCs were performed as described previously (6). Fluorescent staining was used to detect the uptake of Dil-ac-LDL (Molecular Probes; Thermo Fisher Scientific, Inc.) and binding of FITC-UEA-1 (Sigma-Aldrich; Merck KGaA). Briefly, the cells were incubated with Dil-ac-LDL (15 µg/ml) for 4 h, and then stained with FITC-UEA-1 (10 µg/ml) for 1 h and with DAPI for 5 min at room temperature. The cells were washed three times and analyzed under a fluorescence microscope (Olympus Corporation). EPCs at passages 2-4 were used in subsequent experiments.

Proliferation assay of EPCs. The proliferation assay of EPCs was performed to construct a cell proliferation curve after treatment with AMD3100 and SDF-1. Briefly, EPCs were seeded into 96-well plates at a density of 1x10⁴ cells/well (Corning Life Sciences) and cultured in 100 µl microvascular endothelial cell growth medium-2 (Lonza Group Ltd.) supplemented with 5% FBS (Gibco; Thermo Fisher Scientific, Inc.) for 24 h at 37°C. Subsequently, the EPCs were incubated with AMD3100 (MedChemExpress) at different concentrations (10, 1, 0.1, 0.01 and 0.001 µM) or SDF-1α (Cedarlane) at various concentrations (1,000, 100, 10, 1 and 0.1 nM) for 6, 12, 24 and 48 h at 37°C. Then, cells were incubated with Cell Counting Kit-8 solution (Dojindo Molecular Technologies, Inc.) for 2 h according to the manufacturers’ protocols. Saline was used instead of AMD3100 or SDF-1 in the control group. The absorbance was measured at 450 nm in each well using a Synergy Multi-Mode Microplate Reader (BioTek Instruments, Inc.). The 50% effective concentration (EC₅₀) of AMD3100 and SDF-1 was calculated for subsequent experiments based on the proliferative activity at different concentrations following incubation for 24 h. The EC₅₀ value was calculated using GraphPad software (GraphPad Prism 7.00; GraphPad Software, Inc.).

Adhesion assay. The adhesion ability of EPCs to fibronectin (FN; Sigma-Aldrich; Merck KGaA) and human umbilical vein endothelial cells (HUVECs; Yuchi (Shanghai) Biotechnology Co., Ltd.) was assessed by plating EPCs into 24-well plates. Briefly, to investigate the adhesion of EPCs to the extracellular matrix, 24-well plates were pretreated with FN (100 µg/ml) for 2 h at 37°C. Then, EPCs (1x10⁵ cells/well) were added into each well and cultured in microvascular endothelial cell growth medium-2 (Lonza Group, Ltd.) supplemented with AMD3100 (34 nM; group A), SDF-1 (212 nM; group S) or AMD3100 combined with SDF-1 (group AS) for 1 h at 37°C. Unattached cells were washed away three times with PBS. DAPI (10 µg/ml) was used to stain the adherent EPCs for 10 min at 37°C. Adherent EPCs were counted in five randomly selected fields under a fluorescence microscope (magnification, x400; Olympus Corporation).

To assess the adhesion of EPCs to HUVECs, HUVECs (1x10⁵ cells/well) were seeded into 24-well plates to form a monolayer overnight. Unattached cells were washed away using PBS. EPCs (1x10⁵ cells/well) were cultured in microvascular endothelial cell growth medium-2 (Lonza Group, Ltd.) containing Dil stain (4 mg/ml) for 30 min at 37°C, according to the manufacturer's protocol. Dil-labeled EPCs were digested and harvested after washing with PBS three times. Subsequently, Dil-labeled EPCs (1x10⁵ cells/well) were seeded into each well and cultured in medium supplemented with AMD3100 (34 nM), SDF-1 (212 nM) or AMD3100 combined with SDF-1 at 37°C for 2 h. Unattached cells were washed away three times with PBS. DAPI (10 µg/ml) was used to stain the HUVECs for 10 min at 37°C. Adherent EPCs were counted in five randomly selected fields under a fluorescence microscope (magnification, x400; Olympus Corporation).

Confocal immunofluorescence microscopy. CXCR4 and CXCR7 are ligand receptors for SDF-1 and have a role in regulating the biological activities of EPCs. Therefore, the expression levels of CXCR4 and CXCR7 were assessed in each group.

Confocal immunofluorescence microscopy was performed to determine the expression levels of CXCR4 and CXCR7 in EPCs. Briefly, EPCs (1x10⁵ cells/well) were grown on glass coverslips for 12 h, fixed with 4% paraformaldehyde at room temperature for 30 min and incubated with 0.5% Triton X-100 (Sigma-Aldrich; Merck KGaA) for 5 min and 1% BSA (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. Subsequently, EPCs were incubated with anti-human CXCR4 (1:1,000; Abcam; ab197203) and anti-human CXCR7 monoclonal antibodies (1:1,000; Abcam; ab72100) overnight...
at 4°C, and then incubated with Alexa Fluor 488- (1:500; Abcam; ab150077) and Alexa Fluor 647-conjugated secondary antibodies (1:500; Abcam; ab150079) for 2 h at room temperature. Cell nuclei were stained with DAPI for 10 min at room temperature. Confocal immunofluorescence microscopy images were captured using a Leica TCS SP8 confocal microscope (Leica Microsystems GmbH).

Western blot analysis. Western blotting was performed to investigate the effects of AMD3100 and SDF-1 on the expression levels of CXCR4 and CXCR7 in EPCs. Briefly, EPCs (1x10^6 cells/well) were incubated with AMD3100 (34 nM), SDF-1 (212 nM) or AMD3100 combined with SDF-1 for 2 h at room temperature prior to protein extraction. Saline was used instead of AMD3100 or SDF-1 in the control group. Total protein was extracted using a protein extraction kit (Beijing Solarbio Science & Technology Co., Ltd.) and quantified using a bicinchoninic acid protein assay kit (Beijing Solarbio Science & Technology Co., Ltd.). Extracts (50 ng per lane) were subjected to 10% SDS-PAGE (Nanjing KeyGen Biotech Co., Ltd.) and then transferred onto PVDF membranes (Roche Diagnostics) before blocking with 5% skimmed milk for 1 h at room temperature. The membranes were incubated with diluted primary antibodies overnight at 4°C. The following antibodies were used: Rabbit anti-CXCR7 antibody (1:250; Abcam; ab72100), rabbit anti-CXCR4 antibody (1:150; Abcam; ab197203) and rabbit anti-GPDH antibody (1:3,000; Cell Signaling Technology, Inc; 97166). Subsequently, the membranes were incubated with secondary antibody (1:1000; Beijing Boaosen Biotechnology Co., Ltd.; bs-0295D) for 2 h at room temperature. Protein bands were visualized using an Epson photo 1650 (Seiko Epson Corporation). Immunodetection was performed using the Supersignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc., and quantified using ImageJ software (v1.63, National Institutes of Health).

Flow cytometric analysis of the number of EPCs in the peripheral blood of each group. Flow cytometry was performed to detect the number of EPCs in the peripheral blood of rats in each group after treatment. Briefly, mononuclear cells (MNCs) were isolated from the peripheral blood at baseline, and 1, 6, 12, 24 and 48 h after treatment. The blood sample was collected from the jugular vein, and then diluted in PBS at a ratio of 1:1. The MNCs were isolated from cell suspension via gradient centrifugation (1,500 x g) for 10 min at room temperature, and the number of EPCs in the peripheral blood was determined by flow cytometry. EPCs were defined as CD34^+ kinase insert domain receptor (KDR)^+ cells. Briefly, MNCs were first incubated with 0.5% BSA, and then incubated with allophycocyanin (APC)-conjugated anti-mouse CD34 (Abcam; ab155377) and phycoerythrin-conjugated anti-mouse KDR antibodies (Abcam; ab250760) or isotype antibody (10 µl/tube) for 10 min in the dark at 4°C. MNCs were washed once with PBS, followed by flow cytometry using a NAVIOS flow cytometer (Beckman Coulter, Inc.) according to the manufacturer's protocol. The results were analyzed using FlowJo 7.6 software (FlowJo LLC).

Histological assessment. Reendothelialization and neointimal hyperplasia were assessed at 7 and 14 days after treatment.
as described in our previous study (6). All operations were performed under anesthesia with pentobarbital sodium via intraperitoneal injection (30 mg/kg). A pathologist who was blinded to the treatment regimen assessed all specimens. Analysis of the digitalized images was performed using ImageJ 1.63 software (National Institutes of Health).

Neointimal hyperplasia was evaluated using hematoxylin and eosin (H&E) staining. Briefly, following anesthesia with 30 mg/kg pentobarbital sodium, cardiac perfusion was conducted by perfusing PBS via the bilateral jugular vein until the effluent ran clear, followed by fixation with formaldehyde for 5 min. Subsequently, the carotid arteries were excised from the rats, and the specimens were fixed in 10% formalin for 24 h at room temperature. Subsequently, separated vessels (5 mm) were embedded in paraffin and sectioned at 4 µm. Sections were stained with H&E staining kit (Beijing Solarbio Science & Technology Co., Ltd., G1120) for 2 h at room temperature according to the manufacturer's protocol. Neointimal thickness was assessed in terms of the intima/media area ratio, and was measured in H&E-stained axial sections. A pathologist who was blinded to the treatment regimen investigated all specimens. Analysis of the digitalized images was performed using ImageJ 1.63 software (National Institutes of Health).

Reendothelialization was assessed using Evans blue staining. Briefly, 0.5 ml 0.5% Evans blue dye was injected intravenously via the tail vein 30 min before the rats were sacrificed. Subsequently, cardiac perfusion was used to perfuse PBS via the bilateral jugular vein until the effluent ran clear, followed by fixation with 4% formaldehyde for 5 min at room temperature. The common carotid artery was harvested at 4 mm from the bifurcation and opened longitudinally. The areas stained and unstained in blue were measured in the entire injured area, and the rate of reendothelialization (unstained area/total area) was used to determine the difference in reendothelialization among all groups. The analysis of digitalized images was performed using ImageJ 1.63 software (National Institutes of Health).

Statistical analysis. Data are presented as the mean ± standard deviation. One-way ANOVA with Tukey's post hoc test was used to determine statistically significant differences among the same treatment group at different time points in the proliferation assay, or among subgroups. SPSS 20.0 software (IBM Corp.) was used to perform statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of EPCs. EPCs appeared round or spindle-shaped at day 4, and then typical cell clusters appeared after 7-10 days of culture. After 21 days of culture, the cells formed colonies and appeared pebble-shaped. Flow cytometry revealed that the EPCs in the present study were positive for CD34 and KDR, while they were negative for CD45. Furthermore, these cells could take up acetylated LDL and bind to Ulex europaeus agglutinin I. These characteristics identified the cells as EPCs (Fig. 1A-C).

Proliferation of EPCs is attenuated by AMD3100, whereas it is enhanced by SDF-1. Proliferation curves were plotted to reveal the proliferative activity of EPCs after treatment with AMD3100 or SDF-1 at various concentrations and different time points. Furthermore, the EC_{50} of AMD3100 and SDF-1 was calculated for subsequent experiments based on the proliferation curves. The results revealed that AMD3100 reduced the proliferation of EPCs effectively at various concentrations compared with the control group (Fig. 2A). The EC_{50} of AMD3100 was 34 nM (Fig. 2B). By contrast, SDF-1 could promote proliferation of EPCs at various concentrations in a concentration-dependent manner (Fig. 2C). The EC_{50} of SDF-1 was 212 nM (Fig. 2D).

AMD3100 stimulates adhesion of EPCs to HUVECs rather than FN, while SDF-1 stimulates adhesion of EPCs to HUVECs and FN. Adhesion assays demonstrated that fewer EPCs adhered to FN after treatment with AMD3100 [60.3±20.1 (group A) vs. 80.7±16.7 (control group) cells/field, P=0.042; 60.3±20.1 (group A) vs. 105.4±17.1 (group S) cells/field, P=0.007], whereas following treatment with AMD3100, more EPCs adhered to HUVECs compared with the control group [45.2±16.8 (group A) vs. 22.3±4.5 (control group) cells/field, P=0.029]. Additionally, treatment with SDF-1 significantly enhanced the adhesion capacity of EPCs to both FN [105.4±17.1 (group S) vs. 80.7±16.7 (control group) cells/field, P=0.02] and HUVECs [52.7±12.6 (group S) vs. 22.3±4.5 (control group) cells/field, P=0.031]. Furthermore, the adhesion capacities of EPCs to HUVECs in groups S and A were not identified to be significantly different [52.7±12.6 (group S) vs. 45.2±16.8 (group A) cells/field, P=0.23]. The present study further revealed that AMD3100 impaired the SDF-1-mediated adhesion capacity of EPCs to FN [65.6±11.5 (group AS) vs. 105.4±17.1 (group S) cells/field, P=0.015; 65.6±11.5 (group AS) vs. 80.7±16.7 (control group) cells/field, P=0.047]. By contrast, even more EPCs adhered to HUVECs in group AS compared with in the control group [62.7±17.4 (group AS) vs. 22.3±4.5 (control group) cells/field, P=0.029]. The effect of AMD3100 combined with SDF-1 on the adhesion capacity of EPCs to HUVECs was greater than that of AMD3100 [62.7±17.4 (group AS) vs. 45.2±16.8 (group A) cells/field, P=0.031]. The detailed results are shown in Fig. 3A.

AMD3100 only stimulates the expression levels of CXCR7, while SDF-1 upregulates the expression levels of CXCR4 and CXCR7 in EPCs. Both CXCR4 and CXCR7 are involved in regulating the function of EPCs; therefore, the present study evaluated the expression levels of CXCR4 and CXCR7 in EPCs before and after treatment. Confocal immunofluorescence microscopy revealed that both CXCR4 and CXCR7 were expressed by EPCs (Fig. 3B), and AMD3100 treatment upregulated the expression levels of CXCR7 in EPCs. In addition, the expression levels of CXCR4 and CXCR7 were significantly increased after incubation with SDF-1 or AMD3100 combined with SDF-1 for 24 h compared with the control group. Furthermore, upregulation of CXCR7 was identified in group AS compared with in groups A and S. The expression levels of CXCR4 in group S were higher than those in group A. However, the expression levels of CXCR7 were similar in groups S and A (Fig. 3C).

AMD3100 mobilizes circulating EPCs and promotes the incorporation of EPCs into the injury site, while SDF-1 does not. Different treatment regimens were used to reveal the effects of AMD3100, SDF-1 or AMD3100 combined with SDF-1 in vivo.
The circulating EPCs were increased in group A compared with in the control group at 1, 6, 12 and 24 h after treatment. The number of circulating EPCs was significantly increased after 1 h and reached the peak at 6 h after treatment with AMD3100. However, treatment with SDF-1 did not mobilize circulating EPCs (Fig. 4A). An immunofluorescence assay revealed that an increased number of EPCs were recruited to the site of endothelial injury in group A compared with in the control group [16.7±1.2 (group A) vs. 7.3±0.9 (control group) cells/field; P=0.041]. However, treatment with SDF-1 did not recruit more EPCs to the site of endothelial injury [8.7±1.1 (group S) vs. 7.3±0.9 (control group) cells/field, P=0.52] (Fig. 4B). Notably, pretreatment with AMD3100, followed by local administration of SDF-1, recruited significantly more EPCs to the site of vascular injury compared with AMD3100 treatment alone [22.8±1.3 (group AS) vs. 16.7±1.2 (group A) cells/field, P=0.049].

**AMD3100 combined with SDF-1 promotes reendothelialization and inhibits neointimal hyperplasia more effectively than**
**ADM3100 alone.** Increased reendothelialization was identified in groups A and AS at 7 days after treatment compared with in the control group [80.4±6.1 (group A) vs. 46.3±4.2% (control group), P<0.001; 92.7±7.6 (group AS) vs. 46.3±4.2% (control group), P<0.001], and there was a significant difference in reendothelialization between groups AS and A at day 7. By contrast, no significant difference was observed in reendothelialization after 14 days [97.2±5.9 (group A) vs. 92.3±4.7% (control group), P=0.412; 98.5±7.2 (group AS) vs. 92.3±4.7% (control group), P=0.43]. However, early reendothelialization inhibited neointimal hyperplasia after 7 and 14 days of treatment in group A [7 days, 0.35±0.09 (group A) vs. 0.47±0.05 (control group), P=0.023; 14 days, 0.47±0.08 (group A) vs. 0.64±0.07 (control group), P=0.071] and group AS [7 days, 0.24±0.06 (group AS) vs. 0.47±0.05 (control group), P<0.001; 14 days, 0.27±0.05 (group AS) vs. 0.64±0.07 (control group), P<0.001]. The difference in the levels of reendothelialization of group S compared with the control group was not statistically significant at day 7 [51.2±5.4 (group S) vs. 46.3±4.2% (control group), P=0.22] and day 14 [91.1±7.6 (group S) vs. 92.3±4.7% (control group), P=0.517], and neointimal hyperplasia was not attenuated in this group [7 days, 0.45±0.07 (group S) vs. 0.47±0.05 (control group), P=0.049; 14 days, 0.63±0.04 (group S) vs. 0.64±0.07 (control group), P=0.43] (Fig. 5).

**Discussion**

ADM3100 and SDF-1 are extensively used in regenerative medicine, including hematological disease (14,19), angiogenesis (17,20), intimal repairing (16), wound healing (21,22) and brain repair after ischemic stroke (18,23). Furthermore, previous studies have reported that ADM3100 and SDF-1 may be involved in mobilization and recruitment of EPCs (16,17,24,25). These studies indicated that ADM3100 or SDF-1 may be used for endothelial regeneration. The present study assessed the effects of ADM3100 and SDF-1 on endothelial regeneration. Additionally, the effects of ADM3100 and SDF-1 on EPCs were evaluated.
Figure 3. Adhesion capacity, and the expression levels of CXCR4 and CXCR7 are affected by AMD3100 and SDF-1 treatment. (A) Adhesion capacity to FN was impaired by AMD3100 treatment, whereas AMD3100 treatment stimulated the adhesion capacity to HUVECs. A similar tendency was observed after treatment with AMD3100 combined with SDF-1. Scale bar, 25 µm. (B) Confocal immunofluorescence microscopy confirmed that both CXCR4 and CXCR7 were expressed in EPCs. Scale bar, 100 µm. (C) Western blotting revealed that treatment with AMD3100 upregulated the expression levels of CXCR7 but not cXcr4. However, SDF-1 or AMD3100 combined with SDF-1 upregulated the expression levels of cXcr4 and cXcr7. Furthermore, the effects of AMD3100 combined with SDF-1 on the expression levels of cXcr7 were the greatest among the four groups. n=5. *P<0.05; **P<0.001. c, control; a, AMD3100 alone; S, SDF-1 alone; AS, AMD3100 combined with SDF-1; CXCR, C-X-C motif chemokine receptor; EPCs, endothelial progenitor cells; FN, fibronectin; HUVECs, human umbilical vein endothelial cells; SDF-1, stromal cell-derived factor 1.
Figure 4. Number of EPCs in circulation and at the injury site. (A) AMD3100 treatment could effectively mobilize circulating EPCs in a time-dependent manner. However, SDF-1 treatment did not increase the number of circulating EPCs at any time point. Additionally, AMD3100 combined with SDF-1 (group AS) increased the number of circulating EPCs. However, there was no significant difference in circulating EPCs observed between group A and group AS. (B) AMD3100 treatment recruited more EPCs to the injury site. SDF‑1 treatment did not increase the number of EPCs at the injury site. However, pretreatment with AMD3100, followed by local administration of SDF-1 recruited more EPCs to the injury site. The effects of AMD3100 combined with SDF-1 (group AS) on recruitment of EPCs were stronger than those of AMD3100 alone. Scale bar, 50 µm. n=5. *P<0.05; **P<0.001. c, control; a, AMD3100 alone; S, SDF-1 alone; aS, AMD3100 combined with SDF-1; EPCs, endothelial progenitor cells; SDF-1, stromal cell-derived factor 1.
Intravenous or subcutaneous administration of AMD3100 has been reported to effectively induce mobilization of HSCs and EPCs (14,24). Furthermore, a single dose of AMD3100 may mobilize EPCs into peripheral blood (25). Similar to previous studies (13,25), the present study noted that a single dose of AMD3100 was sufficient to mobilize EPCs into circulation. Furthermore, the results of the present study indicated that increased numbers of EPCs were involved in reendothelialization following AMD3100 treatment. The underlying mechanism by which AMD3100 treatment recruits more EPCs to participate in endothelial repair is unclear. The in vivo and in vitro results reported in the present study revealed that endothelial cells (ECs) at the injury site may be crucial for intimal repair after AMD3100 treatment, as it was observed that more EPCs...
adhered to HUVECs compared with FN after AMD3100 treatment. Moreover, more EPCs were recruited into endothelial site after intravenous injection of AMD3100. However, the detailed mechanism should be considered in a further study. In addition, the mismatch between the time circulating EPCs reached their highest level after AMD3100 treatment and the median terminal half-life of AMD3100 may contribute to EPCs ability to mediate intimal repair. Stewart et al. (26) reported that the median terminal half-life of AMD3100 in circulation was 4.6 h. However, in the present study, the number of circulating EPCs reached its peak at 6 h and remained at high levels for 24 h after AMD3100 treatment. The aforementioned inhibitory effect of AMD3100 on EPCs was attenuated before circulating EPCs decreased to baseline levels, as a result, more circulating EPCs were recruited to the arterial injury site.

A previous study demonstrated that a decrease in the level of SDF-1 at the injury site was associated with delayed reendothelialization as fewer EPCs were recruited to the injury site (27). By contrast, local accumulation of fluorescence-labeled EPCs was observed in ischemic muscle after local injection of SDF-1 in an athymic rat hind limb ischemia model (28). Therefore, it may be concluded that local accumulation of SDF-1 is essential for recruitment of endothelial progenitors and in accelerating repair of injury. Thus, the present study also assessed the effects of SDF-1 on intimal repair via local injection. However, the results of the present study indicated that local injection of SDF-1 was ineffective in promoting reendothelialization. Hence, it was revealed that insufficient EPCs in circulation were the main contributing factor for delayed recovery of injury after SDF-1 treatment. Additionally, it was revealed that AMD3100 combined with SDF-1 had superior therapeutic effects compared with AMD3100 alone.

Previous studies have demonstrated that SDF-1 may be involved in regulating the mobilization, proliferation and adhesion capacity of EPCs through binding to CXCR4 and CXCR7 (15,29,30). In accordance with these studies, the present study revealed that SDF-1 treatment stimulated the proliferation and adhesion capacity of EPCs to FN and HUVECs. Furthermore, the present study demonstrated that in EPCs the expression levels of CXCR4 and CXCR7 were upregulated after SDF-1 treatment. The results indicated that SDF-1 exerted its positive regulatory effects on cellular function not only via binding to and activating its receptor, but also by upregulating the expression levels of CXCR4 and CXCR7. However, the molecular mechanism by which SDF-1 can stimulate EPCs to upregulate the expression levels of CXCR4 and CXCR7 needs to be investigated further. The effects of AMD3100 on cellular function of EPCs were also evaluated in the present study. A previous study demonstrated that AMD3100 impaired the proliferation, migration and adhesion capacity of EPCs via blocking the SDF-1/CXCR4 axis (31). The present results demonstrated that AMD3100 could positively modulate adhesion of EPCs to HUVECs via upregulation of the expression levels of CXCR7. It remains elusive how AMD3100 can stimulate upregulation of the expression levels of CXCR7 in EPCs. However, previous studies have demonstrated that CXCR7 may be crucial in regulating cell adhesion capacity, particularly the adhesion capacity to HUVECs (29,30,32). Furthermore, Kalatskaya et al. (33) revealed that AMD3100 may bind to CXCR7 and positively modulate the effect of CXCL12 by inducing β-arrestin recruitment to CXCR7. The aforementioned data indicated that AMD3100 stimulated adhesion of EPCs to HUVECs via upregulation of the expression levels of CXCR7 rather than CXCR4.

The present study demonstrated that the adhesive activity of EPCs was pivotal for EPCs recruitment and EPC-mediated endothelial repair. Furthermore, the in vitro results showed that CXCR4 and CXCR7 molecules were associated with the adhesive activity of EPCs after AMD3100 or SDF-1 treatment. Previous studies identified that other molecules, including P-selectin (34) and E-selectin (35), and very late antigen-4 and its ligand vascular cell adhesion molecule 1 (36) also contribute to the cellular adhesion capacity of progenitor cells. All these results indicated that upregulating these adhesive molecules may contribute to the adhesive activity of EPCs and EPC-mediated intimal repair. However, the present study was still inadequate and these underlying mechanisms should be considered in the future.

There were several limitations of the present study. Firstly, knockdown of CXCR4 and CXCR7 was not performed to identify the detailed molecular mechanism by which SDF-1/CXCR4 and SDF-1/CXCR7 are involved in regulating cellular function of EPCs after treatment with AMD3100 or SDF-1. This should be investigated in future studies. Secondly, the molecular mechanisms by which EPCs exhibited upregulated expression levels of CXCR4 and CXCR7 after treatment with AMD3100 or SDF-1 remain unclear. In addition, only 46.7% cells positive for KDR. The main reason underlying this phenomenon may due to EPCs showing more characteristics of progenitor cells but fewer characteristics of mature endothelial cells.

In conclusion, AMD3100 positively regulated the cell adhesion capacity of EPCs to HUVECs via elevation of the expression levels of CXCR7 rather than CXCR4, whereas SDF-1 stimulated cell proliferation and the adhesion capacity of EPCs to FN and HUVECs by increasing the expression levels of CXCR4 and CXCR7. Treatment with AMD3100 accelerated reendothelialization and inhibited neointimal hyperplasia after endothelial injury, whereas SDF-1 treatment alone failed to promote endothelial regeneration. AMD3100 combined with SDF-1 outperformed AMD3100 alone, promoted early reendothelialization and inhibited neointimal hyperplasia, indicating that early reendothelialization attenuates neointimal hypoplasia following endothelial injury.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Authors' contributions

CI, XM and RL performed the experiments. JZ designed the experiments. HH conducted the statistical analysis of the data. CJ, RL and XM drafted the manuscript. JG and JZ conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures involving human beings and animals were reviewed and approved by the Ethics Committee of Shanghai Sixth People's Hospital (Shanghai, China). All clinical investigations were conducted according to the principles of the Declaration of Helsinki. All patients provided written informed consent prior to the start of the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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