Crucial Role of SDF-1/CXCR4 Interaction in the Recruitment of Transplanted Dermal Multipotent Cells to Sublethally Irradiated Bone Marrow

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Our previous study indicated that dermal multipotent cells (DMCs) could engraft into bone marrow (BM) of rats with sublethal irradiation and promote hematopoietic recovery after being transplanted systemically, but the mechanisms determining the recruitment of DMCs to the irradiation injured BM remain unclear. In the present study, we investigated the role of stromal cell-derived factor-1 (SDF-1)/CXCR4 interaction in this process. Male DMCs were isolated and transplanted into female rats systemically, and by employing quantitative real-time TaqMan polymerase chain reaction for the sex-determining region of Y chromosome, it was found that the amount of DMCs in BM of rats with sublethal irradiation was about 3 times more than that of normal rats (\(P < 0.01\)). Incubation of DMCs with AMD3100 before transplantation, which specifically blocks binding of SDF-1 to its endogenous receptor CXCR4, diminished recruitment of DMCs to the injured BM by 57.2 ± 5.5% (\(P < 0.05\)). In addition, it was confirmed that the expression of SDF-1 in injured BM was up-regulated when compared with that in normal BM, and in vitro analysis revealed that BM extracts from irradiated rats had a strong chemotactic effect on DMCs, which decreased significantly when DMCs were pre-incubated with AMD3100 (\(P < 0.05\)). These data suggest that transplanted DMCs were recruited more frequently to irradiation-injured BM than normal BM and the interactions of SDF-1/CXCR4 played an important role in this process.

INTRODUCTION

Radiation injury occurs after nuclear accidents and nuclear attacks as well as in clinical radiotherapy, which results in bone marrow dysfunction. In recent years, bone marrow mesenchymal stem cells and hematopoietic stem cells transplantation have shown promising effect in rescuing the function of irradiation injured BM. However, the auto-transplantation of bone marrow stem cells is limited when bone marrow is functionally impaired in radiation injury. Under such conditions, stem cells of alternative sources are needed. In our previous study,1 we reported that DMCs could be isolated from newborn rats’ dermis and the isolated DMCs demonstrated multipotent differentiation capacity. After systemic transplantation into sublethally irradiated rats, DMCs could increase white blood cells in peripheral blood. Also, fluorescent in situ hybridization analysis showed that DMCs could engraft into BM of irradiated rats.1 Therein, we were not clear about the mechanisms determining the recruitment of DMCs to the irradiation injured BM. In the present study, we firstly investigated whether the transplanted male DMCs could distribute and engraft more frequently to the injured BM in female recipient rats with sublethal irradiation than that in normal rats by using real-time PCR for the sex-determining region of Y chromosome (SRY), then we clarified the role of SDF-1/CXCR4 interaction in the recruitment of DMCs to irradiation injured BM, since the interaction has been proved to be very important in bone marrow derived stem cells homing to the BM and to involved in stress-induced recruitment of stem cells to the liver and neointima. Using the chemokine receptor CXCR4 antagonist AMD3100, we assessed the effect of blocking the SDF-1/CXCR4 interaction on the recruitment of delivered DMCs. Then we compared the expression level of SDF-1 in irradiation injured BM and normal BM by RT-PCR, immunohistochemistry and Western Blot. In addition, in vitro chemotactic analysis was used to observe the chemotactic effect of...
BM extracts on DMCs, with or without AMD 3100 pre-incubation.

MATERIALS AND METHODS

The experimental protocol was reviewed and approved by the Animal Ethical Committee of the Third Military Medical University, P. R. China.

Materials

Wistar rats were purchased from the Center of Laboratory Animals of the Third Military Medical University. All materials used in DMCs isolation, culture and differentiation tests were of the same origin as previously reported. Reagents for PCR, RT-PCR, and Real-time PCR were obtained from TaKaRa BIO INC. (Kyoto, Japan), and all the primers and probes used were synthesized by Sangon Biological Engineering & Technology services Co., Ltd (Shanghai, China). Tripure™ used to isolate RNA was purchased from Promega Corp. (Madison, Wisconsin, USA). Anti-CXCR4 rabbit polyclonal antibody and anti-SDF-1 rabbit polyclonal antibody were from Santa Cruz Biotech, Inc. (Santa Cruz, California, USA). AMD3100 and phycoerythrin-conjugated anti-rabbit secondary antibody was purchased from Sigma. 52°C for 30 s and 72°C for 45 s. The product was 328 bp. Sterile water and DNA isolated from female rats liver served as control.

The expression of CXCR4 in DMCs was examined by flowcytometry. Total RNA was isolated form cultured DMCs using Tripure™ according to the manufactures instructions and was spectrophotometrically quantified. The cDNA was prepared from 1.0 µg RNA in the presence of 2.5 µM oligo (dT) primer and 200 U moloney murine leukemia virus reverse transcriptase in a total volume of 10 µl. The reaction mixture was incubated for 1 hour at 42°C and stopped by heating for 5 minutes at 99°C. Aliquots (1 µl) of cDNA were subsequently amplified using specific primers for CXCR4 (forward primer was 5'-AGT GGG CAA TGG GTT GGT AAT-3' and reverse primer was 5'-TCA GGG ATA GTC AGG AGG G-3'). The PCR cycles consist of denaturation at 94°C for 45 s, annealing at 56°C for 45 s, and extension at 72°C for 45 s. The PCR product was 354 bp. A 10 µl aliquot of each PCR product was size-separated by electrophoresis on a 2% ethidium-bromide-containing agarose gel and photographed. Sterile water and 293 cells served as control.

Also, the expression of CXCR4 in DMCs was examined by flowcytometry. Immunostaining was performed on the DMCs plated on ploylysine-coated coverslips. The coverslips were blocked with normal serum for 30 minutes, and then incubated with anti-CXCR4 rabbit polyclonal antibody at 4°C overnight. Subsequently, coverslips were incubated in secondary antibodies for 1 hour, and the reaction products were visualized under fluorescent reverse microscope. Control coverslips were incubated as above but without the primary antibody.

Animal group and transplantation procedure

Sixty female Wistar rats, six-week-old and weighing about 150 g, were divided randomly into three groups: group N (non-irradiation plus DMCs transplantation, n = 20), group R (irradiation plus DMCs transplantation, n = 20) and group AMD3100 (irradiation plus transplantation of DMCs incubated with AMD3100, n = 20). Rats in group R and group AMD3100 were irradiated over the whole body with a sublethal dose of 5 Gy of gamma rays from a 60Co source, at which dose gamma rays mainly resulted in BM injury. The absorption rate was 31.02–31.98 cGy/min. DMCs were infused at a dose of 2 × 10⁶ cells in 0.5 ml physiological saline by tail vein injection. In AMD3100 group, DMCs were incubated for 30 minutes at 37°C with 5 µg/mL AMD3100, then washed in phosphate buffered saline before transplantation. All animals were fed with commercial laboratory food and purified tap water and housed under standard conditions. Three days after transplantation, rats were sacrificed and four rats from each group were used in one of the following experiments.

**Quantitative real-time TaqMan PCR**

Quantitative real-time TaqMan PCR for rat’s SRY was employed to determine the amount of transplanted male DMCs in the bone marrow of female recipient. DNA of DMCs or tissues was prepared and dissolved in water. DNA concentration was determined by spectrophotometry, and the total amount of recovered DNA was calculated as [concentration] × [resuspension volume].

Real-time PCR was carried out on DNA Engine Opticon (MJ Research, Inc) by using the above-mentioned PCR primers to SRY and the TaqMan probe 5-FAM- TGC CAA CAC TCC CCT TGC TGC TGT AAT TAMRA-3. The thermal cycle was configured as follows: incubation at 95°C for 5 min, 40 cycles of de-naturation at 95°C and annealing and extension at 60°C for 60 s. In real-time TaqMan PCR, amplification resulted in a fluorescent signal proportional to the amount of PCR product. The template quantity was inversely proportional to the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the reaction. In present study, standard curves were generated by serially diluting male DMCs DNA into female rat genomic DNA as follows: DNA prepared from 0, 5, 50, 100, 500, 1000, 5000, 50 000 or 100 000 DMCs was diluted into DNA prepared from 100 mg female BM. The number of DMCs in bone marrow was determined by the cycle number in the standard curve.

**Examination of the expression of SDF-1 in BM**

The expression of SDF-1 in BM was determined by RT-PCR, immunohistochemistry and Western Blot.

For RT-PCR, the whole BM plugs were obtained by flushing the BM cavity of the femurs and tibiae with a 21-gauge syringe filled with PBS, and then the total RNA was isolated and RT-PCR was performed as above-mentioned method, except the primers used for SDF-1 were 5’-CCA ATC AGA AAT GGG AAC AAG A-3’ (forward) and 5’-GAG GCT TAC AGC ACG AAA CAG-3’ (reverse). And the product was 377 bp. Also, primers (forward primer was 5’-TCA TCA GCG AAA GTG GAA A-3’ and reverse primer was 5’-TGT CTG TCT CAC AAG GGA AGT-3’) to detect hypoxanthine guanine phosphoribosyl transferase (HPRT) were added to each reaction tube as internal control. The RT-PCR product of HPRT was 270 bp.

For immunohistochemistry, 5-micrometer sections of formaldehyde-fixed and paraffin-embedded BM tissue were made. Endogenous peroxidase and biotin were blocked and anti-SDF-1 rabbit polyclonal antibody diluted 1:200 in 1% blocking serum was added and incubated at 4°C overnight. The next day, biotinylated goat anti-rabbit IgG was applied for 30 minutes, and the slides were developed with avidin:biotinylated enzyme complex and were visualized by diaminobenzidine. After slightly counterstained with hematoxylin, the slides were observed in microscope. Between all the steps, the slides were fully rinsed by phosphate buffered saline.

For Western Blot, 100 mg BM plugs were obtained as the above-mentioned method and 1 ml T-PERT™ reagent was added. After homogenization, the sample was centrifuged 10,000 rpm for 5 minutes. The supernatant was collected and were run on 15% SDS-PAGE gels. Proteins were then transferred onto PVDF membrane using the Trans-Blot Semi-Dry Transfer Cell (Bio-Rad Laboratories Inc., California, USA), blocked in 5% skim milk, and probed with anti-SDF-1 rabbit polyclonal antibody (diluted 1:500 in blocking buffer) followed by biotinylated goat anti-rabbit IgG. Then the PVDF membrane were developed with avidin:biotinylated enzyme complex and were visualized by diaminobenzidine.

**In Vitro chemotaxis assay**

**Preparation of BM extracts.** The whole BM were obtained as above-mentioned method and then placed on ice, and the wet weight in grams was rapidly measured. Part of the harvested BM (100 mg) was then homogenized by adding IMDM (150 mg tissue/ml IMDM) and was incubated on ice for 10 minutes. The homogenate was centrifuged at 10,000 g for 20 minutes at 4°C and the supernant was extracted.

**Chemotaxis assay.** Chemotactic activity of BM extracts and recombinant human SDF-1 (rhSDF-1) toward DMCs was evaluated using 12-well microchemotaxis Transwell chamber according to the manufacture’s instructions. To observe the chemotactic effect of rhSDF-1 toward DMCs, 25 µl rhSDF-1 at various concentrations (50,100,150 ng/ml) was placed in the lower wells, and 50 µl re-suspended normal DMCs or AM3100 pre-incubated DMCs (containing 1 × 10^6 cells) were placed in the upper wells. To observe the chemotactic effect of BM extract, 25 µl BM extracts from each group was placed in the lower wells, and cells the same as above-mentioned were placed in the upper wells. The contents of the upper and lower well were separated by a polycarbonate filter (8-µm pore size). After 6 hours incubation at 37°C in 5% CO₂, the upper surface of the membrane was scraped free of cells and debris. The membrane was then fixed and stained using hematoxylin staining. Cells that had migrated though pores and adhered to the lower surface of the membrane were analyzed under high-power (×400) light microscopy and counted in five random high-power fields (HPF). Experiments were performed in triplicate, and data were expressed as means of the numbers of cells per high-power field (cells/HPF) ± standard deviation.

**Statistical analysis**

All data were expressed as means ± standard deviation. Statistical significance was evaluated with an unpaired Student’s t test for comparison between 2 groups or by ANOVA for multiple comparisons. A value of P < 0.05 was considered significant.
RESULTS

Biological characterization of cultured DMCs

When cultured in vitro, less than 0.01% of the dissociated dermal cells adhered to the plastic. After being seeded in 96-well at the density of single cell per well, some of the adherent dermal cells formed colonies. These colonies were selected as DMCs candidates. After expansion, most DMCs candidates demonstrated fibroblast-like cells. DMCs candidates that showed multi-lineage differentiation capacity were used in the following experiments (data and picture not shown, see Ref.1.).

Sex-genotype of DMCs was determined by PCR for SRY. As shown in Fig. 1A, DNA from female rats’ liver and sterile water produced no detectable PCR products, while DNA from isolated male DMCs produced specific PCR products.

We examined the expression of CXCR4, the only known receptor of SDF-1, in isolated DMCs by using RT-PCR and fluorescent immunocytochemistry. Most of the cultured DMCs reacted positively to CXCR4 antibody (Fig. 1B), and expression of CXCR4 at mRNA level also could be detected by RT-PCR (Fig. 1C).

Recruitment of Transplanted DMCs to Sublethally Irradiated Bone Marrow

The amount of transplanted DMCs in BM of rats in group R (rats accepted irradiation and DMCs transplantation), group N (normal rats accepted DMCs transplantation) and group AMD3100 (rats accepted irradiation and transplantation of DMCs incubated by AMD3100) was obtained by real-time TaqMan PCR for rat SRY and was shown in Fig. 2. Three days after transplantation, the amount of DMCs in

![Fig. 1](image1.png)

Fig. 1. Biological characterization of cultured DMCs. (A) Sex-genotype of DMCs was determined by PCR for SRY. DNA from female rats’ liver (lane 1) and sterile water (lane 2) produced no detectable PCR signals, while DNA from isolated male DMCs produced specific PCR signal (328bp, lane 3). (B) Most of the cultured DMCs reacted positively to CXCR4 in fluorescent immunocytochemistry examination (× 200). (C) Expression of CXCR4 in DMCs could be detected by RT-PCR (354bp, lane 3), no PCR signals were found in sterile water control (lane 1) and 293 cells control (lane 2).

![Fig. 2](image2.png)

Fig. 2. Recruitment of transplanted DMCs to bone marrow. The amount of DMCs in BM of group R, group AMD3100 and group N were (1.54 ± 0.11) × 10⁴, (0.66 ± 0.12) × 10⁴ and (0.51 ± 0.08) × 10⁴ respectively. ▲: P<0.05, compared with group R.
BM of group R rats was \((1.54 \pm 0.11) \times 10^4\), significantly greater than that of group N rats \((0.51 \pm 0.08) \times 10^4\) \((P < 0.05)\). In AMD3100 group, the number is \((0.66 \pm 0.12) \times 10^4\). That is to say, incubation of DMCs with AMD3100 before transplantation reduced the recruitment of DMCs to irradiation injured BM by \(57.2 \pm 5.5\%\) \((P < 0.05)\), suggesting that SDF-1/CXCR4 interaction played a crucial role in DMCs recruitment to injured BM.

The expression of SDF-1 in irradiation injured BM was upregulated

By employing RT-PCR, immunohistochemistry and Western Blot, we investigated the expression of SDF-1 in BM of rats in each group (Fig. 3). By employing immunohistochemistry, we found that the number of SDF-1 positive cells in rats of group R and group AMD3100 was greater than that of group N (Fig. 3A–C). And the signals of RT-
PCR products on agarose gel in group R and group AMD3100 were stronger than that in Group N (Fig. 3D). This was further supported by the results of Western Blot (Fig. 3E).

Of note, the expression of SDF-1 in group AMD3100 rats BM was induced mainly by irradiation but not by the interaction of bone marrow and recruited DMCs.

In Vitro chemotaxis assay

The structure of SDF-1 is conservative and the similarity between rats' and human SDF-1 is above 99%, so rhSDF-1 can be used to observe the chemotactic effect on rats’ DMCs. The results showed that the chemotactic effect of rhSDF-1 toward DMCs was dose-dependent (Fig. 4A). The optimal effect was observed at 150 ng/ml, but when DMCs were pre-incubated with AMD3100, the chemotactic effect of rhSDF-1 was almost abolished ($P < 0.01$).

Fig. 4B showed that the number of DMCs migrated to the lower surface of the filter when BM extracts from group R and group AMD3100 were placed in the lower chamber was significantly greater than that of group N rats ($P < 0.01$). However, when DMCs were pre-incubated with AMD3100, the chemotactic effect of BM extracts from group R and group AMD3100 decreased significantly ($P < 0.05$), but did not abolish.

DISCUSSION

Adult stem cells have been isolated in various tissues and showed promising prospects in regenerative medicine. In recent years, bone marrow mesenchymal stem cells and hematopoietic stem cells transplantation have shown excellent effect on rescuing injured BM function. In the case of irradiation-induced BM injury, however, auto-transplantation of bone marrow stem cells is limited since BM is injured itself. Under such conditions, stem cells of alternative sources are needed. Recent studies showed that multipotent stem cells could be isolated from skin.

Given its easy accessibility and relative insensitivity to irradiation, skin-derived stem cells may serve as good alternative in the therapy of irradiation injured BM. In our previous study, we reported that DMCs could be isolated from newborn rats’ dermis and could promote hematopoietic recovery after systemic transplantation. But the mechanisms governing the recruitment of transplanted DMCs to the injured BM, which is a crucial first step of the repair process by transplanted DMCs, are poorly understood.

The chemokine SDF-1, also termed CXCL12, is a powerful chemoattractant of HSCs of both human and murine origin. It is widely expressed in many tissues during development and adulthood, including BM, liver, central nervous system, and skin, et al. And its interaction with CXCR4, the only known receptor of SDF-1, is essential during the BM-derived stem cells homing to BM.

SDF-1/CXCR4 interaction is also reported to involving in recruiting transplanted BM-derived mesenchymal stem cells, CD34+ hematopoietic progenitors, neural stem cells and endothelial progenitor cells to injured sites and participate repair of the injured tissue.

So in the present study, we tested the role of SDF-1/CXCR4 interaction is also reported to involving in recruiting transplanted BM to irradiation-injured BM. We firstly found increased SDF-1 expression in BM following irradiation by using RT-PCR, immunohistochemistry and Western Blot. Then by sex-mismatched transplantation and Real-Time PCR, we found that the amount of DMCs in BM of rats with sublethal irradiation was 3 times more than that of normal rats ($P < 0.01$), but the recruitment were reduced by 57.2 ± 5.5% when DMCs were
incubated with AMD3100 before transplantation, suggesting that SDF-1/CXCR4 interaction play a crucial role in recruiting transplanted DMCs to irradiation-injured BM. This is further supported by in vitro chemotactic analysis. BM extracts from irradiated rats had strong chemotactic effect on normal DMCs, but decreased significantly when DMCs was pre-incubated with AMD3100 (P < 0.05).

Of note, recruitment of stem cells to the injured sites is a 2-step process that begins with binding of circulating stem cells to adhesive complexes in the vasculature around an injury zone, followed by local chemotaxis to the site of engraftment. Increased expression of intercellular adhesion molecule-1, and vascular cell adhesion molecule-1, matrix metalloproteinase-9, vascular endothelial growth factor, monocyte chemoattractant protein-1 and stem cell factor in the injured sites might in concert with SDF-1 to recruit transplanted stem cells to the injured sites.3,10,11 That is why when the interaction of SDF-1/CXCR4 was blocked by AMD3100 incubation, there were still DMCs recruited to irradiation-injured BM (Fig. 4B).

In summary, the present study showed that transplanted DMCs were recruited more frequently to irradiation-injured BM than to normal BM and the interactions of SDF-1/CXCR4 played an important role in this process. The current results are important to our further study. In order to increase the recruitment of transplanted DMCs to injured BM and thus to further enhance the repair effect, CXCR4 were over-expressed in DMCs by transfected with CXCR4 adenovirus expression vector, and it was found that the modified cells were recruited 3.1 times more than normal DMCs to the irradiation-injured BM (our unpublished data).

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