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DFO and DMOG up-regulate the expression of CXCR4 in bone marrow mesenchymal stromal cells

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The C-X-C chemokine receptor 4 (CXCR4) is a receptor of the chemokine stromal cell-derived factor 1. CXCR4/stromal cell-derived factor 1 is essential to the migration of cells. Up-regulated expression of chemokines or their receptors is crucial to enhancing the migration capability of bone marrow mesenchymal stromal cells (BM-MSCs) so that their application in treatment can be optimized. The objective of this study was to determine whether desferrioxamine (1,8-diazafluoren-9-one; DFO) and dimethyloxaloylglycine (DMOG) upregulate the expression of CXCR4 in BM-MSCs. Western blot analysis was used to study the expression of CXCR4 in three groups: DFO group, DMOG group, and control group. Immunofluorescence was also used to determine whether CXCR4 exists in the membrane of BM-MSCs. RESULTS: Compared with that in the control group, the expression of CXCR4 was upregulated in the DFO group. Meanwhile, 500 and 1000 μM DMOG exhibited similar effects on CXCR4. Western blot analysis revealed that the two reagents are correlated with the upregulation of CXCR4 in a dose-dependent manner, whereas immunofluorescence demonstrated that CXCR4 exists in the membrane. In conclusion, DFO and DMOG upregulate the expression of CXCR4, their effects are dose dependent, and CXCR4 is distributed in the membrane of BM-MSCs.

1. Introduction

Bone marrow mesenchymal stromal cells (BM-MSCs), which are also referred to as stromal progenitor cells (Wu et al. 2007), are stem cells isolated from the bone marrow. They differ from hematopoietic stem cells in that their function is to support other cells, not to generate blood cells. BM-MSCs have been used to treat neurodegenerative disorders, because they can be harvested easily for autologous transplantation and do not involve ethical issues (Zhao et al. 2012b). Research has shown that BM-MSCs not only can effectively work in the animal brain but also may improve clinical symptoms (Bjorklund and Lindvall 2000). BM-MSCs are multipotential as they can be differentiated to other cell types, such as neural cells, osteogenic cells, and adipocytes, among others (Bae et al. 2011; Wang et al. 2012a,c). These types of cells have been widely used in the treatment of various diseases.

C-X-C chemokine receptor 4 (CXCR4), which is essential to the migration of cancer cells, is expressed at low levels on the surface of normal BM-MSCs (Shichinohe et al. 2007). The chemokine stromal cell-derived factor 1 (SDF-1) has long been considered to be the specific ligand of CXCR4; however, research has shown that CXCR7, which plays an important role not only in the proliferation of cells but also in the regulation of CXCR4, is also a receptor of SDF-1 (Haege et al. 2012; Harrison et al. 2012). SDF-1 is also referred to as C-X-C chemokine 12 (Singh et al. 2012). The CXCR4/SDF-1 axis plays an important role in the migration of cancer cells and in bone marrow stromal cells (Shi et al. 2009). Compared with BM-MSCs *in vivo*, however, CXCR4 has been reported to be expressed at low levels after the cells were isolated from rats. Thus, identifying ways of up-regulating the expression of CXCR4 is necessary to enhance the therapeutic effect of stem cells.

The fact that CXCR4 plays a pivotal role in the migration of cells, especially in tumor or cancer cells, prompted us to investigate the upregulation of CXCR4 in BM-MSCs (Huang et al. 2012; Shin et al. 2012). Research has shown that the downstream Erk pathway would be activated when SDF-1 is conjugated with CXCR4, which is a seven-transmembrane protein (Zhao et al. 2012a). Moreover, some studies have also proven that LY294002, a specific inhibitor of PI3K, can attenuate the migration of cells. These indicate that the PI3K/Akt pathway is also essential to the CXCR4/SDF-1 axis (Wang et al. 2012b). In spite of this wealth of data, the mechanism by which ligands and receptors regulate the activity of cells remains only partially understood.

Such hypoxia mimics as desferrioxamine (1,8-diazafluoren-9-one; DFO) and dimethyloxaloylglycine (DMOG) are reagents that exhibit the same effects as hypoxia, although their mechanisms are completely different. DFO is an iron chelator that affects cells by chelating Fe^{2+}, whereas DMOG,

Abbreviations: CXCR4, C-X-C chemokine receptor 4; SDF-1, stromal cell-derived factor 1; CXCL12, C-X-C chemokine 12; BM-MSC, bone marrow mesenchymal stromal cell; DFO, desferrioxamine (1,8-diazafluoren-9-one); DMOG, dimethyloxaloylglycine; PHD, prolyl hydroxylase; HIF, hypoxia-inducible factor; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.

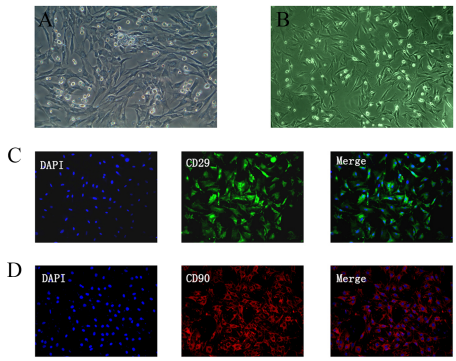

Fig. 1: (A) BM-MSCs at passage 0 under inverted light miscroscope. (B) BM-MSCs at passage 3 under inverted light microscope. (C) Characterization of BM-MSCs with DAPI staining the nuclei and CD29 which are green (FITC) at passage 3. The third photo in C group is the merge of DAPI and CD29. (D) Characterization of BM-MSCs with DAPI staining the nuclei and CD90 which are red (CY3) at passage 3. The third photo in D group is the merge of DAPI and CD90.

a 2-oxoglutarate analog, inhibits the interaction between 2-oxoglutarate and prolyl hydroxylases (PHDs), resulting in decreased PHD activity and the stabilization of hypoxia-inducible factors (HIFs). We chose DFO and DMOG because HIFs can regulate more than 70 genes, including CXCR4, in their downstream region. Moreover, such hypoxia mimics as DMOG are also used to treat post-ischemic injury by enhancing postoperative angiogenesis (Takaku et al. 2012). Studies using animal models have reported that DMOG promotes therapeutic revascularization (Loinard et al. 2009). By contrast, DFO has been used to treat iron (Fe) overload diseases and found to have anti-cancer activity (Lui et al. 2013). In this study, we used DFO and DMOG to upregulate the expression of CXCR4 in BM-MSCs because they have already been confirmed to have effects in animals and cells.

2. Investigations and results

2.1. Characterization of BM-MSCs

BM-MSCs were isolated from femurs and tibias and observed after passage 3 using an inverted light microscope. We focused on identifying large flat as well as oval to spindle-shaped cells (Fig. 1A and B). The purification rates for BM-MSCs were higher than 90% at passage 3 and only 60% at passage 1. Flow cytometry revealed that the cells were detached, and we tested the positive markers CD29 and CD90 as well as the negative markers CD34 and CD45 (Fig. 2) to identify them according to their morphology. As shown in Fig. 1, groups C and D had positive markers (green represents CD29, whereas red represents CD90) in the membrane of BM-MSCs.

2.2. Up-regulation of CXCR4 in treated cells

The results of Western blot analysis showed that the treated groups and control group differed. The average gray value of the treated groups was 0.490 ± 0.022, whereas that of the control group was 0.092 ± 0.031 ($P < 0.01$). Differences among the varying concentrations of DFO and DMOG were also detected ($P < 0.05$). The results showed that the effects of DFO and DMOG are dose dependent (Fig. 3).

2.3. Immunofluorescence

We observed the slices immediately after they were mounted. As shown in Fig. 4, all images were taken under an inverted immunofluorescent microscope. We did not add reagents to the medium in group A such that the expression of CXCR4 was weaker than those in groups B and C. 4,6-Diamidino-2-phenylindole was used to stain the nucleus in all groups, and merged images of CXCR4 that stained green showed that CXCR4 is a membrane protein in BM-MSCs.

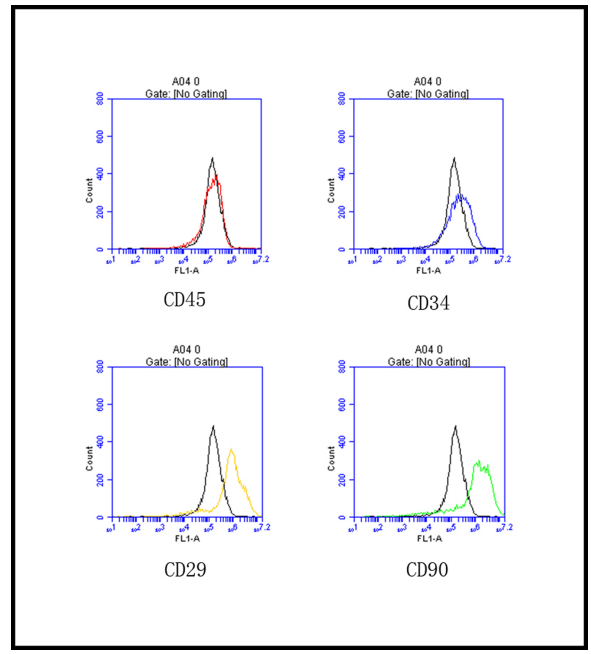

Fig. 2: Flow cytometry shows that CD45 and CD34 are negative and CD29 and CD90 are positive in the membrane of BM-MSCs.

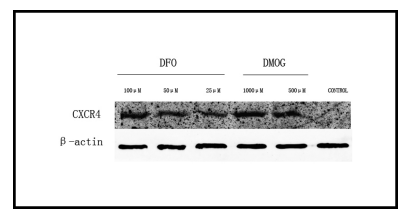

Fig. 3: Expression of CXCR4 between Control group and Treated groups. Compared to the control group, DFO group and DMOG group obviously upregulate the expression of CXCR4 ($P < 0.01$, Student's t-test). To DFO group, we used three different doses (25 μM, 50 μM, 100 μM) to deal with BMSCs. For DMOG group, we used 500 μM and 1000 μM to treat cells. Results shows that the effects of DFO and DMOG are dose-dependent ($p < 0.05$, Student's t-test). Experiments were repeated for 3 times separately.

3. Discussion

This study provides for the first time evidence that DFO and DMOG up-regulate the expression of CXCR4 in BM-MSCs. Compared with that in the control group, the expression of CXCR4 at several concentrations of the reagents was obviously up-regulated. Meanwhile, the effects of DFO and DMOG exhibited dose dependence. CXCR4 in isolated BM-MSCs is widely known to be expressed at low levels (Tong et al. 2011). This indicates that the migration capacity of BM-MSCs transplanted into patients or animal models is limited. Treatment of stem cells before BM-MSC therapy is currently viable, and treatment with DFO or DMOG significantly improves the migration capacity of BM-MSCs. Studies have shown that up-regulating CXCR4 promotes early metastasis in malignant tumors, such as Ewing's sarcoma (Jin et al. 2012). Other research has reported that the migration capacity of BM-MSCs improved because of CXCR4 as tested by transwells (Xie et al. 2012a).

DFO is an iron chelator that has been widely used in clinical practice. Research has shown that DFO ameliorates oxidative

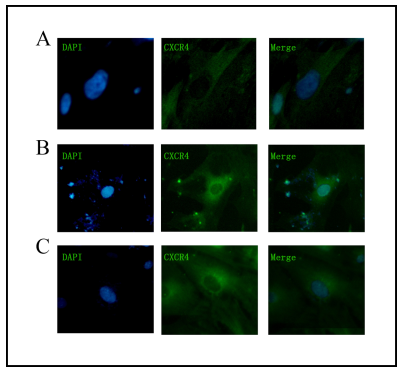

Fig. 4: Immunofluorescence of CXCR4 in the membrane of BM-MSCs(× 400). (A) In control group, we used DAPI to stain the nucleus while FITC-conjugated secondary antibodies marking CXCR4 in membrane of stem cells. (B) After adding DFO (100 μM) into the medium for 72 h, cells were dealed in the same way of group A. (C) Group DMOG (1000 μM), others are the same with A and B. All photos were took at × 400 under inverted immunofluorescence microscope.

stress without limiting the infarct size in myocardial infarction (Chan et al. 2012). In iron-induced cardiomyopathy, DFO has also been proven to be an effective chelating drug. It has also been found to stimulate the JNK and p38 MAPK signaling transduction pathways by iron depletion. On the other hand, DMOG has been applied in inflammatory conditions, with research demonstrating that it attenuates systemic LPS-induced activation of the NF-κB pathway (Hams et al. 2011). By increasing the kidney expression of HIFs, DMOG affects renal protection in chronic kidney disease and may enhance angiogenesis in many diseases (Deng et al. 2010). Thus, we hypothesized that DFO and DMOG up-regulate the expression of CXCR4 in BM-MSCs by increasing the level of HIFs.

Studies have also demonstrated that DFO and DMOG have anti-apoptotic functions (Longpre and Loo 2012; Xie et al. 2012b). One underlying mechanism is that DFO and DMOG inhibit PHD domain protein 3, which is an important component of the DNA damage response, a complex that responds to such environmental cues as changes in ambient oxygen tension. With the function of anti-apoptosis, hypoxia mimics could be extensively applied to treat diseases.

One limitation of our study is that the dose levels at which cells can survive were not determined. We selected doses based on the available literature (Ioannou 2010; Liu et al. 2009; Milosevic et al. 2009).

This study also investigated how the reagents we used up-regulated the expression of CXCR4, with the activation of HIF-1α being one of the probable mechanisms involved (Zou et al. 2011). Hypoxia maintains HIF-1α at high levels in response to low oxygen in the environment (Vaapil et al. 2012). Meanwhile, more than 100 genes in the downstream region, including CXCR4, exist. Thus, we suggested that the up-regulation of CXCR4 is attributable to the activation of HIF-1α.

The mechanisms by which DFO and DMOG up-regulate CXCR4 are absolutely different. DFO is both a hypoxia mimic and an iron chelator (Guo et al. 2012). Iron is necessary for the PHD domain to degrade HIF-1α so that it could activate other genes in its downstream region. By contrast, DMOG maintains HIF-1α at high levels. This reagent is essential to the hydroxylase activity of PHD, which depends on the availability of O2, Fe(II), and 2-oxoglutarate (Takaku et al. 2012). By inhibiting PHD, DMOG ultimately upregulates CXCR4 in stem cells.

In conclusion, DFO and DMOG up-regulate the expression of CXCR4, their effects are dose dependent, and CXCR4 is distributed in the membrane of BM-MSCs.

4. Experimental

4.1. Cells and cell culture

This work was approved by the Science and Technology Commission of Shanghai Municipality (ID: SYXK 2007–0006) with Permit No. 2011-RES1. All animal-related procedures performed were in accordance with the guidelines of the Animal Care and Use Committee of the Tenth People's Hospital of Shanghai. Bone marrow was obtained from 5-week-old male Sprague-Dawley rats weighing 180 g (Chen et al. 2012). The femurs and tibias of the rats were aseptically removed after sterilization in 75% ethanol and euthanasia. With the excision of two ends of bone, the bone marrow was flushed from the marrow cavity using sterile phosphate-buffered saline (PBS), centrifuged at 1500 rpm for 5 min, and then immediately cultured in DMEM (high glucose; Hyclone) with 15% fetal bovine serum of Australian origin and penicillin/streptomycin (100 U/mL to 0.1 mg/mL; Invitrogen) for 3 weeks. As the medium was replaced, non-adherent cells were removed and adherent cells were selected every 3 days. Adherent cells were detached by trypsin–EDTA (0.5–0.2 g/L; Invitrogen) when the cells needed passage. The cells used in our experiments were harvested from passage 3(Chi, et al. 2012).

4.2. Characterization

Flow cytometry was used to identify BM-MSCs. We tested CD29, CD34, CD45, and CD90 to characterize the cells we needed. Some studies may have tested more cell markers to characterize BM-MSCs, but we decided that two positives and two negatives were enough (Takemitsu et al. 2012). Hence, passage 3 BM-MSCs were resuspended in PBS containing 1% bovine serum albumin at the concentration of 1.5×10^6/mL by FACS analysis that included positive assays (1:250) for anti-CD29-FITC (where FITC indicates fluorescein isothiocyanate) and anti-CD90-FITC as well as negative assays (1:250) for anti-CD34 purified and anti-CD45-FITC (all purchased from BD Pharmagen, California, USA). One negative control tube with a cell suspension was used as control.

4.3. Cells and reagents

We tested the expression levels of CXCR4 in three groups: DFO group, DMOG group, and control group. We set three gradient concentrations for the DFO group (25, 50, and 100 μM) and two gradient concentrations for the DMOG group (500 and 1000 μM). All groups were cultivated for 72 h. Both DFO and DMOG were dissolved in DMSO. At passage 3, the cells were treated in DMEM and 10% fetal bovine serum with corresponding concentrations of the two reagents. The control group was treated with DMSO only.

4.4. Western blot analysis

According to the time points (72 h) in the experimental design, cells were collected and lysed in RIPA buffer (50 mM HEPES, pH 7.3, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, and 1 mM NaF) with protease inhibitor cocktail (Roche, Nutley, NJ) for 45 min. Centrifugation was performed at 12,000 rpm and 4 °C for 10 min. The samples were then boiled in boiling water for 3 min with loading and stored at − 80 °C. Before loading was added to the samples, the total protein concentration was tested using a BIO-RAD Synergic 2 Microplate Reader with 5% BSA as a standard. In the 15% SDS-PAGE gradient gels, an equal total amount of protein (45 μg) was loaded and electrophoresed at 80 V for 90 min. After being separated from one another, the protein samples were transferred onto NC membranes for 45 min at 220 mA(Xue, et al. 2012). After being blocked in PBST (PBS with 0.5‰ Tween-20) containing 5% non-fat milk, the membranes were incubated with rabbit monoclonal anti-actin antibodies (1:1000; R&D) and rabbit monoclonal anti-CXCR4 antibodies (1:100; Abcam) overnight at 4 °C. The membranes were washed in PBST three times for 5 min each and then incubated with HRP-marked secondary antibodies (anti-rabbit antibodies) for 1 h at 37 °C. Finally, the membranes were scanned by ODYSSEY and analyzed using ImageJ.

4.5. Immunofluorescence

The cells were fixed in 4% formaldehyde for 20 min and then washed with PBS, which was subsequently incubated with 4% BSA for 1 h. Next, the cells were incubated with primary rabbit monoclonal anti-CXCR4 antibodies (Abcam) at the concentration of 1:150 overnight at 4 °C. On the second day, the cells were washed by PBS three times before being incubated with FITC-marked secondary antibodies (anti-rabbit antibodies; 1:400; Cell Signaling Technology) and 4,6-diamidino-2-phenylindole (1:3000, Sigma) for 30 min (Gehrke et al. 2011). After three washing steps with PBS for 5 min each, the cells were fixed in mounting medium (ProLong Gold). Slices were observed under an inverted immunofluorescent microscope (OLYMPUS BX51).

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