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Chemokine Receptor CXCR7 Mediates Human Endothelial Progenitor Cells Survival, Angiogenesis, but not Proliferation

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ABSTRACT

Stromal cell-derived factor 1 (SDF-1) is a critical regulator of endothelial progenitor cells (EPCs) mediated physiological and pathologic angiogenesis. It was considered to act via its unique receptor CXCR4 for a long time. CXCR7 is a second, recently identified receptor for SDF-1, and its role in human EPCs is unclear. In present study, CXCR7 was found to be scarcely expressed on the surface of human EPCs derived from cord blood, but considerable intracellular CXCR7 was detected, which differs from that on EPCs derived from rat bone marrow. CXCR7 failed to support SDF-1 induced human EPCs migration, proliferation, or nitric oxide (NO) production, but mediated human EPCs survival exclusively. Besides that, CXCR7 mediated EPCs tube formation along with CXCR4. Blocking CXCR7 with its antagonist CCX733 impaired SDF-1/CXCR4 induced EPCs adhesion to active HUVECs and trans-endothelial migration. Those results suggested that CXCR7 plays an important role in human cord blood derived EPCs in response to SDF-1. J. Cell. Biochem. 113: 1437–1446, 2012.

KEY WORDS: CXC CHEMOKINE RECEPTOR 7; STROMAL CELL-DERIVED FACTOR 1; ENDOTHELIAL PROGENITOR CELLS; SURVIVAL; ANGIOGENESIS

Endothelial progenitor cells (EPCs) are considered to control the angiogenic switch of many physiological and pathologic processes [Asahara et al., 1999; Isner and Asahara, 1999; Murohara et al., 2000; Kamihata et al., 2002], such as neovascularization in ischemic tissue and tumorigenesis [Gao et al., 2008]. Local or systemic administration of EPCs derived from bone marrow [Asahara et al., 1999], peripheral blood [Kamihata et al., 2002], or cord blood [Murohara et al., 2000] can enhance ischemic neovascularization and improve function of ischemic tissues in animals with hindlimb or myocardial ischemia. The process EPC participating in neovascularization is regulated by a variety of growth factors, cytokines and chemokines [Yancopoulos et al., 2000]. Among those factors, stromal cell-derived factor 1 (SDF-1) has been confirmed as a principal regulator in mobilization, migration, retention of EPCs [Yamaguchi et al., 2003; De Falco et al., 2004; Pi et al., 2009]. The expression of SDF-1 is up-regulated in the neovascularization sites [Du et al., 2008]. Administration [Yamaguchi et al., 2003] or gene transfer [Hiasa et al., 2004] of SDF-1 enhanced ischemia-induced vasculogenesis and angiogenesis in vivo.

For a long time, SDF-1 was considered to act via its unique receptor CXCR4 [Bleul et al., 1996], but recent studies found it has an alternative receptor, CXC chemokine receptor 7 (CXCR7) [Balabanian et al., 2005; Burns et al., 2006]. CXCR7 is a seven-transmembrane protein widely expressed in hematopoietic system, cardiac microvessels, brain, kidney, and several tumor cell lines [Balabanian et al., 2005; Sierro et al., 2007; Mazzinghi et al., 2008; Sch nemeier et al., 2008]. It has significantly higher binding affinity for SDF-1 than CXCR4 [Burns et al., 2006]. Existing studies have revealed some functions of CXCR7. In $CXCR7^{-/-}$ mouse model,

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CXCR7 was demonstrated to be essential to valve formation, vessel protection, endothelial cell growth and survival [Sierro et al., 2007]. In zebrafish posterior lateral line development, CXCR7 regulated primordial germ cell migration by shaping the distribution of the chemokine SDF-1 in the environment as a decoy receptor [Dambly-Chaudiere et al., 2007; Mahabaleshwar et al., 2008]. In immune system, CXCR7 was reported to be co-receptor for human and simian immunodeficiency viruses [Shimizu et al., 2000], and its expression level correlated with the ability of switch memory B cells differentiating into plasma cells [Infantino et al., 2006]. In the therapeutic homing of renal progenitor cells, CXCR7 mediated SDF-1 induced cells survival, adhesion to endothelial cells exclusively [Mazzinghi et al., 2008]. Moreover, CXCR7 was reported to enhance tumor development [Burns et al., 2006; Maksym et al., 2009; Hattermann et al., 2010; Kollmar et al., 2010; Zheng et al., 2010] and metastasis [Iwakiri et al., 2009].

Although there were a few researches on CXCR7, the exact effects and molecular processes of CXCR7 binding with SDF-1 remained poorly defined, and contradictions existed in previous results. (1): Whether or not CXCR7 is G-protein coupled receptor (GPCR) is debatable [Thelen and Thelen, 2008]. Burns et al. [2006] considered CXCR7 cannot mediate calcium mobilization in MCF-7 cells, but Sierro et al. [2007] found that SDF-1 induced a stronger Ca^{2+} flux in CXCR7 stably transfected HEK293 cells co-expressing CXCR4 and CXCR7 than in HEK293 cells expressing CXCR4 only. (2): Role of CXCR7 in cell survival and proliferation is controversial. CXCR7 was considered to mediate survival but not proliferation of CXCR7-transfected MDA MB 435s cells [Burns et al., 2006] and glioma cells [Hattermann et al., 2010], but mediate cell proliferation instead of survival in CT26 and KEP1 cell lines [Meijer et al., 2008]. (3): Downstream signaling pathways of CXCR7 are still largely indefinite. Hartmann et al. [2008] reported CXCR7 cannot mediate SDF-1 induced Akt or Erk1/2 activation. However, other researches considered that CXCR7 can activate Erk1/2 in glioma cells [Hattermann et al., 2010], AKT pathway in Prostate Cancer [Wang et al., 2008], and both of Erk1/2 and Akt in astrocytes and schwann cells [Odemis et al., 2010]. The combination of those researches revealed that effects of CXCR7 are complex, apparently differ between cell types and need for further research.

Our previous results suggested that CXCR7 was highly expressed on surface of rat bone marrow-derived EPCs and contributed to SDF-1 induced survival, adhesion, trans-endothelial migration (TEM), proliferation, and tube formation alone or along with CXCR4 [Dai et al., 2011]. However, we found CXCR7 was scarcely expressed on human EPCs surface while considerable intracellular CXCR7 was detected, which was different from that in rat EPCs. Considering that effects of CXCR7 are complex and cell type-depending, it is necessary to investigate the role of CXCR7 in human EPCs to facilitate further clinic research.

MATERIALS AND METHODS

UMBILICAL CORD BLOOD SAMPLES

Human umbilical cord blood samples (20–40 ml) from 13 healthy newborns (7 boys and 6 girls; gestational age range, 38–40 weeks) were collected in CPD solution. The Institutional Review Board in The First Affiliated Hospital of Chongqing Medical University approved all protocols, and informed consent was obtained from all parents of newborns.

HUMAN UMBILICAL CORD BLOOD EPCs ISOLATION AND IDENTIFICATION

Human umbilical cord blood EPCs were isolated as previously published protocol [Gulati et al., 2003]. In brief, cord blood (20–40 ml) was diluted 1:1 with Hanks balanced salt solution (HBSS; Invitrogen, NY), then overlaid onto an equivalent volume of Histopaque 1077 (Sigma, MO) and centrifuged at 400*g* for 30 min at room temperature. MNCs were isolated and washed three times with EBM-2 medium (Lonza, Basel, Switzerland), then planted on one well of a six-well plate coated with human fibronectin (2 $\mu g/cm^2$, BD Biosciences, MA) in EGM-2 supplemented with 2%FBS (Sigma). The plate was incubated at 37°C in a humidified environment with 5% CO_2. After 24 h, unattached cells and debris were removed by washing with medium. Medium was changed daily for 7 days and thereafter on alternate days.

At day 7, EPCs were characterized using acetylated low-density lipoprotein uptaking and lectin binding assay. First, cells were incubated with Dilacetylated low-density lipoprotein (Dil-acLDL, final concentration 10 μg/ml, Biomedical Technologies, Milan, Italy) at 37°C for 4 h, and then fixed with 3% paraformaldehyde for 10 min. After washed twice with PBS, cells then reacted with ulex europaeus agglutinin-1 (UEA-1, final concentration 10 μg/ml, Sigma) for 1 h. After staining, samples were viewed with a fluorescence microscope (Olympus IX71, Olympus, Tokyo, Japan). Double positive staining cells were identified as differentiating EPCs. EPCs were further identified by CD133 and vascular endothelial growth factor receptor 2 (VEGFR-2) expression using immunofluorescent staining. In this assay, mouse anti-CD133 antibody (Miltenyi Biotec, CA) and rabbit polyclonal antibody against VEGFR-2 (Santa Cruz, CA) were used.

WESTERN BLOT ANALYSIS FOR CXCR4 AND CXCR7 EXPRESSION

CXCR7 and CXCR4 protein expressions were detected by Western blot assay with HeLa cells as positive control. EPCs and HeLa cells were washed with precooled PBS and lysed in RIPA solution. Protein concentrations were determined for cell lysates clarified by centrifugation 12,000 rpm at 4°C for 30 min. Lysate proteins (40 μg per sample) were resuspended in loading buffer and loaded on a 10% SDS–PAGE. After electrophoresis, protein was transferred onto a polyvinylidene difluoride membrane. For detection of CXCR7 and CXCR4, the membranes were incubated overnight with mouse anti-CXCR7 antibody 11G8 (kindly provided by ChemoCentryx Ltd., CA) or rabbit polyclonal antibody against CXCR4 (1:400, Abcam, MA), respectively. Then, the membranes were washed with Tris-buffered saline with Tween-20 for three times and incubated with peroxidase conjugated goat anti-mouse IgG (1:2,000, Abcam) or goat anti-rabbit IgG (1:2,000, Abcam), respectively, for 1 h and then detected by chromomeric substrate-3, 3′-diaminobenzidine.

FLOW CYTOMETRY ASSAY FOR CXCR4 AND CXCR7 EXPRESSION

CXCR4 surface expression of human EPC was detected by staining with FITC-conjugated anti-CXCR4 mAb (Biolegend, CA). CXCR7

surface expression was identified by staining with 5 μg/ml anti-CXCR7 mAb 11G8 (kindly provided by ChemoCentryx), and then PE-conjugated secondary antibody (Biolegend). For detection of intracellular CXCR7, cells were fixed with 4% paraformaldehyde, and then permeabilized with 0.2% saponin. Thereafter, cells were stained with 11G8 and then PE-conjugated secondary antibodies. Flow cytometric analysis was performed with FACScan (BD FACSCalibur).

EPCs MIGRATION ASSAY

Cells migration assay was performed in 24-well millicell (Millipore, MA) containing microporous (8.0 μm) membrane. In brief, EPCs (1×10^5) were seeded in the upper chambers containing MCDB131 medium supplemented with 0.5% FBS (Sigma) in presence of CCX733 (1 μM, kindly provided by ChemoCentryx Ltd., CA), AMD3100 (2.5 μM, Sigma) or their combination, respectively (cytotoxicity assay showed intrinsic toxicity of either CCX733 or AMD3100 is negligible). The lower chambers (24-well plates) were filled with MCDB131 medium supplemented with 0.5% FBS plus 100 ng/ml SDF-1 (PeproTech, NJ). After incubation for 8 h, non-migratory cells were removed by cotton-tipped swabs, and then the migratory cells were stained with 0.1% crystal violet solution and counted manually in random high magnification fields (10×) in each well.

CELL PROLIFERATION

EPCs were planted in 96-well plates (5×10^3 cells per well), and cultured for 24 h in MCDB131 medium supplemented with 2% FBS plus the specified additives: (1) DMSO, (2) 100 ng/ml SDF-1, (3) SDF-1 and CCX733, (4) SDF-1 and CCX704, (5) SDF-1 and AMD3100, (6) SDF-1 plus CCX733 and AMD3100. Cells were then incubated with 0.5 mg/ml MTT for 4 h at 37°C. After incubation, the medium was aspirated and the formazan reaction products were dissolved in 150 μl DMSO. The optical density of the formazan solution was measured with a microplate reader (Bio-Rad, CA) at 570 nm. Each condition was tested in triplicate.

ASSAY FOR CELL APOPTOSIS

EPC apoptosis induced by serum starvation was detected by annexin V-PI staining (KeyGen Biotech, JiangSu, China) to determine whether CXCR4/SDF-1 or CXCR7/SDF-1 exerted a survival effect on EPCs. Briefly, EPCs were cultured with serum free MCDB131 medium supplemented with SDF-1 (100 ng/ml) in the presence or absence of CXCR4 or CXCR7 antagonist, respectively, for 48 h. After treatment, EPCs were collected and washed for three times. Annexin V-FITC and propidium iodide (PI) were added to the cells (10^6cells/ml in FACS buffer) for 15 min at room temperature in the dark. Cells were analyzed immediately by flow cytometry. Cells with Annexin V positive staining and PI negative staining were identified as apoptosis cells.

ADHENSION ASSAY

HUVECs (5×10^4cells per well) were seeded in 24-well plastic tissue culture plates overnight to form monolayer. The monolayer was activated by treating with 10 ng/ml TNF-α and 10 ng/ml IL-1β for 5 h [Burns et al., 2006]. EPCs were labeled with calcein AM (Invitrogen) for 30 min at room temperature, washed, and then incubated with CXCR7 antagonist CCX733, CXCR4 antagonist AMD3100, or both of them for another 30 min. After washed, EPCs were added onto the HUVEC monolayer for 15 min at 37°C in the presence of 100 ng/ml SDF-1. Non-adherent cells were washed off thoroughly. Adherent cells were counted under fluorescence microscopy.

TRANS-ENDOTHELIAL MIGRATION

The TEM assay was performed in 24-Millicell culture plates containing microporous (8.0 μm) membranes. Briefly, HUVECs (5×10^4 cells/well) were seeded onto millicell for 24 h to form a confluent monolayer. EPCs were labelled with calcein AM and then pretreated with CXCR4 or CXCR7 antagonist, respectively, for 30 min. After that, EPCs were washed, and then added to the top chambers. 100 ng/ml SDF-1 was added to the lower chambers. After incubation for 12 h, non-migratory cells were removed by cotton-tipped swabs. Migratory cells were quantified by fluorescence microscopy on multiple 10× fields.

IN VITRO TUBE-FORMATION ASSAY

The tube formation assay was conducted as described previously [Shao et al., 2008]. Briefly, growth factor-reduced matrigel (BD Biosciences) was thawed in ice water overnight, and then 150 μl Matrigel was added to each well of a 48-well plate and incubated at 37°C for 30 min to polymerize. EPCs were starved in serum free MCDB131 medium for 12 h to remove angiogenic inducing activities of growth factors in EGM-2. Following starving, EPCs (2×10^4 cells/well) were incubated in the presence or absence of CCX733, AMD3100, or their combination for 30 min. Then, 100 ng/ml SDF-1 was added. After culture for 18 h, tube-like structures were recorded under a light microscope (Leica DMI3000B, Leica, Wetzlar, Germany) equipped with a digital camera (Olympus DP25) in a blind manner. The length of tube-like structures in the images was measured using ImageJ (http://rsbweb.nih.gov/ij/). At least six fields were examined per well and experiments were repeated with three independent EPCs cultures.

DETECTION OF NO PRODUCTION

NO production was determined as previous described [Shao et al., 2008]. In brief, EPCs (1×10^6) were cultured for 4 h at 37°C in the same way as described in the proliferation assay. Then, the supernatants were collected and NO concentration was determined using a Nitric Oxide (NO) Colorimetric Assay Kit (4A biotech Co Ltd., Beijing, China) per the manufacturer's protocol. Each condition was tested in triplicate.

STATISTICS

Results are obtained from at least three independent experiments and represented as mean ± SD. Statistical significant differences between groups were performed with Origin 7.5 (OriginLab data analysis and graphing software) with one-way ANOVA and Student's *t*-test analysis. Significance was attributed to a *P*-value of <0.05, and obvious significance was attributed to a *P*-value of <0.01.

RESULTS

CHARACTERIZATION OF EPCs

MNCs isolated from human cord blood were cultured on plates coated with fibronectin. After 4 days of cultivation in EGM-2, MNCs developed spindle-shaped appearance (Fig. 1A left). Typical cell clusters appeared at day 7 (Fig. 1A middle). Colonies with different morphology emerged over 14–21 days of cultivation (Fig. 1A right). Those cells were positive for both DiI-acLDL and UEA-1 stains (Fig. 1B). The double positive cells were recognized as differentiating EPCs. Immunofluorescent staining assay demonstrated that most of the cells were positive for CD133 and VEGFR2 (Fig. 1C), confirmed those cells are EPCs. Cells of passage 2–4 were used for in vitro experiments.

Both CXCR4 and CXCR7 were expressed in human EPCs (Fig. 2A). CXCR4 was expressed on cell surface. However, CXCR7 was scarcely expressed on cell surface, while intracellular CXCR7 was considerable (Fig. 2B).

CXCR4 BUT NOT CXCR7 MEDIATES SDF-1 INDUCED MIGRATION AND PROLIFERATION OF EPCs

The effect of CXCR4 and CXCR7 on cell migration was studied by applying antagonists to the culture. EPC migration was enhanced in the presence of SDF-1 ($182.6 \pm 24.92\%$ vs. $100 \pm 25.31\%$, $P < 0.01$) (Fig. 3A). Promotion of EPCs migration induced by SDF-1 was totally abolished by AMD3100, antagonist of CXCR4 ($85.5 \pm 16.72\%$ vs.$182.6 \pm 24.92\%$, $P < 0.01$), while CXCR7 antagonist CCX733 had no effect on it ($167.4 \pm 15.24\%$ vs. $182.6 \pm 24.92\%$, $P > 0.05$).

Role of CXCR4 and CXCR7 in EPCs proliferation was similar to that in migration. SDF-1 can also promote EPCs proliferation ($151.3 \pm 9.2\%$ vs.$100 \pm 13.6\%$, $P < 0.01$) (Fig. 3B). Promotion of EPCs proliferation induced by SDF-1 was totally abolished by AMD3100 ($107.6 \pm 9.9\%$ vs. $151.3 \pm 9.2\%$, $P < 0.01$). CCX733 had no inhibition on SDF-1 induced proliferation ($151.5 \pm 26.6\%$ vs. $151.3 \pm 9.2\%$, $P > 0.05$). These data suggested that the facilitative effect of SDF-1 on EPC migration and proliferation is mediated by CXCR4 but not CXCR7.

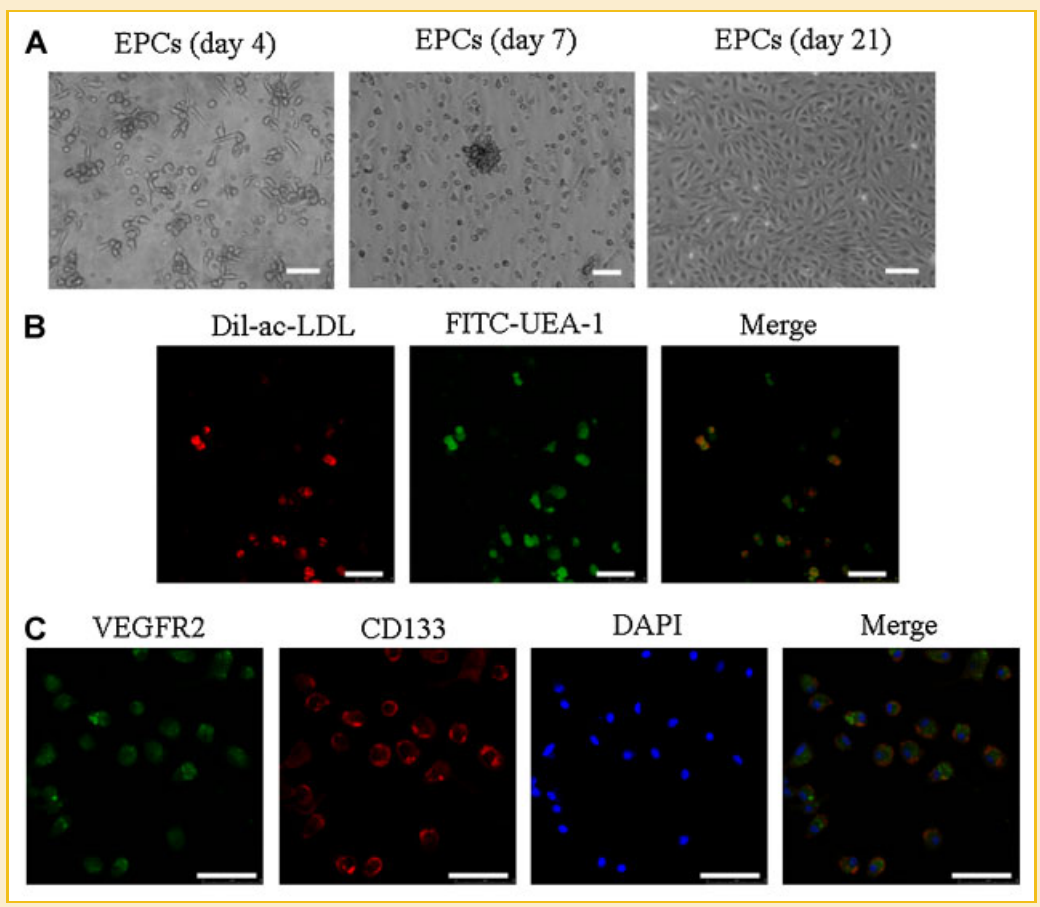

Fig. 1. Characterization of isolated human EPCs. A: Spindle-like cells appeared after 4-day culture in EGM-2 (left). Typical cell clusters appeared at day 7 (middle). Different morphology emerged over 14–21 days of cultivation (right). B: Dil-acLDL and FITC-UEA-1 uptake assay showed that the cells were both DilacLDL/FITC-UEA-1 positive, which indicates that the isolated MNCs are EPC, (C) Cultured MNCs were further characterized by immunofluorescent staining using EPCs specific markers CD133/VEGFR-2 and nuclear maker DAPI. Scale bars represent 50 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

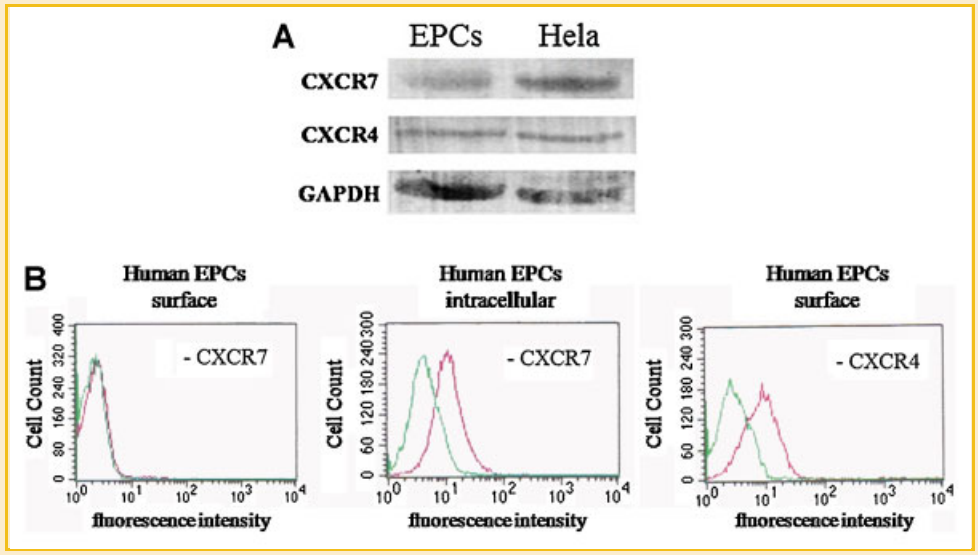

Fig. 2. CXCR7 and CXCR4 expression in EPCs. A: Detecting CXCR7 and CXCR4 expression in EPCs by Western blot. GAPDH was used as loading control and Hela cells were used as positive control. B: Cell surface (left) or intracellular (middle) expression of CXCR7 and cell surface expression of CXCR4 (right) were investigated by flow cytometry. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

CXCR7 BUT NOT CXCR4 MEDIATE SDF-1 INDUCED EPC SURVIVAL

Serum deprivation was used to induce EPC apoptosis. The percentage of apoptotic cells was $29.63 \pm 6.06\%$ when EPCs were cultured in serum free media for 2 days (Fig. 3C). SDF-1 (100 ng/ml) can significantly reduce EPC apoptosis ($12.27 \pm 3.62\%$, $P < 0.05$ vs. control). This anti-apoptotic effect of SDF-1 was almost completely abolished by CXCR7 antagonist CCX733 ($33.03 \pm 2.85\%$ vs. $12.27 \pm 3.62\%$, $P < 0.01$), but remained when blocking CXCR4 with AMD3100 ($10.08 \pm 3.58\%$ vs. $12.27 \pm 3.62\%$, $P > 0.05$). Blocking both CXCR4 and CXCR7 has a similar effect with blocking CXCR7 alone in protection EPCs from apoptosis ($30.46 \pm 4.52\%$ vs. $33.03 \pm 2.85\%$, $P > 0.05$). Collectively, the results suggested that SDF-1 mediates EPC survival predominantly via CXCR7.

CXCR7 BLOCKAGE INTERFERES WITH CXCR4/SDF-1 MEDIATED EPCs TEM AND ADHESION TO HUVECs

Role of CXCR4 and CXCR7 in EPC TEM was investigated in millicell. SDF-1 obviously promoted EPC TEM (51.0 ± 20.8 vs. 6.4 ± 1.7, $P < 0.01$, Fig. 4A). The promotive effect was significantly inhibited by CCX733 (25.8 ± 9.2 vs. 51.0 ± 20.8, $P < 0.05$) and totally abolished by AMD3100 (9.4 ± 5.3 vs. 51.0 ± 20.8, $P < 0.01$; $P > 0.05$ vs. control). The inhibitory effect of blocking both CXCR7 and CXCR4 was similar to that of blocking CXCR4 alone (7.8 ± 3.8 vs. 9.4 ± 5.3, $P > 0.05$). These results suggested that CXCR4 mediates SDF-1 induced EPCs TEM, and blocking CXCR7 can interfere with this process.

As an antecedent step of TEM, EPC adhesion to active HUVEC was investigated using an in vitro cell adhesion assay. SDF-1 also obviously promoted EPCs adhesion to HUVECs (145.0 ± 40.7 vs. 32.6 ± 7.8, $P < 0.01$, Fig. 4B). The promotive effect was significantly inhibited by CXCR7 blockage (58.6 ± 15.0 vs. 145.0 ± 40.7, $P < 0.01$) and totally abolished by CXCR4 blockage (26.4 ± 11.2 vs. 145.0 ± 40.7, $P < 0.01$; $P > 0.05$ vs. control). The inhibitory effect of blocking both CXCR4 and CXCR7 was similar to that of blocking CXCR4 alone (26.0 ± 8.9 vs. 26.4 ± 11.2, $P > 0.05$). These results suggested that CXCR4 mediates SDF-1 induced EPCs adhesion to active HUVECs, and CXCR7 blockage can interfere with this process.

ESSENTIALITY OF BOTH CXCR4 AND CXCR7 FOR SDF-1-INDUCED EPC TUBE FORMATION

To evaluate the roles of CXCR4 and CXCR7 in SDF-1-induced angiogenesis, we investigated effects of CXCR4 antagonist AMD3100 and CXCR7 antagonist CCX733 on EPC tube formation induced by SDF-1. EPCs were cultured in the same media as proliferation assay in 48-well plated coated with 150 μl matrigel (BD Biosciences), and length of tube-like structures was measured 6 h and 18 h after. As shown in Fig. 5, at 18 h, SDF-1 significantly increased the length of tube (1.70 ± 0.17 vs. 1.00 ± 0.25, $P < 0.01$). AMD3100 significantly decreased the length of SDF-1-induced tubes in comparison of the SDF-1 alone group (1.03 ± 0.13 vs. 1.70 ± 0.17, $P < 0.01$); Similarly, CXCR7 antagonist CCX733 also significantly inhibited SDF-1-induced EPCs tube formation (1.11 ± 0.07 vs. 1.70 ± 0.17, $P < 0.01$), while its analog CCX704 have no effect (1.57 ± 0.12 vs. 1.70 ± 0.17, $P > 0.05$). When both CXCR4 and CXCR7 were blocked, the promotive effect of SDF-1 on human EPCs tube formation was completely abolished (0.79 ± 0.07 vs. 1.00 ± 0.25, $P > 0.05$). Tube formation at 6 h showed the same profile (data not shown). These results suggested that SDF-1 induces tube formation of EPCs through both CXCR4 and CXCR7.

CXCR4 BUT NOT CXCR7 MEDIATE SDF-1 INDUCED NO PRODUCTION

The angiogenic effects of SDF-1 involve increased production of NO [Kuhlmann et al., 2005]. 100 ng/ml SDF-1 can significantly enhance NO production in EPCs (9.14 ± 0.22 vs. 5.12 ± 0.50, Fig. 6). This effect was abolished by CXCR4 antagonist AMD3100 (5.32 ± 0.23

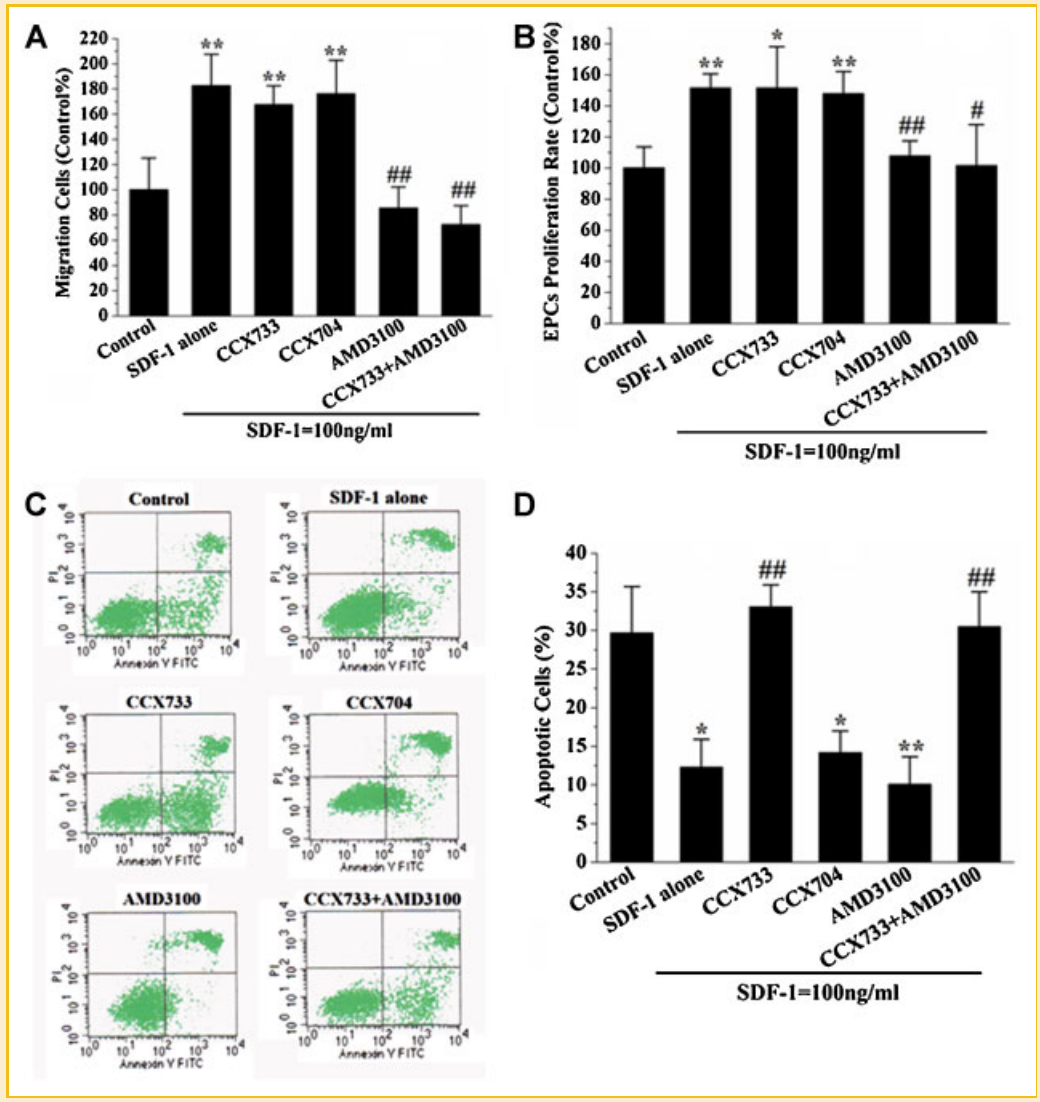

Fig. 3. In vitro effects of CXCR4 and CXCR7 on EPC migration, proliferation and apoptosis. A: EPCs migration. EPC migration was assayed in 24-well millicell containing microporous (8 μM) membranes. EPCs were cultured in the upper chamber with media supplied with (1) DMSO, (2) DMSO, (3) CCX733, (4) CCX704, (5) AMD3100, (6) CCX733 and AMD3100. 100 ng/ml SDF-1 was added to lower chamber of group 2–6. Cells migrated to the underside of the membrane were counted. B: EPC proliferation. EPCs were cultured in the media supplied with (1) DMSO, (2) SDF-1 (100 ng/ml), (3) SDF-1 and CCX733, (4) SDF-1 and CCX704, (5) SDF-1 and AMD3100, and (6) SDF-1 plus CCX733 and AMD3100. Cells number was determined after 48 h by MTT assay. C: EPC apoptosis. EPC apoptosis was detected by Annexin V-PI staining. EPCs were cultured for 48 h in serum-free medium supplemented with factors listed in (B). Cells with Annexin V-FITC positive stain and PI negative stain were defined as apoptotic cells. D: Quantification of EPC apoptosis. Cells with Annexin V-FITC positive stain and PI negative stain were counted and represented as a percentage of apoptotic cells/total cells. $^{*}P<0.05$, $^{**}P<0.01$ versus control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus SDF-1 alone group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

vs. 9.14 ± 0.22, $P < 0.01$), but CXCR7 antagonist CCX733 had no effect on it (8.82 ± 0.46 vs. 9.14 ± 0.22, $P > 0.05$). When blocking both CXCR4 and CXCR7, NO production was similar to that in blocking CXCR4 alone (4.76 ± 0.30 vs. 5.32 ± 0.23, $P > 0.05$). Those results suggested that CXCR4 but not CXCR7 mediates SDF-1 induced enhancement in NO production of EPCs.

DISSCUSSION

CXCR7, the newly identified second receptor of SDF-1, was reported to express in hematopoietic system, cardiac microvessels, brain, kidney, and several tumor cell lines [Balabanian et al., 2005; Sierro et al., 2007; Mazzinghi et al., 2008; Sch nemeier et al., 2008] and have important effects on cardiac development, tumorigenesis and stem cell homing [Balabanian et al., 2005; Sierro et al., 2007; Mazzinghi et al., 2008; Sch nemeier et al., 2008; Maksym et al., 2009]. CXCR7 was high expressed in endothelium of tumor blood vessels, suggesting there might be some correlation between CXCR7 and angiogenesis [Miao et al., 2007]. Our previous study had demonstrated CXCR7 on surface of EPCs derived from rat bone marrow and contributed to SDF-1 induced cell survival, adhesion, TEM, proliferation, and tube formation alone or along with CXCR4 [Dai et al.,

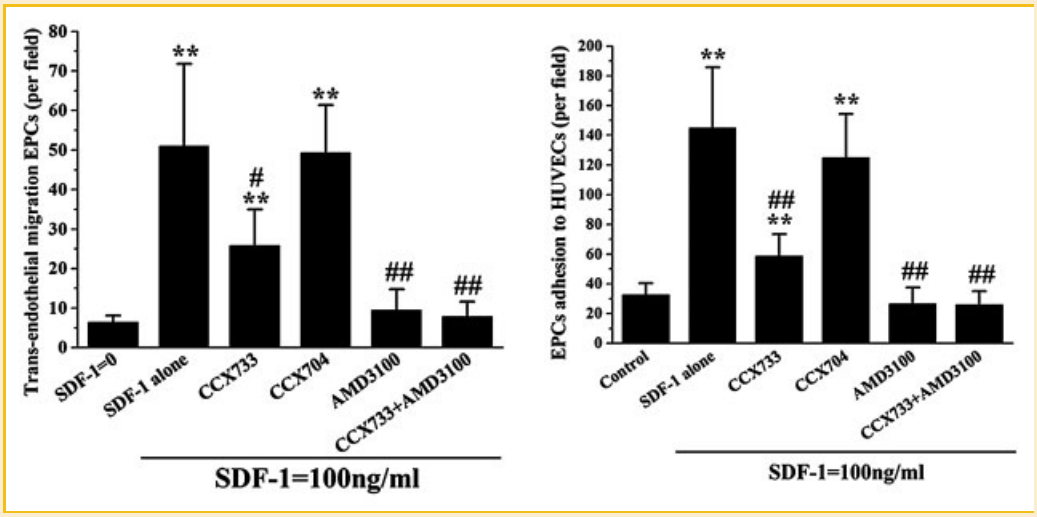

Fig. 4. In vitro effects of CXCR4 and CXCR7 on EPC TEM and adhesion to active HUVECs induced by SDF-1. A: EPC TEM. EPC TEM was assayed in 24-well millicell culture plates containing microporous (8 μM) membranes. 5×10^4 HUVECs were seeded in upper chamber for 24 h to form a confluent monolayer. EPCs pretreated with the same media as described in proliferation assay were washed and then added onto active HUVEC monolayer in the presence of 100 ng/ml SDF-1. After incubation for 12 h, non-migratory cells were removed by cotton-tipped swabs and cells migrated to the underside of the membrane were counted. B: EPC adhesion to active HUVECs. EPCs pretreated with the same media as described in proliferation assay were washed and then added onto active HUVEC monolayer in the presence of 100 ng/ml SDF-1 for 30 min. After that, non-adherent cells were washed-off throughly. Adherent cells were counted in fluorescence microscopic 10 × fields. Data are given as mean±SD ($^{**}P<0.01$ versus control; $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus SDF-1 alone group).

2011]. However, these conclusions are not generally valid for EPCs derived from different species and origins. In present study, we detected only minute level of CXCR7 on cell surface, but considerable level of intracellular CXCR7 in human EPCs derived from cord blood. Our multiple functional assays suggested CXCR7 had no effect on cell migration, proliferation and NO production but exclusively mediated SDF-1 induced cell survival. Besides that, CXCR7 can mediate EPC tube formation along with CXCR4, and blocking CXCR7 can interfere with human EPCs adhesion to active endothelial layer and TEM induced by SDF-1/CXCR4.

Human EPCs were isolated from cord blood for it was rich in EPCs [Murohara et al., 2000] and could be obtained non-invasively. Characterizing the CXCR7 expression of human EPCs by flow cytometry, we detected only minute levels of CXCR7 on cell surface, but considerable levels of intracellular CXCR7 (Fig. 2B), which is in contrast to study on rat bone marrow derived EPCs [Dai et al., 2011], but in conformity with report on $CD34^+$ progenitor cells [Hartmann et al., 2008]. The results revealed that the location of CXCR7 on EPCs might be species and tissue source depending.

CXCR7 was considered to be essential to cell survival, but have no effect on cell migration and proliferation [Burns et al., 2006;

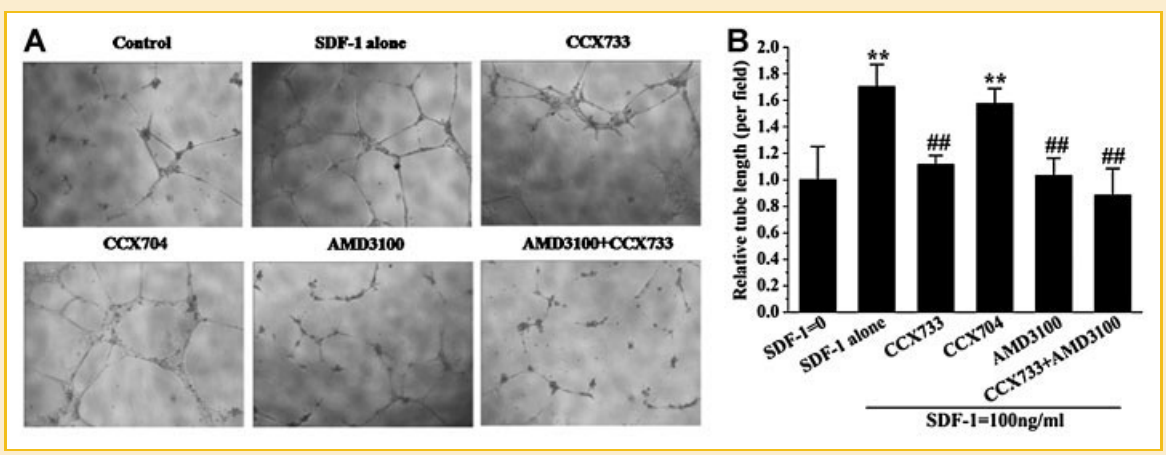

Fig. 5. Effects of CXCR7 and CXCR4 on SDF-1-induced tube formation at 18 h in vitro. Lengths of tube-like structures were measured in a blind manner. EPCs cultured on the matrigel with the same media as described in proliferation assay for 18 h. Representative images of tube-like structures from different groups (A) and quantitative data (B) are presented. Data are given as mean±SD ($^{**}P<0.01$, versus control group; $^{\#\#}P<0.01$, versus SDF-1 alone group).

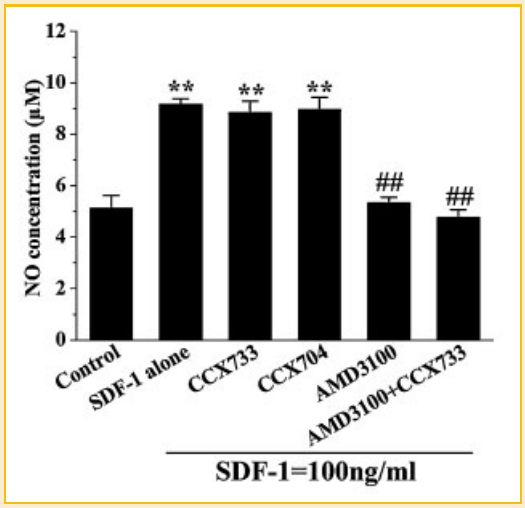

Fig. 6. Effects of CXCR7 and CXCR4 on NO production from EPCs. EPCs were cultured in the same media as proliferation assay. After culture for 4 h, NO concentration in the medium was determined with NO Colorimetric Assay Kit. Data was given as mean ± SD ($^{**}P<0.01$, versus control; $^{\#\#}P<0.01$, versus SDF-1 alone group).

Hattermann et al., 2010]. Our results are consistent with those reports (Fig. 3), and give a new insight view of the mechanism for CXCR7 failed to mediate EPC migration or proliferation. NO was considered to be essential to SDF-1 induced EPCs migration and proliferation but not survival [Shao et al., 2008]. Our results revealed that SDF-1-triggered NO production were abolished entirely by CXCR4 blockage and were fully retained when blocking CXCR7 (Fig. 6). We conclude failure of CXCR7 in mediating EPCs migration, proliferation may attribute to that it is unable to mediate NO production. However, in other study, CXCR7 was reported to mediate cell proliferation in CT26 and KEP1 cell lines [Meijer et al., 2008], and our previous study revealed CXCR7 is essential to proliferation of EPCs derived from rat bone marrow [Dai et al., 2011]. The conflict revealed that role of CXCR7 in cell proliferation depends on not only cell specificity, but also species and tissue origin.

TEM is essential to EPCs mobilizing from bone marrow and engrafting into neo-angiogenesis sites [Shao et al., 2008]. Zabel et al. [2009] confirmed that CXCR7 played an essential role in the SDF-1/CXCR4 mediated TEM of $CXCR4^+CXCR7^+$ human tumor cells. In present study, we found that human EPCs TEM was entirely blocked by CXCR4 inhibition and impaired by CXCR7 blockage, but no additive effect by blocking both CXCR4 and CXCR7 (Fig. 5), implicating CXCR7 blockage can interfere with SDF-1/CXCR4 induced TEM.

EPCs adhesion to endothelial cells is antecedent step of TEM. Mazzinghi et al. [2008] reported that SDF-1 induced renal progenitor cell adhesion to endothelial cells via CXCR7 exclusively, blocking CXCR4 have no effect on cell adhesion. However, we found that SDF-1 induced enhancement of human EPCs adhesion to active HUVECs was attenuated by CXCR7 blockage and totally abolished by CXCR4 blockage (Fig. 3). The contrast may attribute to difference in CXCR7 location. Hartmann et al. [2008] reported that blocking intracellular CXCR7 can attenuate the ability of CXCR4 to properly rearrange by surface-bound SDF-1, thereby interfere with GPCR triggering optimal SDF-1-mediated stimulation of integrin activation, to impair $CD34^+$ cells adhension to endothelial cells. Indeed, CXCR7 and CXCR4 form functional heterodimers with higher responsiveness to SDF-1 than CXCR4 alone [Sierro et al., 2007; Levoye et al., 2009]. We deduced intracellular CXCR7 may give rise to CXCR7/CXCR4 complexes in human EPCs as it did in $CD34^+$ cells [Hartmann et al., 2008]. CXCR7 blockage can interfere with this process, thereby attenuate EPCs adhesion.

We further investigated the role of CXCR7 in the angiogenic potential of EPCs by examining tube formation in vitro. We found that either CXCR7 blockage or CXCR4 blockage can totally abolish EPCs tube formation (Fig. 5). There are two possible mechanisms in which tube formation is impaired: blocking of tube formation directly or induction of tube degeneration [Yancopoulos et al., 2000]. Results revealed that tube length of each group at 18 h (Fig. 5) is similar with that at 6 h (data not shown) post antagonist treatment, which indicated that CXCR7 or CXCR4 blockage impaired EPC tube formation directly but not by induction of tube degeneration. Our data is conformity with the most recent study that both CXCR7 and CXCR4 are required for the angiogenesis of endothelial cells in the rheumatoid arthritis [Watanabe et al., 2010].

Besides engraftment into angiogenesis site to form neo-vessels, EPCs can also promote angiogenesis by secreting pro-angiogenic factors [Ziegelhoeffer et al., 2004]. Existing researches demonstrated that CXCR7 regulated the expression of the pro-angiogenic factors, such as interleukin-8 (IL-8) and VEGF in tumor cells [Wang et al., 2008; Kollmar et al., 2010]. Whether or not CXCR7 regulates human EPCs pro-angiogenic factors secretion needs for further studies.

In conclusion, CXCR7 is scarcely expressed on cell surface of human EPCs derived from cord blood, but intercellular CXCR7 is considerable. CXCR7 has no effect on migration, proliferation, or NO production of EPCs, but exclusively mediates human EPCs survival. Besides that, CXCR7 mediates tube formation of EPCs induced by SDF-1 along with CXCR4. Meanwhile, CXCR7 blockage can interfere with SDF-1/CXCR4 induced human EPCs adhesion to active endothelium and TEM. Though had some conflict with study on EPCs derived from rat bone marrow, these results indicated that CXCR7 plays a critical role in EPC homing and participating in angiogenesis, and may be a potential target molecule in new therapies for angiogenesis-involved diseases.

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