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ORIGINAL ARTICLE

Effects of SDF-1α/CXCR4 on vascular smooth muscle cells and bone marrow mesenchymal cells in a rat carotid artery balloon injury model

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Summary
Bone marrow mensenchyme cells (BMSCs) can differentiate into endothelial progenitor cells which then migrate to injured sites for the repair of neointima, and stromal cell-derived factor-1α (SDF-1α) can mediate the migration of CXCR4 expressing stem/progenitor cells to injured sites for pair. Protein and mRNA expression of SDF-1α and CXCR4 were determined by RT-PCR, Western blot and ELISA. Immediately after common carotid artery balloon injury, the mRNA expression of SDF-1α in vascular smooth muscle cells (VSMCs) first increased and then decreased 7 days later. VSMCs transfected with SDF-1α siRNA did not express SDF-1α mRNA, but after transfection with SDF-1α siRNA, the SDF-1α content in injured VSMCs gradually returned to the baseline level. Normal BMSCs rarely expressed CXCR4 mRNA, but the CXCR4 mRNA expression on BMSCs increased significantly 4 days after common carotid artery injury and was maintained. The migration of BMSCs after artery injury was enhanced when compared with normal BMSCs, but SDF-1α siRNA transfection of VSMCs and AMD3100 treatment remarkably decreased the chemotaxis of BMSCs to VSMCs and SDF-1α, respectively. We conclude that the SDF-1α/CXCR4 axis plays an important role in the migration of BMSCs after balloon injury and can ultimately cause abnormal proliferation of the intima.

Key words: bone marrow mensenchyme cells; vascular smooth muscle cells; stromal cell-derived factor-1α

INTRODUCTION

Vascular smooth muscle cells (VSMCs) have been found to be related to atherosclerosis, restenosis after balloon injury (percutaneous transluminal coronary angioplasty and coronary stent implantation) and hypertension induced vascular remodeling (Schwartz 1994). Experiments confirm that, after vascular injury, mature VSMCs can switch from contractile phenotype to synthetic phenotype, and acquire the capability not only to proliferate and migrate but also to secrete numerous cytokins (Bochaton-Piallat et al. 1996). Bone marrow mensenchyme cells (BMSCs) have multipotentiality, and can differentiate into endothelial progenitor cells after stimulation and migrate from bone marrow to injured sites where they are involved in the repair of neointima through colonization and proliferation.

Stromal cell-derived factor-1α (SDF-1α) is a small cytokine belonging to the chemokine family that is officially designated Chemokine (C-X-C motif)

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ligand 12 (CXCL12). SDF-1α can direct the adherence of inflammatory cells in blood to endothelial cells and mediate the migration of inflammatory cells into subintima where they play an important role (Gleichmann et al. 2000). CXCR4 is a G protein coupled receptor of SDF-1α with seven-transmembrane domains and is widely expressed in numerous cells and non-haematopoietic organs. Studies have demonstrated that CXCR4 plays an important role in the migration of haematopoietic stem cells, and is also involved in cancer metastasis, human immunodeficiency syndrome, inflammation and ischemia (Kucia et al. 2004b). In the present study, a rat carotid artery balloon injury model was established, and then BMSCs and VSMCs were obtained and maintained *in vitro*. The effects of SDF-1α/CXCR4 on the proliferation and migration of these cells were determined.

MATERIALS AND METHODS

Animals, surgical procedures and cell culture

Male Sprague Dawley (SD) rats, 8-week-old, weighing 286±14.3 g, were purchased from the SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed in a temperature-controlled environment (22–24 °C) with a light dark regime of 12/12 hrs. All procedures were approved by the local ethics committee of the School of Medicine, Shanghai Jiao-Tong University (No. JYLL-10002) and animal use conformed to the National Institute of Health guidelines on the ethical use of animals and to the guidelines for caring for laboratory animals issued by the Ministry of Science and Technology of the People's Republic of China.

A rat common carotid artery balloon injury model was established as described in Tulis et al. (2001). Briefly, the rat was intraperitoneally anaesthetized with 2.5% pentobarbital sodium (40 mg/kg) and fixed in the supine position. A midline incision was made in the neck, and then the left common carotid artery and the bifurcation of internal and external carotid arteries were exposed. A "V" incision was made on the external carotid artery followed by insertion of a 2F thrombotic balloon catheter (Edwards Lifesciences, USA) deeply into the common carotid artery, and then the balloon was dilated by infusing 0.10~0.15 ml of normal saline. The catheter was subsequently drawn back to cause damage to the intima. Then, the normal saline was withdrawn and a catheter inserted to the common carotid artery. The procedures were performed twice in order to completely peel off the intima. Finally, an incision suture was performed. The rat was given *ad libitum* access to food and water.

Culture of VSMCs from common carotid artery

VSMCs from the common carotid artery were cultured using the method previously described by Ross et al. (1971). Male SD rats were sacrificed 0, 1 d, 4 d, 7 d, 14 d, and 1 m after common carotid artery balloon injury (S_0, S_{1d}, S_{4d}, S_{7d}, S_{14d} and S_{1m}) and the injured common carotid arteries were obtained. SD rats in the control group (healthy rats) were housed for 2 weeks and sacrificed followed by removal of the common carotid arteries. The arteries were washed with normal saline and opened longitudinally. The intima was removed and the remaining arteries were rinsed with PBS and cut into 1 mm^2 pieces. These pieces were maintained in DMEM (high glucose) (Gibco, USA) containing 10% FBS at 37 °C in humidified air with 5% CO_2 (incubator; Viscon Systems Sdn Bhd, Malaysia). Twenty four hour later, when adherence was observed, the medium was supplemented followed by culture. The medium was refreshed twice weekly.

Culture of BMSCs

BMSCs were cultured according to the method previously described by Law et al. (1996) and Barry and Murphy (2004). SD rats were sacrificed using this method, and bilateral femurs were obtained. The attached muscle and fascia were removed and femurs were fractured in the middle. The femurs were flushed with PBS and washing fluid was filtered through a filter (40 μm, BD Falcon) followed by centrifugation at 1500 rpm for 5 min at room temperature. The cells were resuspended in 1 ml of DMEM (low glucose) and a single cell suspension was prepared. The cells were maintained in dishes at 37 °C in humidified air with 5% CO_2. When adherence was observed (48–72 h later), the adherent red blood cells were removed by PBS washing followed by further culture. The medium was refreshed twice weekly. The acquired cells were detected with flow cytometry; the results showed that these cells were positive for CD44 and negative for CD34 and CD45.

Transfection of SDF-1α siRNA into VSMCs of rats with common carotid artery injury

The 3rd passage VSMCs from rats sacrificed at different time points were seeded in 35 mm dishes at a density of 3×10^5/ml. SDF-1α siRNA (Invitrogen, China) was mixed with FuGENE 6 Transfection Reagent (Roche, Swiss) at a ratio of 1:3 (v/v) followed by incubation for 15 min. Then, this mixture was added to the cells followed by incubation for

24 h. The cells were then harvested (Si_0, Si_{1d}, Si_{4d}, Si_{7d}, Si_{14d} and Si_{1m}).

Detection of cell proliferation by MTT assay

The 3rd passage VSMCs of healthy rats, of those with common carotid artery injury and VSMCs tranfected with SDF-1α siRNA were digested with 0.25% trypsin-EDTA (Amresco, USA) and a single cell suspension was prepared. The cells were seeded in a 96-well plate (100 μl/well) at a density of 2×10^5/ml followed by incubation in an incubator. When adherence was observed, synchronization was performed for 24 h, and MTT solution (5 g/l) (Sigma USA) was added to the cells (20 μl/well) followed by incubation for 4 h. Then, the medium was removed and DMSO (Sigma, USA) was supplemented (100 μl/well) followed by incubation for 10 min with continuous shaking until crystals resolved. Absorbance was determined with a microplate reader (VARIO Thermo Electron Technology, Japan) at 570 nm. The absorbance reflects the proliferation of VSMCs. The experiment was performed in triplicate and data were averaged. The experiment was repeated six times.

Detection of SDF-1α and CXCR4 mRNA expression in VSMCs and BMSCs by RT-PCR

Total RNA was extracted from the 3rd passage VSMCs and BMSCs with TRIzol (Invitrogen, Life Technologies, China), and RT-PCR was performed to detect the mRNA expression of SDF-1α in VSMCs and CXCR4 in BMSCs. The primers were synthesized by Shanghai Sangong Co., Ltd., China.

SDF-1α primers (381 bp):

Forward:
5'-CCAATCAGAAATGGGAACAAGA-3'
Reverse:
5'-GTAGGAGGCTTACAGCACGAA-3'

CXCR4 primers (267 bp)

Forward: 5'-GTGGGCAATGGGTTGGTAAT-3'
Reverse:
5'-GGTGGCGTGGACAATGGCAAGGTAG-3'

The cDNA was denatured at 94 °C for 1 min, followed by 34 cycles of amplification. Each cycle consisted of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 45 sec, with an additional extension for 5 min at 72 °C. The quality of the PCR products was determined by electrophoresis.

Detection of CXCR4 expression in BMSCs by Western blot

Total proteins were extracted from the 3rd passage BMSCs and protein concentration in the supernatant was measured spectrophotometrically at 595 nm. Then, 40 μg of proteins were loaded onto SDS polyacrylamide gel for electrophoresis (Invitrogen, China), and transferred onto PVDF membranes (Millipore, USA). The membranes were incubated with rabbit anti-mouse CXCR4 antibody (1:500; eBioscience, USA) and goat anti-mouse β actin antibody (1:1000; Santa Cruz, USA) overnight at 4 °C. Then, the membranes were incubated with secondary antibodies (donkey anti-rabbit antibody, 800DX 1:5000 eBioscience, USA; donkey anti-goat antibody, 700DX 1:2000, Sigma Chemical Company, USA) for 1 h, followed by detection with an Infrared Fluorescence Imaging and Analyzing System (Odyssey v1.2) (FIAS, Odyssay LI-COR, USA).

Enzyme-linked immunosorbent assay of plasma SDF-1α

The plasma level of SDF-1α was determined by the enzyme-linked immunosorbent assay (ELISA) using an ELISA kit (R&D system, Inc., USA) according to the manufacturer's instructions.

Detection of SDF-1α in the supernatants of VMSCs by ELISA

The 2nd passage VMSCs from healthy rats and injured rats sacrificed at different time points were seeded in a dish at a density of 2×10^5/100 mm. VMSCs in the Si group were transfected with SDF-1α siRNA. Then, 1 ml of supernatants was collected when 90% confluence was observed and stored at –80 °C for later use. The SDF-1α content was determined according to the manufacturer's instructions (Human SDF-1α Immunoassay, USA).

Detection of cell migration

Cells were divided into six groups (Table 1). Cell migration was determined according to the method previously described by Dowell et al (2003). Polycarbonate membranes with 8 μm pore size were inserted into the chambers (Corning, USA). VMSCs were glucose deprived for 24 h and washed with PBS. Cells were digested and re-suspended in DMEM (low glucose) containing 0.4% FBS. Cell density was adjusted to 1×10^6/ml. Cells with and without 200 ng/ml AMD3100 (octahydrochloride, Sigma, USA) treatment for 30 min were added into the upper chamber. Then, 500 μl of DMEM (low glucose) containing 0.4% FBS and with or without 100 ng/ml SDF-1α (Peprotech, UK) were added to the lower chamber. The injured VSMCs were collected when the SDF-1α content in the supernatant reached maximal level (P_{1d}). When 80~90% confluence was observed, the cells were harvested, washed, digested

Table 1. **Grouping and processing of VSMCs and BMSCs.**

Normal group	S group	S+A group	P group	P+A group	P+siRNA group
NMSC	NMSC+S	NMSC+S+A	NMSC+P_{1d}SMC	NMSC+P_{1d}SMC +A	NMSC+P_{1d}SMC +siRNA
	S_0MSC+S	S_0MSC+S+A	S_0MSC+P_{1d}SMC	S_0MSC+P_{1d}SMC +A	S_0MSC+P_{1d}SMC +siRNA
	S_{1d}MSC+S	S_{1d}MSC+S+A	S_{1d}MSC+P_{1d}SMC	S_{1d}MSC+P_{1d}SMC +A	S_{1d}MSC+P_{1d}SMC +siRNA
	S_{4d}MSC+S	S_{4d}MSC+S+A	S_{4d}MSC+P_{1d}SMC	S_{4d}MSC+P_{1d}SMC +A	S_{4d}MSC+P_{1d}SMC +siRNA
	S_{7d}MSC+S	S_{7d}MSC+S+A	S_{7d}MSC+P_{1d}SMC	S_{7d}MSC+P_{1d}SMC +A	S_{7d}MSC+P_{1d}SMC +siRNA
	S_{2w}MSC+S	S_{2w}MSC+S+A	S_{2w}MSC+P_{1d}SMC	S_{2w}MSC+P_{1d}SMC +A	S_{2w}MSC+P_{1d}SMC +siRNA
	S_{1m}MSC+S	S_{1m}MSC+S+A	S_{1m}MSC+P_{1d}SMC	S_{1m}MSC+P_{1d}SMC +A	S_{1m}MSC+P_{1d}SMC +siRNA

S group: SDF-1α treatment group; S+A group: SDF-1α+AMD3100 treatment group; P group: injured VSMCs group; P+A group: injured VSMCs+AMD3100 treatment group; P+siRNA group: injured VSMCs with SDF-1α siRNA transfection group;
NMSC: normal BMSCs; S_0MSC: BMSCs collected immediately after injury; S_{1d}MSC: BMSCs collected 1 d after injury; S_{4d}MSC: BMSCs collected 4 d after injury; S_{7d}MSC: BMSCs collected 7 d after injury; S_{2w}MSC: BMSCs collected 2 weeks after injury; S_{1m}MSC: BMSCs collected 1 month after injury;
S: SDF-1α 100 ng/ml; A: AMD3100 200 ng/ml.

and resuspended in DMEM (low glucose) containing 0.4% FBS. Cell density was adjusted to 1×10^6/ml, and 500 μl of cell suspension were added to the lower chamber. The transwell chamber was incubated for 6 h. The upper chamber was taken out and cells were scrubbed with cottons. Then, the cells were washed with PBS and fixed in ice cold 95% ethanol for 10 min. Staining with hematoxylin was performed for 15 min and cells were counted under a light microscope (×200). Five fields in each section were randomly selected and data were averaged. The experiment was repeated 3 times.

Statistical analysis

Experiments were performed at least thrice and data presented as the mean ± standard deviation (SD). Statistical analysis was performed with SPSS version 13.0 (SPSS Inc., Chicago, USA). The unpaired *t* test was employed for comparison between the two groups and one-way analysis of variance (ANOVA) among multiple groups. Data were statistically evaluated at the significance level of $2\alpha=0.05$.

RESULTS

Proliferation of VSMCs

An MTT assay was performed in the VSMCs from rats with or without common carotid artery injury. Results showed that the proliferation of VSMCs from rats with common carotid artery injury was significantly more active than in those from healthy rats (statistically significant) (Table 2).

mRNA expression of SDF-1α in VSMCs

PCR was performed to detect the mRNA expression of SDF-1α in VSMCs; the results showed the mRNA expression of SDF-1α was markedly decreased in normal VSMCs when compared with that in rats with common carotid artery injury. SDF-1α mRNA expression increased immediately after injury and the enhanced expression of SDF-1α was more evident in the S_{1d}, S_{4d} and S_{7d} groups (Fig. 1A). However, the mRNA expression of SDF-1α was not detectable in VSMCs transfected with SDF-1α siRNA (Fig. 1B).

Table 2. **Proliferation of VSMCs detected by MTT assay.**

N group	S group						Si group					
	S_0	S_{1d}	S_{4d}	S_{7d}	S_{2w}	S_{1m}	Si_0	Si_{1d}	Si_{4d}	Si_{7d}	Si_{2w}	Si_{1m}
0.405± 0.011	0.487± 0.033*	0.574± 0.098*	0.698± 0.075*	0.805± 0.100*	1.317± 0.112*	1.540± 0.173*	0.497± 0.068*	0.593± 0.096*	0.684± 0.116*	0.841± 0.120*	1.392± 0.109*	1.576± 0.097*

* statistically significant versus controls

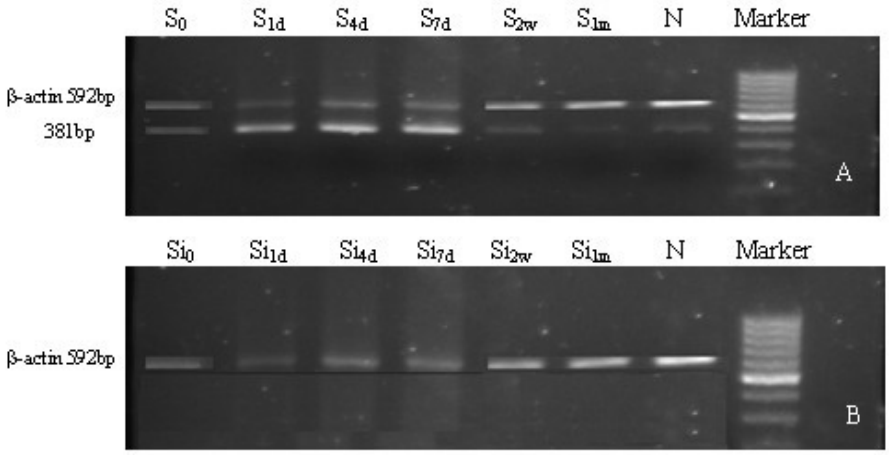

Fig. 1. **mRNA expression of SDF-1α in VSMCs.** *A)* mRNA expression of SDF-1α in VSMCs from rats with common carotid artery injury, *B)* mRNA expression of SDF-1α in injured VSMCs with SDF-1α siRNA transfection.

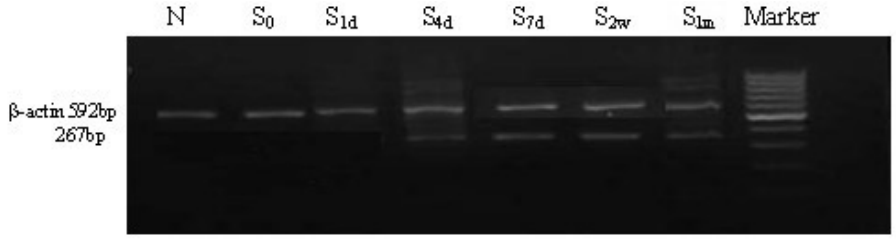

Fig. 2. **mRNA expression of CXCR4 in BMSCs from rats with common carotid artery injury.**

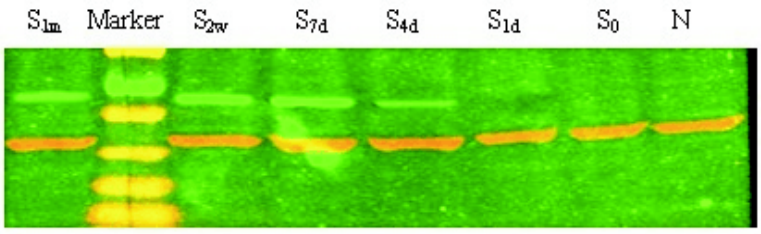

Fig. 3. **Protein expression of CXCR4 in BMSCs of SD rats with common carotid artery injury.**

mRNA expression of CXCR4 in BMSCs
PCR was performed to detect the mRNA expression of CXCR4 in BMSCs and the results indicated that CXCR4 mRNA expression was hardly detectable in NMSCs. However, 4 days after injury, the mRNA expression of CXCR4 increased (Fig. 2).

Protein expression of CXCR4 in BMSCs
The CXCR4 expression in BMSCs was determined by Western blot and results did not show CXCR4 protein expression in NMSCs. However, 4 days after injury, the protein expression of CXCR4 increased (Fig. 3).

Plasma level of SDF-1α after intimal injury
As shown in Table 3, the plasma level of SDF-1α after intimal injury was markedly increased and reached the maximum 1 day after injury (statistically significant) followed by a rapid decrease to the baseline level on day 7. The administration of AMD3100 did not affect the plasma level of SDF-1α.

SDF-1α content in the supernatants of VSMCs
ELISA was performed to detect the SDF-1α content in the supernatants of VSMCs. Results showed that the SDF-1α content in normal VSMCs was lower than that in injured VSMCs. The SDF-1α content increased immediately after common carotid artery injury and reached a maximal level 1 day after injury. However, 7 days after injury, the SDF-1α content decreased to a normal level. In VSMCs transfected with SDF-1 siRNA; the SDF-1α content remained at a baseline level (Table 4 and 5).

Effects of VSMCs on the migration of BMSCs
The effect of VSMCs on the chemotaxis of BMSCs was determined by a Transwell chamber assay. Under normal conditions, there were no chemokines in the lower chamber and no obvious chemotaxis of normal BMSCs was found. When 100 ng/ml SDF-1α was added to the lower chamber, the chemotaxis of BMSCs from injured and normal rats was markedly enhanced when compared with BMSCs without SDF-1α treatment (statistically significant). Furthermore, the chemotaxis of BMSCs from injured rats was more evident than that from normal rats (statistically significant). When 200 ng/ml AMD3100, an antagonist of CXCR4, was supplemented to the upper chamber followed by incubation for 30 min, the chemotaxis of BMSCs was dramatically inhibited. When the injured VSMCs were added to the lower chamber, the chemotaxis of BMSCs was also noted and it was more obvious than that induced by SDF-1α (statistically significant). In addition, after AMD3100 treatment of BMSCs or SDF-1α siRNA transfection of VSMCs, the chemotaxis of BMSCs induced by VSMCs declined, and it was more evident than that induced by SDF-1α after AMD3100 treatment (statistically significant) (Fig. 4A–F) (Table 6).

DISCUSSION

BMSCs have been a hot topic in studies on the treatment of diseases, especially cardiovascular diseases (acute myocardial infarction). Increasing evidence has shown the efficacy of BMSCs. The goal of treatment with BMSCs is to achieve normal and healthy tissues and maintain the whole function. Currently, the treatment of myocardial infarction with stem cells can be performed in three ways: (1) Stem cell transplantation: the mature stem cells are harvested and injected into the area with infarction; (2) Stem cell mobilization: the ability of stem cells to repair the injured heart is enhanced through mobilization of stem cells from bone marrow, and (3) Local treatment with cytokines: stem cells (stem cells in bone marrow and circulation) can regulate the expression of cytokines and growth factors enhancing the ability to repair the injured heart.

Stem cells are characterized by the ability to renew themselves and differentiate into a diverse range of specialized cell types. Tissue-committed stem cells (TCSCs) are cells with multiple differentiation potential in mature individuals. Currently, BMSCs have been found to have the ability to transform. Analysis of the cell cycle of BMSCs shows that about 20% of BMSCs are in the quiescent phase (G0 phase), which suggests that BMSCs have a potent ability to proliferate. The number of BMSCs may be increased by 2~4 fold at each passage. Minguell et al. (2001) speculated that BMSCs are a non-haematopoiesis derived cell population, and have a high requirement for nutrition. The proportion of BMSCs in the bone marrow is extremely low and accounts for about 0.001–0.01% of cells in bone marrow. Therefore, the application of BMSCs should be realized through *in vitro* culture and amplification (Pittenger et al. 1999). Numerous studies have demonstrated the effectiveness of stem cells in ischemic injury (Orlic et al. 2002, Kucia et al. 2004a, Mathur and Martin 2004). Bone marrow is the main stem cell bank in the body, and stem cells in bone marrow can be activated in an appropriate environment, and motivated into peripheral blood. Ratajczak et al. (2004) and Papayannopoulou (2000) speculated that bone marrow not only stores a lot of haematopoietic stem cells (HSCs) but also provides space for the storage of TCSCs from peripheral blood,

Table 3. **Plasma level of SDF-1α after intimal injury** (x±s, ng/ml, n=12 per group)**.**

	Normal group (N group)	Common carotid artery injury group (S group)					
		S_0	S_{1d}	S_{4d}	S_{7d}	S_{1m}	S_{3m}
SDF-1α (ng/ml)	0.312± 0.006	0.885± 0.022*	1.328± 0.009*	1.119± 0.013*	0.323± 0.005	0.320± 0.006	0.309± 0.056

symbols as in Table 2

Table 4. **SDF-1α content in the supernatants of injured VSMCs** from rats sacrificed at different time points.

	Normal group (N group)	Common carotid artery injury group (S group)					
		S_0	S_{1d}	S_{4d}	S_{7d}	S_{2w}	S_{1m}
SDF-1α (ng/ml)	0.692± 0.047	0.324± 0.070*	1.897± 0.058*	1.519± 0.063**	0.711± 0.068	0.706± 0.038	0.676± 0.044

* statistically significant as compared with controls; ** statistically significant vs NMSC group

Table 5. **SDF-1α content in the supernatants of injured VSMCs** transfected with SDF-1α siRNA from rats sacrificed at different time points.

	Normal group (N group)	siRNA transfection group (Si group)					
		Si_0	Si_{1d}	Si_{4d}	Si_{7d}	Si_{2w}	Si_{1m}
SDF-1α (ng/ml)	0.692± 0.047	0.726± 0.057	0.742± 0.061	0.703± 0.024	0.685± 0.037	0.715± 0.049	0.700± 0.041

symbols as in Table 4

including stem cells in the muscle, liver, brain and heart. After stress or injury (such as acute myocardial infarction), TCSCs in the peripheral blood increase significantly and are involved in the repair of injured tissues.

Currently, the mechanism underlying stem cell mobilization is still poorly understood, and may involve numerous cytokines, a variety of proteinases (such as elastase, matrix metalloproteinase and protease G), adhesion molecules and vascular endothelial cells in an haematopoietic microenvironment. These factors interact with each other and finally result in the release of HSCs from bone marrow to peripheral blood (Lapidot and Petit 2002). Many cytokines have been found to stimulate the motivation of BMSCs (mainly HSCs) to peripheral blood including G-CSF, GM-CSF, SCF VEGF, IL-8 and SDF-1 (Misao et al. 2006). The SDF-1α could enhance the binding of tissues expressing SDF-1α to CXCR4 on the circulation progenitor cells. In addition, the number of $CD34^+CXCR4^+$ cells is related to the SDF-1α level after CP/CPB. However, this relationship was not noted before CP/CPB and 4 days after CP/CPB. These findings suggest that the motivation of progenitor cells and production of key cytokines occurred a few hours after injury. Misao et al (2006) also demonstrated that AMD3100, an antagonist of CXCR4, could block the interaction between $CXCR4^+$ cells and SDF-1α, and also counteract the improvement of myocardial function by G-CSF in acute myocardial infarction.

VSMCs have been shown to exist in two phenotypic states: contractile phenotype and synthetic phenotype. Normal VSMCs are mainly characterized by a differentiated phenotype. When vessels are

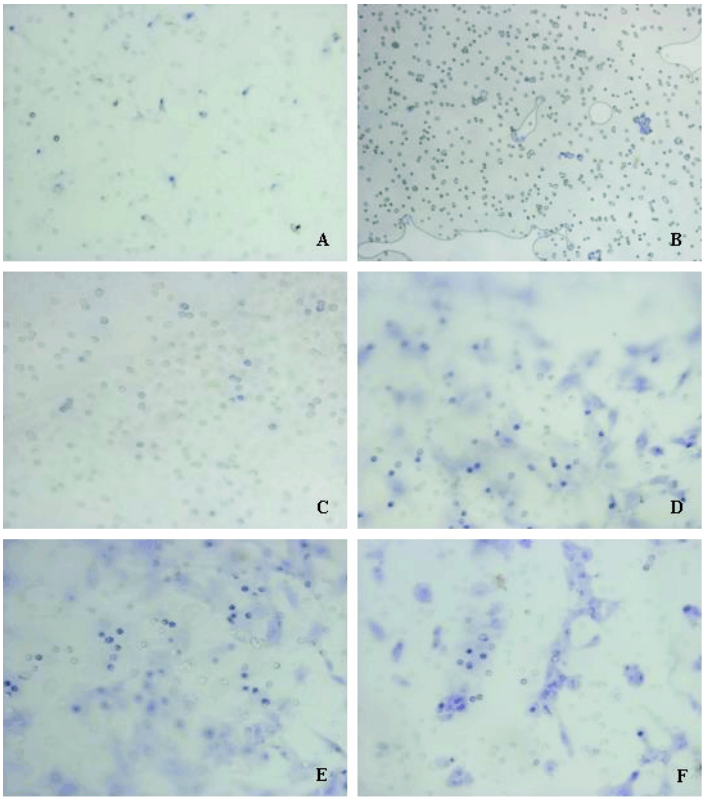

Fig. 4. **Effects of VSMCs on the migration of BMSCs.** *A)* normal BMSCs (NMSC group), *B)* SDF-1α treatment group (S group), *C)* SDF-1α+AMD3100 treatment (S+A group), *D)* intervention with injured VSMCs (P group), *E)* intervention with injured VSMCs and AMD3100 (P+A group), *F)* injured VSMCs transfected with SDF-1α siRNA (P+siRNA group).

injured or these cells are treated with certain growth factors, vasoactive substances or neurotransmitters *in vitro*, the phenotype is switched from differentiated to dedifferentiated and these cells are able to proliferate. Zhang et al. (2008) found that, after culture of vessels with endothelial cell injury, the VSMCs started to proliferate and the proliferation increased over time. Not only were VSMCs switched from contractile phenotype to synthetic phenotype, but an obvious proliferation of VSMCs was noted. These findings are also observed in atherosclerosis plaque and restenosis after percutaneous transluminal angioplasty. In the present study, the endothelial cells of the common carotid artery were injured and VSMCs were maintained *in vitro*. These VSMCs were characterized by synthetic phenotype and active proliferation. In addition, the proliferation of injured VSMCs was not affected by SDF-1α siRNA transfection.

After injury, SDF-1 expression can be detected in the injured intima and media, and CXCR4 expression is also detectable in the intima. Gao and Li (2007) transplanted the abdominal aorta of Wistar rats to SD rats, and the results showed that the proliferation of neointima in the abdominal aorta graft of SD rats was almost completely suppressed after SDF-1 antibody treatment. Furthermore, the thickness of neointima was significantly related to the SDF-1 level.

Our study showed that the mRNA expression of SDF-1α increased immediately after common carotid artery injury and that the increase of mRNA expression continued until 4 days after injury. However, the mRNA expression of SDF-1α 7 days, 2 weeks and 1 month after injury was similar to that before injury. ELISA was performed to detect the SDF-1α content in the plasma and in the cell culture supernatants respectively, and the results showed that the SDF-1α content increased immediately after common carotid artery injury both *in vivo* and *in vitro*. The SDF-1α content reached a maximal level 1 day after injury and returned to normal level 7 days after injury. There was a relationship between SDF-1α expression in VSMCs and SDF-1α content in the supernatants, and our results were consistent with

Table 6. **Effect of VSMCs on the chemotaxis of BMSCs determined by Transwell chamber assay.**

Group		Number of cells
NMSC group		6.21±0.35
SDF-1α treatment (100 ng/L S group)	NMSC+S	13.42±0.58*
	S_0MSC+S	22.67±0.61**#
	S_{1d}MSC+S	25.71±0.34**#
	S_{4d}MSC+S	24.85±0.64**#
	S_{7d}MSC+S	25.19±0.73**#
	S_{2w}MSC+S	26.01±0.26**#
	S_{1m}MSC+S	25.85±0.17**#
SDF-1α+AMD3100 treatment (AMD3100 200 ng/ml S+A group)	NMSC+S+A	6.24±0.54
	S_0MSC+S+A	6.66±0.35
	S_{1d}MSC+S+A	6.24±0.19
	S_{4d}MSC+S+A	6.37±0.48
	S_{7d}MSC+S+A	6.69±0.71
	S_{2w}MSC+S+A	7.05±0.63
	S_{1m}MSC+S+A	7.15±0.27
injured VSMCs (P group)	NMSC+P_{1d}	12.94±0.56*
	S_0MSC+P_{1d}	30.69±0.47**##
	S_{1d}MSC+P_{1d}	34.46±0.73**##
	S_{4d}MSC+P_{1d}	32.58±0.61**##
	S_{7d}MSC+P_{1d}	34.82±0.39**##
	S_{2w}MSC+P_{1d}	33.73±0.54**##
	S_{1m}MSC+P_{1d}	33.15±0.81**##
injured VSMCc+AMD3100 treatment (P+A group)	NMSC+P_{1d}+A	10.56±0.11*^
	S_0MSC+P_{1d}+A	11.25±0.51*^
	S_{1d}MSC+P_{1d}+A	11.68±0.48*^
	S_{4d}MSC+P_{1d}+A	10.96±0.39*^
	S_{7d}MSC+P_{1d}+A	11.23±0.16*^
	S_{2w}MSC+P_{1d}+A	10.33±0.53*^
	S_{1m}MSC+P_{1d}+A	10.98±0.72*^
injured VSMCs transfected with SDF-1α siRNA (P+siRNA group)	NMSC+P_{1d}+siRNA	10.25±0.23*^
	S_0MSC+P_{1d}+siRNA	10.54±0.67*^
	S_{1d}MSC+P_{1d}+siRNA	11.67±0.53*^
	S_{4d}MSC+P_{1d}+siRNA	10.98±0.28*^
	S_{7d}MSC+P_{1d}+siRNA	11.61±0.36*^
	S_{2w}MSC+P_{1d}+siRNA	10.88±0.17*^
	S_{1m}MSC+P_{1d}+siRNA	11.58±0.64*^

* statistically significant as compared with controls
** statistically significant vs NMSC group
\# statistically significant vs normal BMSCs at the same group
^ statistically significant vs S+A group

previous reports (Suda et al. 1987). These findings suggest that injury stimulates the transcription and translation of SDF-1α and finally leads to increased protein expression of SDF-1α.

SDF-1 can specifically bind to CXCR4 and then induce the migration of monocytes, lymphocytes and endothelial cells, which play important roles in the embryonic development. In humans and rats, SDF-1 expression is sustained and can be regulated by the changes in the surrounding environment. The SDF-1 gene is extremely conservative among different species. Human SDF-1 gene is highly homologous

with murine SDF-1 gene (99%), and only one amino acid was different between human SDF-1 gene and murine SDF-1 gene. CXCR4, a receptor of SDF-1, is coupled with a G protein, and an orphan receptor. The CXCR4 gene is 32% homologous with the IL-8 receptor gene (Loetscher et al. 1994). CXCR4 is constitutively expressed in a lot of cells, especially on haematopoietic stem/progenitor cells. SDF-1 is the unique ligand of CXCR4 which is the exclusive receptor of SDF-1 having definite physiological functions; the important difference between SDF-1/CXCR4 and other chemokines/chemokine receptors. When compared with other chemokines, SDF-1 has more extensive biological activities and expressions of SDF-1/CXCR4 vary from different systems including the nervous system, the vascular system and the haemopoietic system. Studies have shown that SDF-1/CXCR4 play crucial biological roles in the development of the haemopoietic system, nervous system, the cardiovascular system and HIV infection (Feng et al. 1996). SDF-1 can be constitutively produced in many organs, but in bone marrow, SDF-1 is mainly expressed on endothelial cells and immature osteoblasts. SDF-1 is a highly basic protein, and can bind to the heparan sulfate on stromal cells through a series of basic amino acid residuals. The signal area in the N-terminal is then exposed and this facilitates the binding between SDF-1 and CXCR4. The SDF-1 that binds to heparan sulfate is then fixed on the cell membrane, which is the active form of SDF-1 (Amara et al. 1999). The binding between SDF-1 and CXCR4 is the basis of the biological functions of SDF-1.

SDF-1 can confer potent chemotactic effects on the *in vitro* $CD34^+$ BMSCs in a dose dependent manner (Yamaguchi et al. 2003). After intravenous injection of adenovirus carrying SDF-1α plasmids, HSCs were significantly motivated (Hattori et al. 2000, Moore et al. 2001), which however could be suppressed by the neutralizing antibodies of CXCR4 and SDF-1 (Petit et al. 2002). After transfection with the lentivirus carrying CXCR4 gene, $CD34^+$ BMSCs had an over-expression of CXCR4, which could improve the chemotaxis induced by low dose SDF-1, and prolong the survival time of progenitor cells with CXCR4 over-expression. In addition, the SDF-1/CXCR4 axis plays an important role in the chemotaxis of bone marrow-derived cardiac progenitor cells (Suda et al. 1987) in acute myocardial infarction. Therefore, the SDF-1/CXCR4 axis is an important participant in the motivation of bone marrow stem cells and in the direction of stem/progenitor cells to tissues.

In the present study, a Transwell chamber assay was performed to evaluate the effects of SDF-1α and injured VSMCs on the chemotaxis of BMSCs. Results showed that the chemotaxis of normal BMSCs was almost undetectable without SDF-1α treatment. After SDF-1α treatment, the chemotaxis of normal BMSCs was remarkably enhanced. Furthermore, the chemotaxis of BMSCs from rats with artery injury was more evident than that of normal BMSCs after SDF-1α treatment. After treatment with AMD3100, the chemotaxis of BMSCs from rats with artery injury and normal BMSCs was alleviated significantly. In addition, the normal BMSCs and BMSCs from rats with artery injury were co-cultured with injured VSMCs, and the results showed that the chemotaxis of BMSCs was enhanced, and more obvious than that of BMSCs induced by SDF-1α alone. However, when injured VSMCs were transfected with SDF-1α siRNA, the chemotaxis of normal BMSCs and BMSCs BMSCs from rats with artery injury was attenuated, but still more evident than that of BMSCs induced by SDF-1α alone. After treatment with AMD3100 to block the interaction between SDF-1α and CXCR4, the chemotaxis of BMSCs induced by injured VSMCs was alleviated. These findings suggest that the interaction between SDF-1α and CXCR4 was involved in the chemotaxis of BMSCs induced by the injured VSMCs.

Moreover, the mRNA and protein expressions of CXCR4 were determined in BMSCs. Results showed the mRNA expression of CXCR4 gradually increased and sustained after common carotid artery injury. This result suggested the proportion of $CXCR4^+$ cells was increased after the BMSCs were stimulated, which may be as a result of release of a variety of cytokines and inflammatory factors. At the early stage of injury, apoptosis and necrosis may induce the chemotaxis of numerous inflammatory cells including neutrophils and macrophages. A large number of cytokines and growth factors are then released from inflammatory cells, induce the migration of circulation TCSCs to injured sites and are involved in the repair. In the angiogenesis after injury, endothelial cells can originate in two ways: (1) the adjacent vascular endothelial cells can form new vessels through sprouting or migration (Folkman and Shing 1992); (2) the endothelial progenitor cells (EPCs), a subpopulation of BMSCs, can form new vessels through transdifferentiation (Shi et al. 1998). Thus, BMSCs play an important role in angiogenesis. Evidence has demonstrated that BMSCs could aggregate in the post-ischemic hind legs of mice, where angiogenesis has occurred, and could promote angiogenesis (Asahara et al. 1997, Takahashi et al. 1999). In the migration of BMSCs to injured sites, cytokines play a critical role. Numerous factors are involved in the whole process of repair including stem cell motivation related factors (G-CSF,

GM-CSF, SCF (Metcalf and Nicola 1983, Anderson et al. 1990), SDF-1, IL-8 (Ulich et al. 1991, Laterveer et al. 1995, Fibbe et al. 2000) and VEGF (Asahara et al. 1999, Hattori et al. 2001, Paul and Steven 2002, Simons and Ware 2003). In the present study, the effect of SDF-1α on the chemotaxis of BMSCs was detected through a Transwell chamber assay, and the results demonstrated that SDF-1α could induce the chemotaxis of BMSCs which could be blocked by AMD3100, an antagonist of CXCR4. These findings suggest that SDF-1α could induce the chemotaxis of $CXCR4^+$ cells among BMSCs. After injury, SDF-1α expression increases gradually and a concentration gradient of SDF-1α forms accompanied by increased CXCR4 expression on cells. These changes may promote migration of BMSCs to the injured sites and their involvement in repair.

RNA interference (RNAi) is a potent tool. siRNA is synthesized *in vitro*, and can specifically bind to a target gene resulting in the silence of the target gene and the suppressed expression of the target gene. In the present study, SDF-1α siRNA was synthesized and transfected into injured VSMCs. The results showed that the mRNA and protein expressions of SDF-1α were markedly decreased in VSMCs after transfection with SDF-1α siRNA accompanied by suppressed migration of BMSCs induced by injured VSMCs, which may finally decrease the repair of injured intima and result in subsequent intimal thickening.

In the present study, VSMCs and BMSCs were collected from normal rats and rats with common carotid artery injury; the protein and mRNA expression of SDF-1α and CXCR4 were determined. In addition, the effects of VSMCs, SDF-1α siRNA and AMD3100 on the chemotaxis of BMSCs were investigated. The results showed that SDF-1α siRNA could decrease the mRNA and protein expression of SDF-1α, and AMD3100 treatment could inhibit the chemotaxis of BMSCs induced by SDF-1α or injured VSMCs. Additionally, the SDF-1α expression increased in VMSCs after common carotid artery injury and SDF-1α could induce the chemotaxis of BMSCs. Based on these findings, we speculated that increased SDF-1α expression in VMSCs after common carotid artery injury could promote the migration of BMSCs to injured sites where they are involved in the repair of injury. Therefore, increasing the circulating SDF-1α level at the early stage of injury may recruit the $CD34^+CXCR4^+$ cells and facilitate the endothelialisation of the injured artery leading to construction of an integrated normal endothelial barrier which can block the action of circulating cytokines on the injured sites. Additionally, at a late stage of injury, blocking the interaction between SDF-1α and CXCR4 may prevent the $CD34^+CXCR4^+$ cells from accumulating in the injured sites and inhibit the post-traumatic hyperplasia of vascular smooth muscle cells which is also beneficial for the proliferation of newly generated intimal cells.

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