Cloning, expression and identification of an isoform of human stromal cell derived factor-1α

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Abstract. Human stromal cell derived factor-1α (hSDF-1α), a chemotactic factor of stem cells, regulates inflammation, promotes the mobilization of stem cells and induces angiogenesis following ischemia. Six SDF-1 isoforms, SDF-1α, SDF-1β, SDF-1γ, SDF-1δ, SDF-1ε and SDF-1φ, which all contain a signal peptide at the N-terminus, have been reported. In the present study a special isoform of hSDF-1α is described that does not contain the N-terminal signal peptide sequence. The *hSDF-1α* gene was cloned with the recombinant plasmid pCMV-SPORT6-hSDF1 as the template, and the prokaryotic expression vector pET15b-hSDF-1α was constructed. This hSDF-1α was successfully expressed as an inclusion body in *Escherichia coli* BL21(DE3). The recombinant hSDF-1α was refolded *in vitro* and separated by cation exchange chromatography. Following these two steps the purity of the hSDF-1α was able to reach >85%. The recombinant hSDF-1α was then purified by size-exclusion chromatography. SDS-PAGE analysis demonstrated that the purity of the hSDF-1α was >95%, which meets almost all the requirements of a protein experiment. Chemotactic activity of the recombinant hSDF-1α was analyzed by Transwell migration assay and it was found that the recombinant hSDF-1α was able to stimulate THP-1 cell migration. These data suggest that the procedure of producing recombinant hSDF-1α proteins with chemotactic activity was feasible and the N-terminal signal peptide of hSDF-1α has little effect on the chemotactic activity of hSDF-1α.

Introduction

Ischemic cerebrovascular disease is a disease with a high incidence worldwide. Progress has been made in the treatment of ischemic cerebrovascular disease, and the application and curative effect of stem cells has become an area of increasing interest. Stromal cell derived factor 1 (SDF-1) is currently the strongest known chemotactic factor associated with stem cell mobilization. It can activate a series of signal transduction pathways through binding to its specific receptor CXC chemokine receptor 4 (CXCR4), and thus it plays a role in the regulation of inflammation, mobilization of stem cells and induction of angiogenesis following ischemia of tissues. Voermans *et al* (1) demonstrated that human SDF-1 (hSDF-1α) induced the aggregation of intracellular actin in $CD34^+$ precursor cells, stimulated the tyrosine phosphorylation of focal adhesion proteins and changed cytoskeletal structures, and activated the migration of hematopoietic stem cells, by rhodamine staining and fluorescence-activated cell sorting. The combined effects of SDF-1 and vascular endothelial growth factor can markedly improve the survival time of endothelial cells. SDF-1 has been found to increase $CD34^+$ cell proliferation, inhibit apoptosis and promote the differentiation of $CD34^+$ cells (2,3), which indicates that SDF-1 plays an important role in the proliferation of hematopoietic stem cells and the process of homing and mobilization; therefore, studies on SDF-1 have attracted growing attention. SDF-1 was first found in cytokines secreted by the mouse bone marrow stromal cell line pA6 in 1994 (4). Since four conserved cysteine residues in the C-terminal and two cysteines in the N-terminal of its amino acid sequence are separated by another amino acid, SDF-1 is classified into the CXC subfamily of chemokines, and is also known as CXCL12 (5). The *hSDF-1* gene is located in chromosome 10q11.1 (6) and encodes different proteins due to its different splicing modes. The Gen Bank accession numbers for these different cDNAs and their associate proteins are SDF-1α, SDF-1β, SDF-1γ, SDF-1δ, SDF-1ε and SDF-1φ. hSDF-1α is an 89-amino-acid protein while SDF-1β, SDF-1γ, SDF-1δ, SDF-1ε and SDF-φ encode 93, 119, 120, 90 and 100-amino-acid proteins, in all of which the first 89 amino acids are identical to those of SDF-1α (7,8). The full-length cDNA of *hSDF-1α,* which encodes 89 amino acids including the N-terminal signal peptide, is ~270 bp. A signal peptide cleavage site exists between the twentieth and twenty-first amino acids of the N-terminal of hSDF-1α (9). Crump *et al* (10) studied the NMR structure of SDF-1 in different solution conditions. The results showed that chemokine tertiary structure consists of a flexible N-terminus connected by an extended 'N-loop' and a single turn of a 3_{10}-helix to a three-stranded β-sheet and a

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C-terminal α-helix. The functional domain of the C-terminal is important to maintain SDF-1 conformation and the β-helix to regulate the activity of the SDF-1 in its interaction with glycosaminoglycans. The structure of the N-terminal is also important in its interactions with CXCR4; however the effect of the signal peptide on the activity of hSDF-1α is unknown.

In the present study, the process and technology of cloning and expressing the hSDF-1α protein without the N-terminal signal peptide was reported, and the chemotactic activity of the recombinant hSDF-1α was identified. This study may help to understand the technology of the recombinant and purified hSDF-1α, the diverse physiological functions of hSDF-1α, the activity of hSDF-1α and the effects of N-terminal signal peptide on the activity of hSDF-1α.

Materials and methods

HSDF-1α cloning and expression vector construction. Primers were designed according to the *hSDF-1α* cDNA sequence provided by the National Center for Biotechnology Information (NM_199168). The gene, which had mature peptide sequences of 213 bp without the signal peptide, was cloned from the total DNA of the pCMV-SPORT6-SDF1α plasmid (GeneCopoeia, Guangzhou, China) using polymerase chain reaction (PCR) amplification with sense primer 5'-GAT GCCATGGACGGGAAGCCCGTCAGC-3' and antisense primer 5'-CGCGGATCCTTACTTGTTTAAAGCTTTCTC CAGGT-3' (*Nco*I and *Bam*HI restriction sites are underlined) (11). Primers were synthesized by the Beijing Dingguo Changsheng Biotechnology Co. Ltd. (Beijing, China). The PCR amplification conditions were as follows: 98˚C for 5 min, 94˚C for 30 sec, 60˚C for 30 sec, 72˚C for 30 sec with a total of 30 cycles, and 72˚C for 10 min for extension. PCR products were identified by 1.5% agarose gel electrophoresis, retrieved and purified with a gel extraction kit (Omega Bio-Tek, Inc., Norcross, GA, USA). The retrieved target fragments and vector pET-15b (Beijing Dingguo Changsheng Biotechnology Co. Ltd.) were double digested with *Nco*I and *Bam*HI restriction enzymes (Takara Bio Inc., Otsu, Japan) at 37˚C for 6 h, respectively. Digested fragments and plasmids were retrieved with a gel extraction kit (Takara Bio Inc.) and incubated with T4 ligase (Takara Bio Inc.) at a constant temperature of 16˚C overnight. The ligation products were then transformed into competent cells of *Escherichia coli* (*E.coli*) DH5α (Beijing Dingguo Changsheng Biotechnology). The transformed DH5α cells were cultured on Luria-Bertani (LB) solid medium containing 100 μg/ml ampicillin sodium salt (Sigma-Aldrich, St. Louis, MO, USA) overnight to screen the positive clones that were sent to Shanghai Biological Engineering Co. Ltd. (Shanghai, China) for sequencing. The recombinant plasmid was verified as correct by sequencing and named as pET-15b-hSDF-1α.

Inducible expression and analysis of recombinant protein hSDF-1α. The recombinant hSDF-1α was expressed in the *E. coli* cell strain BL21(DE3) (Beijing Dingguo Changsheng Biotechnology). Bacterial cells transformed with the pET-15b-hSDF-1α plasmid were grown in LB fluid medium supplied with 100 μg/ml ampicillin sodium salt at 37˚C with shaking at 220 rpm for 12 h (DDHZ-300; TaiCang Experimental Equipment Factory, Suzhou, China). The following day, 1 ml culture broth was inoculated in 100 ml fresh LB fluid medium supplied with 100 μg/ml ampicillin sodium salt and grown at 37˚C with shaking at 220 rpm for 3 h. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mmol/l to induce gene expression for ~8 h at 30˚C with shaking at 220 rpm until the optical density at 600 nm was 0.7. Bacterial cells were subsequently collected by centrifugation at 4,444 x g for 20 min at 4˚C and frozen at -20˚C. The expression of target proteins was analyzed by Tris-Tricine-SDS-PAGE electrophoresis (12).

Refolding of recombinant hSDF-1α protein. The bacterial cell precipitate was mixed with ultrasound lysis buffer (5 mmol/l EDTA disodium salt and 10 mmol/l Tris-HCl, pH 8.0) at a ratio of 1:10 (w/v). The precipitate containing recombinant hSDF-1α was collected following ultrasonication centrifugation at 8,888 x g for 10 min and dissolved by denaturing buffer (6 mol/l guanidine hydrochloride, 10 mmol/l β-mercaptoethanol, 5 mmol/l EDTA disodium salt and 10 mmol/l Tris-HCl, pH 8.0) at a ratio of 1:5 (w/v). The supernatant containing denatured recombinant hSDF-1α was then collected by centrifugation at 8,888 x g for 30 min and refolded by dropwise dilution at 4˚C with stirring into refolded buffer (10 mmol/l Tris HCl, pH 8.0, 5 mmol/l EDTA disodium salt, 1 mmol/l reduced glutathione and 0.2 mmol/l oxidized glutathione) to yield a final protein. Following stirring overnight at 4˚C the refolded hSDF-1α was isolated by centrifugation at 8,888 x g for 30 min and the supernatant containing the refolded recombinant hSDF-1α was collected.

Separation and purification of recombinant refolded protein hSDF-1α. Cation exchange was necessary as the next step in purification (13). Elution buffer A (0.3 mol/l NaCl, 10 mmol/l Tris HCl, pH 8.5) was first used to equilibrate the chromatographic column. The supernatant containing the refolded recombinant hSDF-1α was loaded onto a 5 ml sulfopropyl (SP) Sepharose fast flow (FF) column (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) at 2 ml/min and then refolded recombinant hSDF-1α was eluted from the SP Sepharose FF column in elution buffer B (1 mol/l NaCl and 10 mmol/l Tris HCl, pH 8.5). The hSDF-1α was detected by Tris-Tricine-SDS-PAGE electrophoresis.

Purification of recombinant refolded protein hSDF-1α by size-exclusion chromatography. Size-exclusion chromatography was carried out on a Sephadex G-75 (GE Healthcare Bio-Sciences) column (1.5/60) with a flow rate of 1 ml/min, while monitoring the absorbance at 280 nm. The elution buffer was 10 mmol/l phosphate buffer (pH 7.0) which was used to equilibrate the Sephadex G-75 column and elute hSDF-1α protein. The hSDF-1α was detected by Tris-Tricine-SDS-PAGE electrophoresis.

Determination of recombinant hSDF-1α activity. A Transwell migration assay with a pore diameter of 8 μm was used to detect the chemotactic migration of THP-1 monocytes to recombinant hSDF-1α (14,15). THP-1 cells cultured for 3-5 generations were further cultured using serum-free medium RPMI-1640 (Hyclone, Logan, UT, USA) containing

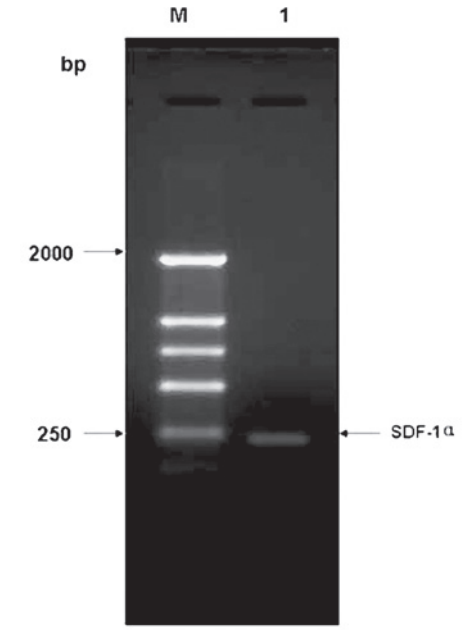

Figure 1. Agarose gel electrophoresis analysis of *hSDF-1α* gene cloned from pCMV-SPORT6-hSDF1 by PCR. Lane M, DL2000 (DNA marker); Lane 1, PCR analysis of hSDF-1α cDNA prepared from pCMV-SPORT6-SDF1α. hSDF, human stromal cell-derived factor; PCR, polymerase chain reaction.

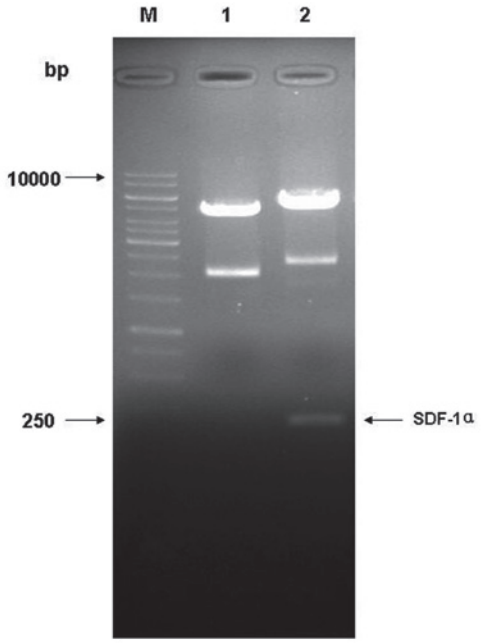

Figure 2. Identification of recombinant plasmid pET-15b-SDF1α digested by restriction enzymes *Nco*I and *Bam*HI. Lane M, middle marker; lane 1, pET-15b doubly digested with *Nco*I and *Bam*HI; lane 2, pET-15b-SDF1α doubly digested with *Nco*I and *Bam*HI. SDF, stromal cell-derived factor.

0.2% bovine serum albumin (BSA; Sijiqing; Zhejiang Tianhang Biotechnology Co. Ltd., Beijing, China) for 12 h. The cells were harvested and resuspended in RPMI-1640 containing 0.5% fetal bovine serum (FBS) at a concentration of $3x10^5$ cells/ml. hSDF-1α was prepared with concentrations of 0, 10, 100 and 500 ng/ml in 10 mmol/l phosphate buffer (pH 7.0). A 600-μl amount of hSDF-1α at each of the aforementioned concentrations was taken and added to 24-well plates (Beyotime Institute of Biotechnology, Jiangsu, China) in triplicate. A chamber insert was placed into the 24-well plate and 200 μl cell suspension with a concentration of $3x10^5$ cells/ml was added to each chamber. The cells were then incubated in a CO_2 incubator for ~10 h and the cell numbers in each well were counted and calculated under a microscope (magnification, x200; TE2000-U; Nikon Corporation, Tokyo, Japan).

Determination of protein concentration. Standard protein assay procedures using the Rio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and BSA protein standards were used to determine the hSDF-1α protein concentration.

Results

Construction and identification of pET-15b-hSDF-1α. The six known SDF-1 isoforms, SDF-1α, SDF-1β, SDF-1γ, SDF-1δ, SDF-1ε and SDF-1φ share the same first three exons but contain different fourth exons and have the same signal peptide at their 5'-end; therefore, the *SDF-1* gene was amplified without the N-terminal signal peptide sequences. Sense and antisense primers were designed to clone the *hSDF-1α* gene. The amplified target fragment identified by 1.5% agarose gel electrophoresis was ~213 bp (Fig. 1), indicating that the *hSDF-1α* gene was successfully amplified, and was consistent with the theory. The amplified target fragment was cloned into the pET-15b vector. The double-enzyme digestion results demonstrated a band of ~213 bp in lane 2, suggesting that the target gene was successfully inserted into vector pET-15b (Fig. 2). The positive recombinants identified by double-enzyme digestion were further sequenced, and the alignment results revealed that the sequence of the constructed recombinant vector and reading frame were correct, indicating that the recombinant prokaryotic expression vector named pET-15b-hSDF-1α was successfully constructed.

Expression of recombinant protein hSDF-1α. The recombinant vector pET-15b-hSDF-1α was transformed into *E. coli* BL21(DE3) to express the *hSDF-1α* gene. The hSDF-1α protein was analyzed with Tris-Tricine-SDS-PAGE following induction. A band was clearly observed with a molecular weight corresponding to that estimated from the deduced amino acid sequence, and the recombinant hSDF-1α appeared in the precipitate of cell lysates (Fig. 3), suggesting that the recombinant hSDF-1α was successfully expressed in the form of an inclusion body in *E. coli* BL21(DE3). Due to the inactivity of the aggregated form of hSDF-1α, it was necessary for the hSDF-1α to be dissolved and refolded *in vitro,* to provide biologically active hSDF-1α. Optimal refolding efficiency of hSDF-1α required optimal conditions of refolding; thus the inactive hSDF-1α was refolded under optimized refolding conditions. Bradford method analysis following refolding revealed a refolding rate of 27%. Cation exchange chromatography required preparation of the refolded hSDF-1α sample in a low-salt-containing buffer prior to loading on the cation exchange column to remove

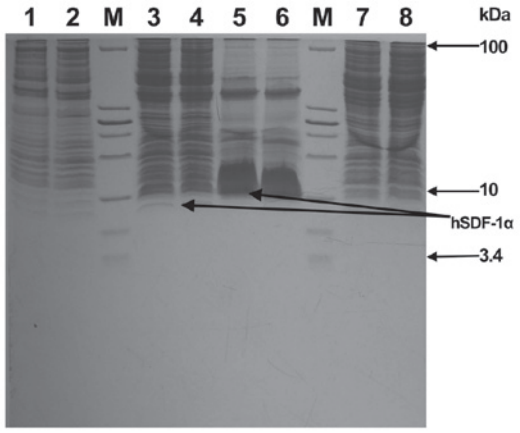

Figure 3. Expression of recombinant bacteria *E.coli* BL21(DE3)/pET15b-SDF1α. Lane M, low protein molecular weight marker (from top to bottom the molecular weights are 100, 30, 25, 20, 15, 10, 5 and 3.4 kDa); Lanes 1 and 2, bacteria were cultured at 37˚C with shaking at 220 rpm for 2.5 h and then cultured at 30˚C with shaking at 220 rpm for 6 h. 1 ml bacteria was boiled and analyzed by Tris-Tricine-SDS-PAGE electrophoresis; Lanes 3 and 4, bacteria were cultured at 37˚C with shaking at 220 rpm for 2.5 h and then cultured at 30˚C with shaking at 220 rpm for 6 h following the addition of isopropyl β-D-1-thiogalactopyranoside. 1 ml bacteria was boiled and analyzed by Tris-Tricine-SDS-PAGE electrophoresis; Lanes 5 and 6, the precipitation of bacterial lysates was analyzed by Tris-Tricine-SDS-PAGE electrophoresis; Lanes 7 and 8, The supernatant of the bacterial lysates was analyzed by Tris-Tricine-SDS-PAGE electrophoresis. hSDF, human stromal cell-derived factor.

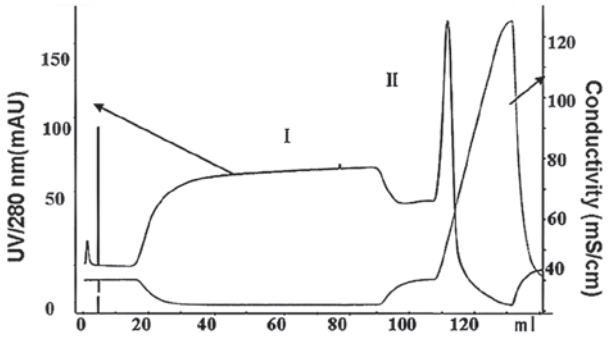

Figure 4. Chromatogram of the refolding solution from a SP Sepharose FF column. Recombinant hSDF-1α samples were purified with an ion exchange SP Sepharose FF prepacked column following refolding. SP, sulfopropyl; FF, fast flow; hSDF, human stromal cell-derived factor.

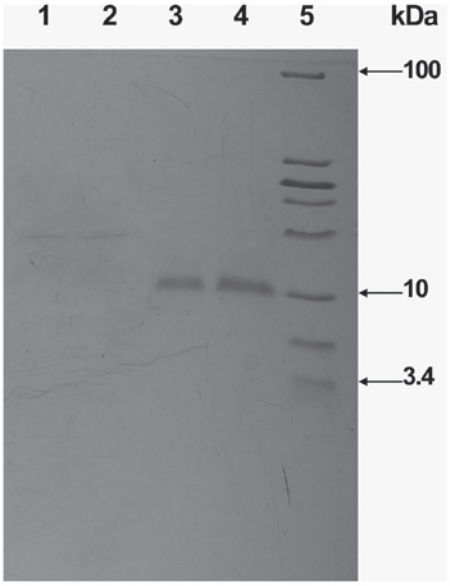

Figure 5. SDS-PAGE analysis of the protein fractions collected from the SP Sepharose FF column. Lanes 1 and 2, proteins which did not bind to the column (Peak I in Fig. 4); Lanes 3 and 4, protein eluted by elution liquid B (Peak II in Fig. 4); Lane 5, molecular weight marker (from top to bottom the molecular weights are 100, 30, 25, 20, 15, 10, 5 and 3.4 kDa, respectively).

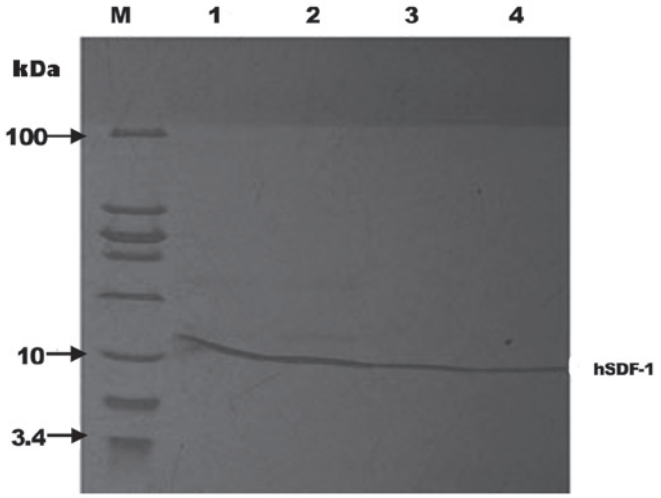

Figure 6. SDS-PAGE analysis of the protein fractions collected from strong cation exchange and size-exclusion chromatography. Lanes 1 and 2, proteins collected from strong cation exchange chromatography; Lanes 3 and 4, proteins collected from size-exclusion chromatography; Lane M, molecular weight marker (from top to bottom the molecular weights are 100, 30, 25, 20, 15, 10, 5 and 3.4 kDa, respectively). hSDF, human stromal cell-derived factor.

any salt or contaminating materials which would inhibit binding of hSDF-1α to the column. The refolding process only dilutes the concentration of salt and does not remove other impurities.

Separation and purification of the recombinant refolded protein hSDF-1α. A low concentration of refolded hSDF-1α protein with a high level of impurity necessitated strong cation exchange and size-exclusion chromatography. Strong cation exchange chromatography under refolding conditions was employed to remove the majority of the truncated product, accomplished by thorough washing with cation exchange buffer (elution buffer A). hSDF-1α was eluted from the strong cation exchange column in cation exchange buffer (elution buffer B). During the elution process of 0-100% B, peaks I and II were observed in the chromatogram of the refolding solution. Tris-Tricine-SDS-PAGE analysis following strong cation exchange showed that hSDF-1α existed in peak II with an impurity at ~15% abundance (Figs. 4 and 5). Strong cation exchange was followed by size-exclusion chromatography under refolding conditions. This step removed higher and lower molecular mass impurities. Tris-Tricine-SDS-PAGE analysis following size-exclusion chromatography showed that

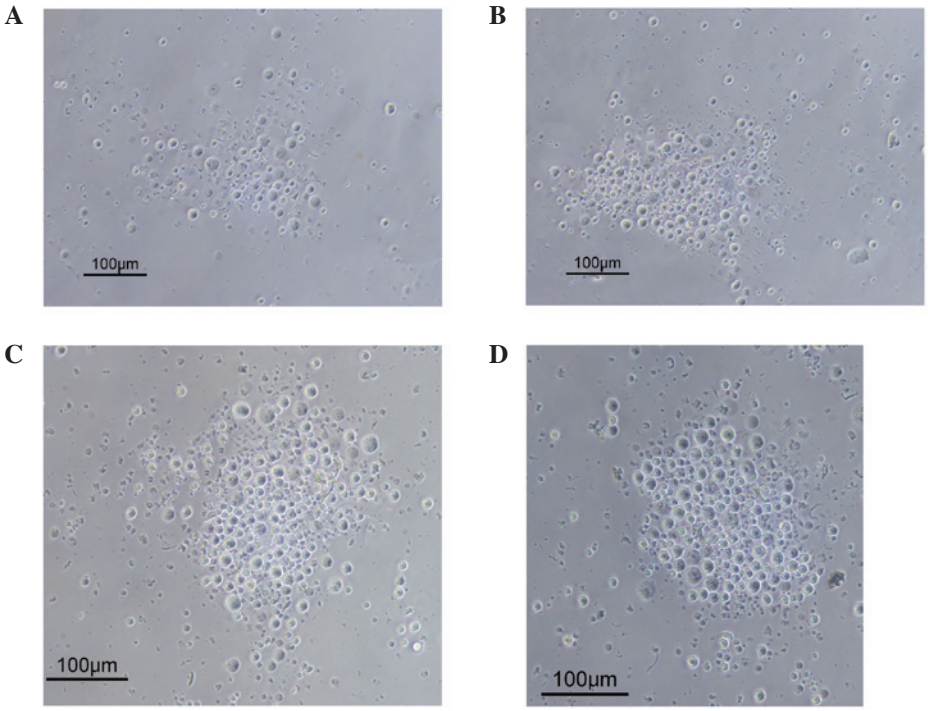

Figure 7. THP-1 cell migration stimulated by purified recombinant hSDF-1α. The different concentrations of the recombinant hSDF-1α are (A) 0 ng/ml, (B) 10 ng/ml, (C) 100 ng/ml and (D) 500 ng/ml. hSDF, human stromal cell-derived factor.

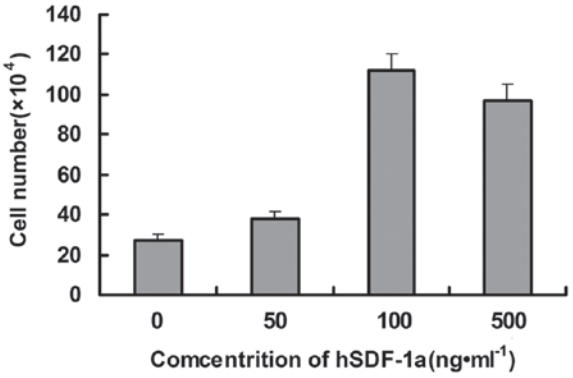

Figure 8. Chemotaxis assay. THP-1 cell migration stimulated by purified recombinant hSDF-1α. Mean values and standard deviations are calculated from data of triplicate wells. hSDF, human stromal cell-derived factor.

there were few impurities, indicating that the target protein could be obtained by purification using strong cation exchange and size-exclusion chromatography (Fig. 6).

Determination of recombinant hSDF-1α activity. The effects of recombinant hSDF-1α on the chemotactic activity of THP-1 cells were investigated by Transwell cell migration assay, with RPMI-1640 containing 0.5% FBS as a control. The number of cells that migrated to the lower chamber with recombinant hSDF-1α protein concentrations of 10, 100 and 500 ng/ml was significantly higher than that of the control group, indicating that a chemotactic effect of the purified recombinant mutant hSDF-1α protein occurred in the THP-1 cells (Figs. 7 and 8), demonstrating that the cloned, expressed, refolded and purified recombinant mutant hSDF-1α in the present study had high biological activity.

Discussion

SDF-1α is a protein that plays an important role in the migration, proliferation, differentiation and adhesion of endothelial progenitor cells (EPCs) *in vivo*. CXCR4 is a specific receptor of SDF-1, and is a G protein-coupled receptor composed of 352 amino acids and containing a seven transmembrane helical structure. CXCR4 is widely expressed in bone marrow mononuclear cells and stem cells (16). The interaction between CXCR4 and SDF-1 not only regulates the release of stem cells from bone marrow into the peripheral circulation but also regulates the recruitment and residence of stem cells in ischemic tissues (17,18), which plays an important role in the application of stem cell therapy for hypoxia, ischemic heart disease and brain injury (19,20). Crystal structure and NMR studies have demonstrated that hSDF-1α binds to CXCR4 in its monomeric form, and the eight amino acids of the N-terminus form an important receptor binding region, whereas the C-terminus of SDF-1 is not involved in receptor binding (7). Yu *et al* (8) studied NEK293 cells transfected with SDF-1δ, which has an additional 50 amino acids on the C-terminals, and therefore is >50% longer than hSDF-1α. The study showed the presence of secreted protein at ~14 kDa, the correct molecular size to be the intact protein, suggesting that the C-terminals of SDF-1δ were not cleaved. This demonstrated that SDF-1δ was also able to stimulate CXCR4-mediated clathrin-mediated endocytosis cell migration in a similar manner to hSDF-1α. This research showed that the activity of hSDF-1α was associated with its structure.

In the present study, hSDF-1α without a signal peptide sequence was created and an efficient protocol for cloning, expression and purification of the recombinant protein was developed. The *hSDF-1α* cDNA sequence which did not

contain the N-terminal signal peptide sequence was successfully constructed in a pET-15b vector, and hSDF-1α was efficiently expressed in *E. coli* BL21(DE3) cells in the form of an inclusion body. Prior to being separated and purified by strong cation exchange chromatography, the recombinant hSDF-1α was refolded with oxidized glutathione and reduced glutathione. This process not only diluted the concentration of salt but also removed certain impurities and improved the subsequent separation efficiency. The purity of the recombinant hSDF-1α reached >85% following cation exchange chromatography, which meets the requirements of general protein experiments. Size-exclusion chromatography was then used to separate out the hSDF-1α. Tris-Tricine-SDS-PAGE analysis showed that the purity of the recombinant hSDF-1α reached >95%, which meets the requirements of all protein experiments. To determine if the recombinant hSDF-1α was functional, a chemotaxis assay evaluating the ability of hSDF-1α to stimulate the migration of cells expressing the CXCR4 receptor, was performed using THP-1 cells. The recombinant hSDF-1α stimulated THP-1 cell migration, showing that the recombinant hSDF-1α had bioactivity, and indicating that the N-terminal signal peptide of hSDF-1α had little effect on the activity of hSDF-1α. Further studies are required to determine if there are quantitative differences in chemotaxis activities; however the present study laid a good foundation for further study of the *hSDF-1α* gene, the function of the recombinant hSDF-1α protein and the mechanism of interaction of hSDF-1α with its specific receptor CXCR4.

Acknowledgements

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