Recombinant stromal cell-derived factor-1 protein promotes neurite outgrowth in PC-12 cells

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Abstract. Stromal cell-derived factor-1 (SDF-1) is a chemokine involved in neuronal differentiation, as well as proliferation and migration. In the present study, the effects of recombinant SDF-1 on neurite outgrowth for nerve regeneration and engineering were evaluated in PC-12 cells. The effects of purified SDF-1 protein on cell toxicity, proliferation and migration were also assessed. SDF-1 significantly augmented cell proliferation in a dose-dependent manner, with low cytotoxicity in PC-12 cells. Cell migration also increased in the presence of SDF-1. SDF-1 significantly increased neurite number and length, compared with the control (untreated cells). Neurofilament mRNA levels, which are involved in neuronal differentiation, were also significantly upregulated in the presence of SDF-1. These results suggested that SDF-1 might prove useful for tissue engineering through induction of neuronal differentiation.

Introduction

Stromal cell-derived factor-1 (SDF-1), also known as C-X-C motif chemokine ligand 12 (CXCL12), is a chemokine expressed in several types of cells and tissues (1). SDF-1 is composed of two forms, SDF-1α and SDF-1β, by alternate splicing. SDF-1 is a well-characterized chemokine that induces a chemotactic response in several cell types, such as hematopoietic cells and mesenchymal stem cells (MSCs) (1-5). For instance, SDF-1α induces focal adhesion assembly and turnover through F-actin reorganization in MSCs (3). Zhu *et al* (4) demonstrated that icariin could induce hypoxia-inducible factor-1α expression and regulate CXCR4 expression, leading to bone marrow stromal cell migration (4). In ectopic ossification of human spinal ligaments, SDF-1 and its receptor promote chemotaxis in MSCs (5).

SDF-1 also promotes neurite outgrowth and induces neurogenesis in bone-marrow derived cells and neural stem cells (6-8). Shyu *et al* (6) suggested that intracerebral administration of SDF-1α in a rat model of stroke exerted a neuroprotective effect by suppressing neurotoxicity. In addition, Hu *et al* (3) demonstrated that SDF-1α stimulated neuronal differentiation of MSCs by increasing nestin and β-III tubulin expression. In a rat model of Parkinson's disease, SDF-1, and its receptor C-X-C motif receptor 4 (CXCR4), exerted a therapeutic effect following neural stem cell (NSC) transplantation (7). Interestingly, previous studies have mostly focused on the endogenous or exogenous expression of the CXCL12 gene, rather than the SDF-1 protein it encodes. For instance, Kim *et al* (9) reported that intermittent hydrostatic pressure increased endogenous SDF-1 concentration and promoted migration of mesenchymal stem cells (9). Moreover, endogenous SDF-1/CXCR4 expression is also significantly increased in a Parkinson's disease rat model following NSC transplantation (7). Similarly, the migration of transplanted MSCs in cerebral infarction can be improved by controlling CXCL12/CXCR4 signaling (10). Sheu *et al* (11) reported that the administration of SDF-1α increased the migration of $CD34^+$ cells, which improved peripheral nerve regeneration. Therefore, in light of these previous findings, the aim of the present study was to evaluate the effect of a recombinant SDF-1 protein on neuronal differentiation.

PC-12 is a cell line derived from rat adrenal medulla and is a mixture of neuroblastic cells and eosinophilic cells that can differentiate into neuron-like cells (12). Thus, PC-12 cells are widely used for neuronal differentiation studies. In neuronal differentiation studies using PC-12 cells, nerve growth factor (NGF) and dexamethasone are commonly used to induce neuronal differentiation. Several other compounds have previously been reported to induce neuronal differentiation of PC-12 cells. For example, Seow *et al* (13) suggested that 6-shogaol, a compound derived from ginger (*Zingiber officinale var officinale*), induced neurite outgrowth. In another study, *Momordica cochinchinensis* seed displayed NGF-mimetic activity and induced neurite outgrowth in PC-12 cells (14). Moreover, the neuronal differentiation effect of growth factors has been extensively studied. For instance, fibroblast growth

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factor (FGF)-1 and -2 also induce neuronal differentiation in the PC-12 cell lines (15,16).

In the present study, it was hypothesized that a recombinant SDF-1 protein might induce neuronal differentiation of PC-12 cells. A recombinant SDF-1 protein constructed in our previous study was isolated and purified in *Escherichia coli* (*E. coli*) (17). Cell toxicity and the proliferative effect of SDF-1 protein in PC-12 cells were measured. Also, the effect of SDF-1 protein on cell migration was also measured using a wound healing assay. To investigate the effects of recombinant SDF-1 protein on neurite outgrowth, neurite number and length was measured. Neurite morphology was also observed under a confocal laser scanning microscope (CLSM) and a scanning electron microscope (SEM). Neurofilament (NF) mRNA levels were quantified by reverse transcription-quantitative PCR (RT-qPCR). SDF-1 protein was found to induce neurite growth, thus indicating its potential in nerve tissue engineering.

Materials and methods

Construction of expression plasmids. Recombinant SDF-1 protein was constructed as described previously (17). Briefly, the human SDF-1 sequence was amplified by PCR (Perkin Elmer, Inc.) using primers flanked with restriction sites for *Xho*I and *Kpn*I (forward, 5'-*Xho*I-AACCTCGAGAGCGAT GGCAAACCGGTG-3'; reverse, 5'-*Kpn*I-GTTGGTACCTTA TTTGTTCAGCGCT-3'). The thermocycling conditions consisted of 30 cycles at 94˚C for 1 min, 55˚C for 1 min and 72˚C for 1 min. Following digestion with *Xho*I and *Kpn*I at 37˚C for 60 min, ligation with the pBAD/HisA vector at 20˚C for 4 h was performed to produce the pBAD/HisA-SDF-1 construct.

Protein expression and purification of recombinant SDF-1 protein. Ligation mixture was spread in Luria-Bertani medium (LPS Solution Co., Ltd.) containing 100 μg/ml ampicillin after heat-shock at 42˚C for 1 min. Following bacterial transformation, TOP10 *E. coli* cells (Invitrogen; Thermo Fisher Scientific, Inc.) were cultured in Luria-Bertani medium containing 100 μg/ml ampicillin overnight at 37˚C. Once the optical density value at 600 nm reached 0.6, bacterial cells were incubated with 0.1% (w/v) L-arabinose at 20˚C for 6 h. Cells were centrifuged at 6,000 x g at for 10 min at 4˚C, then lysed (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). After centrifugation at 14,000 x g at 4˚C for 30 min, the soluble protein was purified from the supernatant by affinity to a nickel-nitrilotriacetic acid resin (Invitrogen; Thermo Fisher Scientific, Inc.). The purified recombinant SDF-1 protein was analyzed via 10% SDS-PAGE followed either by Coomassie Brilliant Blue staining or western blotting. The protein concentrations were measured using the Bradford method (Bio-Rad Laboratories, Inc.). The purity of SDF-1 protein was determined by Coomassie Brilliant Blue staining at room temperature for 30 min, and the molecular size was confirmed by western blotting. For western blotting, SDF-1 protein (50 μg/lane) was electrophoresed via SDS-PAGE on a 10% gel, and subsequently transferred to nitrocellulose membrane. Following which, membranes were blocked with 1% BSA at room temperature for 1 h, washed and then incubated with the mouse anti-His antibody (1:1,000; cat. no. sc-8036; Santa Cruz Biotechnology, Inc.) at 4˚C overnight. The blotted membrane was washed with Tris-buffered saline with 0.5% Tween-20 and incubated with HRP-conjugated anti-mouse secondary IgG (1:2,000; cat. no. sc-2371; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. Bands were detected using ECL detection reagent (Immobilon® ECL Ultra Western HRP Substrate; EMD Millipore).

Table I. Sequences of primers used for RT-qPCR.

Target gene	Sequence (5'-3')
GAPDH	F: TGGAAGGACTCATGACCACA
	R: TTCAGCTCAGGGATGACCTT
NF	F: TGGAAGGACTCATGACCACA
	R: GGAGACGTAGTTGCTGCTTCTT

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NF, neurofilament; F, forward; R, reverse.

Cell culture. The PC-12 rat pheochromocytoma cell line was purchased from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 5% penicillin-streptomycin solution in a 5% CO_2 atmosphere at 37˚C.

Cell viability and proliferation assay. Cell viability was evaluated using an MTT assay. PC-12 cells were seeded at a density of $1x10^4$ cells/well and incubated for 24 h at 37˚C with 5% CO_2. The cells were washed three times with PBS, then incubated for 24 h at 37˚C with 5% CO_2 in the presence of 0.5, 1 and 5 μg/ml recombinant SDF-1 protein. Untreated cells were also used as a control. Subsequently, 500 μl MTT was added to each well and incubated at 37˚C for 4 h. Then, the media was removed and dissolved with DMSO. Optical density at 540 nm (OD_{540}) was read using a microplate reader. Cell proliferation activity was similarly evaluated. PC-12 cells ($1x10^3$ cells/well) were incubated for 3 and 5 days with various concentration of SDF-1 protein. Cell proliferation was also measured by reading at OD_{540}.

In vitro wound healing assay. Cell migration was evaluated using an in vitro wound healing assay (18). PC-12 cells were seeded at a density of $1x10^6$ cells/well to create a confluent monolayer. Following overnight serum starvation at 37˚C with 5% CO_2, a scratch was made using a 200 μl pipette tip. Cells were incubated for 6 days in the presence of 5 μg/ml. Untreated cells were used as a negative control. Cell migration was evaluated at day 0, 2, 4 and 6 (magnification, x10) under a CKX41 light microscope (Olympus Corporation).

Neurite outgrowth assay. PC-12 cells were seeded at a density of $1x10^3$ cells/well and incubated with 5 μg/ml SDF-1 protein for 5 days at 37˚C with 5% CO_2. Untreated cells were also used as a negative control. On the fifth day, neurite number

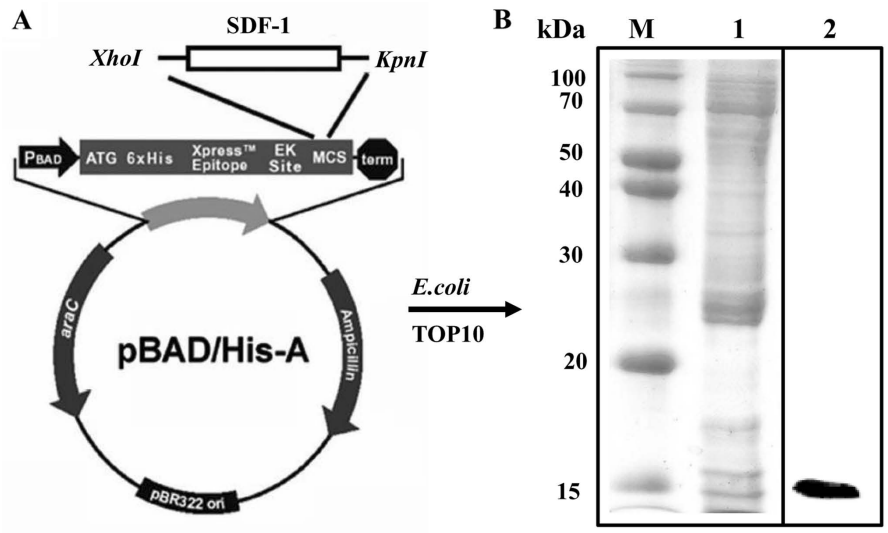

Figure 1. Engineered and purified recombinant SDF-1 protein. (A) SDF-1 construct. (B) SDS-PAGE (lane 1) and western blotting (lane 2). The molecular ladder for SDS-PAGE is displayed in lane M. The molecular weight of the SDF-1 protein is ~15 kDa. SDF-1, stromal cell-derived factor-1; araC, L-arabinose operon regulator; *E. Coli*, *Escherichia coli*; ori, origin.

and length were examined under a CKX41 light microscope (magnification, x10). Three images per well were captured in each three experiments. To evaluate neurite number, cell images were captured and the neurite number in the field was counted. Neurite number was normalized with the control and expressed as a percentage. To evaluate neurite length, neurite with 1.5 times longer than the cell body were scored.

CLSM analysis. Neurite morphology was examined under a CLSM. Cells were treated as aforementioned, and subsequently fixed with 10% formalin for 30 min and stained with Alexa Fluor 488 conjugated phalloidin and DAPI for 30 min (Invitrogen; Thermo Fisher Scientific, Inc.). Then, neurite morphology was observed under an LSM700 confocal microscope (magnification, x10; Carl Zeiss AG).

SEM analysis. Neurite morphology was also examined under SEM. Cells were fixed with 2.5% glutaraldehyde at room temperature for 30 min, and serially dehydrated with ethanol solution (50, 70, 90, 95 and 100%) for 10 min each. Specimens were then treated with hexamethyldisilazane (Sigma-Aldrich; Merck KGaA) and coated with platinum (magnification, x250).

RT-qPCR analysis. PC-12 cells were seeded at density of $1x10^3$ cells/well and incubated for 5 days at 37˚C with 5% CO_2. Total RNA was extracted using an Easy-spin Total RNA Extraction kit (Intron Biotechnology, Inc.). Then, cDNA was synthesized at 50˚C for 50 min and 85˚C for 5 min using SuperScript® IV-First Strand Synthesis System (Invitrogen; Thermo Fisher Scientific, Inc.). The expression of the NF gene was quantified by RT-qPCR. The reaction mixture contained 0.1 μm of each primer, 10 μl of 2X SYBR-Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc), including AmpliTaq Gold™ DNA Polymerase with Buffer, dNTPs mix, SYBR-Green I dye, Rox dye and 10 mm $MgCl_2$, and 1 μl template cDNA. PCR was conducted with activation (94˚C for 10 min), followed by 40 cycles of denaturation (94˚C for 15 sec), and annealing and extension (60˚C for 1 min). Primer sequences are listed in Table I. The Cq value for each gene was normalized to GAPDH using the formula: $dCq = Cq_{(GAPDH)} - Cq_{(NF)}$ (19).

Statistical analysis. All experiments were conducted three times. Data are expressed as the mean ± SD. Date was analyzed using Student's t-test or one-way ANOVA followed by Duncan's multiple range test. Statistical analysis was carried out using GraphPad Prism 7 software (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression and purification of recombinant SDF-1 protein in E. coli. Recombinant SDF-1 protein was constructed and purified from *E. coli*. The yield for purified SDF-1 protein from a 1 L culture was 0.7 mg/ml. The molecular weight of the purified recombinant SDF-1 protein was ~15 kDa (Fig. 1).

Effect of recombinant SDF-1 protein on cell viability. Cell viability was measured to evaluate the potential cytotoxic effects of the recombinant SDF-1 protein on PC-12 cells. PC-12 cells incubated with SDF-1 concentrations ranging from 0.5, 1 and 5 μg/ml retained >95% cell viability, compared with untreated cells (Fig. 2A).

Effect of recombinant SDF-1 protein on cell proliferation. PC-12 cell proliferation was measured after incubation for 3 or 5 days in the presence of 0.5, 1 or 5 μg/ml SDF-1. At both timepoints, cell proliferation significantly increased in a dose-dependent manner (Fig. 2B). In particular, cell proliferation in the presence of 5 μg/ml SDF-1 increased ~1.5-fold. Based on cell viability and proliferation findings, SDF-1 was used at a concentration of 5 μg/ml in subsequent experiments.

Effect of recombinant SDF-1 protein on cell migration. Cell migration in PC-12 cells in the presence of SDF-1 was assessed using an in vitro scratch assay over a 6-day time course. At

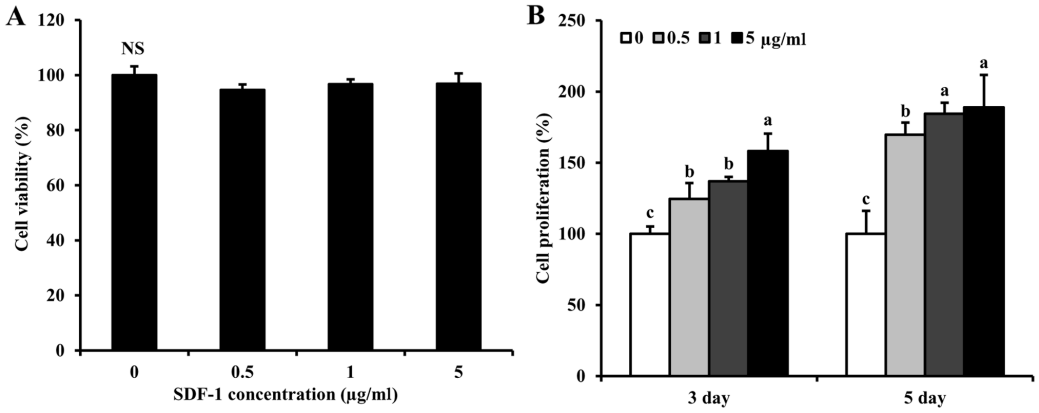

Figure 2. Effect of SDF-1 protein on cell toxicity and proliferation in PC-12 cells. (A) Cell toxicity was evaluated in PC-12 cells following 24 h incubation in the presence of 0.5, 1 or 5 μg/ml SDF-1. (B) PC-12 cell proliferation was measured after incubation for 3 or 5 days in the presence of 0.5, 1 or 5 μg/ml SDF-1. Untreated cells were used as a control. Data are presented as the mean ± SD (n=3). Different lower letters indicate significant differences on the same day among different concentrations [a]P<0.05 vs. b; [b]P<0.05 vs. c. SDF-1, stromal cell-derived factor-1; NS, not significant.

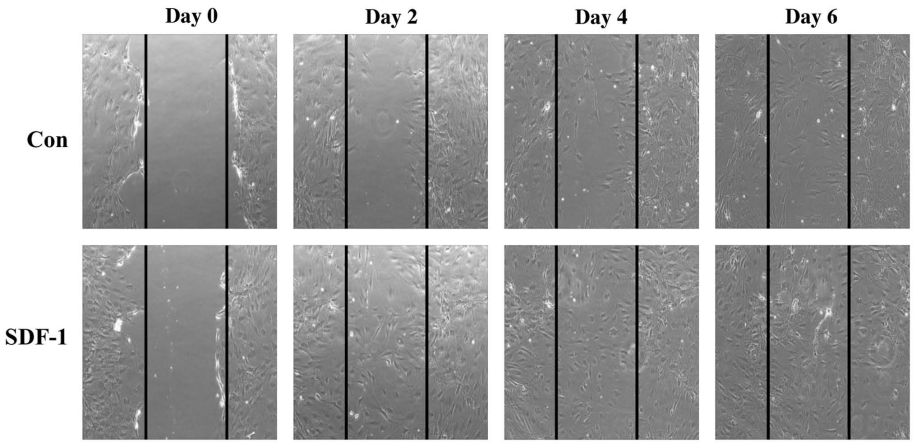

Figure 3. Effect of SDF-1 protein on wound healing activity in PC-12 cells after 6 days. Confluent PC-12 cells were scratched with a 200 μl pipette tip after overnight serum starvation at 37˚C with 5% CO_2. Cells were incubated for 6 days in the presence of 5 μg/ml SDF-1. Untreated cells were used as a negative control. SDF-1, stromal cell-derived factor-1, Con, control.

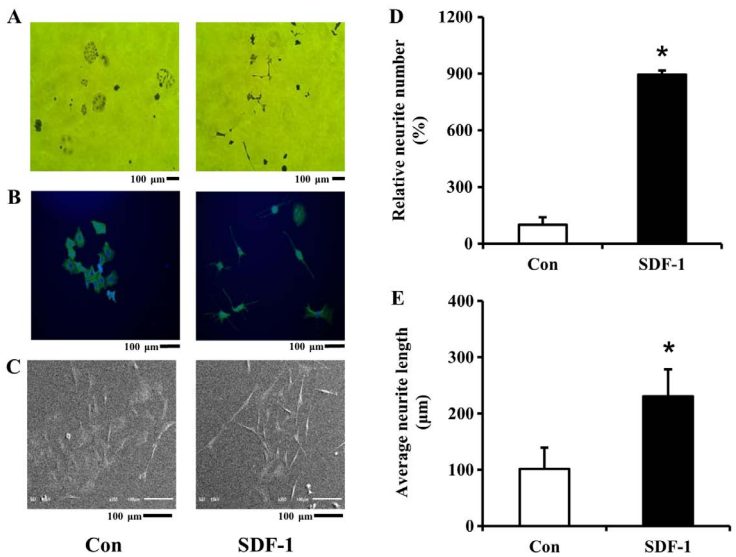

Figure 4. Effect of SDF-1 protein on neurite outgrowth in PC-12 cells. (A) Microscopic image (magnification, x10; scale bar, 100 μm). (B) CLSM image (scale bar, 100 μm). (C) SEM image (scale bar, 100 μm). (D) Relative neurite number. (E) Average neurite length. PC-12 cells were incubated with 5 μg/ml SDF-1 protein for 5 days at 37˚C with 5% CO_2. Untreated cells were also used as a negative control. On the fifth day, each image was obtained under a light microscope, CLSM and SEM. Data are presented as the mean ± SD (n=3). Different lower letters indicate significant differences between Con and SDF-1 *P<0.05. SDF-1, stromal cell-derived factor-1; Con, control; CLSM, confocal laser scanning microscope; SEM, scanning electron microscope.

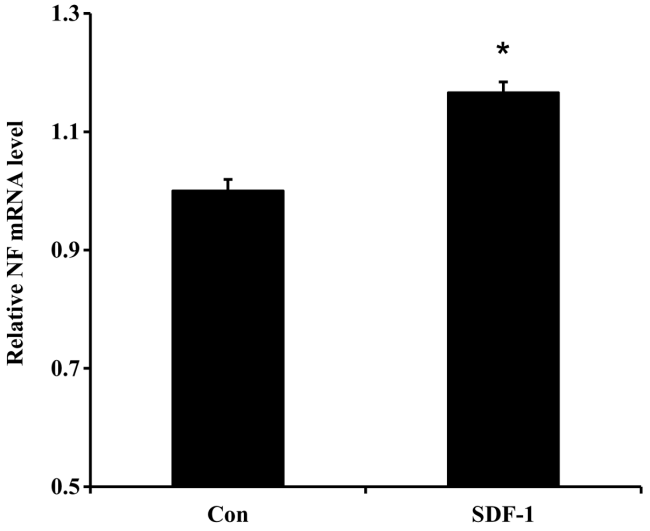

Figure 5. Effect of SDF-1 protein on NF mRNA level in PC-12 cells. NF gene expression was measured using reverse transcription-quantitative PCR. Data are presented as the mean ± SD (n=3). Different lower letters indicate significant differences between Con and SDF-1 *P<0.05. SDF-1, stromal cell-derived factor-1; Con, control; NF, neurofilament.

day 2, SDF-1 induced PC-12 cell migration from the edge of the scratch at 2 days, compared with the initial time point (Fig. 3). However, at day 6, cell migration in the presence SDF-1 was similar to the control. These results confirmed the chemoattractant effect of SDF-1 in PC-12 cells.

Effect of recombinant SDF-1 protein on neurite outgrowth. Neurite number and length in PC-12 cells were determined from microscopy images (Fig. 4). In all examined microscopic images, SDF-1 induced neurite outgrowth in PC-12 cells (Fig. 4A-C). This stimulating effect of SDF-1protein on neurite outgrowth was observed in images obtained from an optical microscope, as well as CLSM and SEM. In addition, SDF-1 significantly increased neurite number in PC-12 cells 8.97-fold, compared with the control (Fig. 4D). Moreover, SDF-1 also significantly increased the average neurite length in the SDF-1-treated cells, compared with the control (230.56±47.91 vs. 101.39±37.74 μm, respectively; Fig. 4E). Therefore, SDF-1 stimulates neurite outgrowth in PC-12 cells. Collectively, these results suggest that recombinant SDF-1 protein might be utilized for nerve regeneration by inducing neurite outgrowth.

Effect of recombinant SDF-1 protein on NF gene expression. NF gene expression levels were significantly upregulated in PC-12 cells treated with 5 μg/ml SDF-1 protein, compared with control cells (Fig. 5). These results suggested that SDF-1 might influence neurite outgrowth in part by upregulating the NF gene.

Discussion

Previous studies have documented the use of the SDF-1 gene, and recombinant SDF-1 protein is well-characterized. Based on the chemotactic properties of SDF-1, recombinant SDF-1 protein has been extensively utilized as a therapeutic agent in various tissue engineering studies involving the bones, nerves and brain (20-22). In the present study, a 15-kDa recombinant SDF-1 protein was initially purified in *E. coli* in order to evaluate its effects on cell viability, proliferation, migration and neuronal differentiation.

Cytotoxicity analysis is needed because even purified recombinant proteins can be toxic to cells. However, similarly engineered recombinant proteins have been demonstrated to be nontoxic in several studies. For instance, Jo *et al* (23) reported that, in the presence of oxidative-stress, Tat-DJ-1 promoted cell survival in HepG2 cells without cytotoxicity. Similarly, recombinant human dentin phosphoprotein also demonstrated low toxicity in our previous study (24). In the present study, purified recombinant SDF-1 protein was not toxic to PC-12 cells.

In the present study, 5 μg/ml SDF-1 significantly increased cell proliferation. This cell proliferative effect of SDF-1 is proposed to be due to its chemoattractant properties. Li *et al* (25) demonstrated that endothelial progenitor cells engineered to express the CXCL12 gene promoted the proliferation of oligodendrocyte progenitor cells in a murine ischemic stroke model. Tang *et al* (21) also reported that recombinant human SDF-1α enhanced MSC proliferation and recruited MSCs in injured eyes for corneal epithelium regeneration.

As aforementioned, SDF-1 is a powerful chemokine, which directly induces cell migration in various cell types including NSC, MSC and so on. In our previous study, SDF-1 loaded scaffolds significantly enhanced cell migration (17). Similarly, SDF-1 significantly enhanced the migration of PC-12 cells in the present study. These cell migration and recruiting activities of SDF-1 is expected to be more effective in damaged tissue. For instance, the combination of SDF-1α) and transforming growth factor-β1-loaded silk fibroin-porous gelatin scaffold enhanced MSCs migration, leading to the promotion of cartilage regeneration and repair (26).

Furthermore, recombinant SDF-1 enhanced neurite outgrowth by increasing neurite number and length. The neuronal differentiation effect of SDF-1 was confirmed under SEM and CLSM examination. These results suggested that recombinant SDF-1 might replace NGF for inducing neuronal differentiation of PC-12 cells if comparative studies with NGF in the future were performed. Several studies have evaluated neurite outgrowth in PC-12 cells (27-29). For instance, Eik *et al* (27) reported that *Lignosus rhinocerus* aqueous extract increased neurite number and length in differentiated PC-12 cells. Similarly, *Ziziphus jujube* water extract induces neurite outgrowth in PC-12 cells (28). The effect of recombinant SDF-1 on the neuronal differentiation of PC-12 cells has not yet been evaluated. Thus, to the best of our knowledge, the present study is the first to report the effect of SDF-1 on PC-12 neuronal differentiation.

To confirm the neuronal differentiation of recombinant SDF-1 protein, NF mRNA levels were measured. NF is typically expressed in neuronal cells, is closely involved in neurite outgrowth, and is considered as a leading marker for neuronal differentiation (30). Hence, NF expression has been investigated in the neuronal differentiation study (31-33). In the present study, SDF-1 upregulated NF expression, compared with the control. The neuronal differ-

entiation effect of SDF-1 on PC-12 cells is similar to that of NGF, as it increases the number and length of neurites. NGF binding to the tropomyosin receptor kinase A receptor activates RAS and RAF, respectively. Subsequently, MEK and ERK are activated, leading to neurite outgrowth of PC-12 cells (34). In a similar study, 6-shogaol demonstrated the mimic effect of NGF in the neuronal differentiation of PC-12 cells (13). Moreover, Yao *et al* (35) reported that staurosporine (a protein kinase inhibitor) activated JNK and ERK and contributed to neurite outgrowth in PC-12 cells. The mechanistic basis of the effects of SDF-1 in PC-12 cells was not evaluated in the present study and remains to be determined in the future.

In conclusion, PC-12 cells exposed to a purified SDF-1 protein retained high cell viability at all concentrations tested, thus demonstrating low cytotoxicity. In addition, SDF-1 increased cell proliferation and migration. SDF-1 also significantly induced neurite growth, compared with control cells. Thus, SDF-1 could be used in nerve tissue engineering.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JHJ made substantial contributions to the conception of the present study. YRY performed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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