SDF1/CXCR4 axis facilitates the angiogenesis via activating the PI3K/AKT pathway in degenerated discs

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Abstract. Symptomatic degenerative disc disease (DDD) is considered the leading cause of chronic lower back pain (LBP). As one of the main features of intervertebral disc degeneration (IDD), vascular ingrowth plays a crucial role in the progression of LBP. Stromal cell-derived factor 1 (SDF1) and its receptor C-X-C receptor 4 (CXCR4) were reported to be overexpressed in the degenerated intervertebral discs, suggesting that they may be involved in the pathogenesis of IDD. Moreover, SDF1 has been identified to induce neovascularization in rheumatoid arthritis disease. However, the roles of the SDF1/CXCR4 axis in the neovascularization of IDD remain unclear. Therefore, the objective of the present study was to elucidate whether the SDF1/CXCR4 axis takes part in neovascularization in degenerated intervertebral discs and its underlying mechanisms. Adenovirus infection was used to upregulate SDF1 expression in primary nucleus pulposus cells (NPCs). The effects of SDF1 on the proliferation and angiogenesis of vascular endothelial cells (VECs) were assessed by Cell Counting Kit-8 and tube formation assays after VECs were treated with the supernatants derived from SDF1 overexpressed or not treated NPCs. Transwell chambers using the supernatants from NPCs as chemokines were applied to assess VEC migration and invasion. An MD3100, MK-2206 and SF1670 were used to antagonize CXCR4, aKT serine/threonine kinase 1 (aKT) and phosphatase and tensin homolog (PTen) in VECs. The results revealed that SDF1 overexpression significantly increased the ratio of phosphorylated aKT to aKT and decreased PTen expression in NPCs, as well as enhanced the proliferation, migration, invasion and angiogenesis abilities of VECs. However, these effects induced by SDF1 overexpression in NPCs were all reversed when VECs were pretreated with AMD3100 or MK-2206, whereas enhanced by SF1670 treatment. Collectively, the present study indicated that enhancement of the SDF1/CXCR4 axis in NPCs can significantly accelerate angiogenesis by regulating the PTEN/phosphatidylinositol-3-kinase/AKT pathway.

Introduction

Chronic lower back pain (LBP) is a type of non-specific LBP that refers to pain lasting for more than one year and rarely allowing normal activity (1). It is estimated that the cost of chronic LBP in the United States alone is as high as $12-90 billion annually (2). Although continuous improvements have been made in treatment and prevention guidelines, the incidence of chronic LBP is still increasing (3). Symptomatic degenerative disc disease (DDD) is considered as the leading cause of chronic LBP and is characterized by material composition changes (4), annulus fibrosus tears (5), granulation tissue and vascular nerve ingrowth (6,7). Intervertebral discs, as the largest avascular organs in the human body will be changed in the internal material metabolism and microenvironment when neovascularization occurs, indicating that neovascularization has a significant promoting effect on intervertebral disc degeneration (IDD) progression (8).

Stromal cell-derived factor 1 (SDF1) belongs to the chemokine superfamily. Through specifically binding to the G protein-coupled receptor C-X-C receptor 4 (CXCR4), SDF1 plays an important role in a range of physiological and pathological activities (9). Zhang et al (10) have reported that SDF1 and CXCR4 were highly expressed in degenerated intervertebral discs when compared with the normal human or rat discs, which suggested that the SDF1/CXCR4 axis may be involved in the pathogenesis of IDD. Additionally, SDF1 has been identified as able to induce neovascularization in the motor system in rheumatoid arthritis disease (11). As discs belong to the motor system, the SDF1/CXCR4 axis was speculated to also play a role in the vascularization process in the degenerated intervertebral discs. To this end, the present study used primary nucleus pulposus cells (NPCs) isolated from degenerated intervertebral disc tissues to elucidate the effect of the SDF1/CXCR4 axis on the vascularization of vascular endothelial cells (VECs), and explore the underlying mechanisms.
Materials and methods

**NPC isolation and culture.** NPCs were isolated from the degenerated intervertebral disc tissues obtained from 12 patients with lumbar DDD (diagnosed with disc herniation, spondylolisthesis and spinal stenosis) from June 2017 to December 2017 in The First Affiliated Hospital of Chongqing Medical University. Consent was signed by each patient and this study was approved by the Ethics Committee of Chongqing Medical University. All specimens used in the present study were at least grade III, classified according to Pfirrmann grading (12) (Table I).

For cell isolation, specimens from discectomy were first washed into phosphate-buffered solution (PBS). After being mashed into pieces using mortar, 2% type II collagenase (HyClone; GE Healthcare Life Sciences) was used to digest the NP tissue debris at 37˚C for 8 h. The primary NPCs were obtained after the aforementioned mixture was filtered and centrifuged at 500 x g for 5 min at room temperature. The primary NPCs positively expressed aggrecan and type II collagen. NPCs were resuspended and cultured in DMEM/F12 medium (HyClone; GE Healthcare Life Sciences), supplemented with 15% fetal bovine serum (FBS; CellMax; cellmaxcell.com/), 100 U/ml penicillin and 100 µg/ml streptomycin (Beyotime Institute of Biotechnology) at 4˚C overnight. After blocking with 5% goat serum (Sigma‑Aldrich; Merck KGaA) for 1 h at room temperature, NPCs were incubated with the corresponding secondary antibodies, including anti-rabbit FITC fluor-conjugated secondary antibody (1:200; cat. no. ab155090; Abcam), anti-aggrecan antibody (1:200; cat. no. AF6126; Beyotime Institute of Biotechnology) and anti-type II collagen antibody (1:200; cat. no. AF6528; Beyotime Institute of Biotechnology) at 4˚C overnight. After washing with PBS, the cells were incubated with the corresponding secondary antibodies, including anti-rabbit FITC fluor-conjugated secondary antibody (1:200; cat. no. Po0186; Beyotime Institute of Biotechnology) and anti-rabbit DyLight 549-conjugated secondary antibody (1:200; cat. no. ab96885; Abcam) at 37˚C for 1 h. Cell nuclei were stained with DAPI (Beyotime Institute of Biotechnology) for 5 min at 4˚C. Images were obtained using a fluorescence microscope (Leica Microsystems GmbH).

**VEC culture and treatments.** VECs (EA.hy926; The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) were cultured in DMEM (HyClone; GE Healthcare Life Sciences), supplemented with 5% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. VECs were incubated for 30 min with small molecule inhibitors, including AMD3100 (inhibitor of CXCR4), MK-2206 [inhibitor of AKT serine/threonine kinase 1 (AKT)] and SF1670 [inhibitor of phosphatase and tensin homolog (PTEN)], all purchased from Selleck Chemicals at a concentration of 10 µM. To suppress SDF1, VECs were treated with anti-SDF1 antibody (100 µg/ml; cat. no. ab155090; Abcam) for 24 h.

**Conditioned medium preparation.** The conditioned medium derived from NPCs was prepared as previously described (13). Briefly, NPCs (5x10^5) were seeded in a T25 flask and maintained in 4 ml culture medium. After 3 days, the medium was collected and centrifuged at 12,880 x g for 10 min room temperature to obtain the supernatant, which served as the conditioned medium of VECs. The conditioned medium was frozen at −80˚C and used for experiments within 2 weeks.

**Adenovirus transfection.** The adenovirus vector (OE-SDF1) used to upregulate SDF1 expression and the negative control OE-NC were obtained from Shanghai GenePharma Co., Ltd. NPCs were first incubated with OE-SDF1/OE-NC adenovirus solution (MOI=100) for 6 h at 37˚C. Subsequently, the medium was replaced with fresh medium for further culture at 37˚C for 24 or 48 h. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed 24 h post transfection to verify SDF1 mRNA expression, and western blot analysis was performed 48 h later to verify SDF1 protein expression.

**RT-qPCR.** Total RNA was extracted from NPCs using the TRIzol® reagent (Sigma‑Aldrich; Merck KGaA). Then, 1 µg of total RNA was used to synthesize cDNA using a one-step method according to the instructions of a reverse transcription kit (Thermo Fisher Scientific, Inc.). RT-qPCR was performed on an ABI-7500 PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR Select Master Mix (Thermo Fisher Scientific, Inc.). The reaction system was as follows: SYBR Select Master Mix, 5 µl; primers (10 µM), 1 µl; cDNA (5 µg/ml), 1 µl; and enzyme-free water, 3 µl. The SDF1 primer sequences were as follows: Upstream, 5'-TCAGCCTTGAGCT ACAGATGCC-3', and downstream, 5'-TCTGAGGCGCAC AGTTTGAGG-3'. The GAPDH primer sequences were as follows: Upstream, 5'- CCGAGTCAAGGATTCGGTCC TAT-3', and downstream, 5'-AGCCTTCCATGGTGGAA GAC-3'. The reaction conditions were as follows: Activation at 95˚C for 2 min and 95˚C for 2 min, followed by 40 cycles of denaturation at 95˚C for 3 sec and annealing/extension at 60˚C for 30 sec. The expression data were calculated by the 2^ΔΔCq method (14) and normalized to GAPDH. The experiments were independently repeated three times.

**Western blot analysis.** NPCs and VECs were lysed with radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology) on ice for 30 min. Then, the lysate was centrifuged at 12,880 x g for 10 min at 4˚C, and the supernatant was collected. After the protein concentration had been determined with a bicinchoninic acid kit (Beyotime Institute of Biotechnology), 50 µg protein from each group, including control, NC, OE-SDF1, OE-SDF1 + AMD3100, OE-SDF1 + MK-2206 and OE-SDF1 + SF1670 was loaded into the 10% SDS-PAGE gel (Beyotime Institute of Biotechnology) and separated by electrophoresis. Then, the proteins were transferred onto the polyvinylidene fluoride (PVDF) membranes (EMD Millipore). Subsequently, the PVDF membranes were blocked with 5% skimmed milk for 1 h, followed by incubation with the primary antibodies, including...
10 µl of cc K-8 reagent was added to each well and incubated in an incubator at 37˚C for 24 h. Subsequently, 100 µl mixed medium (1:1 ratio of conditioned:complete medium) was added and incubated at 37˚C for another 4 h. The absorbance was measured at 490 nm.

Enzyme linked immunosorbent assay (ELISA). The supernatant was collected from cultured NPCs after administration with various concentrations of anti-SDF1 antibody (0, 10, 50, 100, 200 and 500 µg/mol) for 12, 24 or 48 h. Then, the levels of SDF1 was tested by using an ELISA kit (cat. no. ab100637; Abcam) according to the manufacturer's instructions.

Cell Counting Kit-8 (CCK-8). A total of 3,000 VECs were seeded into each well of a 96-well plate and left to incubate overnight. After pretreatment with inhibitors (AMD3100, MK-2206 or SF1670) for 30 min, VECs were resuspended in a medium containing 10% FBS and incubated for 4 h. The absorbance was measured spectrophotometrically at 450 nm.

Transwell migration and invasion assay. First, NPCs (5x10⁴/well) were seeded into 24-well plates and incubated at 37˚C overnight. On the second day, VECs (1x10⁵) were resuspended with DMEM containing 1% FBS and seeded into the upper chamber of the Transwell plate (Corning, Inc.) which was placed in the 24-well plate. After an additional co-culture period of 24 h, the upper chambers were removed, and the non-migrated cells in the upper layer of the semipermeable membrane were removed using swabs. After fixation in 4% paraformaldehyde for 10 min at room temperature, the migrated cells were stained with 0.1% crystal violet solution for 10 min at room temperature and then rinsed with PBS. Five random field of views were selected and images were captured from each chamber. A similar procedure was carried out to detect the invasion ability of VECs except for the chambers being coated with Matrigel and co-cultured for 48 h.

Tube formation assay. Plates (96-wells) were coated with Matrigel (BD Bioscience) and incubated at 37˚C to solidify. Following pretreatment with the inhibitors (AMD3100, MK-2206 or SF1670) for 30 min, VECs were resuspended in a mixed medium (1:1 ratio of conditioned:complete medium). Then, 2x10⁴ VEC cells in 100 µl was added into the aforementioned prepared 96-well plates. Tube-like structures were observed approximately 4 h post incubation at 37˚C. Five random field of view of images were captured using an inverted light microscope (Leica Microsystems GmbH) from each well (magnification, x100). ImageJ software (version 1.8.0; National Institutes of Health) was used for analysis.

Statistical analysis. Statistical analysis was performed using SPSS 19.0 software (IBM Corp.). All data are presented as the mean ± standard deviation. An unpaired Student's t-test was used for comparisons between two groups, while a one-way ANOVA followed by Dunnett's post hoc test was used for comparisons between multiple groups (≥3 groups). P<0.05 was considered to indicate a statistically significant difference.

Results

Upregulation of SDF1 regulates the activation of PTEN/AKT signaling. To explore the effects of the SDF1/CXCR4 axis in the progression of IDD, NPCs were isolated from degenerated intervertebral disc tissues, which were identified by immunofluorescence assays through staining of type II collagen and aggrecan as previously described (5). As revealed in Fig. 1A, NPCs presented with both aggrecan and type II collagen expression. Compared with the control group, the mRNA and protein expression levels of SDF1 were both significantly increased in OE-SDF1-treated NPCs (Fig. 1B and C). Similarly, immunofluorescence assays revealed that OE-SDF1 transfection notably enhanced the fluorescence intensity (Fig. 1D). To explore the mechanisms of SDF1 in IDD, the conditioned supernatant derived from NPCs in the presence or absence of OE-SDF1 transfection was used to culture VECs. The results revealed that SDF1 overexpression increased the relative p-AKT expression and decreased PTEN expression in VECs (Fig. 1E). These results demonstrated that the activation of phosphatidylinositol-3-kinase (PI3K)/AKT signaling in VECs induced by SDF1 may be involved in the progression of IDD.

CXCR4 inhibition impairs SDF1 roles in VEC proliferation, migration, invasion and angiogenesis promotion. The study proceeded to explore SDF1 roles in the proliferation, migration,
invasion and angiogenesis of VECs. The content of SDF1 in the cultured supernatant of NPCs was significantly reduced when NPCs were treated with anti-SDF1 antibody (100 µg/ml) for 24 h (Fig. 2A and B). The study proceeded with 100 µg/ml anti-SDF1 antibody for the subsequent assays, as this was the minimum concentration required to induce this effect.

Compared with the control group (conditioned medium from control NPCs), the tube formation, migratory and invasive abilities of VECs were all significantly enhanced when cells were treated with the conditioned medium derived from OE-SDF1-transfected NPCs (Fig. 2C-E). However, anti-SDF1 antibody administration significantly neutralized these
effects induced by SDF1 overexpression in NPCs (Fig. 2C-E), confirming a vital role of SDF1 in promoting VEC migration, invasion and angiogenesis.

The present study then proceeded to explore the roles of the SDF1/CXCR4 axis in the proliferation, migration, invasion and angiogenesis of VECs. Suppression of CXCR4 in VECs with
AMD3100 administration significantly inhibited the expression of p-AKT in VECs as compared with the OE-SDF1 group (Fig. 3A). VECs treated with cultural supernatant derived from NPCs with SDF1 overexpression revealed enhanced proliferation (Fig. 3B), angiogenesis (Fig. 3C), migratory (Fig. 3D) and invasive capacities (Fig. 3E). However, these effects induced by SDF1 upregulation were all weakened when VECs were incubated with AMD3100 to antagonize CXCR4 (Fig. 3B-E). These results demonstrated that SDF1 induced angiogenesis in a CXCR4-dependent manner.
AKT inhibition impairs SDF1 roles in the promotion of VEC proliferation, migration, invasion and angiogenesis. Next, the study explored the role of PI3K/AKT signaling in the proliferative, migratory, invasive and angiogenic capabilities of VECs. The results indicated that the p-AKT expression was significantly decreased when VECs were treated with MK-2206, an inhibitor of AKT when compared with the OE-SDF1 group (Fig. 4A). In addition, MK-2206 administration significantly
reversed the OE-SDF1-mediated promotion of cell proliferation (Fig. 4B), angiogenesis (Fig. 4C), migration (Fig. 4D) and invasion (Fig. 4E).

As PTEN is a negative regulator of PI3K/AKT signaling (15), the study then explored the effects of SFI670, an inhibitor of PTEN on VEC proliferation, migration, invasion and angiogenesis. p-AKT expression was significantly increased when VECs were treated with SF1670 compared with the OE-SDF1 group (Fig. 5A). In contrast to MK-2206 treatment, SF1670 treatment also significantly enhanced the
effects of OE-SDF1 on the promotion of VEC proliferation, angiogenesis, migration and invasion (Fig. 5B-E). These results demonstrated that SDF1 promoted angiogenesis by activating PI3K/AKT signaling.

Discussion

Accumulating evidence has indicated that the degree of disc degeneration is closely associated with the occurrence of neovascularization: The greater the number of new blood vessels, the greater the degree of disc degeneration (16). The new blood vessel, as a pioneer, can secrete nerve growth factor after entering the interior of the intervertebral disc and eventually induce the growth of painful nerve fibers into the intervertebral disc along the path of blood vessel growth (17), eventually leading to long-term chronic pain. In a previous study, it was demonstrated that SDF1 could facilitate the angiogenesis of IDD via CXCRI7, another affinity receptor of SDF1 (18). The present study focused on exploring the effect and mechanism of the SDF1/CXCR4 axis on angiogenesis, and the results indicated that SDF1/CXCR4 exerted a pro-angiogenic role through activation of the PI3K/AKT pathway.

It has previously been reported that the SDF1/CXCR4 axis has a crucial role in tumor metastasis (19), hematopoietic stem cell homing (20), organ development (21) and angiogenesis (22). In particular, a role in angiogenesis has been identified in various tissue damage-repair processes. For instance, Ziegler et al (23) reported that the SDF1/CXCR4 axis was closely involved in the repair of myocardial ischemia and played a role in the process of neovascularization. Wang et al (24) revealed that the SDF1/CXCR4 axis was strongly implicated in angiogenesis when a cerebral ischemic stroke occurred. Du and Liu (25) demonstrated that the SDF1/CXCR4 axis promoted angiogenesis and lymphangiogenesis in the sutured cornea. Additionally, Zhang et al (10) have reported that SDF1 expression was increased as the disc degeneration grade increased. As fissure formation after IDD and the subsequent vascular ingrowth are also body injury repair responses, the present study speculated that the SDF1/CXCR4 axis may be involved in the blood vessel ingrowth in IDD. The present study adopted adenovirus transfection to upregulate SDF1 expression in NPCs and found that the OE-SDF1 vector significantly increased the expression of SDF1 at both mRNA and protein levels. Notably, it was observed that SDF1 upregulation significantly increased cell viability, tube formation, the migratory and invasive abilities of VECs, which indicated that SDF1 exerts a pro-angiogenic role. However, the angiogenic ability of SDF1 was significantly reversed when CXCR4 was inhibited with AMD3100, which demonstrates that CXCR4 is necessary for the function of SDF1 in conducting downstream activity. Collectively, the present experimental results demonstrated that the SDF1/CXCR4 axis plays a crucial role in neovascularization during IDD, which is consistent with the research by Rätsep et al (16).

PI3K/AKT is an important downstream pathway of the SDF1/CXCR4 axis (26). When signals are delivered to the G protein-coupled receptor CXCR4, the two subunits of PI3K, P85 and P110, will undergo a conformational change to activate, leading to AKT phosphorylation and the subsequent signaling activation (26). However, PTEN is a negative regulator of the PI3K/AKT signaling pathway, which can dephosphorylate PIP3 and inhibit the phosphorylation of AKT (27). The present study observed that VECs that were incubated with the conditioned medium from OE-SDF1 NPCs exhibited a significant increase in the expression of p-AKT and a reduction in PTEN expression, which suggests that PI3K/AKT signaling may be involved in SDF1-induced angiogenesis. To this end, MK-2206 and SF1670 were recruited to suppress AKT and PTEN, respectively. The results revealed that MK-2206 treatment significantly reversed the promoting role of SDF1 in cell proliferation, migration, invasion and tube formation, which suggested that the SDF1/CXCR4 axis promotes the angiogenesis through activation of the PI3K/AKT signaling pathway. These results are consistent with a previous study (28). PTEN, as a tumor suppressor gene encoding protein, has been identified to have an important role in inhibiting cancer cell proliferation, metastasis, apoptosis and angiogenesis (29). When SF1670, a PTEN inhibitor was added to VECs, the inhibitory effect of PTEN was weakened with an increased amount of AKT phosphorylation. Moreover, SF1670 treatment significantly strengthened SDF1-mediated enhancements in VEC viability, migration, invasion and tube formation. This phenomenon is similar to the proangiogenic effect of PTEN suppression in the tumor microenvironment (30).

In summary, the present study, using the in vitro experiments, demonstrated that NPCs isolated from degenerated intervertebral disc tissues can induce VEC angiogenesis through the SDF1/CXCR4 signaling axis via the regulation of the PTEN/PI3K/AKT pathway. However, the present study has limitations. For example, the culture environment of the cells failed to simulate the hypoxic and high-pressure conditions experienced in vivo, and further downstream factors were not evaluated. Further experiments will be performed in order to clarify the role of SDF1/CXCR4 in an animal model. Overall, neovascularization ingrowth in degenerated intervertebral discs is an important hallmark of the pathological process of IDD. The in-depth study underlying its mechanisms may hopefully provide new insights and methods for the treatment of DDD.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HZ performed most of the experiments and wrote the manuscript. PW collected the clinical data and along with XZ and WZ conducted further experiments. HR performed data analysis, and ZH contributed to the conception of the study and critically revised the manuscript for important
intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Experiments involving human samples were performed in the light of the Helsinki Declaration and was approved by the Ethics Committee of the Chongqing Medical University. All patients signed informed consent forms.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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