Abstract: Objective: This study explores the correlations of stromal cell-derived factor-1 (SDF-1) and CXC chemokine receptor 4 (CXCR4) levels with caspase-3 expression in the retina of rats after optic nerve injury. Materials and methods: A total of 24 adult healthy specific pathogen-free Sprague-Dawley rats were selected and randomly divided into an optic nerve injury group (n=16) and an optic nerve sham-injury group (n=8). The optic nerve injury group was further sub-divided into a 3 d group (n=8) and a 7 d group (n=8) after their injuries. In the optic nerve injury group, the left eye of each rat was removed and prepared for the optic nerve injury model using the optic nerve clamping method. In the sham-injury group, the optic nerve in the left eye was only exposed without being clamped. The rats were sacrificed at 3 d and 7 d after their optic nerve injuries, and the retina was isolated. The expressions of SDF-1, CXCR4, and caspase-3 in the retina of the rats in each group were measured using an immunohistochemical method. Messenger ribonucleic acid (mRNA) and the protein expressions of SDF-1, CXCR4, and caspase-3 (cleaved caspase-3) in the retinas of rats were measured using quantitative real-time polymerase chain reaction (qPCR) and western blotting, respectively. Moreover, the correlations of the expression of SDF-1 and CXCR4 with caspase-3 expression were analyzed using the Spearman method. The apoptosis of retinal ganglion cells of rats in each group was observed using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Results: Immunohistochemistry of the retinas revealed that, compared with those in the sham-injury group, the expressions of SDF-1 and CXCR4 in the retina in the 3 d group and the 7 d group were gradually increased. Caspase-3 expression was significantly elevated at 3 d after the injuries, but obviously decreased at 7 d after the injuries. The results of qPCR showed that the relative expression levels of SDF-1 and CXCR4 mRNA in the retina at 3 d and 7 d after optic nerve injuries were also significantly higher than those in the sham-injury group (P<0.01), and the caspase-3 mRNA expression was initially increased at 3 d but reduced at 7 d after the injuries (P<0.01). Western blotting for the detection of the SDF-1, CXCR4 and caspase-3 proteins indicated changes similar to those of the qPCR. Spearman analysis results demonstrated that there was a positive correlation between the SDF-1 and CXCR4 expressions, but the expressions of SDF-1 and CXCR4 had negative correlations with caspase-3 expression. TUNEL staining showed that apoptosis of the retinal cells was increased in the 3 d group but significantly decreased in the 7 d group. Conclusion: After optic nerve injury, the continuous increase of the SDF-1 and CXCR4 levels suppresses the apoptosis of retinal cells and repairs the retina by inhibiting the cleavage activation of caspase-3. This provides new insights for the ongoing treatment of optic nerve injury.

Keywords: Optic nerve injury, SDF-1, CXCR4, caspase-3, apoptosis

Introduction

The death of retinal ganglion cells and the irreversible visual injury of visual functional units after optic nerve injury are the main causes of blindness, and so far there have been no effective treatments [1].

Stromal cell-derived factor-1 (SDF-1) belongs to α chemokine family and represents a type of chemokine protein with a small molecular weight [2, 3]. CXC chemokine receptor 4 (CXCR4) is widely expressed in a variety of tissues and organs and is the only specific receptor of SDF-1. Various biologic functions can be exerted when SDF-1 binds to CXCR4 specifically [4]. When inflammation, hypoxia, ischemic injury, and other lesions occur in tissues, the SDF-1 protein expression is increased significantly and is involved in the repair of damaged tissues and cells [5]. SDF-1 and CXCR4 play key roles in the repair processes of the myocard-
um, nerves, liver, and kidneys [6-9]. Also, it has been indicated that SDF-1 protein expression in the retina is significantly increased in cases of optic nerve injury [10]. Moreover, the SDF-1 protein can reverse the apoptosis of endothelial progenitor cells induced by serum-free culture and can also inhibit the cleavage activation and protein expression of caspase-3 [11]. In this study, we aimed to investigate the possible mechanisms of SDF-1, CXCR4, and caspase-3 in regeneration and repair after optic nerve injury.

Materials and methods

Reagents

The SDF-1, MMP-9, and caspase-3 rabbit polyclonal antibodies and horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). The primer synthesis and an RT-qPCR kit were provided by TaKaRa (Dalian, Liaoning, China). The bicinchoninic acid (BCA) protein quantification kit and the tissue lysis solution were bought from the Beyotime Institute of Biotechnology (Nantong, Jiangsu, China). The immunohistochemical staining kit SP-9001 was purchased from Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd. (Beijing, China).

Laboratory animal grouping and establishment of the optic nerve injury model

A total of 32 healthy female SD rats of clean grade weighing 180-220 g were randomly divided into an optic nerve injury group (the left eye was determined to be the injured eye, n=16) and the optic nerve sham-injury group (the left eye was set as the sham-injured eye, n=8). The optic nerve injury group was further divided into a 3 d group (n=8) and a 7 d group (n=8), according to different time points after the optic nerve injury. After the rats were anesthetized using an intraperitoneal injection of 10% chloral hydrate, the rats’ eyes were cleaned and disinfected, and the upper fornical conjunctiva were cut and isolated bluntly to expose the optic nerves. The optic nerves were clamped for 10 s using a medium non-invasive vascular clamp at 2 mm behind the eyeballs until there was a dilation of the pupils and no local ischemia in the ocular fundus. The conjunctiva was sutured and an antibiotic ointment was applied. The rats in the optic nerve sham-injury group were anesthetized using the same method, and the optic nerve of the each rat’s left eye was only exposed without being clamped.

Collection of retinal tissue specimens

Immunohistochemical specimens: 4 rats were taken at 3 d and 7 d after the optic nerve injury and anesthetized using an intraperitoneal injection of chloral hydrate, followed by cardiac perfusion using 4% paraformaldehyde. The left eyeballs were taken out, and the retinal tissues were isolated and fixed in 4% paraformaldehyde overnight, dehydrated with gradient alcohol, and embedded in paraffin to be prepared as 4 nm-thick pathological sections. The sections were fixed on the anti-off glass slide, baked for 30 min and stored at 4°C.

Western blotting and RT-PCR specimens: 4 rats were taken at 3 d and 7 d after their optic nerve injuries and anesthetized, after which the left eyeballs were quickly removed. From each rat, the entire retina was taken out under a microscope, and quickly stored in a refrigerator at -80°C for standby application.

Detection of SDF-1, CXCR4 and caspase-3 expressions in retinal tissues using immunohistochemistry

The paraffin sections of the retinal tissues in each group were dewaxed, and endogenous peroxidase was inactivated using 3% \( \text{H}_2\text{O}_2 \), followed by antigen retrieval in a microwave oven. Then sections were sealed with 10% goat serum and the corresponding primary antibody was added (diluted at 1:100) for incubation in the refrigerator at 4°C overnight. After that, the sections were washed with phosphate buffered saline with Tween-20 (PBST) for 3 times (5 min per time), and the biotin-labeled secondary antibody was added and the sections were incubated for 1 h, and then the sections were washed again with PBST 3 times (5 min each time), followed by color development with diaminobenzidine (DAB), hematoxylin counterstaining, sealing with neutral gum, and photography under the microscope.

Measurement of the SDF-1 and CXCR4 mRNA expressions in retinal tissues by RT-qPCR

After the retinal tissues of the rats in each group were taken from the eyeballs, the total
RNA was extracted, and the qualified RNA was used as a template to synthesize complementary deoxyribonucleic acid (cDNA) by reverse transcription. The specific reaction conditions were as follows: incubation at 42°C for 15 min, incubation at 95°C for 3 min, then cooling on ice. After the corresponding primers (Table 1) were added, the mRNA expression of each gene was measured by fluorescence quantitative RT-PCR, with the β-actin gene as an internal reference. The reaction conditions were as follows: 94°C for 5 min, 94°C for 30 s, 57°C for 30 s, 75°C for 30 s, and amplification for a total of 45 cycles, 72°C for 5 min. The relative expression was calculated using the 2^ΔΔCt method, ΔΔCt = Ct target gene − Ct β-actin of same specimen.

**Table 1.** SDF-1, CXCR4 and caspase-3 mRNA primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Primer sequence</th>
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<tr>
<td>SDF-1</td>
<td>Forward</td>
<td>5’-GACGGTAAGCCAGTCAGCCT-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CAGGTACTCTTGATCCACT-3’</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Forward</td>
<td>5’-ACGCAGACGTCCTCCAGTA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CCACCTGTTCAACTCCTCC-3’</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Forward</td>
<td>5’-ACCTCAGAGACATTAC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-CCCCACTCCAGTCATTT-3’</td>
</tr>
<tr>
<td>β-actin</td>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-TGCTTGCTGATCCATCTG-3’</td>
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**Detection of SDF-1, CXCR4 and caspase-3 protein expressions in retinal tissues by western blotting**

Approximately 50 mg of frozen retinal tissues were taken from the rats in each group and lysed using the tissue lysis solution to extract the total protein. The concentration of the extracted protein was determined using the BCA method, and 30 μg of protein were loaded into each well for electrophoretic separation. Then the protein was transferred onto a membrane using the wet method, sealed with skim milk powder, and then the corresponding primary antibody (diluted at 1:1000) was added to the protein for incubation at 4°C overnight. It was fully washed with tris-buffered saline with Tween-20 (TBST) and incubated with the secondary antibody at room temperature for 2 h, followed by image development.

**Detection of apoptosis in retinal tissues by TUNEL**

The paraffin sections in each group were routinely dewaxed, washed with PBS and then terminal deoxynucleotidyl transferase and a digoxin-labeled dUTP reaction solution were added dropwise for incubation at 37°C for 2 h. After being washed with PBS 3 times (3 min each time), a biotinylated anti-digoxin antibody then was added to the sections dropwise for incubation at 37°C for 30 min, and washed again with PBS 3 times (3 min per time), followed by color staining.
SDF-1 and CXCR4 versus caspase-3 after optic nerve injury

development by DAB, reaction in a dark place at room temperature for 10 min, and photography under a fluorescence microscope and analysis.

Statistical processing

All the data are presented as the mean ± standard deviation. Statistical Product and Service Solutions (SPSS) 17.0 software was used for the statistical data processing. A t test was used for the comparison among groups, and the continuous data from multiple groups were analyzed using one-way ANOVA, with Tukey’s post hoc test. P<0.05 suggested that a difference was significant.

Results

SDF-1, CXCR4, and caspase-3 protein expressions in the retinal tissues of rats

According to immunohistochemistry, the SDF-1, CXCR4, and caspase-3 proteins were expressed less in the retinal tissues of rats in the sham-injury group, but the levels showed increasing trends in the retinal tissues of the rats in the injury group. The caspase-3 protein expression was increased first at 3 d; however, it decreased at 7 d after injury, suggesting that the continuous increase of the SDF-1 and CXCR4 protein expressions may have led to the decline in the caspase-3 protein expression (Figure 1).

SDF-1, CXCR4, and caspase-3 mRNA expressions in the retinal tissues of rats

Compared with those in the sham-injury group, the SDF-1 and CXCR4 mRNA expressions in the retinal tissues of the rats in the 3 d group and the 7 d group were gradually increased (P<0.01). Moreover, compared with its expression in the sham-injury group, the caspase-3 mRNA expression was significantly elevated in retinal tissues of the rats in the 3 d group (P<0.01) but significantly reduced in the 7 d group (P<0.01) (Figure 2).

SDF-1, CXCR4 and cleaved caspase-3 protein expressions in the retinal tissues of rats

The SDF-1, CXCR4, and cleaved caspase-3 protein expressions in the retinal tissues of the
rats after optic nerve injury were detected using western blotting. The results revealed that the SDF-1 and CXCR4 protein expressions in the retinal tissues of rats in the 3 d and 7 d groups were obviously increased compared with those in the sham-injury group ($P<0.01$). The cleaved caspase-3 protein expression in the retinal tissues of the rats in the 3 d group was significantly higher than it was in the sham-injury group ($P<0.01$), but it obviously decreased in the retinal tissues of the rats in the 7 d group compared with its level in the 3 d group ($P<0.01$), indicating that the growing expressions of SDF-1 and CXCR4 may inhibit the cleavage activation process of the caspase-3 protein (Figure 3).

**Correlation analyses** showed that there was a positive correlation between the SDF-1 and CXCR4 expressions, but the expressions of SDF-1 and CXCR4 were negatively associated with the caspase-3 level ($r=-0.637$, $P<0.01$) (Table 2).

**Apoptosis in the retinal tissues of the rats**

Compared with the sham-injury group, the number of apoptotic cells in the retinal tissues of the rats was obviously increased in the 3 d group, but significantly downregulated in the 7 d group, suggesting that SDF-1 and CXCR4 may resist the effects of optic nerve injury on the retina by inhibiting the apoptosis of the retinal cells (Figure 4).

**Discussion**

SDF-1 and its receptor CXCR4 play key roles in the immune system, inflammation, tumors, AIDS and other diseases, the former of which, according to studies, is of great significance in injury repair of a variety of tissues [12-14]. With the deepening of research on optic nerve injury repair, the effects of SDF-1 and CXCR4 therein have also been gradually recognized, and SDF-1 has been found to be able to enhance the viability of retinal ganglion cells [15]. According to clinical findings, SDF-1 improved the survival rate of photoreceptor cells in patients with retinal detachment [16].

It was believed previously that when the axon or axon of an animal's optic nerve is damaged, irreversible injury occurs in optic nerve and retinal ganglion cells, leading to their death. Therefore, the approach to protect patients with traumatic optic neuropathy is to inhibit the apoptosis of retinal ganglion cells [17]. The cysteine protease family significantly participates in the apoptosis process, and caspases are key factors for the initiation and execution of apoptosis [18, 19]. Optic nerve injury induces the cleavage activation of caspase-3 protein in retinal ganglion cells, and retinal ganglion cell apoptosis can be prevented when caspase-3 inhibitors are applied [20]. Therefore, this study aims to clarify the changes in SDF-1, CXCR4 and caspase-3 protein expressions in the process of optic nerve injury and to study the correlations among them, so as to illuminate the possible molecular mechanisms of SDF-1 and CXCR4 in protecting optic nerve injury.

In this experiment, the SDF-1, CXCR4, and caspase-3 expressions in the retinas of the rats in each group were measured using immunohistochemistry, western blotting, and qPCR. Compared with those in the sham-injury group, the expressions of SDF-1 and CXCR4 in the retinas in the 3 d and 7 d groups were gradually increased, and the caspase-3 level was primarily elevated at 3 d but reduced at 7 d after injury. Through a Spearman analysis, we demonstrated that there was a positive correlation between the SDF-1 and CXCR4 expressions, but the expressions of SDF-1 and CXCR4 were negatively associated with the caspase-3 level. Furthermore, TUNEL staining showed that the apoptosis of the retinal cells was increased in the 3 d group but significantly decreased in the 7 d group, revealing the inhibitory effects of SDF-1 and CXCR4 on the apoptosis of the retinal cells according to the previous finding that an optic nerve injury induces the cleavage activation of caspase-3 protein in retinal ganglion cells. Retinal ganglion cell apoptosis can

<table>
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<th>Gene</th>
<th>SDF-1</th>
<th>P</th>
<th>CXCR4</th>
<th>r</th>
<th>P</th>
</tr>
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<td>CXCR4</td>
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<tr>
<td>Caspase-3</td>
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<td>&lt;0.01</td>
<td>-0.637</td>
<td>&lt;0.01</td>
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be prevented when caspase-3 inhibitors are applied [20]. Of note, our preliminary data revealed that the SDF-1 and CXCR4 levels were persistently upregulated in the optic nerve injury model, which remarkably reversed the rising expression of caspase 3 in the course of the disease. However, the molecular mechanisms of SDF-1 and CXCR4 in inhibiting caspase-3 expression and cleavage activation are still elusive and need to be elucidated in future studies.

Taken together, the expressions of SDF-1 and CXCR4 are increased after optic nerve injury and show a negative correlation with the expression of caspase-3. Rising levels of SDF-1 and CXCR4 inhibit the apoptosis of retinal cells and repair the retina by suppressing caspase 3, a finding that provides fundamental leads for treatment of retinal injury.

Disclosure of conflict of interest

None.

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