Original Article
Protease nexin-1 inhibits apoptosis of nucleus pulposus cells induced by TNF-α

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Abstract: Objective: The study aimed to analyze the expression of protease nexin-1 in human degenerative nucleus pulposus (NP) cells of intervertebral disc and its effect on apoptosis. Method: Nucleus pulposus tissue and cells were collected from 12 cases with degenerative disc disease (group A) and 10 cases with vertebra fractures (group B, control). The expression of protease nexin-1 (PN1) mRNA and PN-1 protein were determined by qRT-PCR and Western blot. The nucleus pulposus cells were treated with 100 ng/mL TNF-α solution. The proliferation of nucleus pulposus cells treated with TNF-α was tested by CCK-8. LV-PN-1 lentiviral expression plasmid or LV-NC empty plasmid were transfected into nucleus pulposus cells. The mRNA expression of PN-1 in nucleus pulposus cells was tested by qRT-PCR. The apoptosis of nucleus pulposus cells was tested by flow cytometry. The expression of X-linked inhibitor of apoptosis protein (XIAP) in nucleus pulposus cells was tested by Western blot. Result: PN-1 mRNA and protein expression in intervertebral group A was significantly lower than that in group B (P<0.001). TNF-α induced significant inhibition on cell proliferation. LV-PN-1 transfection increased PN-1 mRNA and protein expression. TNF-α induced cell apoptosis and XIAP protein expression was inhibited by V-PN-1 transfection but not LV-NC transfection. Conclusion: PN-1 could inhibit apoptosis of nucleus pulposus cells induced by TNF-α which might be related to decreased expression of XIAP.

Keywords: Protease nexin-1, nucleus pulposus cells, apoptosis, mechanism

Introduction

Intervertebral disc degeneration (IVDD) was characterized by lumbar disc herniation, spondylolisthesis, discogenic low back pain (DLBP) and degenerative spinal stenosis, causing pain in the waist, back and legs [1, 2]. About 10% of the 50-year-olds suffered from intervertebral disc degeneration while 60% of the 70-year-olds suffered from severe disc degeneration [3]. Previous studies showed that the excessive apoptosis of cells was a leading factor for a decrease in cells of intervertebral discs [4]. The nucleus pulposus cells, inactive and highly differentiated, play an important role in the process of intervertebral disc degeneration [5]. During the process, fibrinogen polypeptide fragments are significantly increased due to the destruction of fibrin in the extracellular matrix. Mediated by protease hydrolysis, the activity of MMPs increased, causing intervertebral disc degeneration [6]. Therefore, the pathological changes of protease activity may be one of the mechanisms that induces degeneration of the intervertebral disc [7]. Regulation of protease may be an important method to alleviate intervertebral disc degeneration.

Protease nexin-1 (PN-1) is a serine protease inhibitor that prevents cartilage degeneration by inactivating various proteases, such as plasminogen, urokinase [8, 9]. PN-1 can regulate the activity of matrix enzymes through the interaction of intracellular cartilage components and extracellular matrix [10]. The intervertebral disc tissue belongs to cartilage connective tissue, which is similar to physiological and pathological characteristics with knee osteoarthritis [11]. Similarly, PN-1 may play an important role in the occurrence and development of intervertebral disc degeneration.

Some scholars suggested that matrix metalloproteinase-9 exerted an influence on invasion and metastasis of prostate cancer cells by reg-
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The study is aimed at the mechanism of PN-1 on the apoptosis of human degenerative disc nucleus pulposus cells.

Materials and methods

General data

Samples of nucleus pulposus tissues and cells were collected from patients who were treated with spine surgery in our hospital. Degenerative nucleus pulposus tissue and cells were from 12 cases who received spine surgery (the intervention group), including 7 males and 5 females aged 44-62, with an average age of 53.47±10.68 years old. Patients were diagnosed as lumbar disc herniation through vital signs and imaging methods before surgery [15]. According to Pfirrmann, surgical intervertebral disc performed at III-IV levels, and scoliosis (Cobb angle <90°). Normal nucleus pulposus tissue and cells were from 10 cases who received spine surgery due to fracture of lumbar vertebra, including 5 males and 5 females aged 32-51, with an average age of 35.61±8.64 years old [16].

Exclusion criteria were patients with history of severe liver and renal dysfunctions, congenital heart disease, neurological diseases, endocrine and metabolic disease, mental disorders, malnutrition and tumors. The study was approved by the medical ethics committee of Juye People’s Hospital. Patients and their family members signed the formed consent.

Experimental materials, instruments and reagents

The following materials and instruments were used, including DMEM/F12 cell culture medium (Hyclone, Logan, UT, USA), CCK-8 kit (Jiangsu Beyotime Biotechnology Co., Ltd., China), Fetal bovine serum, Penicillin-streptomycin solution (Sigma Chemical Company, USA), TNF-α (PeproTech, Rocky Hill, NJ, USA), Polyclonal anti-PN-1 (AbCam, Cambridge, Cambridgeshire, UK), TurboFect Transfection Reagent (Cat #RO-0531, R0532), SYBR® Gree qRT-PCR, RNA extraction Trizol kit, Annexin V-FITC apoptosis kit, LV-PN-1 Lentiviral Expression Plasmid, LV-NC empty plasmid (Invitrogen, Carlsbed, CA, USA), M-MLV transcription kit (Fermentas, Burlington, Ontario, Canada), Mouse anti-human β-actin IgG, BCA kit (Beijing BioTeke Coporation, China), 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), Elx-800 MTT (BioTek, Winooski, VT, USA), FACSCanto flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and PN-1 mRNA and GAPDH primers (Shanghai GenePharma Co., Ltd) (Table 1).

Isolation and culture of nucleus pulposus cells [17]

The collected nucleus pulposus tissue is placed in a sterile culture dish, sealed and stored in an ice box, and transported to the laboratory. The nucleus pulposus tissue was washed by PBS (phosphate buffered saline) buffer, and gelatinous nucleus pulposus was separated. The non-fibrous and soft tissue was placed in the centrifugal tube. After adding trypsin, the tissue was placed in a water bath at 37°C for 30 min. After the centrifugation for 5 min with force of 800× g and radius of 13.5 cm, the supernatant was discarded and placed in type II collagenase (2 g/L) at 37°C for 4 hours, and filtered through 200 um cell strainer. Cell precipitation was taken after centrifugation. The cells were planted in a T25 flask at a concentration of 5×10^5/mL, and then cultured in a DMEM/F12 medium with 5% CO_2 at 37°C. When cells fused to about 80%, the third generation of nucleus pulposus cells was taken for follow-up experiments.

Apoptosis model of nucleus pulposus cells

Nucleus pulposus cells were digested with 0.25% trypsinase and centrifuged for 5 minutes with 800× g and radius of 13.5 cm. Cell precipitation was taken and added to DMEM/F12 medium with 5% CO_2 at 37°C. The cells were washed and centrifuged, and resuspended in a fresh medium with 5% CO_2 at 37°C.
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F12 medium. Cell suspension was prepared to inoculate in 96-well plates at a concentration of 1×10⁴, 100 µL in each well, and cultured in cell incubator with 5% CO₂ at 37°C for 24 hours. The cells were stimulated with 100 ng/mL TNF-α solution [18] to obtain the same volume nucleus pulposus as the negative control group (TNF-α-NG). Medium nucleus-free cells were set up as the control group and cultured in cell box with 5% CO₂ at 37°C for 24 hours.

Cell proliferation

The cells were seeded in 96-well plates and the number was controlled at 1×10⁵/mL/per well. 10 µL CCK-8 solution was added to 100 µL RPMI-1640 medium, and cultured in a 5% CO₂, 37°C cell incubator for 4 hours. The OD value of each well was measured at 450 nm and repeated 4 times for each group. The amount of absorbance is directly proportional to the ability of cell proliferation. Relative proliferation activity = OD value of intervention group/OD value of control group.

Transfection of cells

Nucleus pulposus cells were inoculated into 6-well plates at 3×10⁵/well, and cultured when confluence reached 80-90%. Cells were transfected with TurboFect transfection reagent. The constructed LV-PN-1 lentiviral expression plasmid and LV-NC empty plasmid were transfected into nucleus pulposus cells. LipofectamineTM 2000 6 µL was mixed with 150 µL of Opti-MEM. 2 µg of LV-PN-1 lentiviral expression plasmid, LV-NC empty plasmid and 2 µg of TNF-α, TNF-α+LV-PN-1 and TNF-α+LV-NC was transfected into Opti-MEM mixed at room temperature for 20 min. Then, the mixture was placed in the nucleus pulposus cell culture medium for 6 hours, and replaced by normal medium and transfected for 48 h. After that, the supernatant was filtered and stored in a refrigerator at -80°C. After 24 hours, the nucleus pulposus cells were inoculated into 6-well plates at 2×10⁵ cells/well. Virus supernatant and cell culture medium were added to each well at a concentration of 8 µg/mL. Nucleus pulposus cells without treatment were cultured as the untransfected group cultured in 5% CO₂ at 37°C for 24 hours.

Apoptosis of cells

After 48 hours, the transfected cells were digested with trypsin, washed with cold 0.01 mol/L PBS, and centrifuged at 111.8 (×g) at 25°C. The supernatant was discarded and the cells were re-suspended with 100 µL of 1× binding buffer, and then transferred to flow cytometry. By adding 5 µL 7AAD and 5 µL PE Annexin V in each tube, the cells were reacted at room temperature for 15 min, and then 400 µL of 1× binding buffer was added for flow cytometry within 1 hour. Each sample was tested three times. The apoptotic cells are displayed in Q2 and Q3.

qRT-PCR

The collected tissues and cells were used for the measurement of total RNA with EasyPure miRNA Kit. The total RNA was tested by ultraviolet spectrophotometer and AGE (agarose gel electrophoresis) to determine the purity, concentration and integrity. Reverse transcription of the total RNA was operated by using the M-MLV kit accordingly. Subsequent amplification of PCR was performed. PCR reaction system was as follows: 1 µL cDNA, 1 µL upstream and downstream primers, 10 µL of Mix, 1 µL of CDNA, and ddH₂O was added to 20 µL. PCR reaction conditions were as follows: pre-denaturation at 95°C for 5 min, denaturation at 94°C for 30 s, annealing at 61°C for 30 s, extension at 72°C for 45 s, for a total of 40 cycles. Each sample with three repetitive wells was tested for three times. In this study, GAPDH was used as an internal reference, and 2⁻ΔΔct was used to analyze the data.

Western blot detection

Tissues and cells were collected to extract the total protein by RIPA pyrolysis. And BCA was used to determine the protein concentration, which was adjusted to 4 µg/µL, and was separated by 12% SDS-PAGE electrophoresis. After that, the membrane was transferred to PVDF, and dyed using ponceau liquid. PBST was used for 5 min and 5% skimmed milk powder for 2 hours. Primary antibody (1:1000) was added at 4°C overnight. The membrane was removed and sheep anti-rat secondary antibody (1:5000) labeled with HRP (horse radish peroxidase) was incubated at 37°C for 1 hour. TBST was rinsed three times for 5 minutes. The images were displayed in a dark room. Excess liquid was absorbed by using filter paper. ECL emits light and develops. The protein bands were scanned and the gray values were analyzed using Gel-Pro_
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Table 2. Expression of PN-1 mRNA and PN-1 protein in two groups of intervertebral disc tissues (X ± sd)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>PN-1 mRNA</th>
<th>PN-1 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>12</td>
<td>0.637±0.224</td>
<td>0.518±0.137</td>
</tr>
<tr>
<td>Group B</td>
<td>10</td>
<td>1.357±0.193</td>
<td>1.126±0.207</td>
</tr>
<tr>
<td>t</td>
<td>-</td>
<td>7.984</td>
<td>7.936</td>
</tr>
<tr>
<td>P</td>
<td>-</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Figure 1. Expression of PN-1 mRNA and PN-1 in the intervertebral disc tissue of the two groups. Expression of PN-1 mRNA in intervertebral disc tissue of the two groups (A); Western blot results of PN-1 protein (B); expression of PN-1 protein in intervertebral disc tissue of the two groups (C). Note: Compared with group A, *P<0.001.

Statistical method

SPSS20.0 (IBM Corp, Armonk, NY, USA) and GraphPad Prism 7 were used for statistical analysis. The measurement data were expressed as mean ± standard deviation (mean ± sd). The one-way analysis of variance was performed for comparison among multiple groups with post hoc Dunnett-t test. P<0.05 indicates statistically significant.

Results

Expression of PN-1 mRNA and PN-1 in intervertebral discs of two groups

Group A showed significantly lower expression of PN-1 mRNA than that in group B (P<0.001).

Effect of pretreatment with TNF-α on apoptosis of nucleus pulposus cells

Based on the effect of PN-1 transfection on the apoptosis of nucleus pulposus cells, the proportion of pretreatment with TNF-α on apoptosis of nucleus pulposus cells was tested by flow cytometry. The results showed that the apoptosis of nucleus pulposus cells in TNF-α-LV-PN-1 group, TNF-α-LV-NC group, TNF-α group and the untransfected group were 10.62±2.167%, 8.96±0.109 and 7.35±0.118. There was no significant difference in the expression of PN-1 in nucleus pulposus cells between LV-NC group and the untransfected group (P>0.05). Compared with LV-NC group and the untransfected group, the expression of PN-1 in LV-PN-1 group was significantly increased (P<0.001) (Figure 3).

Effect of pretreatment with TNF-α on proliferation of nucleus pulposus cells

Indicated by CCK-8, the OD values of TNF-α group, TNF-α-NG group and the control group were 52.71±2.53%, 93.46±4.25% and 94.45±4.31%. There was no significant difference in OD value between TNF-α-NG and the control group (P>0.05). The OD value of TNF-α group was significantly lower than that of TNF-α-NG group and the control group (P<0.001) (Figure 2).
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In order to analyze the biological mechanism of PN-1 regulating apoptosis of nucleus pulposus cells, the expression of XIAP in nucleus pulposus cells pretreated with TNF-α was tested by Western blot. The results showed that there was no significant difference in the relative expression of XIAP between TNF-α-LV-NC group and TNF-α group (P>0.05). The relative expression of XIAP in nucleus pulposus cells of TNF-α-LV-PN-1 group was lower than that of TNF-α-LV-NC group and TNF-α group (P<0.001) (Figure 5).

Discussion

Intervertebral disc degeneration, the main cause of pain in the waist, legs and back, is one of the main risk factors for disability [19]. With multiple factors and complex process, the treatment of degeneration of intervertebral disc is still not good [20, 21]. Excessive apoptosis of nucleus pulposus cells plays an important role in the process of degeneration of intervertebral discs [22], so the targeted therapy of controlling apoptosis in intervertebral disc degeneration is a hot research topic in recent years [23, 24].

The intervertebral disc is composed of nucleus pulposus, annulus fibrosus and cartilage endplate. Excessive apoptosis can reduce the number of nucleus pulposus cells and degenerate annulus fibrosus, which inhibits the production of extracellular matrix. Thus, nucleus pulposus cells are gradually replaced by connective tissue. The aging and reduction of extracellular matrix cause intervertebral disc degeneration [22, 25]. Previous studies revealed that the expression of serine protease significantly increased and participated in the process of inflammation during intervertebral disc degeneration [20]. Therefore, the change of serine protease determines the evolution of intervertebral disc [26]. PN-1, located in the surface of cells, is a serine protease inhibitor with a molecular structure of 45-50-kDa glycoprotein, of which combination with glycosaminoglycan inhibits the hydrolysis of endoproteins [27]. PN-1, involved in the process of fibrin, can inhibit the activity of protease in a highly complex and irreversible way [9].

In the study of Boukais et al. [28], vascular smooth muscle cells could prevent the injury of the arterial wall caused by proteolysis by producing anti-protease, such as PN-1. PN-1, a serine protease inhibitor expressed by vascular smooth muscle cells, could inhibit the activity of plasmin by forming the complex to increase
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Figure 4. Effect of pretreatment with TNF-α on apoptosis of nucleus pulposus cells. Note: Compared with TNF-α-LV-NC group and TNF-α group, *P<0.001; compared with the untransfected group, #P<0.001.

Figure 5. Effect of pretreatment with TNF-α on the expression of XIAP in nucleus pulposus cells. Note: Compared with TNF-α-LV-NC group and TNF-α group, *P<0.001; compared with the untransfected group, #P<0.001.

the expression of PN-1, which can protect the artery wall from protease damage. In the study of Xin et al. [29], PN-1 expression was low in human B-cell lymphoma. Upregulation of PN-1 could inhibit the signal of sonic hedgehog, thereby preventing the growth of human B-cell lymphoma. Therefore, the up-regulation of PN-1 may be beneficial to cure disease. Our results showed that the expression of PN-1 in degenerative intervertebral disc is significantly reduced, indicating that PN-1 may be involved in the occurrence and development of disc degeneration. Serine protease is up-regulated in the process of intervertebral disc degeneration, during which PN-1, a serine protease inhibitor, may gradually decrease during the course of disease.

TNF-α, an important proinflammatory cytokine, plays an important role in the pathological changes of intervertebral disc degeneration, which can promote the apoptosis of nucleus pulposus cells and inhibit the expression of extracellular matrix in nucleus pulposus [30]. In a study of Liu et al. [31], latent membrane protein-1 (LMP-1) inhibited TNF-α induced intervertebral disc degeneration by inhibiting the production of extracellular mechanism of nucleus pulposus and the expression of matrix metalloproteinase. Wang et al. [32] revealed that miR-494 could protect nucleus pulposus cells from TNF-α induced apoptosis by inhibiting JunD. PN-1 may inhibit the apoptosis of nucleus pulposus cells in degenerative intervertebral disc. According to apoptosis model of nucleus pulposus cells prepared by using TNF-α, the proliferation of cells pretreated with TNF-α was significantly inhibited. The follow-up experiments also proved that the apoptosis in TNF-α-LV-PN-1 group was significantly lower than that in TNF-α-LV-NC group and TNF-α group, indicating that up-regulation of PN-1 in nucleus pulposus cells could inhibit TNF-α induced apoptosis of nucleus pulposus cells. PN-1 could inhibit apoptosis of degenerative disc nucleus pulposus cells.

XIAP, an inhibitor of apoptosis protein, is targeted for caspases causing cell death [33]. XIAP, combined with caspase 3, 7 and 9, promotes apoptosis of mitochondria and death receptors [34]. High levels of XIAP associated with malignant tumors and are proved to be associated with decreased survival and poor prognosis in clinic [35]. XIAP has many biological functions in apoptosis [36, 37]. McKee et al. [13] suggested that excessive expression of PN-1 could enhance apoptosis of prostate cancer cells through inhibiting the expression of XIAP. In order to analyze the biological mechanism of PN-1 in regulating apoptosis of nucleus pulposus cells, Western blot was used to test the expression of XIAP in nucleus pulposus cells of PN-1 pretreated with TNF-α. The results showed that the relative expression of XIAP in nucleus pulposus cells in TNF-α-LV-PN-1 group was lower than that in TNF-α-LV-NC group and
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TNF-α group. Therefore, PN-1 may inhibit the apoptosis of nucleus pulposus cells by inhibiting the expression of XIAP. Wu et al. [38] showed that PN-1 inhibited IL-1β-induced apoptosis of nucleus pulposus cells by regulating NF-κB signaling path [16]. In this study, up-regulation of PN-1 can inhibit the apoptosis of nucleus pulposus cells. However, the mechanism of PN-1 in the process of intervertebral disc degeneration is limited. The regulatory mechanism has yet to be demonstrated.

In this study, excessive expression of PN-1 can inhibit the apoptosis of degenerative disc nucleus pulposus cells, which has been proved. Some defects still existed. The relationship between PN-1 and XIAP has not been proved by the dual-luciferase reporter system. And the effect of PN-1 on the regulation path of nucleus pulposus cells has not been further analyzed. More studies need to be conducted to prove the result in this study.

In conclusion, PN-1 may be involved in the occurrence and development of intervertebral disc degeneration. PN-1 can inhibit TNF-α induced apoptosis of nucleus pulposus cells through inhibiting the expression of XIAP. Thus, PN-1 is expected to be a biological target for the prevention and treatment of intervertebral disc degeneration.

Disclosure of conflict of interest

None.

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References


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