Original Article
Integrin β1 overexpression protects nucleus pulposus cells from apoptosis and attenuates intervertebral disc degeneration

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Received February 2, 2017; Accepted February 26, 2017; Epub May 1, 2017; Published May 15, 2017

Abstract: Intervertebral disc (IVD) degeneration is a chronic pathologic process characterized by excessive apoptosis of intervertebral disc cells. The purpose of the current study was to detect the effect of integrin β1 on apoptosis of NP cells and IVD degeneration. Integrin β1 overexpressing lentivirus vector (LV-ITG β1) was constructed and transduced to NP cells, and its effect on cell apoptosis were studied in vitro. The underlying mechanism was subsequently studied. In addition, the in vivo effect of integrin β1 on IVD degeneration was studied by intradiscal injection of LV-ITG β1 in a rat IVD degeneration model. In vitro study showed that integrin β1 overexpression protected NP cells from levofloxacin-induced apoptosis, and LV-ITG β1 could significantly upregulate the expression of both FAK and p-FAK and downregulate the expression of P53 measured by western blot. Intradiscal injections of LV-ITG β1 suppressed NP cell apoptosis tested by TUNEL staining, and attenuated disc degeneration assessed by histological and MRI examination. Our findings suggested that integrin β1 could be used as an effective gene therapy target for IVD degeneration due to its significant effect on preventing NP cell apoptosis both in vitro and in vivo, and FAK-P53 signaling axial was involved in the protective effect of integrin β1 on NP cells.

Keywords: Integrin β1, apoptosis, nucleus pulposus cells, intervertebral disc degeneration

Introduction
Back pain is one of the most common causes of disability worldwide [1]. Back pain etiology is typically associated with intervertebral disc (IVD) degeneration. The treatment currently used for back pain only alleviates symptoms without altering the natural process of IVD degeneration. Biological treatment approaches show encouraging results in the treatment of IVD degeneration and resultant back pain.

Integrins are heterodimeric transmembrane adhesion receptors composed of two subunits [2]. The β1 subunit assembles with many others α-subunits to form different heterodimers that transmit extracellular survival signals across the cell membrane [3, 4]. Integrin β1 subunit expression is downregulated during NP cell apoptosis, indicating that integrin β1 may be involved in the regulation of NP cell survival [5]. However, whether overexpression of integrin β1 would protect NP cells from apoptosis has not been investigated.

In this study, a lentivirus vector overexpressing integrin β1 (LV-ITG β1) was constructed and transduced to NP cells, and its effect on cell apoptosis was studied in vitro. The underlying mechanism was subsequently studied. In addition, the in vivo effect of integrin β1 on IVD degeneration was studied by intradiscal injection of LV-ITG β1 in a rat IVD degeneration model.

Materials and methods

Ethical statement
Animal experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animal.
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Culture of rat NP cells

Immediately after euthanasia, rat IVDs from the lumbar spine were harvested from six-week-old male Sprawl-Darley (SD) rats. NP tissues were separated from the specimen and digested in 0.25% trypsin for 10 min and, subsequently, in 0.025% type II collagenase for 4 h at 37°C. NP cells were cultured in high-glucose DMEM medium containing 10% FBS (Gibco, San Diego, CA, USA) and 1% penicillin/streptomycin. Passage 2-3 cells were used throughout the experiments.

Lentivirus vectors and transfection

Recombinant integrin β1 overexpressing lentivirus vector (LV-ITG β1) was constructed by Genechem Co. Ltd. (Shanghai, China). The coding sequence of integrin β1 (Genbank ID: NM_017022) was cloned with the forward primer 5′-GAGGATCCCCGGGTACCGGTCGCCACATGAATTTGCAACTGGTTTTC-3′ and reverse primer 5′-TCCTTGTAGTCCATACCTTTTCCCTCATACTTCGGATTGAC-3′. The 2438 bp sequence was subsequently inserted into Agel/Agel multiple cloning site of GV358 (Ubi-MCS-3FLAG-SV40-EGFP-IRESPuromycin). The lentivirus vector overexpressing a scrambled sequence was used as a control (LV-CON). Multiplicity of infection (MOI) was determined by a trial test and MOI = 50 was used for NP cell transfection.

Hoechst 33258 staining

Cells were divided into four groups based on the treatment received. Group I: normal NP cells were set as negative control; Group II: cells were treated with 60 μg/mL levofloxacin for 24 h and set as positive control; Group III: cells were transduced with LV-CON and then treated with levofloxacin; Group IV: cells were transduced with LV-ITG β1 and then treated with levofloxacin. Before treatment, all cells were cultured in serum-free medium for 24 h, and after treatment, NP cells were harvested for Hoechst 33258 staining. Briefly, cells were washed with PBS, fixed in 4% paraformaldehyde for 10 min, and incubated with 5 μg/mL Hoechst 33258 working solution for 1 min. The stained cells were visualized under a fluorescence microscope.

Flow cytometry

Apoptosis was detected by using APC annexin-V/7-ADD apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer’s protocol. The processed cells were harvested and washed with cold PBS twice. After resuspension in binding buffer, cells were incubated with APC Annexin V and 7-AAD for 15 min. Cells were then analyzed by flow cytometry (BD FACSCalibur, BD Biosciences) immediately after staining.

Real-time PCR analysis

Cells were harvested for Real-time PCR analysis after treatment. Total RNA was extracted using TRizol reagent (Invitrogen, Carlsbad, CA, USA) and quantified by spectrophotometry. Complementary DNA was reverse transcribed from total RNA using TaKaRa RNA PCR Kit Ver. 2.1 (TaKaRa Bio, Tokyo, Japan). The sequences of the primers used are as follows: integrin β1: 5′-GACGAAAGTGCTCTAACA-3′, 5′-CTGAAGGACCACCTCTAC-3′; Gapdh: 5′-TGCTGAGTATGTGGTGGAGT-3′, 5′-GTCTTCTGAGTGGCAGTGAT-3′. Real-time PCR was performed using the SYBR Premix ExTaq kit (TaKaRa) with ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) according to manufacturer’s protocol. The 2−ΔΔCt method was used to measure gene expression.

Western blot analysis

Total protein was extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) with a mixture of protease inhibitors, separated by 10% SDS-PAGE, and electrotransferred to a PVDF membrane (Millipore, Billerica, MA, USA). After blocking nonspecific protein binding, the membranes were incubated at 4°C overnight with rabbit monoclonal antibody against integrin β1 (Abcam, Cambridge, MA, USA, ab179471, 1:1000 dilution), focal adhesion kinase (FAK; Abcam, ab40794, 1:1000), p-FAK (Abcam, ab81298, 1:1000), and p53 (Abcam, ab90363, 1:1000). The membranes were then washed with TBST and incubated with HRP-conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA, USA 1:2000) at room temperature. An Odyssey
Infrared imaging system (LI-COR, Lincoln, NE, USA) was used to detect the bands of interest.

Caspase-3 activity assay

Caspase-3 activity was determined with a caspase 3 activity assay kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer’s instructions. The absorbance of each well at 405 nm was measured using a microplate spectrophotometer (Biotek, Winooski, VT, USA), and caspase-3 activity was expressed as the fold changes in enzyme activity over control.

Animal model and lentivirus vector injection

Forty eight-week-old male SD rats were used. L4/5 and L5/6 IVD degeneration model was developed by destroying both dynamic (muscles) and static (bony and ligamentous architecture) restraints as previously described [6]. Intradiscal injection of lentivirus vector was performed one week after the initial surgery. The forty rats were randomly divided into two groups and injected with LV-ITG β1 or LV-CON. A ventral approach described by Rousseau et al. was applied [7]. The rats were placed in the supine position after anesthesia with a single intraperitoneal injection of 90 mg/kg ketamine and 10 mg/kg xylazine. A 4-cm long abdominal midline incision was performed to open the peritoneal cavity. The stomach and intestines were pulled out and protected with saline-soaked gauze. The retroperitoneum on the right was exposed and vertically torn lateral to the posterior vena cava. The ventral aspect of the lumbar spine was exposed by removing the muscle attachment. The lentivirus vector (2.5 mL) mixed with 50 μg/mL of polybrene (Genechem Co., Ltd. Shanghai, China) was slowly injected into the L4/5 and L5/6 intervertebral discs using a microsyringe (5 μL, Hamilton).

X-ray examination

All rats received X-ray examination before surgery, before injection, and 1, 3, 5, and 7 weeks after injection. Digital radiographs were analyzed by ImageJ software. Disc height was expressed as the disc height index (DHI), and changes in the DHI of injected discs were expressed as % DHI and normalized to the measured preoperative IVD height (normalized % DHI = postoperative DHI/preoperative DHI × 100%) [8].

MRI examination

Seven weeks after injection, MRI examinations were performed on a 3.0 T scanner (Trio, SIEMENS, München, Germany). T2 spin-echo weighted images were captured in the sagittal plane in the following settings: fast spin echo sequence with time to repetition (TR) of 4000 ms and time to echo (TE) of 120 ms; 256 (h) × 128 (v) matrix; field of view of 260; and 4 excitations. The slice thickness was 0.8 mm. Two independent blinded observers evaluated the degree of disc degeneration in the MRI image using a modified Thompson classification [9].

Histological staining

After MRI examination, the rats were anesthetized and euthanized by cervical dislocation. The L3-L6 discs were dissected out and carefully kept intact. The L3/4 IVD of the two groups was set as a control. The tissues were fixed in 4% paraformaldehyde for 48 h and then decalciﬁed in 20% ethylenediaminetetraacetic acid (EDTA) solution for 3 weeks. The samples were embedded in paraffin and sectioned (5-μm thickness) along the mid-sagittal plane. The sagittal sections were stained with hematoxylin and eosin (H&E) to assess IVD degeneration and Safranin O-fast green to assess the relative proteoglycan content.

TUNEL staining

Cell apoptosis was tested by TUNEL staining using MK1020 Apoptosis Detection Kit (Boster Biotechnology, Wuhan, China) according to the manufacturer’s instruction. Total and TUNEL-positive cells on two serial sections per discs were counted under 400× magnification in the NP and outer or inner anulus fibrosus (AF) region, separately. The percentage of TUNEL-positive cells compared with total disc cells was then calculated on five noncontiguous fields.

Statistical analysis

The experimental values are expressed as mean ± standard deviation. All in vitro experiments were repeated at least three times. Statistical analyses were performed using the IBM SPSS Statistics 22 software (SPSS, Chicago, IL, USA). ANOVA with subsequent LSD and S-N-K test was used for multiple comparisons between observed means. Mann-Whitney U test was used to compare in vivo data
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**Figure 1.** LV-ITG β1 inhibited levofloxacin-induced NP cell apoptosis. (A) Shows the transfection efficiency of LV-ITG β1 on NP cells at MOI = 50. Integrin β1 expression in LV-ITG β1-transfected NP cells was analyzed by western blotting (B and C) and RT-PCR (D). (E) Shows the effect of LV-ITG β1 on levofloxacin-induced NP cell and nucleus morphology changes, observed under an inverted phase contrast microscope and fluorescence microscope, respectively. The number of Hoechst 33258 staining-positive NP cells was determined in three different fields in each group. Integrin β1 significantly decreased the incidence of NP cell apoptosis induced by levofloxacin (G). (F) Showed typical FACS plots with annexin V-APC and 7-AAD double staining. The apoptosis rate increased in levofloxacin-treated NP cells, while

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Integrin β1 overexpression suppresses levofloxacin-induced caspase-3 activation

As shown in Figure 2A, after treatment with 60 μg/mL levofloxacin for 24 h, caspase-3 activity increased 4-fold. LV-ITG β1 decreased caspase-3 activity to 3.3-fold relative to that of control cells, while LV-CON had no obvious effect on levofloxacin-induced caspase-3 activity.

The FAK-P53 signal axis is involved in the regulation of apoptosis in NP cells

To evaluate the mechanism underlying the effects of integrin β1 on levofloxacin-induced NP cell apoptosis, FAK and P53 expression was determined by western blot (Figure 2B). The results showed that levofloxacin decreased the expression of integrin β1 and p-FAK and upregulated P53 expression. However, no obvious effect was observed on levofloxacin-induced caspase-3 activity.

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Intradiscal LV-ITG β1 injection attenuates disc degeneration based on MRI, but fail to restore disc height based on X-ray

One week after the induction of IVD degeneration in our rat model, the lumbar disc space height decreased by about 30%. After LV-ITG β1 and LV-CON injection, the lumbar spine showed malalignment and endplate calcification in both groups. No significant difference in disc space height was detected between the two groups (P > 0.05). Disc degeneration on MRI was graded using the Thompson score. The score was significantly lower (degenerate lightly) in the LV-ITG β1 group than in the LV-CON group (P < 0.05). (Figure 3A, 3C).

Lentivirus-mediated integrin β1 overexpression attenuates histological disc degeneration

As shown in Figure 4A, in the control disc, the disc structure was well organized. In the LV-CON group, the AF was ruptured and showed fissure formation and serpentine appearance, the NP matrix was condensed, and the border between the AF and NP was interrupted. In the LV-ITG β1 group, the disc showed less ruptured fibers, slight condensation of the NP matrix, and minimally interrupted AF and NP border. According to Koichi’s histological grading scale, the score was significantly decreased in the LV-ITG β1 group when compared to that in the LV-CON group at 7 weeks after injection (Figure 4C) (P < 0.05) [8].

We used Safranin O-fast green staining to analyze IVD histologically for proteoglycan content [10]. As shown in Figure 4B, in the normal control IVD, the AF and NP presented an intense red staining. In the LV-CON group, the IVD showed a low staining with large areas of red washed out in the AF, while in the LV-ITG β1 group, the IVD presented a uniform red staining in the NP and AF, but the staining was less intense than that in the normal control group.

Lentivirus-mediated integrin β1 overexpression suppresses NP cell apoptosis in vivo

TUNEL staining indicated that, in the IVD, apoptosis was observed in the NP, inter annulus fibrosus (IAF), and outer annulus fibrosus (OAF)
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Figure 4. Histological analysis for the assessment of IVD degeneration. A. To assess IVD degeneration, sagittal sections were stained with hematoxylin and eosin. B. Safranin O-fast green staining was used to analyze IVD histologically for proteoglycan content. The staining was obviously low in the LV-CON group, while the staining improved in the LV-ITG β1 group. C. According to Koichi’s histological grading scale, the score was significantly decreased in the LV-ITG β1 group when compared to that in the LV-CON group at 7 weeks after injection (P < 0.05). The L3/4 IVD from the LV-ITG β1 and LV-CON groups was set as the normal control group. *P < 0.05.

zone, separately (Figure 5A). There were very few TUNEL positive cells in normal control IVD, while apoptosis was highly evident in all three zones of the discs from the LV-ITG β1 and LV-CON groups. A decrease in the apoptosis rate was observed in the NP and IAF zone in the LV-ITG β1 group compared to that in the LV-CON group (P < 0.05), while there was no significant difference in the apoptosis incidence in the OAF zone between the two groups (Figure 5B).
Excessive apoptosis of NP cells is one of the characteristics of early stage IVD degeneration [11, 12]. Because NP cells express higher levels of collagen type II and proteoglycan compared to other cells within the IVD, excessive apoptosis of NP cells would significantly disrupt the dynamic balance between the extracellular matrix (ECM) synthesis and catabolism, resulting in ECM remodeling, biomechanical changes, and ultimately IVD degeneration [13, 14]. Due to the important role played by apoptosis in IVD degeneration, the suppression of apoptosis is commonly targeted as a potential therapeutic strategy for intervertebral degeneration disease (IDD) [15, 16].

It is noteworthy that integrin beta 1 can be highly upregulated in many epithelial cells originating from solid tumors and has the ability to enhance cell survival [17]. Our previous study showed that artificially upregulated expression of integrin β1 reduced mechanical stress-induced AF cell apoptosis [18]. Additionally, accumulating evidence suggests that apoptosis plays an important role in IVD degeneration. Thus, we studied the effect of lentivirus-mediated integrin β1 overexpression on the apoptosis of NP cells and IVD degeneration.

The levofloxacin-induced NP cell apoptosis model was used in the in vitro study. Levofloxacin treatment alone did not induce apoptosis of NP cells cultured in complete medium, but
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enhanced the effect of serum deprivation on NP cell apoptosis. This model simulated NP cell apoptosis in vitro, as the NP receives very little nutrition in IVD [19]. In addition, the expression level of integrin β1 is downregulated by levofloxacin-induced NP cell apoptosis [5]. As expected, integrin β1 had a protective effect on levofloxacin-induced NP cell apoptosis. This effect was confirmed by cell and nucleus morphology and flow cytometry. Moreover, our results suggest that integrin β1 regulates cell apoptosis by controlling caspase-3 activation.

The FAK-P53 crosstalk pathway plays a role in integrin-mediated survival signaling regulation in tumor and embryonic cells [20]. Both FAK and P53 are cancer-related genes. Cells artificially overexpressing FAK are resistant to apoptosis induced by ionizing radiation and hydrogen peroxide [21]. P53 is commonly regarded as a classical senescence marker and a powerful apoptosis inducer. FAK is activated by integrin signaling and suppresses p53-directed apoptosis in a kinase-independent manner [22]. Both FAK and p53 are expressed in human NP cells [23-25]. However, there is no previous report on the relationship between integrin β1 and the FAK-p53 signaling axis in NP cells. To further study the mechanism underlying the inhibitory effect of integrin β1 on NP cell apoptosis, we examined the involvement of the FAK-p53 signaling axis. While the p-FAK protein expression levels were decreased by levofloxacin, total FAK expression level was stable and unresponsive to pathological stimuli. These results are consistent with previous studies [23]. However, integrin β1 upregulated FAK activation and expression. Moreover, our study showed that p53 expression was negatively correlated with the expression of integrin β1 and FAK. These results indicate that integrin β1 inhibits levofloxacin-induced apoptosis through upregulation of the expression and phosphorylation of FAK and downregulation of P53 expression.

Gene therapy is a promising method for the treatment of IVD degeneration. Early degenerated disc with sufficient number of viable cells can be treated with modification of target gene expression. Regulating the expression of CHOP, TGF β1, LMP-1, BMP-2, TIMP-1, ADAMTS-5, and Sox-9 presents therapeutic effects for IVD degeneration [6, 26-30].

The unbalanced dynamic and static force-induced rat lumbar IVD degeneration model was used for our in vivo experiments. The IVD degeneration in this model was induced by pathological mechanical force, but not by direct IVD injury. Thus, we considered that this model closely mimics clinical IVD degeneration [6, 31, 32]. In the early stage of IVD degeneration, the populations of viable cells are sufficient, allowing gene therapy. By transferring the target genes to recipient cells; we can expect a long lasting biological effect, which benefits chronic IVD degeneration [33]. The encapsulated avascular structure makes direct injection the only reliable method for delivering lentivirus vectors to IVDs [22, 34]. We found that direct injectionLV-ITG β1 into the disc decreased the cell apoptosis incidence in the NP and inner AF zone. However, in the outer AF zone, no significant difference was observed in term of apoptosis incidence between the LV-ITG β1 and LV-CON groups. It is possible that the compact structure of AF prevents the lentivirus vector to permeate into the outer AF.

Additionally, LV-ITG β1 improved the histological score of IVD degeneration, which coincided with the degree of degeneration on MRI. Furthermore, Safranin O-fast green staining was improved in the LV-ITG β1 group, which suggested that integrin β1 decreased the decomposition of proteoglycan, probably by suppressing NP cell apoptosis. However, LV-ITG β1 failed to restore the disc height. One possible explanation is that the disc height was significantly decreased one week after molding surgery, the low nutrition and oxygen environment in IVD adversely affected the restoration of the pathologic changes that already occurred. Thus, we propose that gene therapy using LV-ITG β1 might only have a preventive effect on early stage IVD degeneration, but may not have the ability to restore pathologic changes that already occurred.

This study provided a new strategy for IVD degeneration treatment: to upregulate the expression of cancer-related genes. Because integrin β1 and FAK are highly expressed in invasive and metastatic tumors [17], it is possible that normal NP cells function as cancer-like cells to block physical apoptosis. Although the incidence of tumors that originate from NP cells is low, the risk of malignant transforma-
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In conclusion, the present study confirmed the effectiveness of lentiviral-mediated integrin β1 overexpression on apoptosis of NP cells in vitro and in vivo. The FAK-p53 signaling pathway is involved in integrin β1-mediated regulation of NP cell apoptosis. In a rat IVD degeneration model, intradiscal injection of LV-ITG β1 attenuated IVD degeneration by suppressing apoptosis of IVD cells. Gene therapy using LV-ITG β1 represents a promising new therapeutic approach and should be further investigated for the treatment of IVD degeneration.

Acknowledgements

This work was partly supported by National Natural Science Foundation of China (8157-2168, 81272038).

Disclosure of conflict of interest

None.

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