

Effect of Survivin gene therapy via lentivirus vector on the course of intervertebral disc degeneration in an *in vivo* rabbit model

BIN YUE*, YAZHOU LIN*, XUEXIAO MA, GUOQING ZHANG and BOHUA CHEN

Department of Orthopedic Surgery, The Affiliated Hospital of Qingdao University, Qingdao, Shandong 266003, P.R. China

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Abstract. The aim of the current study was to use gene therapy to attenuate or reverse the degenerative process within the intervertebral disc. The effect of survivin gene therapy via lentiviral vector transfection on the course of intervertebral disc degeneration was investigated in the current study in an *in vivo* rabbit model. A total of 15 skeletally mature female New Zealand White rabbits were randomly divided into three groups: Punctured blank control group (group A, n=5), punctured empty vector control group (group B, n=5) and the treatment group (group C, n=5). Computed tomography-guided puncture was performed at the L3-L4 and L4-L5 discs, in accordance with a previously validated rabbit annulotomy model for intervertebral disc degeneration. After 3 weeks, a lentiviral vector (LV) carrying survivin was injected into the nucleus pulposus. The results demonstrated that through magnetic resonance imaging, histology, gene expression, protein content and apoptosis analyses, group A and B were observed to exhibit disc degeneration, which increased over time, and no significant difference was observed between the two groups ($P>0.05$). However, there was reduced disc degeneration in group C compared with the punctured control groups, and the difference was statistically significant ($P<0.05$). Overall, the results of the present study demonstrated that injection of the LV carrying survivin into punctured rabbit intervertebral discs acted to delay changes associated with the degeneration of the discs. Although data from animal models should be extrapolated to the human condition with caution, the present study suggests potential for the use of gene therapy to decelerate disc degeneration.

Introduction

Approximately 80% of the aging population suffer from lower back pain at some point in their lives (1-3). Back pain associated with intervertebral disc degeneration (IDD) clearly affects quality of life and work productivity, and significantly impacts health care spending (4). However, the currently used first-line treatments, including activity modification, analgesic and anti-inflammatory medications, physical therapy and surgery only relieve symptoms temporarily and do not target repair or deceleration of the underlying degenerative progress (5,6). Alternative and less invasive treatment methods such as gene therapy are being developed, aiming to slow or reverse the degenerative process of IDD.

Developing approaches to stimulating regeneration of disc tissue or slowing the degeneration progression requires an understanding of the causes of the degeneration. Previous studies have demonstrated that the number of nucleus pulposus (NP) cells reduces and the composition of the extracellular matrix associated with these cells is altered in degenerative discs (7-9). *In vitro* and *in vivo* studies have suggested that the cellular loss attributed to the excessive apoptosis of disc cells serves an important role in disc degeneration (10,11). These mechanisms provide direction and fundamental information for gene therapy.

Survivin protein, a baculoviral inhibitor of apoptosis, serves an important role in the regulation of mitosis progression and apoptosis inhibition (12). In osteoarthritis and rheumatoid arthritis, survivin stimulates chondrocyte proliferation and inhibits apoptosis (13,14). Furthermore, preliminary studies have indicated that survivin is expressed in fetal disc tissue, and that there is a differential expression of survivin between degenerated NP tissue and comparatively normal NP tissue (15,16). In a previous study, it was demonstrated that survivin is re-expressed in disc degeneration disease and is required for degenerated NP cell proliferation and anti-apoptosis *in vitro* (17). Thus, it is suggested that the use of survivin as a target gene for gene therapy for IDD may be efficacious. However, to the best of our knowledge, at present no studies investigating gene therapy with survivin via a lentiviral vector (LV) and the course of intervertebral disc degeneration *in vivo* have been conducted.

The present study investigated the use of gene therapy in improving IDD by administering LV-survivin into disc cells. This validated IDD model was used to determine whether

Correspondence to: Dr Bohua Chen, Department of Orthopedic Surgery, The Affiliated Hospital of Qingdao University, 16 Jiangsu Road, East, Qingdao, Shandong 266003, P.R. China
E-mail: bhchen@hotmail.com

*Contributed equally

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treatment with LV-survivin can ameliorate the course of NP cell apoptosis. The results provided information for gene therapy to decelerate IDD in future clinical application.

Materials and methods

Materials. A total of 15 skeletally mature female New Zealand White rabbits (3-months-old, weighing 2-2.5 kg; Chongqing Kangda Juxin Rabbit Co., Ltd., Qingdao, China) were randomly divided into three groups: Group A, punctured blank control group (degeneration and placebo injection, $n=5$); group B, punctured empty vector control group (degeneration and an empty LV injection, $n=5$); and group C, treatment group (degeneration and LV-survivin injection, $n=5$). LV-survivin and empty LV were purchased from Genechem Co., Ltd. (Shanghai, China). The titers of LV-survivin and empty LV were 2×10^8 and 1×10^9 TU/ml, respectively. The Animal Care and Use Committee of the Affiliated Hospital of Qingdao University (Shandong, China), approved all animal experimental protocols, which followed the principles expressed in the National Institute of Health Guide. The rabbits were maintained separately at room temperature with 16:8 h light:dark cycle, and were fed with rabbit feed (Qingdao Kangda Food-stuffs Co., Ltd., Qingdao, China).

Puncture surgery. Computed tomography (CT)-guided percutaneous needle puncture technology was used to establish this model (18). Magnetic resonance imaging (MRI) and histology highlighted alterations that resembled the hallmarks of human disc degeneration. In brief, rabbits were anesthetized with Su Mian Xin II (0.15 ml/kg intramuscularly; Kangda Medical Products Co., Ltd., Shanghai, China) and atropine sulfate (0.05 mg/kg intramuscularly; Jiangsu Lianshui Pharmaceutical Co., Ltd., Lianshui, China), the position of the target discs (L3-L4 and L4-L5) were identified using CT, and then a 16-gauge needle was inserted toward the center of the disc, with CT guidance. The needle pinpoint was confirmed to be positioned in the disc center by CT (Fig. 1). Postoperatively, the rabbits were housed in individual cages and injected intramuscularly with penicillin at 80×10^5 U to prevent infection.

Injection surgery. At 3 weeks post-puncture surgery, groups A, B and C were injected with equivalent phosphate-buffered saline (PBS; 50 μ l), empty LV ($5 \times 10^6/50$ μ l; Genechem Co., Ltd., Shanghai, China), and LV-survivin ($5 \times 10^6/50$ μ l; Genechem Co., Ltd.), respectively, using CT-guided percutaneous needle injection technology. This time point was selected due to the fact that it is the earliest that MRI could identify changes of degeneration with this model (18). An 100 μ l microinjector (Hamilton Bonaduz AG, Bonaduz, Switzerland) was used for therapeutic injections into the center of the NP.

MRI. MRIs were obtained at time-point 0 (prior to puncture), 3 weeks post-puncture (prior to injection), and 12 weeks post-puncture (prior to sacrifice) for all rabbits. A 3-T standard human knee coil was used to obtain T2-weighted images (repetition time=2120 ms, echo time=113 ms, slice thickness=0.6 mm). The rabbits were anesthetized and placed in the knee coil in the supine position.

Table I. Details of nucleotide sequences of sense and anti-sense primers, RT-qPCR amplification product.

Gene	Primer	PCR product (bp)
Survivin	F: CAGATGACGACCCCATAGAGGA R: CCTTTGCAATTTTGTCTTGGC	141 bp
GAPDH	F: GGATTGGTTCGTATTGGG R: GGAAGATGGTGTATGGGATT	205 bp

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; bp, base pairs.

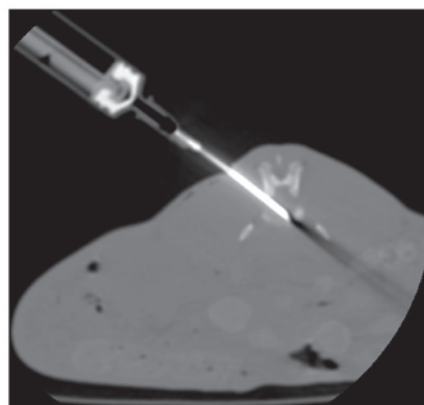


Figure 1. Computed tomography-guided percutaneous needle puncture technology.

Histological processing. All rabbits were sacrificed by air injection into the ear vein 12 weeks subsequent to the initial puncture surgery, following the final MRI scan. Immediately after sacrifice, spines were dissected out *en bloc*. The L3-L4 discs ($n=5$ per group) were prepared for histology. Two discs from each group were fixed and then dehydrated using a histology tissue processor and embedded in paraffin. Next, the discs were sectioned at a thickness of 5 μ m in the coronal plane. The sections were stained with hematoxylin and eosin (H&E; Beijing Leagene Biotechnology Co., Ltd., Beijing, China). The other discs were embedded in Tissue-Tek™ (Sakura, CA, USA) and then sectioned at a thickness of 5 μ m in the coronal plane in a freezing microtome (Leica CM1950; Leica Microsystems GmbH, Wetzlar, Germany). The sections were stained for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Nanjing KeyGEN Biotech Co., Ltd., Nanjing, China) and were imaged using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan).

Gene expression and protein content of survivin. A total of 3 L4-L5 discs from each group ($n=5$ per group) were prepared for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. mRNA was extracted from NP tissue using TRIzol™ (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the absorbance at was measured at 260 and 280 nm for

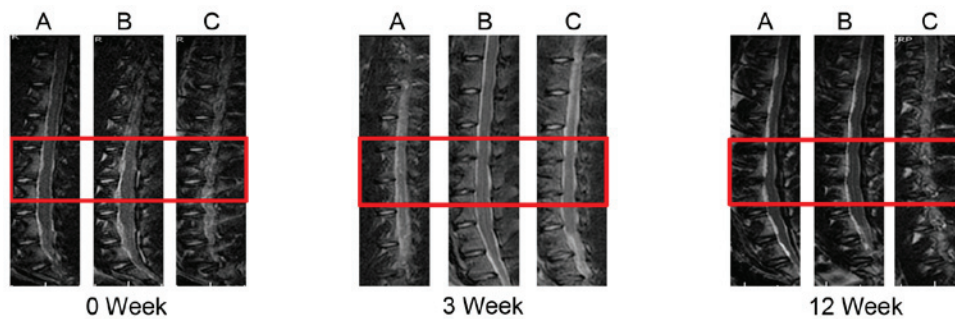


Figure 2. T2-weighted midsagittal magnetic resonance imaging of different groups at different time points. A, punctured blank control group; B, punctured empty vector control group; C, treatment group.

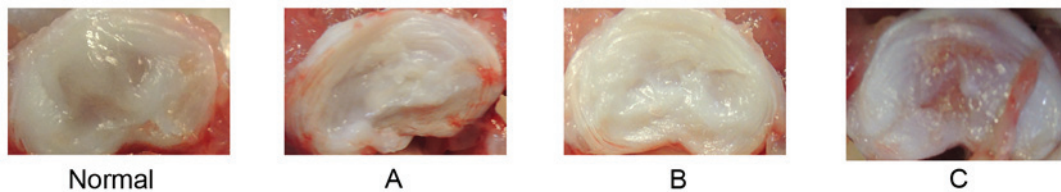


Figure 3. Disc morphology of the different groups. A, punctured blank control group; B, punctured empty vector control group; C, treatment group.

quantification and quality control. A total of 1 μ g mRNA was reverse transcribed to cDNA using PrimeScript™ RT reagent kit (cat no. DRR037A; Takara Bio, Inc., Otsu, Japan), and the reaction product was treated with RNase-free DNase I. PCR was conducted using the cycling conditions (LightCycler 480 Instrument II) as stated by the manufacturer's instructions. Primers were designed and purchased from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China) (Table I). Another specific primer pair for human GAPDH was used as an internal control. In each experiment, samples were analyzed in duplicate. The normalized target gene expression was determined through the comparative Cq method ($\Delta\Delta Cq$ method) (19).

NP tissues were washed with ice-cold PBS, weighed and then ground using a pestle and mortar. Lysis was performed on ice for 45 min using 200 μ l radioimmunoprecipitation assay buffer with 2 μ l phenylmethanesulfonylfluoride for every 100 μ g of tissue. Subsequently, the lysis solution was centrifuged at 5,700 \times g at 4°C for 20 min. For the western blot analysis of survivin, GAPDH was used as an internal control, and proteins were resolved through sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% gel, prior to transferring onto Immobilon P membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% fat-free dried milk and probed with a monoclonal rabbit anti-survivin antibody (1:1,000; ab76424; Abcam, Cambridge, MA) and anti-GAPDH antibody (1:2,000; ab9485; Abcam) for 8 h at 4°C. Subsequent to incubation with horseradish peroxidase-conjugated secondary antibodies (1:2,000; CW0103; Beijing Kangwei Century Biotechnology Co., Ltd., Beijing, China) for 4 h at room temperature, the positive bands were visualized using chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL, USA).

Cell culture and caspase-3 activity assay. The remaining L4-L5 discs were prepared for measuring caspase-3 activity. NP cells were cultured as described in a previous study (20). In brief, the NP tissue was cut into small pieces (~ 1 mm²)

and then digested with 0.25% trypsinase (HyClone™; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C under gentle agitation for 20 min. Subsequently, 0.5% collagenase type II (MP Biomedicals, LLC, Santa Ana, CA, USA) was used at 37°C for approximately 4 h. The cells were transferred to a 12.5 cm² culture flask at a density of 10⁵ cells/cm². The cells were cultured in a CO₂ incubator (SANYO Electric Co., Ltd., Osaka, Japan) at 37°C with humidity, and grown in Dulbecco's modified Eagle's medium (DMEM)-F12 containing 15% fetal calf serum. The experiments were analyzed in duplicate. For ischemic conditions, NP cells were cultured in DMEM culture medium (glucose deprivation) in a CO₂ incubator at 37°C with 1% oxygen and 95% humidity. These cells were subsequently used for analyzing caspase-3 activity. The caspase-3 activity was measured using the Caspase-3 Colorimetric Assay kit (BioVision, Inc., Milpitas, CA, USA). The NP cells were counted and pelleted at 1.5 \times 10⁶ cells. The cells were then re-suspended in cell lysis buffer, and 50 μ l 2X reaction buffer (containing 10 mM DTT) and 5 μ l of DEVE-pNA were added. The samples were incubated for 90 min at 37°C and their optical densities were read at 405 nm using a microtiter plate reader (Sunrise™; Tecan Group, Ltd., Männedorf, Switzerland).

Statistical analysis. All values were presented as the mean \pm standard error. Student's t-test and one-way analysis of variance with a post hoc Fisher's least significant difference test were applied to measure the statistical significance of the differences. Statistical analyses were performed using SPSS software for Windows (version 19; SPSS, Inc., Chicago, IL, USA).

Results

Imaging. The T2-weighted midsagittal MRI scans of groups A and B were darkened and reduced in area from 0-12 weeks, consistent with the degeneration. Group C demonstrated

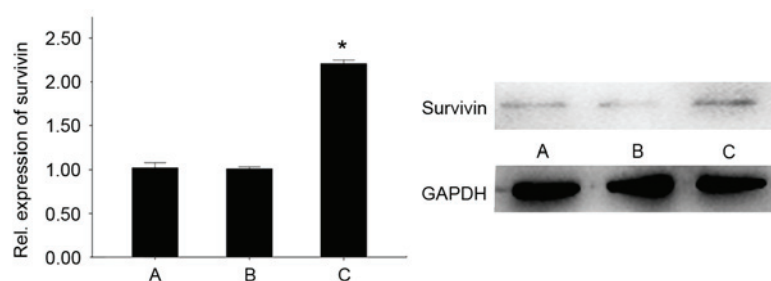


Figure 4. Gene expression and protein content of survivin. * $P < 0.05$ vs. group A and B. A, punctured blank control group; B, punctured empty vector control group; C, treatment group.

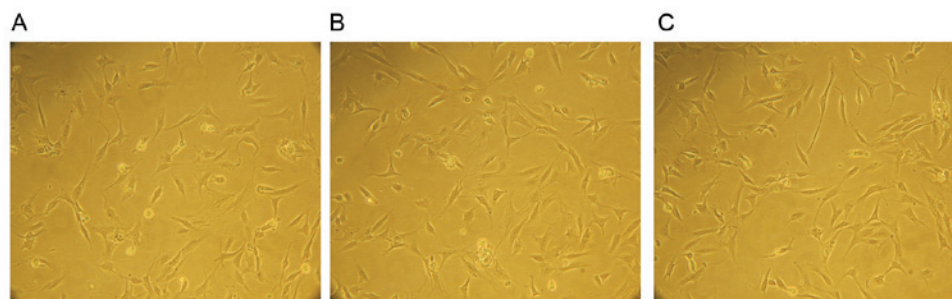


Figure 5. Morphology of nucleus pulposus cells in the different groups (magnification, $\times 100$). A, punctured blank control group; B, punctured empty vector control group; C, treatment group.

reduced qualitative evidence of degeneration when compared with groups A and B; treated NPs did not darken and reduce in area as much as the punctured discs (Fig. 2). The morphology of normal discs indicated an abundance of water and ECM. Discs from groups A and B were reduced in the quantity of both water and ECM, and exhibited fibrotic morphology, consistent with the degree of degeneration. However, the quantity of water and ECM in group C were between that of the normal group (group A) and the punctured group (group B) (Fig. 3).

Gene expression and protein content of survivin. The gene and protein content of each group were analyzed by RT-qPCR and western blot analysis. Gene expression and protein content of survivin in group C demonstrated a significant increase compared with groups A and B ($P < 0.05$). No significant difference was observed between groups A and B (Fig. 4).

The morphology of NP cells. The NP cells were attached to the culture dish after 3-5 days of culture. The cell morphology gradually elongated and became triangular or polygonal, and the cytoplasm became plump and equally distributed, and the attached cells exponentially increased. Subsequently, 10-15 days later, 90% of the cells formed colonies. No significant differences of cell morphology were observed among the three groups (Fig. 5).

Caspase-3 activity reflected apoptosis of NP cells. The caspase-3 activity of NP cells was assayed. Under the normal culture conditions, group C did not exhibit significantly altered caspase-3 activity compared with groups A and B ($P = 0.082$ and $P = 0.539$, respectively). When exposed to ischemia *in vitro* (1% oxygen, glucose deprivation), the caspase-3 activity of all

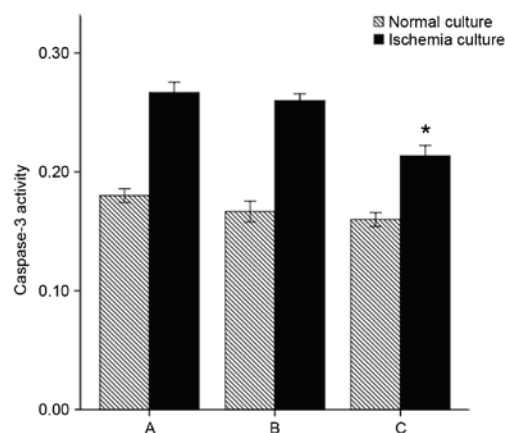


Figure 6. Caspase-3 activity reflected the apoptosis of nucleus pulposus cells. * $P < 0.05$ vs. group A and B. A, punctured blank control group; B, punctured empty vector control group; C, treatment group.

groups increased compared with the normal culture conditions ($P = 0.00$). However, caspase-3 activity remained significantly reduced overall for group C (group C + *in vitro* ischemia vs. group A + *in vitro* ischemia and group B + *in vitro* ischemia: $P = 0.00$ and $P = 0.01$, respectively) (Fig. 6).

Histological staining. The histological analysis of NP tissues was performed using H&E staining. Under the highest magnification, the number of NP cells in group C was significantly increased compared with groups A and B (Fig. 7).

Apoptosis analysis of NP tissues was performed using TUNEL staining. The TUNEL-positive NP cells (brown cells) in group C were markedly reduced compared with groups A and B (Fig. 7).

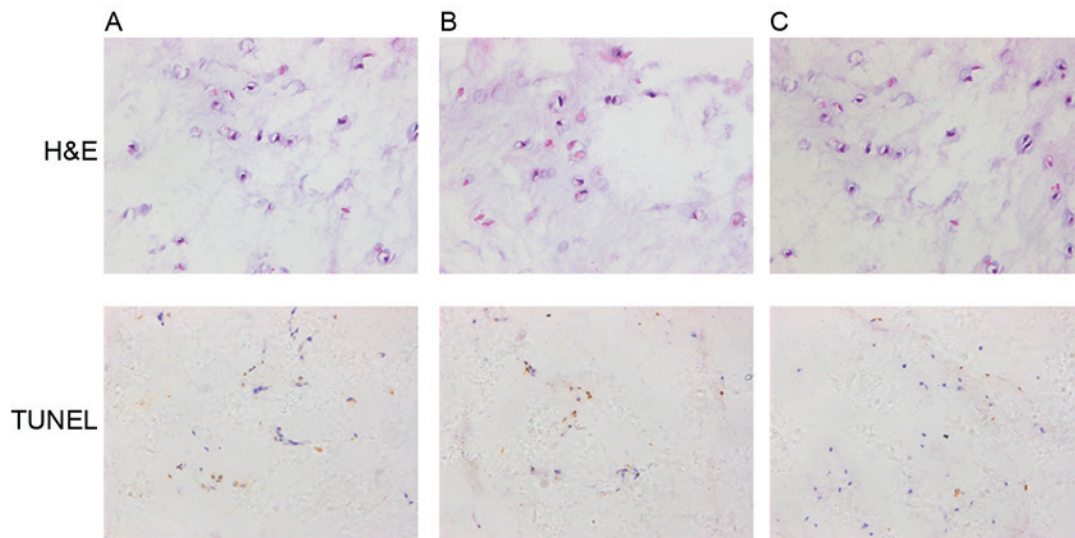


Figure 7. H&E and TUNEL staining in the different groups. H&E, hematoxylin and eosin; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling. A, punctured blank control group; B, punctured empty vector control group; C, treatment group.

Discussion

The current study demonstrated that LV-survivin injection into the intervertebral discs may slow the process of IDD *in vivo*. Results from MRI, histology, gene expression, protein content and apoptosis analyses all demonstrated a slowing of the course of injury-induced degeneration.

The degeneration model was established using CT-guided percutaneous needle puncture technology. Although an injury model for disc degeneration does not truly reflect the complex biochemical cascade of human degenerative disc disease, it does suggest that LV-survivin may be a possible treatment method to ameliorate the process of injury-induced degeneration. Similarly, injection surgery was accomplished with CT guidance; however, a microinjector with a needle small enough not to exacerbate further degeneration was used (21). Furthermore, 3 weeks subsequent to the puncture, there was an alteration in the NP visible by MRI, and the degeneration was in an early phase. In the phase, the cellular state might be able to mount a healing response for LV injection, so this time point was chosen for the LV injection (22).

In the current study, the MRI imaging of all groups at the 0 week time point exhibited normal intervertebral disc morphology. A total of 3 weeks after the puncture, the imaging of all groups demonstrated the same degree of degeneration. A total of 12 weeks after the puncture, treated NPs did not darken and reduce in area as much as the punctured discs. The anatomical morphology of the discs following sacrifice were consistent with that of MRI imaging. These results indicated that LV-survivin injection aided in the preservation of the intervertebral discs.

The gene expression and protein content of survivin in NP tissues of group C were significantly increased compared with that of groups A and B. These results indicated that there was a stable expression of survivin in NP cells following *in vivo* LV-survivin injection into the rabbits. Thus, based on the effect and function of survivin, a reduction in caspase-3 activity and apoptosis of NP cells, and an increase in the number of NP cells

was detected, compared with the punctured control groups. This observation may elucidate the effect of LV-survivin gene transfection on the deceleration of the degeneration process *in vivo*.

Caspase-3 activity was analyzed using the caspase-3 activity assay, and no alteration between the caspase-3 activities of groups A, B and C was observed in the unstressed NP cells. However, in *in vitro* ischemic cultures (1% oxygen, glucose deprivation), the caspase-3 activity of the NP cells significantly increased compared with unstressed NP cells, and the increase of group C caspase-3 activity was statistically reduced compared with that of groups A and B. These results were consistent with a previous study (17), and indicated that ischemic culture conditions induced NP cell apoptosis, and that overexpression of survivin may contribute in the reduction of caspase-3 activity for the anti-apoptotic function in the degeneration process.

In order to identify NP cell numbers and apoptotic alterations occurring in the different groups, H&E staining and TUNEL were used. These results indicated that LV injection with survivin slowed NP cell apoptosis according to the results of caspase-3 activity, and similarly demonstrated that overexpression of survivin may serve a role under ischemic conditions *in vivo* or *in vitro*.

Furthermore, in the primary culture of NP cells, no significant different of cell morphology were observed among the three groups. The results were different from the previous studies, with a previous study reporting that *in vitro*, the morphology of degenerative NP cell subsequent to transfection with LV-survivin was significantly changed (23). The difference may have resulted from the different transfection conditions. The *in vitro* condition of NP cells is complex, and numerous factors and pathways influence each other, thus the regulation of cell morphology is complicated.

Although the current study identified a number of statistically significant observations, there were limitations. All imaging, gene expression, protein content, apoptosis and histology results demonstrated that the LV injection with survivin had a weak effect on slowing degeneration.

It is also possible that the 12-week course of the present study may have been insufficient to demonstrate the full therapeutic effect in the degenerative cascade. This may be due to the fact that in the model used, degeneration has been demonstrated to continue through to a minimum of 24 weeks (24), therefore longer time points may have demonstrated more statistically significant alterations in signal intensity between the treated and punctured discs.

The present study demonstrated, to the best of our knowledge, for the first time that LV injection may slow disc degeneration. Although the results may not necessarily translate to human degenerative disc disease, and the results are preliminary compared with clinical treatment, they provide a potential direction for gene therapy as a treatment for disc degeneration.

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