Ad/CMV- hTGF-β1 Treats Rabbit Intervertebral Discs Degeneration *in Vivo* *

ZHAN Zirui (詹子睿), SHAO Zengwu (邵增务)△, XIONG Xiaoqian (熊晓芊), YANG Shuhua (杨述华),
DU Jingyuan (杜靖远), ZHENG Qixin (郑启新), WANG Hong (王 洪), GUO Xiaodong (郭晓东), LIU Yong (刘 勇)
Department of Orthopedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 130022, China

Summary: To investigate therapeutic efficiency of Ad CMV- hTGF-β1 gene for rabbit intervertebral disc degeneration model. 60 Japanese white rabbits were selected to form the L5-L6 Anterior-Lateral-Anulus-Fibrosus-Incision-Induced model in order to simulate human intervertebral disc degeneration. 36 rabbits, whose corresponding intervertebral discs were injected with 20 μl (10×10^6 pfu)of Ad CMV- hTGF-β1 gene, constituted the therapy group. 12 were injected with 20 μl (10×10^6 pfu)of Ad CMV-LacZ gene as comparison group, while 12 were only injected with equivalent capacity of saline for empty comparison group. 3 weeks after injection, examples were taken for investigation of HE staining, MRI, Western Blotting and immunohistochemical research TGF-β1. Wide distribution of TGF-β1 was detected by immunohistochemical research in the degenerated annulus fibrosus after injection. Western Blotting research showed significant increase of TGF-β1 content in intervertebral discs treated with TGF-β1 gene than comparison groups. MRI signal transformed from low to comparatively high and that intervertebral disc pathological degree improved. Ad CMV- hTGF-β1 gene transfection is a potential method to increase TGF-β1 content and reverse intervertebral disc degeneration.

Key words: intervertebral disc degeneration; TGF-β1; rabbit; genetic therapy

Intervertebral disc (IVD) degeneration, which is closely concerned with insufficiency of growth factors in intervertebral discs, is the main cause of intervertebral disc related diseases[1]. TGF-β1 (Transforming growth factor-β1) is one of the key growth factors in IVD degeneration, which is capable of stimulating differentiation of IVD cells, accelerating synthesis of proteoglycan and collagen, and inhibiting activities of matrix metalloproteinase. In this study, Ad/CMV-hTGF-β1 gene was injected to an animal mode of IVD degeneration. Then investigations of histological observation, Magnetic Resonance Imagination (MRI), immunohistochemical research for TGF-β1 and Western Blotting research for hTGF-β1 were carried out to assess the possibility and efficiency of TGF-β1 gene therapy for IVD degeneration.

1 MATERIALS AND METHODS

1.1 Materials

Ad/CMV-hTGF-β1、Ad/CMV-LacZ gene used in this study were kindly presented by Heidelberg University, Deutschland. Other reagents were described when mentioned below.

1.2 Estahlishment of IVD Degeneration Model

Sixty Japanese white rabbits (weighting from 4.8 to 5.2 kg, offered by experimental animal department of Tongji Medical College) were selected to form the AAII model (Anterior-Lateral Anulus Fibrosus Incision Induced model of IVD degeneration). Each rabbit was laid on its back after anesthetized with Ketamine(1.0 mg/kg). An left-extra-peritoneal route was adopted to expose left anterior-lateral part of L4-5、L5-6、L6-7 IVDs. An incision was operated on the anterior-lateral part of anulus fibrosus (AF) of L5-6 IVD, and it was carefully confirmed that nucleus pulpous (NP) obviated exposure from the AF when the incision was being made. Penicillin of 1×10^6 U/d was intromuscularly injected for 3 days preoperatively and postoperatively. The rabbits were fasting for 1 day after the operation, and were freely released in the cage.

1.3 Injection of TGF-β1 Gene

Eight weeks after the incision were made, another operation was made through the original route to access the corresponding IVDs for intranucleus-pulpous injection. 36 rabbits were injected with Ad/CMV-hTGF-β1 20 μl (10×10^6 pfu) of as the therapy group, 12 were injected with 20 μl (10×10^6 pfu) of Ad/CMV-LacZ as the comparison group, while 12 were injected with 20 μl of saline as empty comparison group. 3 weeks after the injection, the animals were executed with air embolism, and corresponding IVDs were collected for further investigation.

1.4 MRI Analysis

Three weeks after the injection, axial MRI at T2 weight scan was adopted to evaluate the degeneration degree of IVDs. Total point of each IVD was the sum of points of anterior, middle and pos-

ZHAN Zirui, male, born in 1970 Associate Professor
△Corresponding author: SHAO Zengwu
Tel: 86-13971021748, Fax: 86-027-85805503
E-mail: szwjj@medmail.com.cn

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terior part of the IVD. As to each part, 0 point for high intensity signal, which manifests white color, while 1 point for gray, 2 for dark and 3 points for black. Therefore IVD degeneration can be divided into 3 degrees according to the total point: 1—3 points as mild degeneration, 4—6 as degeneration, 7—9 as severe degeneration.

1.5 Histological Observation

Examples of IVDs were collected with 0.5 cm thick superior and infra vertebral bodies. The examples were immersed in 40 % Formaldehyde solution for 4 h for fixation, then decalcified with 10 % calcium nitrate for 5 days. IVDs were then sliced into segments of 6 μm, and stained with hematoxylin and eosin.

1.6 Immunohistochemical Research for TGF-β1

Freshly collected examples were immersed overnight in 40 % Formaldehyde solution, decalcified with 10 % calcium nitrate, embed with paraffin and then sliced into segments. The segments were subsequently de-waxed, hydrated with gradient alcohol, immersed with hydrogen peroxide solution, then 1 % bovine serum albumin (Boster Company, Wuhan) was added in drip . The examples were then placed into wet package of TGF-β1McAB reaction (Jingmei Company, Wuhan) at 4 C overnight, and processed according to user's manual of the package, in short, the examples were eventually blocked with gum. 10 visual fields of each sheet were randomly selected for observation, and the rate of positive stained chondrocyte-like cells was recorded: negative for no positive cells, 0—5 % for doubtfully positive, 5 %—25 % for rather positive, 25 %—50 % for positive and >50 % for strongly positive.

1.7 Western Blotting of TGF-β1

Examples were cut into small pieces, pulverized with ultrasound, split with splitting solution into chyliform, then further pulverized with ultrasound. An SDS-PAGE electrophoresis was made. The examples were shifted to a film, sealed with non-specific antigens. 1 : 1000 Goat-anti-Rabbit TGF-β1 antibody was (Boster Company, Wuhan) then added and the examples were incubated at 4 C overnight; 1 : 1500 Biotin-labeled second antibody (Boster Company, Wuhan) was added and incubated at 37 C for 2 h, followed by reacting with indication solution (Boster Company, Wuhan) for 15 min, and the reactions were finally ceased with tap water.

1.8 Statistics

All results were assessed with SPSS version 11.0.

2 RESULTS

2.1 Axial MRI at T2 Weight Scan for Intervertebral Disc Degeneration

Eight weeks after operation, NP signal significantly turned lower, and boundary between NP and AF became irregular. Vacuum phenomenon was observed in some IVDs. After the injection, signal intensity of therapy group obviously turned higher than comparison group and empty comparison group (fig. 1). There were significant difference between therapy group and empty comparison group or comparison group (Wilcoxon test, $P<0.001$).

2.2 Histological Observation

Eight weeks after operation, grossly the examples showed: 1. Inner 2/3 part of the incision remained unhealed, and part of NP protruded into the incision; 2. The NPs turned shrunken and smaller. Microscopically: 1. Small vessel appeared in the injury area of AF, and granular tissue emerged near the incision; 2. Original layer structure of AF disorganized, and concentric fissure appeared in almost each layer of the AF; 3. Notochord cells in NP decreased, while chondrocyte like cells and fibroblast like cells increased; 4. Abnormal cell phenomena, such as cell melting, apoptosis appeared.

Three weeks after the injection, injured IVDs showed reparation. Lots of small vessels extended into the incisions, and the injury almost healed. AF partially regained layer structure. NP cells proliferated actively, and the mess array restored in order (fig. 2).

2.3 TGF-β1 Immunohistochemical Research

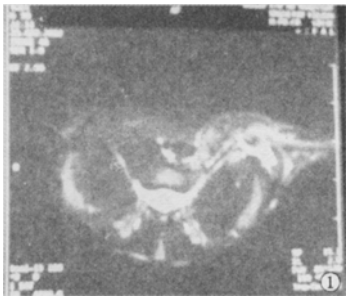
TGF-β1 staining was positive in most normal IVDs examples, while obviously decreased in degenerated IVDs. Fig. 3 shows the immuno-staining of TGF-β1 of therapy group, which demonstrates strongly positive staining of TGF-β1. And the immuno-staining of Empty comparison group shows barely positive staining, just like the comparison group. The difference among the three group is significant (Wilcoxon test, $P<0.001$).

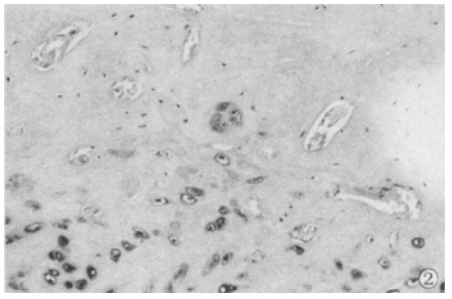
2.4 TGF-β1 Western Blotting

Fig. 4 demonstrates the results of TGF-β1 Western blotting of normal IVDs, therapy group, comparison group and empty comparison group. TGF-β1 bands of the two comparison groups are rather weake, while the band of therapy group is obvious. It exhibits the obvious increase of TGF-β1 content in treated IVDs than the two comparison groups, and that the content of TGF-β1 in therapy group was close to the normal IVDs.

3 DISCUSSION

Occurring and development of intervertebral disc degeneration is rather long-termed and complicated. During the process, various cytokines play important rolls. Inflammatory cytokines, like IL-1 (Interleukin-1), can strongly inhibit synthesis of matrix ingredients (like proteoglycan), accelerate matrix proteolysis, and induce IVD cell apoptosis. Growth factors, like TGF-β1, CDGF (Cartilage derived growth factor), BMP (Bone morphogene-





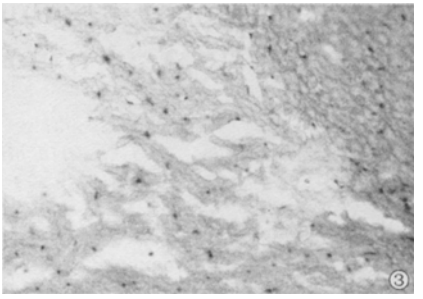


Fig. 1 Three weeks after injection. Axial MRI at T2 weight scan signal showed recovery of IVD. Axial MRI at T2 weight scan (Rabbit intervertebral disc L5-6)

Fig. 2 Three weeks after TGF-β1 gene injection. NP cells proliferated a lot and laid in order. Water content of NP was increased much. AF regained layer structure (Rabbit intervertebral disc L5-6. HE × 200)

Fig. 3 Three weeks after TGF-β1 gene injection. lots of brown granular presented. which mean a strong expression of TGF-β1. (Rabbit intervertebral disc L5-6. Immunohisto chemical staining × 200)

sis protein) are capable of stimulating synthesis of matrix ingredients and cell differentiation. However. the content of the growth factors decreases during IVD degeneration. and IVD cells react to the growth factors more weakly [1].

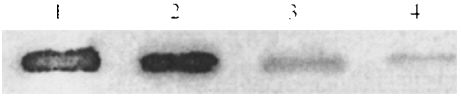


Fig. 4 Three weeks after injection. Western Blotting revealed that TGF-β1 was increased significantly
1: Normal IVD; 2: Therapy group; 3: Comparison group; 4: Empty comparison group

TGF-β1. which belongs to the transforming growth factor super family. can regulate proliferation and differentiation of mesenchyme derived cells. like chondrocyte. osteoblast and Schwann cell. *In vitro* studies have demonstrated its excellent characteristics for IVD degeneration therapy [4]. However. because of their high differentiation. inactivity. poor nutrition and slow metabolism. IVD cells need a rather long term for reparation. Therefore. highly concentrated. and long termed TGF-β1 is necessary to support recovery of IVD degeneration [1,5].

Adenovirus vector is currently the most effective vector for IVD genetic therapy. CMV (Cytomegalovirus) promoter of the vector can strongly enhance expression of the target gene. and prolong its expression time. And the relatively close environment inside the IVD enhances transfection efficiency and local concentration of target gene. So. genetic therapy with adenovirus vector is generally acknowledged as the most hopeful method for IVD degeneration [6,7].

Here we extend the Ad/CMV- hTGF-β1 gene transfection from *in vitro* study into the rabbit AAII model. so as to investigate the therapeutic efficiency *in vivo*. As. described above. the model perfectly simulated degeneration of IVD matrix and cells in human IVDs. We also observed decreased content of TGF-β1 in degenerated IVDs. which is consensus with TGF-β1 insufficiency of human IVDs degeneration and re-accentuates the necessarily of TGF-β1 compensation in degenerated IVDs [1,8].

MRI is now the most convenient and sensitive way to assess IVD degeneration. The assessment criterion utilized in this study is raised by our research group based on MRI signals changes. MRI T2 weight scan (axial) can sensitively demonstrate water loss. which is mainly caused by IVD matrix degradation by changes of signal intensity. Therefore. decrease of MRI signal in our study showed water loss in ANII model. and indirectly manifested degradation of the matrix. Correspondently. the obvious increase of signal intensity after the injection clearly indicated the promotion of matrix quality induced by TGF-β1.

After injection. even in severely degenerated IVDs. strong staining of TGF-β1 granular could be observed in AF and NP cells. and the TGF-β1 content increased much. It proves the high transfection efficiency of Ad/CMV- hTGF-β1 gene in this IVD degeneration model. and that the gene can increase TGF-β1 content significantly. And we also confirmed that time-efficiency of Ad/CMV- hTGF-β1 gene can last longer than 3 weeks [1].

Histological observation indicated improvements of IVD matrix and matrix cells. We also carried out intensive and consecutive observation to investigate changes of IVDs after injection: 1 week after injection. small vessels appeared at the injured area of AF. and AF cells proliferated and rearrayed in order; 2 weeks after injection. majority of AF incision healed. and cells of endplates and NP proliferated actively; 3 weeks after injection. layer structure of AF regained. endplate nearly re-

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remains unsettled, accumulating evidence suggests that insulin is capable of stimulating ovarian androgen synthesis[9]. In this study, it was verified that the concentrations of serum testosterone were higher in DHEA-treated rats than in control rats ($P<0.001$). It appears that elevated testosterone is an important characteristic of ovarian cyst development. To clarify the regulatory role of resistin produced by adipose tissue in PCOS rats with IR, which was proved by higher fasting serum glucose, insulin, and INS/GC ($P<0.001$, $P<0.05$, $P<0.001$), we measured expression of resistin mRNA level in adipose tissue of two groups. The result showed that expression of resistin mRNA in DHEA-treated group was significantly higher than that in control group ($P<0.05$). These results suggest that increased resistin mRNA expression in adipose tissue may be one of the important factors responsible for IR in women with PCOS.

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covered. The water content of NP increased much and NP cells proliferated a lot.

In this study, Ad/CMV- hTGF-β1 gene exhibits preferable transfection efficiency and time-efficiency in the AAII model. It is able to stimulate reparation of IVD matrix and matrix cells, and improve degree of IVD degeneration much. It is a potential method for clinical therapy of IVD degeneration.

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