Transforming growth factor β1 (TGF-β1) regulates the expression of extracellular matrix genes in a concentration-dependent manner.

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Abstract

Here, we investigated the effects of different concentrations of transforming growth factor β1 (TGF-β1) on the regulation of aggrecan (*ACAN*), collagen type II (*COL2A1*), and SRY-box 9 (*Sox9*) gene expression in human nucleus pulposus cells *in vitro*. Second-generation human nucleus pulposus cells were grown in culture media containing 4 different concentrations of TGF-β1 (0.1, 1.0, 10, and 100 μg/L). The control group was not treated with TGF-β1. Then, reverse transcription polymerase chain reaction was used to measure the gene expression of *ACAN, COL2A1*, and *Sox9*. There were no significant differences in expression between the control group and the group treated with 0.1 μg/L TGF-β1. However, significant increases in the expression of *ACAN, COL2A1*, and *Sox9* were observed as the concentration of TGF-β1 increased, and expression was highest following treatment with 10 μg/L TGF-β1. Thus, TGF-β1 induced the expression of *ACAN, COL2A1*, and *Sox9* in nucleus pulposus cells, suggesting that TGF-β1 may play an important role in the prevention and treatment of intervertebral disc degeneration.

Introduction

Disc degeneration is a major cause of common conditions such as cervical spondylosis and low-back pain [1]. Current therapies, including conservative treatments and invasive procedures, can only relieve the pain of disc degeneration, but cannot restore disc structure and function [2,3]. Therefore, effective therapies for intervertebral-disc degeneration are urgently needed. The extracellular matrix of the nucleus pulposus is composed of COL2A1 and proteoglycans, which function to withstand shearing force and conductive pressure. ACAN, the most common proteoglycan, plays an important role in maintaining disc height and withstanding compression [4]. In the process of degeneration, activation of matrix metalloproteinase and aggrecanase lead to a rapid decrease in matrix-like collagen II and ACAN; this causes marked damage to and degeneration of intervertebral discs [5]. Therefore, regeneration of the nucleus pulposus is of great interest for designing new therapies to maintain the function of the intervertebral disc [1,6].

Nucleus pulposus cells are the major matrix-secreting cell type. However, only a few of these cells exist in normal nucleus pulposus tissues, and they are easily differentiated *in vitro*,

causing them to lose their normal cellular phenotype and limiting their application in biological therapy [7]. Therefore, stimulation of human nucleus pulposus cells to synthesize extracellular matrix and maintain the normal cell phenotype through the application of growth factors or gene therapy as a means to reverse disc degeneration is emerging as an interesting therapeutic approach [8]. SOX9, COL2A1, and ACAN could be identified based on expression level despite of their resemble phenotype in nucleus pulposus cells and articular chondrocytes [9]. The expression of these chondrogenic proteins and transcription factors has been widely characterized as the nucleus pulposus phenotype [10]. Transforming growth factor β1 (TGF-β1) is involved in cell proliferation and extracellular matrix synthesis, and these functions have been confirmed in both cartilage and endothelial cells [11]. In this study, we demonstrated the effects of TGF-β1 on the expression of *ACAN, COL2A1*, and *Sox-9* in normal human nucleus pulposus cells to explore effective methods to stop or reverse early stage disc degeneration.

Materials and Methods

Cell collection and culture

The study protocol was approved by the ethics committee of our hospital. After obtaining written informed consent, nucleus pulposus samples were obtained from 3 donors with scoliosis who underwent anterior correction surgery. The donors were 8-19 years old, with a mean age of 13.8 years. Nucleus pulposus tissues were sealed in a sterile container, transported to the laboratory within 20 minutes, and screened immediately on a super clean bench. Then, the fibrous ring and adjacent tissues were removed with surgical scissors. The core nucleus pulposus tissues were separated from the vertebral cartilage under a microscope. Remnant tissues were washed twice with phosphate-buffered saline (PBS; HyClone, Logan, UT, USA) to remove debris, cut into pieces (1-2 mm^2) with a scalpel, and digested with 0.25% tryptase (HyClone) at 37°C for 30 min, with shaking every 5 min. Next, the tissues were centrifuged at $1,000 \times g$ for 5 min and then digested with 0.2% collagenase type II (Gibco-Invitrogen, USA) at 37°C for 4 h after removing the supernatant. Samples were filtered through a nylon screen and centrifuged at $1,000 \times g$ for 5 min. The above procedures were repeated 3 times. Finally, the human nucleus pulposus cells were diluted in 2 mL of F12-10% FBS (HyClone) and counted using counting plates (Corning, NY, USA). The cells were seeded into culture bottles (Corning) at a density of 1×10^4 cells/mL in F12-10% FBS and incubated at 37°C and 5% CO_2. The medium was changed every 3 days. Cells were grown until 80% confluence and then digested with 0.25% tryptase and split into 5 culture bottles for subculturing (the first passage). After reaching 80% confluence, cells were cultured and passaged again by the same method. Average cell viability was 96%, as detected by trypan blue staining. According to the regulations governing the administration of medical institutions issued by the state council of the PRC, patients were informed of the study protocol and related risks before this study, and they signed consent forms.

Test groups

Human nucleus pulposus cells from the second passage, grown to 80% confluence, were centrifuged at $1,000 \times g$ for 5 min. The supernatant was removed, and F12 medium containing 10% fetal bovine serum (FBS; HyClone) was added to obtain a cell density of 1×10^4 cells/mL. The cells were split into 5 culture bottles. After 24 h incubation, the medium was removed, and 10% FBS/F12 medium containing 0.1, 1.0, 10, or 100 μg/L of TGF-β1 (Peprotech, Rocky Hill, NJ, USA) was added (the treatment groups). Medium without added growth factor was used for the control group.

Morphological observation of human nucleus pulposus cells

Cells from each group were observed by inverted phase contrast microscopy every day during this study, and differences in morphology and growth status were compared among groups.

Cell survival rate determination

Cells in all groups were cultured for 72 h and then digested with 0.25% tryptase. Then, the cell survival rate was determined by cell counting using trypan blue exclusion.

Total RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Cells were cultured for 72 h and total RNA was extracted using TRIzol one-step (Gibco, Grand Island, NY, USA), according to the manufacturer's instructions. RNA purity and concentration were assessed using an ultraviolet spectrophotometer. Next, 2 μg of total RNA was reverse transcribed with AMV revertase (Takara, Shiga, Japan) according to the manufacturer's instructions. The cDNA for *ACAN, COL2A1*, and *SOX-9* was amplified by real-time PCR. Primers were synthesized by Beijing Aoke Bio-Tech Co., Ltd. (Beijing, China), as previously described [8]. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal reference. Table 1 shows the sequences of the primers for *ACAN, COL2A1*, and *Sox9* [12]. The reaction conditions were 30 cycles of 3-stage PCR (denaturation at 94°C for 30 s, annealing at 52°C for 45 s, and extension at 72°C for 45 s). The amplified cDNA products were analyzed by 1.5% agarose gel electrophoresis. Electrophoresis photographs were analyzed using the IBAS2.5 image processing system. The absorbance ratios of *ACAN, COL2A1*, and *Sox9* to GAPDH were used to determine the relative expression levels.

Table 1. *Oligonucleotide primers used for real-time PCR.*

Gene	Primer type	Sequence
Collagens type II	Primer fw (5'-3')	AAG AAA CAC ATC TGG TTT GGA GAA A
	Primer rev (5'-3')	TGG GAG CCA GGT TGT CAT C
Aggrecan	Primer fw (5'-3')	CCA ACG AAA CCT ATG ACG TGT ACT
	Primer rev (5'-3')	GCA CTC GTT GGC TGC CTC
SOX-9	Primer fw (5'-3')	AAC GCC GAG CTC AGC AAG
	Primer rev (5'-3')	ACG AAC GGC CGC TTC TC

Statistical analysis

Each experiment was repeated 3 times. Statistical analysis was performed using SPSS15.0 software (SPSS, Inc., Chicago, IL, USA), and the results are presented as the mean ± SD. One-way analysis of variance was used to compare the control and treatment groups. The t-test was used to compare 2 groups. A P value less than 0.05 was considered statistically significant.

Results

Morphological observations

When grown in monolayer culture with medium containing 10% serum, the human nucleus pulposus cells in the control group were fusiform with 2 cytoplasmic processes (Figure 1).

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Compared to the control group, the cells in the treatment groups showed no significant morphological differences when cultured for less than 72 h. However, at 72 h, the cells in the groups treated with 10 or 100 µg/L TGF-β1 were short and spindle-shaped or polygon-shaped with short cytoplasmic processes, abundant cytoplasm, and powerful refractivity, compared to the those in the control group. The morphology of cells exposed to 10 µg/L TGF-β1 showed the greatest differences (Figure 2). There were no striking differences among the 0.1 µg/L TGF-β1, 1.0 µg/L TGF-β1, and control groups at 72 h.
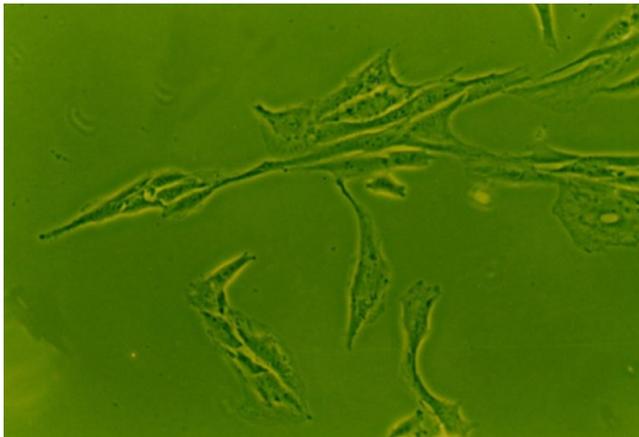
Figure 1. *Normal nucleus pulposus cells (X100).*
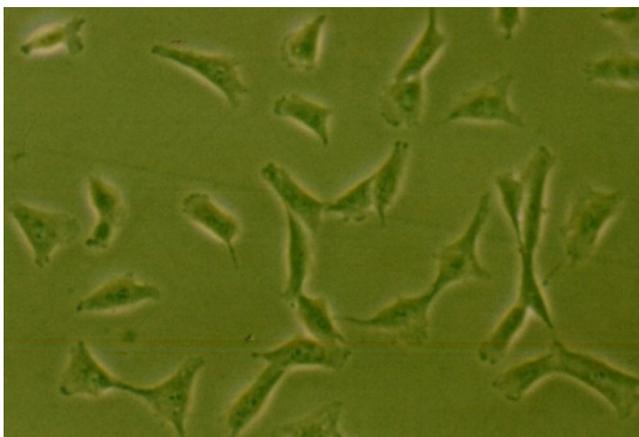
Figure 2. *10 µg/L of TGF-β1 (X100).*

Table 2. *The mRNA expressions of Aggrecan, collagen type II and SOX-9 (n=3, $\bar{x} \pm s$).*

TGF-β1 group	Aggrecan	Collagen type II	SOX-9
Control group	0.775 ± 0.024	1.312 ± 0.036	1.186 ± 0.019
0.1 µg/L group	0.828 ± 0.008	1.497 ± 0.022	1.247 ± 0.035
1.0 µg/L group	1.361 ± 0.016[*]	1.939 ± 0.031[*]	2.352 ± 0.031[*]
10 µg/L group	1.814 ± 0.029[**]	2.862 ± 0.038[**]	3.374 ± 0.056[**]
100 µg/L group	1.663 ± 0.027[**]	2.715 ± 0.024[**]	3.161 ± 0.037[**]

Note: Compared with control group, *P<0.05, **P<0.01

Cell survival

In the presence of 10% FBS, the survival rate of cells in the control group was 89%. Exposure to TGF-β1 increased the cell survival rate, and the cell survival rates of the 0.1 µg/L, 1.0 µg/L TGF-β1, and 10 µg/L TGF-β1 groups were 91%, 94%, and 96%, respectively. However, the cell survival rate decreased to 93% at the highest concentration of TGF-β1 (100 µg/L).

Effects of TGF-β1 on the gene expression of ACAN, COL2A1, and SOX-9

The absorbance ratios of *ACAN* to *GAPDH* in the control and 0.1 µg/L TGF-β1 groups were similar at 0.775 ± 0.024 and 0.828 ± 0.008, respectively. The absorbance ratios of ACAN to GAPDH in the 1 µg/L, 10 µg/L, and 100 µg/L TGF-β1 groups, at 1.361 ± 0.016, 1.814 ± 0.029, and 1.663 ± 0.027, respectively, were significantly higher than that in the control group (P<0.05). Similarly, the absorbance ratio of *COL2A1* to *GAPDH* was 1.312 ± 0.036 in the control group, which was not significantly different from that of the 0.1 µg/L TGF-β1 group. However, compared to the control group, the *COL2A1* to *GAPDH* ratios of the other TGF-β1 treatment groups were obviously increased. The most striking effect of TGF-β1 treatment was observed in the 10 µg/L TGF-β1 group, in which the *COL2A1* to *GAPDH* ratio was 2.18 times higher than that of the control group (P<0.05). Additionally, there was no significant difference in *Sox-9* gene expression between the control group and the 0.1 µg/L TGF-β1 group (P>0.05), whereas there was a significant difference between the control group and the 1 µg/L TGF-β1 group (P<0.05). Compared to the control group, Sox-9 gene expression levels in the 10 and 100 µg/L TGF-β1 groups were significantly higher (P<0.01). These data showed a marked elevation in Sox-9 gene expression following exposure to 10 µg/L TGF-β1 when compared to the expression level in the control group (P<0.01). However, the gene expression of *ACAN*, *COL2A1*, and *SOX-9* showed a tendency to decrease in response to higher TGF-β1 concentrations (Table 2).

Discussion

Degenerative disc disease has high rates of incidence and disability and frequently causes neck, shoulder, low-back, and leg pain. Nucleus pulposus tissues play a primary role in this disease [13]. Normal nucleus pulposus tissue is tremellose composed of proteoglycan and COL2A1. Compared to cartilage tissue, there is more proteoglycan in nucleus pulposus tissues, and the ratio of proteoglycan to *COL2A1* is 27:1 in the nucleus pulposus and 2:1 in cartilage [14]. Within the nucleus pulposus, proteoglycan is embedded in grids composed of *COL2A1*. *ACAN*, the main proteoglycan component, shows powerful adsorption ability, and the water content in normal pulposus tissues is as high as 80%, which imparts various characteristics, such as flexibility, viscous-elastic behavior,

crushing resistance, and shock absorption [15]. Because there is no vascular tissue in the nucleus pulposus, nutrition is supplied through diffusion and dispersion from adjacent tissues [16], which makes the nucleus pulposus prone to degeneration when subjected to pressure for an extended time period. During disc degeneration, *ACAN* and *COL2A1* levels decrease, while collagen type I (*COL1A2*) levels increase, which promotes gradual fibrosis and degeneration of the fibrous ring and cartilage endplate.

Current therapies for degenerative disc disease include conservative treatment and surgery. However, these methods only relieve the symptoms but do not eliminate the cause of disc degeneration, and the long-term effects are not ideal [17]. With the advent of tissue engineering and regenerative medicine, cell-based therapies for the repair of degenerative discs have gained attention [18,19]. Cytokines are used to improve the activity of nucleus pulposus cells and promote the synthesis of normal extracellular matrix, enabling the reversal of disc degeneration. TGF-β1 is a 2-chain polypeptide with a molecular weight of 25 kDa. It is a secreted protein that performs many cellular functions, including control of cell proliferation and cell differentiation, and promotion of extracellular matrix synthesis [20]. Vadala et al. [21] used a scaffold and directly incorporated TGF-β1 into a polymeric solution to culture bovine annulus fibrosus cells. After 3 weeks, glycosaminoglycan and total collagen levels were significantly higher in the treatment group than in the control group, indicating that TGF-β1 could be used to treat disc degeneration.

A previous study showed that TGF-β1 could promote cell proliferation in the presence of 10% serum, and there was a dose-effect relationship from 1 to 10 μg/L TGF-β1 [22]. Because nucleus pulposus cells lack specific cell markers, some studies evaluated *ACAN, COL2A1*, and *Sox-9* expression as specific markers. Sox-9 promotes cartilage cell differentiation and maintains the cartilage cellular phenotype, which is closely related to disc degeneration. *Sox-9* increases the synthesis of *ACAN* and *COL2A1* in the disc matrix [23].

Our results showed that TGF-β1, at 1-100 μg/L, clearly promoted the expression of *ACAN, COL2A1*, and *SOX-9*. These results confirm that TGF-β1 can promote and maintain the expression of the nucleus pulposus cell phenotype *in vitro*, and 10 μg/L TGF-β1 showed the strongest effects.

The mechanisms of TGF-β1 activity are unclear; thus, studies examining the effect of TGF-β1 on the expression of genes in the extracellular matrix of the nucleus pulposus are needed. First, TGF-β1, which promotes the expression of proteoglycan and COL2A1, should be directly injected into degenerative nucleus pulposus tissues to improve the activity and proliferation of nucleus pulposus cells. However, some researchers have suggested that since growth factors have short half-lives and disc degeneration is a chronic process, it is difficult to obtain satisfactory effects through injection [24]. Interestingly, some studies have found that proteoglycan and COL2A1 levels in discs were increased when TGF-β1 was injected into a degenerative disc model (P<0.01) [25]. These

results indicated that TGF-β1 promotes the synthesis of extracellular matrix, thus delaying disc degeneration. Additionally, fabrication of a tissue-engineered injectable scaffold to treat degenerative disc disease may be possible [26,27]. Nucleus pulposus cells and stem cells cultured *in vitro* were used as seed cells. Stem cells are common and show multi-directional differentiation; however, an effective method for differentiating stem cells into nucleus pulposus cells has not been established. Furthermore, the number of nucleus pulposus cells is limited, and they show low activity. The results of this study indicate that in nucleus pulposus cells cultured *in vitro*, TGF-β1 promotes the expression of the normal cell phenotype, making it possible to culture nucleus pulposus cells *in vitro* for use as seed cells in disc engineering.

Authors' Contribution

Dr. Rongfeng Zhang performed the experiments and prepared the manuscript. Dr. Rongfeng Zhang and Dr. Jia-gang Xu took charge of the experimental design. Dr. Xiang-kun Wu and Zhen-tai Liu helped assess the experimental design and prepare the manuscript.

Acknowledgements

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