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.

Keywords: TGF-β1, Nucleus pulposus cells, Gene expression, Aggrecan, Collagen type II, SRY-box 9.

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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 aggreganase 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 Sox9 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 mm²) with a scalpel, and digested with 0.25% trypsin (HyClone) at 37°C for 30 min, with shaking every 5 min. Next, the tissues were centrifuged at 1,000 x 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 x 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 x 10⁴ cells/mL in F12-10% FBS and incubated at 37°C and 5% CO2. The medium was changed every 3 days. Cells were grown until 80% confluence and then digested with 0.25% trypsin 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 x 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 x 10⁴ 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% trypsin. 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 reverse transcriptase (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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagens type II</td>
<td>Primer fw (5'-3')</td>
<td>AAG AAA CAC ACT TGG TTT GGA GAA A</td>
</tr>
<tr>
<td></td>
<td>Primer rev (5'-3')</td>
<td>TGG GAG CCA GGT TGT CAT C</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>Primer fw (5'-3')</td>
<td>CCA ACG AAA CCT ATG ACG TGT ACT</td>
</tr>
<tr>
<td></td>
<td>Primer rev (5'-3')</td>
<td>GCA CTC GTT GGC TGC CTC</td>
</tr>
<tr>
<td>SOX-9</td>
<td>Primer fw (5'-3')</td>
<td>AAC GCC GAG CTC AGG ACT</td>
</tr>
<tr>
<td></td>
<td>Primer rev (5'-3')</td>
<td>ACG AAC GGC CGC TTC TC</td>
</tr>
</tbody>
</table>

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).
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 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.

### 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 ± 0.016 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,
Our results showed that TGF-β1, at 1-100 μg/L, clearly 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.

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References

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