



ORIGINAL ARTICLE

Knockdown of asporin affects transforming growth factor- β 1-induced matrix synthesis in human intervertebral annulus cells



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KEYWORDS

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TGF- β 1

Summary *Background/Objective:* Asporin is associated with osteoarthritis and lumbar disk degeneration. Previous studies in chondrocytes showed that asporin can bind to transforming growth factor- β 1 (TGF- β 1) and downregulate matrix biosynthesis. However, this has not been studied in intervertebral disk (IVD) cells. This study aimed to inspect the expression of asporin under TGF- β 1 stimulation and its effect on TGF- β 1-induced matrix biosynthesis in human intervertebral annulus cells.

Methods: Human intervertebral annulus cells were obtained from the pathological region of IVD in eight patients. After primary culture and redifferentiation in alginate beads, cells were reseeded and treated with different concentrations (5 ng/mL, 10 ng/mL, and 15 ng/mL) of TGF- β 1 for up to 24 hours. Total RNA extracted from the cells and those with asporin knockdown were subjected to real-time polymerase chain reaction analysis to examine the expression of asporin and extracellular matrix genes.

Results: TGF- β 1 stimulation induces asporin transcription significantly in a dose- and time-dependent manner. Knockdown of endogenous asporin leads to the upregulated expression of collagen II alpha 1 and aggrecan.

Conclusion: Our results have verified a functional feedback loop between TGF- β 1 and asporin in human intervertebral annulus cells indicating that TGF- β 1-induced annulus matrix biosynthesis can be significantly upregulated by knockdown of asporin. Therefore, asporin could be a potential new therapeutic target and inhibition of asporin could be adopted to enhance

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the anabolic effect of TGF- β 1 in human intervertebral annulus cells in degenerative IVD diseases.

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Introduction

Degenerative disk disease (DDD) is the most prevalent disease affecting the human spine, and represents a leading cause of low back pain in aging populations [1]. DDD is characterised by the destruction of the normal tissue structure from an imbalance between catabolism and anabolism. Physiologically, the disk cells actively regulate the homeostasis of intervertebral disk tissue under normal conditions. These cells maintain a balance between anabolism and catabolism by modulating their activity [2]. However, the underlying molecular mechanism is not clearly elucidated. Several noncollagenous extracellular matrix (ECM) components may contribute to the degenerative process of DDD, and among them is asporin [3].

Asporin is a member of the class I small leucine-rich proteoglycan (SLRP) protein family, which has been found to be expressed in the ECM of osteoarthritic cartilage [4], degenerated intervertebral disk (IVD) [3], and pathological skin scars [5,6]. The name of asporin is derived from the combination of aspartate (asp-) and decorin (-orin), due to the unique stretch of aspartate residues (D-repeat) at its N-terminus and the overall similarities with decorin [7]. Polymorphisms were identified in the D-repeat domain, and a significant association was found between D14 allele and osteoarthritis in Chinese and Japanese populations [4,8,9]. Recent studies showed that the D14 allele is also significantly associated with lumbar disk degeneration in Asian individuals [3]. Moreover, asporin is mainly found in the outer annulus cells, and its increased expression is associated with the level of disk degeneration [10].

An *in vitro* study showed that, like several other SLRP members, transforming growth factor- β 1 (TGF- β 1) upregulated the expression of asporin [11], but asporin itself forms a functional feedback loop through binding directly to TGF- β 1, thereby preventing the interaction of TGF- β 1 with its *in vivo* type II receptor, and the TGF- β 1-induced expression of cartilage matrix genes and chondrogenesis [12]. However, the results from chondrogenic cell models have not been verified in human IVD cells.

In this study, we examined the expression of asporin under TGF- β 1 stimulation in cultured human intervertebral annulus cells and inspected the effect of the knocking down of asporin on the expression of aggrecan (ACAN) and collagen II alpha 1 (COL2A1).

Materials and methods

Isolation and primary culture of human intervertebral annulus cells

The experimental study of human disk specimens was approved and supervised by the Human Subjects

Institutional Ethical Review Board at the Beijing Jishuitan Hospital, Beijing, China. All patients signed the informed consent form for the use of their disk specimens in research, and the confidentiality of all patients' personal information was carefully maintained. Annulus specimens from the pathological region of IVD were derived from a routine disk procedure performed on eight patients (aged 11–28 years; 2 women and 6 men) with lumbar disk herniation. After careful debridement and washing, the specimens were minced and incubated at 37°C in 0.25% trypsin (Sigma-Aldrich Corporation, Lenexa, KS, USA) for 2 hours followed by 0.2% collagenase II (Sigma-Aldrich Corporation) overnight digestion until the ECM was completely dissolved. Cells were harvested per plate at a density of 1×10^5 cells/mL in Dulbecco's modified eagle medium (DMEM)/F12 1:1 (Gibco-BRL Life Technologies, Grand Island, NY, USA) supplemented with 10% foetal bovine serum, 1% (volume/volume) penicillin and streptomycin (Sigma-Aldrich Corporation) at 37°C, 5% carbon dioxide, and 90% atmospheric humidity.

Three-dimensional culture of human intervertebral annulus cells in alginate beads

Monolayer cultured human annulus cells were collected and resuspended at a concentration of approximately 4×10^5 cells/mL in a 1.2% low-viscosity alginate sodium solution (Sigma-Aldrich Corporation). The alginate suspension was added in 102mM CaCl₂ dropwise through a 22-gauge needle. The beads were polymerised in the CaCl₂ solution and then washed twice with 0.15M NaCl. Then, the beads were resuspended (7–15 beads/mL) in DMEM/F12 growth medium. The medium was changed every 3 days for 2 weeks. At the end of the culture period, the beads were depolymerised by adding three volumes of 55mM sodium citrate/0.15M NaCl, pH 6, and incubated at 37°C for 10 minutes. The cell pellets were centrifuged (300g), washed twice with phosphate-buffered saline, reseeded, and cultured in a six-well plate at a density of 1×10^5 cells/well in the DMEM/F12 growth medium until they reached confluence.

Induction of asporin by TGF- β 1

When the cells cultured in the six-well plate reached confluence, the culture medium was replaced with DMEM/F12 containing 0.2% foetal bovine serum. After 12 hours, the cells were treated with different concentrations of TGF- β 1 (Sigma-Aldrich Corporation; 5 ng/mL, 10 ng/mL, and 15 ng/mL) and examined at different time points (6 hours, 12 hours, 18 hours, and 24 hours).

RNA isolation and real-time polymerase chain reaction assays

The induction of asporin, ACAN, and COL2A1 by TGF- β 1 was examined. The total RNA from cultured cells was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and reverse transcribed into cDNA using the ImProm-II Transcription System (Promega Corporation, Madison, WI, USA). Quantitative real-time polymerase chain reaction (PCR) was performed using the SYBR Green method. Gene-specific primers were designed using the Primer Express 2 software (Applied Biosystems, Foster City, CA, USA; Table 1). The reactions were conducted on an ABI PRISM 7300 system (Applied Biosystems) using the Power SYBR Green PCR kit (Applied Biosystems) according to the manufacturers' instructions. Two microlitres of each reverse transcription reaction mixture was used as a template for the real-time PCR. PCR cycling conditions were set as follows: 94°C for 15 minutes, followed by 40 cycles at 94°C, 55°C, and 72°C for 30 seconds. The 2- Δ Ct and 2- $\Delta\Delta$ Ct methods were used to calculate the relative expression of each target gene, and the values were normalized to glyceraldehyde-3-phosphate dehydrogenase levels.

Knockdown of asporin by small interfering RNA

Predesigned small interfering RNA (siRNA) was purchased from Invitrogen Life Technologies with targeting sequences of 5'-AAGUGAAGCUCCAAUAAAGUUGGUG-3' and 5'-CACCAA-CUUUAUUGGAGCUUCACUU-3'. As a negative control, a scrambled siRNA with similar guanine–cytosine content (Invitrogen Life Technologies) was transfected under the same conditions. The siRNAs were transfected into cultured human annulus cells using Entranster-R transfection reagent (Engreen Biosystem Corporation Limited, Beijing, China), and the transfection system was optimised according to the manufacturers' instructions. Twenty-four hours after transfection, cells were treated with TGF- β 1 (10 ng/mL) for 24 hours, and subjected to real-time PCR analysis.

Western blot analysis

Western blot analysis was performed as described previously [13]. Equal protein samples of 50 μ g from the cell lysate were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene

difluoride membranes (Millipore Corporation, Billerica, MA, USA). Primary antibody for asporin (1:500 dilution; Santa Cruz Biotechnology Inc., Paso Robles, CA, USA) and secondary horseradish peroxidase–conjugated donkey antgoat immunoglobulin G antibody (1:2000 dilution; Proteintech Group Inc., Rosemont, IL, USA) were used. Immunoreactive signal bands were visualised using an enhanced chemiluminescence kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), exposed to an X-ray film, and analysed using a computer-assisted image analysis system (Gene Genius Bio Imaging System, Syngene, Cambridge, UK).

Statistical analysis

All data were shown as mean \pm standard deviation. The statistical analysis was performed using SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA). The differences among the groups were analysed using the one-way analysis of variance test. A p value $<$ 0.05 was considered statistically significant.

Results

Patients

The removed intervertebral disk tissues of eight patients (aged 11–28 years; 2 women and 6 men), who underwent intervertebral disk fusion surgery in the hospital after confirmation of disease history and magnetic resonance imaging (MRI) examination, were used in this study. One of



Figure 1 Representative magnetic resonance image of the intervertebral disk. A 20-year-old patient with moderate to severe intervertebral disk degeneration affecting L4–5 (Grade IV). Sagittal magnetic resonance imaging scan (Magnetom 1.5T; Siemens AG, Berlin, Germany) was performed with a slice thickness of 5 mm. A T2-weighted image with a repetition of 2500 ms and an echo time of 90 ms of the intervertebral disc was taken in all the participants.

Table 1 Primers for real-time polymerase chain reaction.

Primers	Sequences (5' to 3')
Asporin F	CTGGGCCTAGGAAACAACAA
Asporin R	TTCATCTTTGGCACTGTTGG
Aggrecan F	TCTACCGCTGCGAGGTGAT
Aggrecan R	TGTAATGGAACACGATGCCCTT
Collagen II alpha 1 F	GGAAGAGTGGAGACTACTGGATTGAC
Collagen II alpha 1 R	TCCATGTTGCAGAAAACCTTCA
GAPDH F	ACCACAGTCCATGCCATCAC
GAPDH R	TCCACCACCCTGTTGCTGTA

GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

the representative MRI images of the participants is presented in [Figure 1](#).

Induction of asporin by TGF- β 1

After redifferentiation in alginate beads, the human annulus cells were cultured in a six-well plate and treated with 5 ng/mL, 10 ng/mL, and 15 ng/mL of TGF- β 1. Using real-time PCR, the expression of asporin was examined every 6 hours up to 24 hours. No obvious difference was found among different groups at the beginning of the treatment. The asporin expression increased with the induction time and the TGF- β 1 concentration ([Figure 2](#)). At 24 hours after stimulation, a significant difference between groups was observed ($p < 0.003$).

Knockdown of asporin by siRNA

The effect of asporin expression levels on the TGF- β 1-induced matrix biosynthesis was investigated. Transient transfection assays were performed to knock down the expression of asporin using siRNA. A low-cytotoxicity transfection reagent was chosen for this experiment. The transfection system was optimised according to the manufacturers' instructions. For the liposome-based transfection, the reagents had a low efficiency in some primary cultured cells. Only a 60–70% knockdown effect was observed at both protein and mRNA levels ([Figure 3](#)).

The effect of asporin on TGF- β 1-induced matrix biosynthesis

After confirmation of the knockdown of asporin, the cells were incubated with 10 ng/mL of TGF- β 1 for 24 hours, and

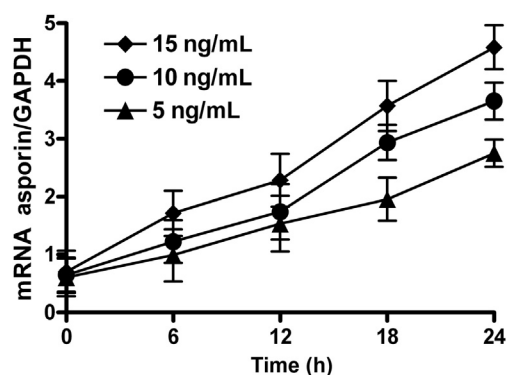


Figure 2 Transforming growth factor- β 1 regulates the expression of asporin in human intervertebral annulus cells. The human intervertebral annulus cells were cultured in a six-well plate. When they reached confluence, culture medium was replaced and cells were treated with transforming growth factor- β 1 at the assigned concentrations (5 ng/mL, 10 ng/mL, and 15 ng/mL) and examined at the indicated time points (6 hours, 12 hours, 18 hours, and 24 hours). Expression of asporin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified using real-time polymerase chain reaction. The asporin message is normalised with GAPDH. The data are shown as mean \pm standard deviation of triplicate assays.

then the total mRNA from the cells was subjected to real-time PCR to compare the difference in the expression of ECM *COL2A1* and *ACAN*. The results clearly showed that TGF- β 1-induced expression of *COL2A1* and *ACAN* was upregulated by the knockdown of asporin ([Figure 4](#))

Verification in a Beagle dog intervertebral disk degeneration animal model

A Beagle dog animal model using the percutaneous puncture induction of intervertebral disk degeneration under x-ray fluoroscopy was conducted, and MRI was performed to confirm the degradation of intervertebral disks in the animal model before removing the intervertebral disk tissues. Western blotting was used to inspect the levels of

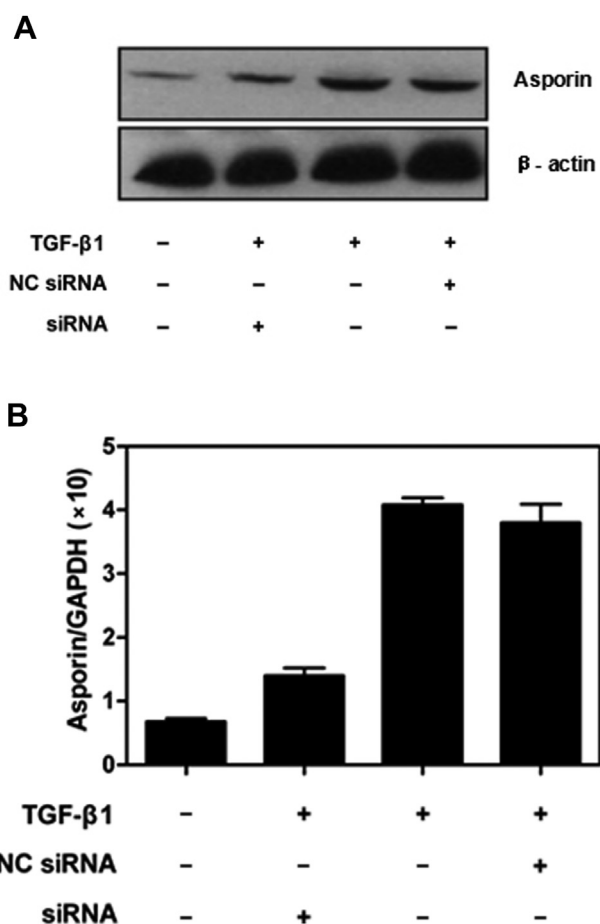


Figure 3 Knockdown of asporin expression by small interfering RNA (siRNA). siRNA (100nM) targeting asporin or control siRNA (NC siRNA) was transfected into cultured annulus cells. Twenty-four hours later, the cells were treated or untreated with 10-ng/mL transforming growth factor- β 1 (TGF- β 1) for another 24 hours. (A) The knockdown efficiency was detected using Western blotting and cell lysates were probed with asporin antibody with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as a loading control. (B) The knockdown efficiency was detected with real-time polymerase chain reaction and real-time polymerase chain reaction for asporin mRNA level normalised to that of GAPDH. The data are shown as mean \pm standard deviation of triplicate assays. * $p < 0.05$.

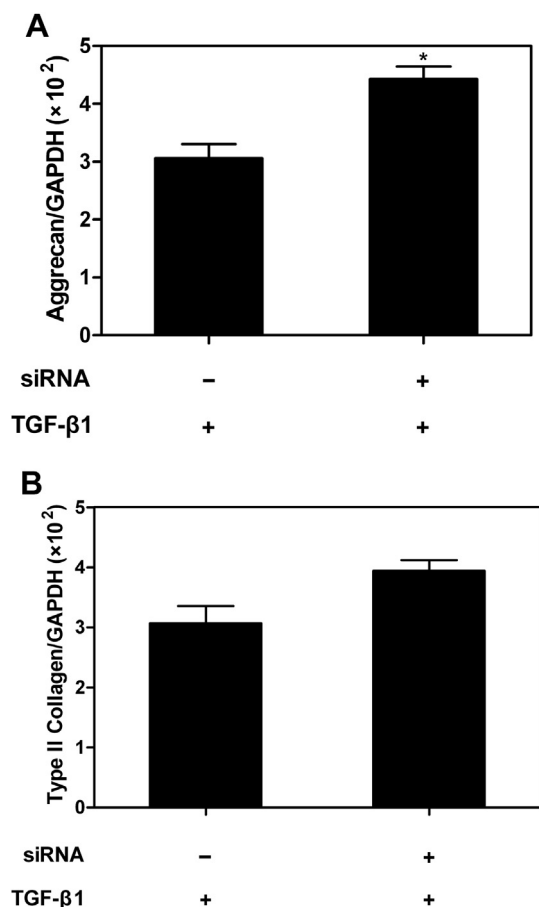


Figure 4 The effect of aspirin on transforming growth factor- β 1 (TGF- β 1)-induced matrix biosynthesis. The cultured annulus cells were transfected with aspirin siRNA or control small interfering RNA (siRNA; 100nM). After 24 hours, the cells were incubated with 10 ng/mL of TGF- β 1 for another 24 hours. The expression levels of (A) aggrecan and (B) type II collagen were determined using real-time polymerase chain reaction. The results were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are from triplicate assays. * $p < 0.05$.

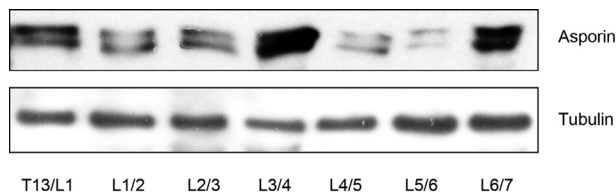


Figure 5 Level of aspirin inside intervertebral disk in a Beagle animal model. Percutaneous puncture induction of intervertebral disk degeneration animal model under X-ray fluoroscopy was conducted, and Western blotting was used to inspect the levels of aspirin inside the intervertebral disk at the annulus regions. Tubulin was adopted as a loading control (T13/L1: intervertebral disk between thoracic vertebrae 13 and lumbar vertebrae 1; L1/2: intervertebral disk between lumbar vertebrae 1 and 2.) L3/4 and L6/7 were confirmed to have lumbar disk herniation by magnetic resonance imaging.

aspirin inside the intervertebral disk. In the degraded intervertebral disks, the levels of aspirin were obviously higher compared with that from the normal ones (Figure 5).

Discussion

DDD is characterised by the abnormal intervertebral disk structure incurred from an imbalance between catabolism and anabolism and the disk cells in the disk plays a critical role in maintaining the balance [2]. In this study, we investigated the relationship between the expression of aspirin and TGF- β 1 controlled annulus biosynthesis process and confirmed a functional feedback loop between TGF- β 1 and aspirin in human intervertebral annulus cells.

We found that the transcription of aspirin increased significantly in a dose- and time-dependent manner in response to TGF- β 1 stimulation in the disk cells from patients. This is in agreement with the results obtained in chondrocytes [12]. Several studies suggest the overall similarity between the chondrocytes and IVD cells in response to many cytokines [14], and our result provides evidence in support of this postulation.

SLRP family members, including aspirin, have the ability to bind to TGF- β 1 and inhibit the interaction of TGF- β 1 with its *in vivo* type II receptor [15]. However, an *in vitro* study showed that such an effect had to be mediated by heparin or heparan sulfate in the presence of type II collagen [15]. These findings suggested the existence of a regulatory network in the ECM components for the interaction between TGF- β and its receptors. New therapeutic interventions such as expression or injection of TGF- β 1 into IVD resulted in the stimulation of matrix biosynthesis [16–18]. Our results indicate that TGF- β 1-induced expression of collagen II alpha 1 and aggrecan can be significantly enhanced by knocking down the expression of endogenous aspirin. This suggests that the inhibition of aspirin could be potentially used to improve the anabolic effect of TGF- β 1 in human intervertebral annulus cells in IVD patients. It would be intriguing to first test in animal models of IVD for the effect of TGF- β 1 in combination with the knockdown of aspirin.

In summary, this study confirmed a functional feedback loop between TGF- β 1 and aspirin in human annulus cells. It highlighted the potential use of aspirin as a new preventive or therapeutic target to enhance the anabolic effect of TGF- β 1 in degenerative IVD diseases.

Conflicts of interest

The authors have no conflicts of interest to declare.

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