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Cross talk between Smad transcription factors and TNF-α in intervertebral disc degeneration

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Abstract

The transforming growth factor-β (TGF-β) and the tumor necrosis factor-α (TNF-α) families are known to play important roles in intervertebral disc degeneration (IVD). However, molecular interactions between the TGF-β and TNF-α signaling pathways have yet to be elucidated. The purpose of this study was to analyze the expression patterns of Smad transcription factor signaling associated with IVDs with aging and to examine the modulation of Smad signaling by TNF-α in IVD cells using SD rats. According to these experimental results, BMP signals in the TGF-β family were more likely to be a key factor in IVD degeneration by aging, and it was predicted that besides the involvement of catabolic factors like MMPs and ADAMS-TS, there may be a decrease in expression of anabolic factors through cross talk of signaling between TNF-α and TGF-β pathway in pathogenesis of disc degeneration.

Keywords: Intervertebral disc; Disc degeneration; Nucleus pulposus; TNF-alpha; Smad

Low back pain, a common locomotor dysfunction, occurs because of intervertebral disc (IVD) degeneration resulting from aging, kinetic load, or social factors, such as smoking [1,2].

Clinically, spinal fusion is frequently used to treat disc disorders; however, postoperative problems, such as instability of the adjacent intervertebral discs, often occur. Motion preservation, through the development of a new treatment method inhibiting IVD degeneration prior to surgery, has been investigated and various biological strategies aimed at the restoration and regeneration of IVD degeneration have been suggested [3]. These strategies currently include intradiscal injections of cytokines or growth factors, gene transfection of IVD cells, artificial IVDs using tissue engineering, and cell transplantation therapy [4–7]. These reports demonstrate that growth factors belonging to the transforming growth factor-β (TGF-β) super family activate IVD cells and are also involved with bone, cartilage and stem cell differentiation. TGF-β super family proteins increase the synthesis of the extracellular matrix and PGs essential to maintaining IVD homeostasis and are key factors in IVD degeneration and regeneration.

The intracellular effectors of TGF-β signaling are members of the Smad family of proteins and consist of five receptor-regulated Smads (R-Smads): Smad1, Smad2, Smad3, Smad5, Smad8, one cooperating Smad (Co-Smad): Smad4, and two inhibitory Smads (I-Smads): Smad6, Smad7 [8]. Among the R-Smads, Smad2 and Smad3 are phosphorylated, mainly by stimulation by TGF-β, whereas Smad1, Smad5 and Smad8 are phosphorylated mainly by stimulation by the bone morphogenetic proteins (BMPs) [9]. Moreover, among the I-Smads, Smad6 selectively inhibits BMP signals, whereas Smad7 inhibits both TGF-β and BMP signals [8,9]. In addition, Smads are activated by a receptor transfer into nuclei where they modulate transcription [8,9]. This pathway is relatively simple, but other signal-transducing pathways further modulate the activation and functions of the Smads. To elucidate the molecular biological process of IVD degeneration, it is

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necessary to analyze the differentiation of IVD cells, the transcription factors that are important for degeneration, and the cross talk between transcription factors.

It is known that an inflammatory cytokine, tumor necrosis factor-α (TNF-α), inhibits the expression of PGs and type II collagen by increasing the expression of matrix metalloproteinases (MMPs) and ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs), which are among the major factors in IVD degeneration [10]. However, there are no reports in the literature describing intracellular signaling cross talk between the TGF-β family and the TNF-α signal pathway, including in the disc degenerative process.

Therefore, the purposes of this study were to analyze the genes related to intracellular Smad signaling in the NP and AF of IVDs, to study the influences of the TGF-β super family and Smads on IVD degeneration due to aging (Study 1), to study the influence of TNF-α on Smad gene expression and to compare cross talk between a TGF-β family and TNF-α signals from the perspective of Smad gene expression (Study 2).

Materials and methods

The animal experiments were carried out according to a protocol approved by the Animal Experimentation Committee at our institution.

Study 1: disc harvest. A total of 64 female Sprague–Dawley (SD) rats were used for the aging study. The "young group" consisted of 32 female Sprague–Dawley (SD) rats that were 12 weeks old; the "old group" consisted of 32 female SD rats that were 32 weeks old. All animals were purchased from Japan Crea (Tokyo, Japan). The rats were euthanized by injection of an excess amount of pentobarbital sodium (Nembutal®, Abbott. Lab) (100 mg/kg). Following euthanasia, coccygeal intervertebral discs were harvested under sterile conditions (each rat yielded one sample that included three vertebrae). The tissues from the three vertebrae dissected from each rat were pooled to produce one sample each for the nucleus pulposus (NP) and annulus fibrosus (AF). These pooled NP and AF tissues were used for biochemical analyses (six samples each), histology (six samples each), and reverse transcription polymerase chain reaction (RT-PCR) analysis (20 samples each).

Measurement of proteoglycan content. The NP disc tissues ($n = 6$ samples each per group) harvested for the biochemical analyses were digested with papain (Sigma–Aldrich, MO, USA). The papain digests were then analyzed to determine the total sulfated PG content by the dimethylmethylene blue (DMMB) dye-binding method [11]. The ratio of the absorbance measured at 530 and 595 nm by a plate reader (Spectra MAX250, Molecular Devices) was calculated to determine the PG content of each sample. The total amount of PG per NP disc was normalized to the total mass per NP disc (wet weight). All assays were performed in duplicate.

Histological examination. The three spinal segments were harvested together with their cranial and caudal vertebral bodies. The spinal segments from each animal ($n = 6$) in each group (young and old) ($n = 18$ IVDs per group) were fixed, and then calcified. Each specimen was cut longitudinally at the center of the disc for histologic evaluation. Sample discs ($n = 18$) from each group (young and old) were processed individually for paraffin wax embedding. These were stained with hematoxylin and eosin and Safranin-O for evaluation. Two histologists who are familiar with human and animal IVD specimens performed the evaluation of these sections. The intra-observer reliability, based on readings at two time intervals one month apart, was $\kappa = 0.90$, showing excellent agreement.

Reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was used to determine the level of gene-specific PCR primers for the following in Table 1. β-Actin expression was used as the internal control. Briefly, total RNA was extracted from the separately NP and AF tissues from 20 rats in each age group with an SV Total RNA Isolation System (Promega, Madison, WI). PCR amplification was carried out using a two-step protocol, comprising 10 min of preheating at 95 °C for activation of AmpliTaq Gold DNA polymerase (Applied Biosystems), followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The PCR products were separated by electrophoresis on non-denaturing 1.2% TBE polyacrylamide gels and then stained with ethidium bromide. The gels were subsequently scanned with a UV Densitograph System (Atto Biotechnologies, Inc., Tokyo, Japan) and band intensities were quantified by densitometry and normalized for β-actin gene levels using the CS Analyzer software program (Version 2.01, Atto). The semi-quantitative data are representative of three independent experiments ($n = 3$).

Statistical analysis. The results are expressed as means ± standard deviation (SD) of the experiments. Statistical significance was determined using a two-way analysis of variance (ANOVA) with Fisher's PLSD test as a post hoc test. The Statview program was used for statistical analyses. Significance was accepted at $p < 0.05$. Error bars in the figures represent the standard deviation (SD).

Study 2: disc harvest and culture. The NP and AF were macroscopically dissected from the coccygeal discs of 12-week-old female SD rats ($n = 12$; Japan Crea) after euthanasia. NP tissues were first digested with 0.025% Trypsin–EDTA for 15 min; the isolated cells were then cultured in monolayer with Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen, Corp., Carlsbad, CA) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C, 5% CO_2. AF tissues were first digested with pronase E (0.04%) (Kakenkagaku, Tokyo, Japan) for approximately 30 min and then with 0.125% collagenase P (Boehringer Mannheim, Mannheim, Germany) for approximately 1 h. The digested tissue was filtered with a 100-μm cell strainer, and then spread onto culture plates. The cells were cultured in monolayer with DMEM supplemented with 10% FBS and antibiotics at 37 °C, 5% CO_2 [12]. The cultures were maintained for two weeks to permit the formation of tissues with properties similar to those of native NP and AF. TNF-α protein (#24783, Upstate, NY) was added to the cultures at a concentration of 50 ng/ml for either 24 or 48 h before harvesting.

Reverse transcription polymerase chain (RT-PCR) reaction. At harvesting, total RNA was isolated by first mincing the cells using a sterile scalpel. The pieces were then snap-frozen and pulverized in liquid nitrogen and the tissue powder was homogenized with Isogen reagent (Nippongene, Tokyo, Japan) and then further purified using RNeasy spin columns (Qiagen, Valencia, CA). RNA samples were then reverse transcribed to cDNA using oligo dT primer and Multiscribe Reverse Transcriptase followed by the SV Total RNA Isolation System. RT-PCR analysis was carried out as mentioned above. (The upstream and downstream primer sequences for each primer are as mentioned above) (Table. 1).

Results

Study1: proteoglycan content. The biochemical analysis showed that the PG content of the NP tissues of young rats differed significantly from that of the old rats (young: 1.48 ± 0.35 μg/mg mass ratio; old: 0.84 ± 0.20 μg/mg mass ratio ($p < 0.05$, two-way ANOVA) (Fig. 1A).

Histological examination. The histological structure and morphology of the NP and AF were well preserved in both age groups. However, an age-related decrease in sulfated PGs stained by Safranin-O was detected in the NP region of old rat discs compared to that in young rat discs (Fig. 1B). The histological structure exhibited a decreased number of notochordal cells with aging. The notochordal