

Laboratory Investigation

The Effects of TWEAK, Fn14, and TGF- β 1 on Degeneration of Human Intervertebral Disc

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Objective : The purpose of this study is to explain the effect and reciprocal action among tumor necrosis factor (TNF) like weak inducer of apoptosis (TWEAK), fibroblast growth factor-inducible 14 (Fn14), and transforming growth factor- β 1 (TGF- β 1) on degeneration of human intervertebral disc (IVD).

Methods : Human intervertebral disc tissues and cells were cultured with Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F-12) media in 37°C, 5% CO₂ incubator. When IVD tissues were cultured with TWEAK, Fn14 that is an antagonistic receptor for TWEAK and TGF- β 1, the level of sulfated glycosaminoglycan (sGAG) was estimated by dimethyl methyleneblue (DMMB) assay and sex determining region Y (SRY)-box 9 (Sox9) and versican messenger ribonucleic acid (mRNA) levels were estimated by reverse transcriptase polymerase chain reaction (RT-PCR).

Results : When human IVD tissue was cultured for nine days, the sGAG content was elevated in proportion to culture duration. The sGAG was decreased significantly by TWEAK 100 ng/mL, however, Fn14 500 ng/mL did not change the sGAG production of IVD tissue. The Fn14 increased versican and Sox9 mRNA levels decreased with TWEAK in IVD tissue TGF- β 1 20 ng/mL elevated the sGAG concentration 40% more than control. The sGAG amount decreased with TWEAK was increased with Fn14 or TGF- β 1 but the result was insignificant statistically. TGF- β 1 increased the Sox9 mRNA expression to 180% compared to control group in IVD tissue. Sox9 and versican mRNA levels decreased by TWEAK were increased with TGF- β 1 in primary cultured IVD cells, however, Fn14 did not show increasing effect on Sox9 and versican.

Conclusion : This study suggests that TWEAK would act a role in intervertebral disc degeneration through decreasing sGAG and the mRNA level of versican and Sox9.

KEY WORDS : Intervertebral disc · TWEAK · Fn14 · TGF- β 1 · Sox9 · Versican.

INTRODUCTION

Lumbar disc degeneration is a common problem that is a leading cause of low back pain in humans. It has been widely held that symptoms of lumbar disc disease are the result of either herniation of the nucleus pulposus through a mechanically weak annulus fibrosus or from tearing of the annulus itself^{1,5}. The normal intervertebral discs have three components : end plate, nucleus pulposus and annulus fibrosus. The end plate is formed from cartilage that resembles articular cartilage and vertebral cortex. The nucleus pulposus consists of chondrocytes within a matrix of type II col-

lagen and proteoglycan and annulus fibrosus comprises dense sheet of highly orientated type I collagen fibers containing fibroblast-like cells¹⁰. Disc degeneration accompany with several phenomena such as decreased aggrecan and type II collagen productions, or elevated type I collagen synthesis and type II collagen denaturation¹³.

Tumor necrosis factor (TNF) like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily originally identified as a weak inducer of apoptosis in certain tumor cell lines². As with other members of the TNF superfamily TWEAK has pleiotropic effects including proangiogenic effects on vascular endothelial cells, proinflammatory activities on epithelial and endothelial cells, as well as proliferation enhancing effects on endothelial cells and astrocytes¹⁷.

TWEAK receptor named fibroblast growth factor-inducible 14 (Fn14) is a member of the TNF receptor superfamily that expressed by nonlymphoid cell types. TWEAK/

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Fn14 interaction plays important roles in proliferation, migration, inflammatory responses, and survival in a variety of cell types, including endothelial, epithelial, immune and some tumor cells¹⁾.

Transforming growth factor- β 1 (TGF- β 1) is known to modulate cell differentiation, bone formation, angiogenesis, hematopoiesis, cell cycle progression, cellular migration and extracellular matrix production and especially involved in proteoglycan synthesis in intervertebral disc cells¹⁶⁾. TGF- β 1 treatment increased mitogen-activated protein kinases (MAPK) activity and sex determining region Y (SRY)-box 9 (Sox9), aggrecan, and collagen type II gene expression¹⁹⁾. Sox9, a transcription factor, belongs to the SRY (sex-determining region on the Y chromosome) family and plays a role in chondrogenesis and type II collagen expression⁷⁾. The loss of expression of Sox9 in some of the annulus cell population may play a role in disc aging and degeneration, possibly by decreased modulation of the expression and production of type II collagen by disc cells⁵⁾.

In current study, the change of sulfated glycosaminoglycan (sGAG) amount was examined in various culture conditions or after treatment of TWEAK, its receptor Fn14 and TGF- β 1. The messenger ribonucleic acid (mRNA) levels of Sox9 and versican were estimated in cells treated with TWEAK, Fn14 and TGF- β 1.

MATERIALS AND METHODS

Materials

Human intervertebral disc tissue specimens were granted from hospitals in Seoul. These tissue samples were obtained from fifteen patients and stored in DMEM-F12 media (Jeil Biotechservices Inc., Seoul, Korea) containing 10% fetal bovine serum (Jeil Biotechservices) and 1% antibiotic-antimycotic solution (Jeil Biotechservices) in the entire study period, and the sample of 5-8 gram obtained from one or two patients was used in one experiment.

Human intervertebral disc primary cell and tissue culture

Human intervertebral disc tissues were minced by surgical blade and pincette in DMEM-F12 media. And disc tissue culture was incubated in 37°C, 5% CO₂ incubator. Minced disc tissue was sequentially digested with 0.2% pronase and 0.025% collagenase (SIGMA-ALDRICH., St. Louis, MO, USA) in DMEM/F-12 media. Separated cell solution was centrifuged at 1,200 rpm, for 5 min, and precipitated cells were resuspended in media. Resuspended cell solution was poured to 100 mm culture dish and incubated in 37°C, 5% CO₂ incubator. The minced intervertebral

disc tissue was incubated with DMEM-F12 media containing 10% fetal bovine serum and 1% antibiotic-antimycotic solution in 37°C, 5% CO₂ incubator.

Dimethylmethylene blue assay for glycosaminoglycan quantification

The amount of sulfated sGAG in the culture media was determined by invoking Ferndale's method⁴⁾. DMMB assay reagent solution (16 mg DMMB in 1 L distilled water containing 2.37 g NaCl, 3.04 g glycine, 95 mL HCl) (SIGMA-ALDRICH) was prepared and stored at room temperature. The amount of sGAG in the samples was estimated via method that 900 mL DMMB solution was added to a 100 mL sample media in a 1.5 mL tube and mixed and then transferred to cuvette. The rate of absorbance was immediately read at 525 nm. The assay was calibrated by use of reagent blanks and standards containing up to 1.8 μ g/mL of chondroitin sulfate (SIGMA-ALDRICH).

Reverse transcription polymerase chain reaction for the estimation of Sox9 and versican mRNA levels

Total RNA was extracted by the TriZol reagent (Invitrogen., Carlsbad, CA, USA) according to manual. Complementary DNA was synthesized by SuperScript III (Invitrogen) reverse transcriptase from total RNA, and polymerase chain reactions (PCR) for Sox9 and versican were administered with PCR PreMix kit (iNtRON Biotechnology., Gyeonggi, Korea). The primer sequences used for RT-PCR are as follows : Sox9 forward primer; 5'-TTTCCAAGAC ACAAACATGA-3', Sox9 reward primer; 5'-AAAGTCCA GTTTCTCGTTGA-3', versican forward primer; 5'-CTG CCCCAGCCTTTCT-3', versican reward primer; 5'-GCGGCTTATTGCAGTTTGG-3'.

Statistical analysis

Data are presented as mean \pm SEM (Standard Error of Measures). Statistically significant differences between two groups were calculated by the Student's t-test and one-way ANOVA was used to certify the statistical differences among over three groups. A value of $p < 0.05$ was considered significant.

RESULTS

The production amount of sGAG in cultured intervertebral disc tissue during culture duration

To investigate the change of sGAG production amount during culture duration, intervertebral disc tissue was cultured for 9 days. After 2, 4, 6 or 9 days cultivation, the sGAG

was gradually increased with culture duration, and it was especially the largest on ninth day after culture initiation (Fig. 1). The sGAG production was more increased to 21% at ninth day after culture compared to second day in culture system ($p < 0.05$).

The change of sGAG in the condition of TWEAK or Fn14 treatment

In order to speculate the effect of TWEAK and Fn14 on sGAG production in intervertebral disc tissue culture, we treated TWEAK of various concentration (50-100 ng/mL) and Fn14 (100-1000 ng/mL) to cultured intervertebral disc tissues during three days. The production amount of sGAG in TWEAK 100 ng/mL treated group was decreased more than control group ($p < 0.005$). But, in disc cultures treated with TWEAK inhibitor (Fn14) of various concentration (100, 500 and 1,000 ng/mL), sGAG production was

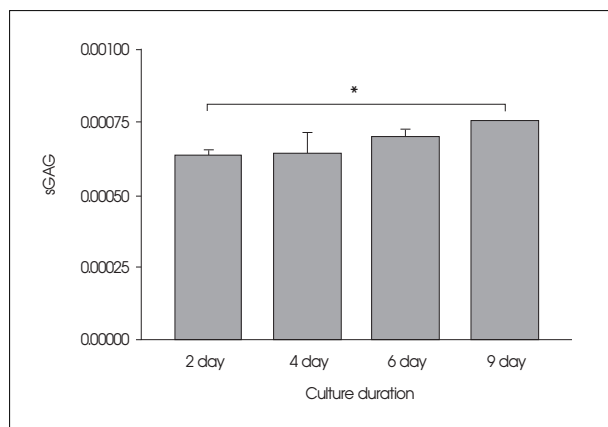


Fig. 1. The relation of sulfated glycosaminoglycan (sGAG) production amount with culture duration in intervertebral disc culture. The sGAG was gradually increased with culture duration, and it was the largest on nine day after culture initiation (* $p < 0.05$).

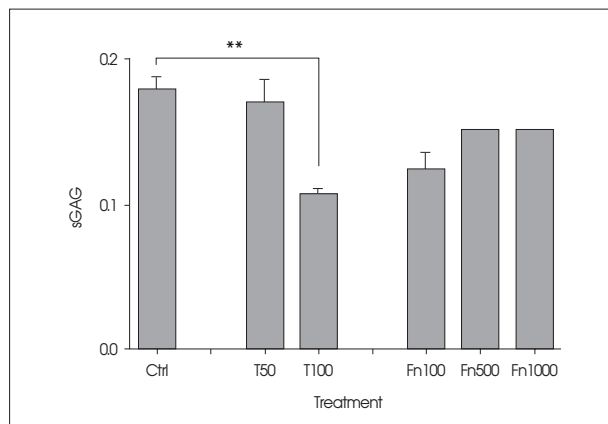


Fig. 2. The change of sGAG by TWEAK or Fn14 in intervertebral disc culture. TWEAK 100 ng/mL decreased the sGAG compared to control group, but TWEAK inhibitor, Fn14 500 ng/mL did not have an effect to sGAG production (** $p < 0.005$).

not changed compared to control group (Fig. 2).

The effect of TGF-β1 on sGAG production in intervertebral disc culture

TGF-β1 20 ng/mL increased sGAG concentration to 40.8% compared to control group ($p < 0.05$) (Fig.3).

The change of sGAG production amount in intervertebral disc tissue culture treated with TWEAK, Fn14 and TGF-β1

TGF-β1 and Fn14 showed a tendency that sGAG amount decreased with TWEAK was increased through the treatment of Fn14 or TGF-β1 but the result was insignificant (Fig. 4).

The mRNA expression levels of versican and Sox9 in intervertebral disc tissue treated with TWEAK and Fn14

In order to examine the expression rates of versican and

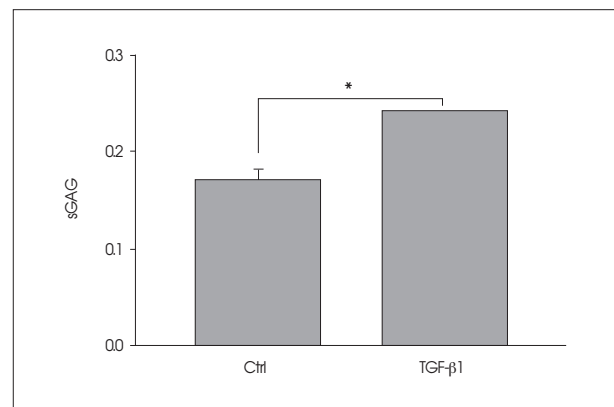


Fig. 3. The increase of sGAG production rate with TGF-β1. TGF-β1 20 ng/mL increased more sGAG concentration than control group (* $p < 0.05$).

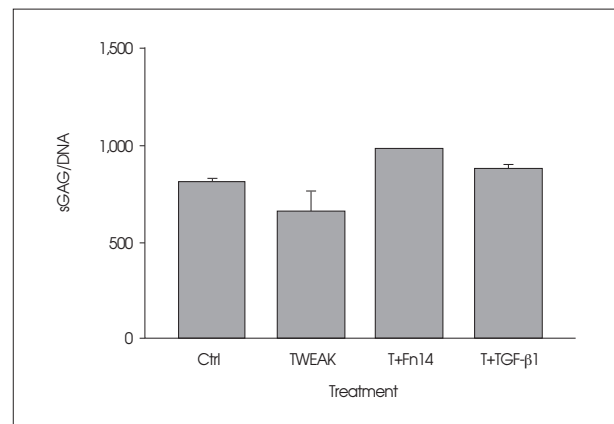


Fig. 4. Sulfated glycosaminoglycan (sGAG) production rate in intervertebral disc tissue treated with TWEAK, Fn14, and TGF-β1. TGF-β1 and Fn14 showed a tendency that sGAG amount decreased with TWEAK was increased through the treatment of Fn14 or TGF-β1.

Sox9 mRNA in intervertebral disc tissue treated with TWEAK and Fn14, we treated the TWEAK of serial concentrations (100-250 ng/mL), Fn14 500 ng/mL. And, we studied the co-treated effect of TWEAK and Fn14 on mRNA expression levels of versican and Sox9. In TWEAK 100 ng/mL treated condition, the mRNA levels of versican and Sox9 genes were reduced to 95% and 82% in each, also they were decreased to 99% and 78%, respectively, in the condition of 250 ng/mL TWEAK treatment. The mRNA expression rates of versican and Sox9 were more increased 120% and 116%, respectively, than control group in Fn14 500 ng/mL treated condition.

In the co-treated condition of TWEAK 100 ng/mL and Fn14 500 ng/mL, versican and Sox9 mRNA levels were significantly increased to 142% and 156% in each. But, Fn14 did not increase the mRNA levels in TWEAK 250

ng/mL treated condition. The increasing capability of Fn14 was prominent in the condition of co-treatment with TWEAK 100 ng/mL (Fig. 5). TGF- β 1 20 ng/mL increased the Sox9 mRNA expression to 180% in co-treated condition with TWEAK 100 ng/mL compared to control group. Both Fn14 and TGF- β 1 increased the mRNA expression of Sox9 decreased by TWEAK (Fig. 6).

The mRNA level of Sox9 and versican in primary cultured intervertebral disc cells treated with TWEAK, Fn14 and TGF- β 1

Sox9 mRNA expression was decreased with TWEAK, however, it was more increased with TGF- β 1 treatment than only TWEAK treated group in primary cultured intervertebral disc cells (Fig. 7). But, Fn14 did not show increasing effect on Sox9 mRNA expression rate. Versican mRNA

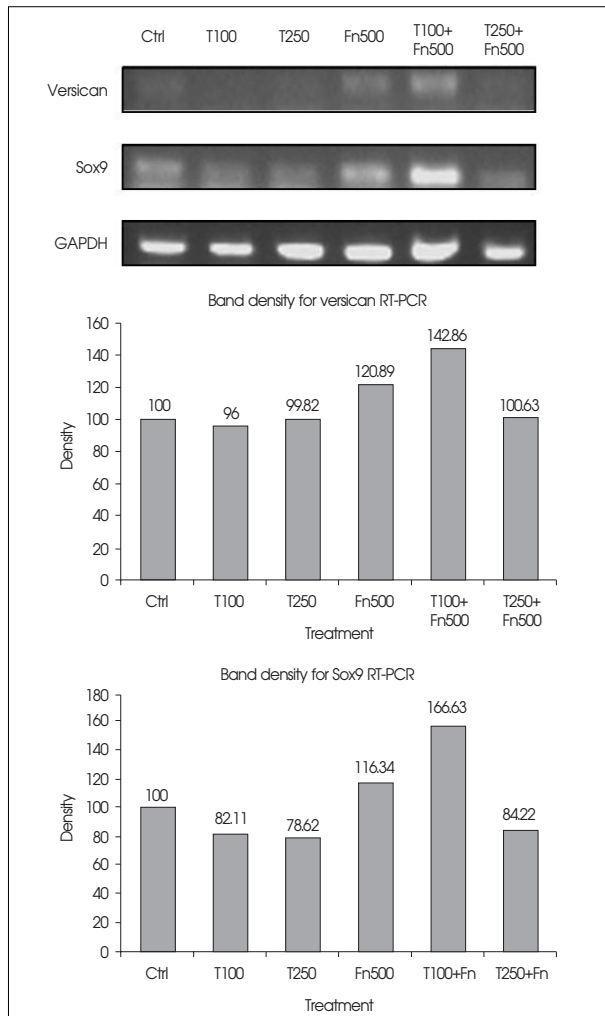


Fig. 5. The mRNA levels of versican and Sox9 in cultured intervertebral disc with treatment of TWEAK and Fn14. The expression rates of versican and Sox9 decreased by TWEAK were increased through Fn14 treatment. The increasing capability of Fn14 was prominent in the condition of co-treatment with TWEAK 100 ng/mL.

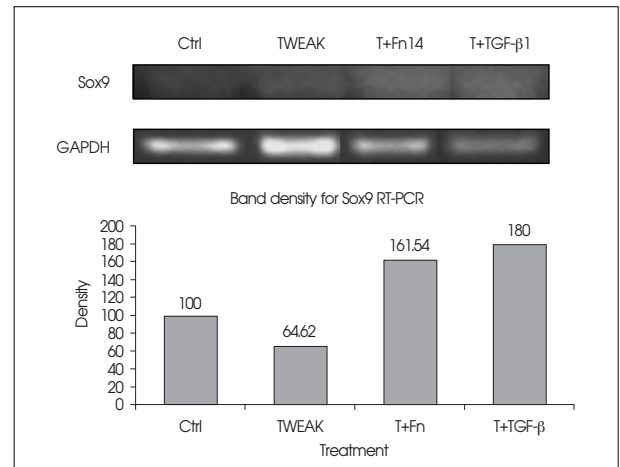


Fig. 6. Sox9 mRNA level in intervertebral disc culture in the condition of TWEAK, Fn14, and TGF- β 1 treatment. Both Fn14 and TGF- β 1 increased the mRNA expression of Sox9 decreased by TWEAK.

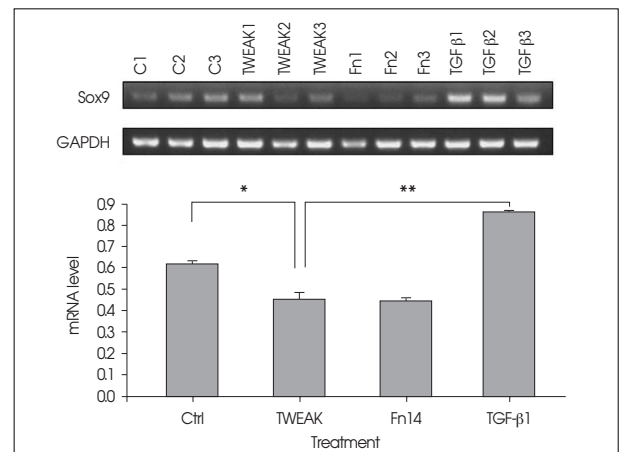


Fig. 7. Sox9 mRNA level in primary cultured intervertebral disc cells treated by TWEAK, Fn, and TGF- β 1. Sox9 mRNA expression was decreased with TWEAK and more increased with TGF- β 1 than only TWEAK treated group (* $p < 0.05$, ** $p < 0.005$).

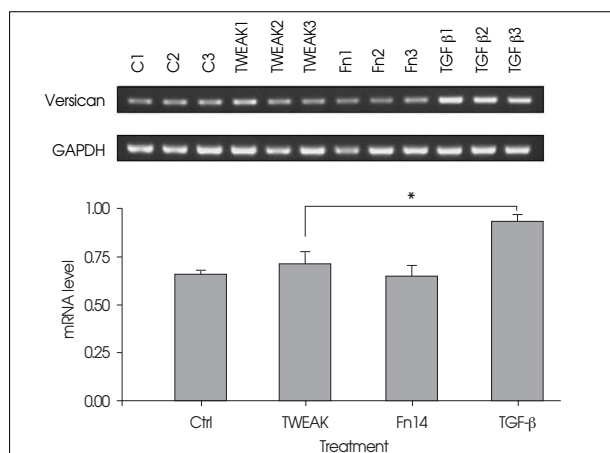


Fig. 8. Versican mRNA level in primary cultured intervertebral disc cells treated by TWEAK, Fn, and TGF- β 1. Versican mRNA expression was increased more with TGF- β than only TWEAK treated group (* $p < 0.05$).

expression was elevated more with TGF- β 1 treatment than only TWEAK treated group (Fig. 8).

DISCUSSION

Herniation of spinal discs (HD) is a common problem that is responsible for symptoms in up to 40% of all patients with low back pain³. Although the primary cause of low back pain has not been identified, disc degeneration seems to be related to the intensity of low back pain due to loss of mechanical stability in the spine.

TWEAK stopped endogenous repair by blocking differentiation of osteoblastic and chondrocytic precursors¹⁷. In disc tissues, TWEAK plays a role in matrix metalloproteinases (MMP)-3 upregulation and aggrecan downregulation, resulting in proteoglycan degradation and promotion of disc degeneration²¹.

The degeneration of the intervertebral disc is related to a variety of inflammatory mediators including nitric oxide (NO), interleukins, MMP, prostaglandin E2 (PGE2), tumor necrosis factor (TNF) alpha and other cytokines¹⁸.

It was reported that MMP-3 and MMP-7 play crucial roles in IVD degradation of *in vitro* murine culture model of the acute HD⁶. TNF-alpha and interleukin (IL)-1beta both caused an increase in protease transcription [MMP-3, MMP-13, a disintegrin and matrix metalloproteinase with thrombospondin motif (ADAMTS) 4 and ADAMTS5] and in pro-inflammatory enzymes, inducible nitric oxide synthase and cyclooxygenase (COX)-2, as well as a decrease in matrix protein transcription, including collagen II, aggrecan, fibromodulin and link protein (IL-1 beta only), and an increase in MMP-3 and MMP-9 secretion²⁰.

The positive immunofluorescence stained cell numbers

for aggrecan, type I-II collagen, bone morphogenetic protein (BMP)-2 and BMP-7 (members of TGF- β family) were also increased after each TGF- β 1 and recombinant human BMP-2 treatment, and also more increased significantly in the aggrecan, type I, II collagen, BMP-2 and 7 when they were used jointly⁸.

Among IL-1 inhibitors, IL-1 receptor antagonist (IL-1ra) might be a candidate for preventing IVD degeneration¹². Indeed, Le Maitre and colleagues⁹ have shown in monolayer and three-dimensional alginate-cultured resident cells from degenerate IVDs that IL-1ra down-regulates metal-dependent proteases and, delivered directly or by gene therapy in explants of degenerated human IVDs, almost completely eliminates enzyme activity, thereby decreasing extracellular matrix degradation¹¹.

BMP-2 significantly increased aggrecan and collagen type II mRNA expression 8.3 and 4.61 times, respectively, and decreased versican mRNA expression 0.54 times as compared with control. BMP-2 significantly increased TGF- β 1 and BMP-7 mRNA expression 2.32 and 2.45 times, respectively, compared with control. There was no significant change in BMP-6 mRNA expression¹⁴.

In this study, TWEAK 100 ng/mL decreased the mRNA expression levels of Sox9 and versican, however, those were more increased with Fn14 500 ng/mL than control in intervertebral disc tissue culture. The TWEAK decreased sGAG production, but Fn14 and TGF- β 1 increased it above normal level. Transforming growth factor- β 1 increased sGAG production and elevated Sox9 mRNA level decreased by TWEAK more than Fn14 in intervertebral disc tissue culture. In other report, TWEAK induced disc degeneration via the elevation of MMP-3 expression, and Fn14 antagonized the effect of TWEAK²¹. Our study confirmed the reciprocal reaction of TWEAK and Fn14 on disc degeneration and had a meaning showed an interrelation between TWEAK, Sox9, versican and TGF- β 1. It was suggested that TWEAK has a negative effect on sGAG production via lowering the mRNA expression of Sox9 and versican participated in the production of sGAG and other proteoglycan in nucleus pulposus from our results. In intervertebral disc cell culture TWEAK decreased the mRNA level of only Sox9, however, Fn14 didn't have an effect to the decreased condition by TWEAK. But TGF- β 1 elevated the mRNA expression of both Sox9 and versican more than only TWEAK treated group. It was postulated that TGF- β 1 has a stronger effect on TWEAK than Fn14.

CONCLUSION

This study suggests that TWEAK would play a role in

intervertebral disc degeneration through decreasing sulfate glycosaminoglycan (sGAG) and mRNA expression levels of versican and Sox9, and the negative effect of TWEAK on intervertebral disc integrity should be reversed with Fn14 and TGF- β 1.

It is supposed that the antagonistic relation of TWEAK and TGF- β 1, or Fn14 has an important role on intervertebral disc homeostasis.

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