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Laboratory Investigation

The Relation Between Sox9, TGF-β1, and Proteoglycan in Human Intervertebral Disc Cells

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Objective : The aim of this study is to elucidate the effects of transforming growth factor-β (TGF-β)1 and L-ascorbic acid on proteoglycan synthesis, and the relationship between Sox9, proteoglycan, and TGF-β1 in intervertebral disc cells.

Methods : Human intervertebral disc tissue was sequentially digested to 0.2% pronase and 0.025% collagenase in DMEM/F-12 media and extracted cells were cultured in 37℃, 5% CO_2 incubator. When intervertebral disc cells were cultured with TGF-β1 or L-ascorbic acid, the production level of sulfated glycosaminoglycan (sGAG) was estimated by dimethyl methyleneblue (DMMB) assay. The changes of Sox9 mRNA and protein levels via TGF-β1 were detected by RT-PCR and Western blot analysis in each.

Results : The amount of sGAG was increased with the lapse of time during incubation, and sGAG content of pellet cultured cells was much larger than monolayer culture. When primary cultured intervertebral disc cells in monolayer and pellet cultures were treated by TGF-β1 20 ng, sGAG content of experimental group was increased significantly compared to control group in both cultures. L-Ascorbic acid of serial concentrations (50-300 ug/ml) increased sGAG content of mono layer cultured intervertebral disc cells significantly in statistics. The co-treatment of TGF-β1 and L-ascorbic acid increased more sGAG production than respective treatment. After treating with TGF-β1, Sox9 mRNA and protein expression rates were significantly increased in disc cells compared with the control group.

Conclusion : This study suggests that TGF-β1 would increase sulfated glycosaminoglycan (sGAG) and other proteoglycans such as versican by elevating Sox9 mRNA and protein expressions in order.

KEY WORDS : Intervertebral disc · Proteoglycan · TGF-β1 · Sox9.

INTRODUCTION

Intervertebral disc has an articulation having buffer function, and constitutes nucleus pulposus as inner part and annulus fibrosus in outer part. Nucleus pulposus constitutes mainly of type II collagen, but annulus fibrosus constitutes primarily of type I collagen. Back pain is related to degenerative changes of the intervertebral disc. Disc degeneration accompanied with several phenomena such as decreased aggrecan and type II collagen productions, or elevated type I collagen synthesis and type II collagen denaturation. Transforming growth factor-β (TGF-β) is multifunctional cytokine that regulates cell differentiation, osteogenesis, angiogenesis, haematopoiesis, cell cycle progression, cellular migration, and extracellular matrix production[10]. It was reported that TGF-β1 involved in proteoglycan synthesis in intervertebral disc cells and can be an alternative curing method for degenerative intervertebral disease[13]. TGF-β treatment increased mitogen-activated protein kinases (MAPK) activity and Sox9, aggrecan, and collagen type II gene expression[12]. Therefore, TGF-β may be used as a therapeutic alternative to degenerated disc disease. Sox9 that is related to chondrogenesis and osteogenesis plays an important role in mesenchymal condensation and chondrocyte differentiation. Sox9 is a transcription factor for the activation of type II collagen production, or 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 cells[2]. The continuous expression of Sox9 via a retrovirus improves collagen II expression in monolayer culture of late passage human articular chondrocytes and also enhances their ability to respond to 3-dimensional cultures[16]. It was reported that L-ascorbic acid stimulates the proliferation of chondrocytes and maintains the chondrogenic properties of the cells in an alginate beads culture.

In this study, the change of sulfated glycosaminoglycan (sGAG) amount was examined in

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various culture conditions, or after treatments of TGF-β1 and L-ascorbic acid. Sox9 mRNA and protein levels were estimated in cells treated with TGF-β1. Furthermore, we investigated the effect of TGF-β1 on mRNA expression of versican that is a large aggregating proteoglycan mainly distributed in nucleus pulposus. It was reported that the carboxy-terminal G3 domain of versican appears to influence intervertebral disc cell adhesion and proliferation in vitro[18].

MATERIALS AND METHODS

Materials

Human disc specimens were granted from Seoul Medical Center (Seoul, Korea). Cell culture reagents were purchased from Jeil Biotechservices Inc. (Seoul, Korea) and GIBCO (Carlsbad, California, USA). Collagenase, pronase, and dimethylmethylene blue were obtained from SIGMA-ALDRICH (St. Louis, MO, USA). Rabbit polyclonal anti-Sox9 antibody was purchased from Chemicon (Billerica, MA 01821, USA). Bovine anti-rabbit secondary antibody was obtained from Santa Cruz Biotechnology, Inc. (California, USA). TRIzol® reagent and SuperScript III reverse transcriptase were purchased from Invitrogen (Carlsbad, California, USA). Maxime PCR PreMix kit, protein extraction kit and chemiluminesence Western blot detection kit were obtained from iNtRON Biotechnology (Gyeonggi-do, Korea).

Human intervertebral disc primary cell culture

The intervertebral disc tissue samples were minced by surgical blade and pincette in DMEM-F12 media. Minced disc tissue was sequentially digested to 0.2% pronase and 0.025% collagenase in DMEM/F-12 media. Separated cell solution was centrifuged at 1,200 rpm, for 5min, and precipitated cells were resuspended in media. Resuspended cell solution was poured to 100 mm culture dish and incubated in 37°C, 5% CO_2 incubator.

Dimethylmethylene blue (DMMB) assay for glycosaminoglycan quantification

The amount of sulfated glycosaminoglycan in the culture media was determined by invoking Farndale's method[1]. DMMB assay reagent solution (16 mg DMMB in 1 L distilled water containing 2.37 g NaCl, 3.04 g glycine, 95 ml HCl) 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 ug/ml of chondroitin sulfate.

RT-PCR for the estimation of Sox9 and versican mRNA levels

Total RNA was extracted by the TriZol reagent according to manual. Complementary DNA was synthesized by SuperScript III reverse transcriptase from total RNA, and polymerase chain reactions (PCR) for Sox9 and versican were administered with PCR PreMix kit.

The primer sequences used for RT-PCR are as follows : Sox9 forward primer; 5'-TTTCCAAGACACAAACATG-A-3', Sox9 reward primer; 5'-AAAGTCCAGTTTCTCG-TTGA-3', versican forward primer; 5'-CTGCCCCGAG-CCTTTCT-3', versican reward primer; 5'-GCGGCTTA-TTGCAGTTTGG-3'.

Western blot analysis for Sox9 protein expression

The protein amounts of protein extracts were estimated by Bradford method. Extracted proteins (10 ug) were loaded on 10% SDS-PAGE gels. Western blot analysis was performed using a polyclonal antibody against Sox9. Images were taken by X-ray film processor.

Statistical analysis

Data are presented as mean ± SEM (Standard Error of Measures). Statistically significant differences between groups were calculated by the Student's t-test or one-way ANOVA. A value of $p < 0.05$ was considered significant.

RESULTS

The amount of sGAG production in monolayer and pellet cultures during cultivation

To understand the effect of culture method on sGAG production, while intervertebral disc cells were cultured for 8 days,

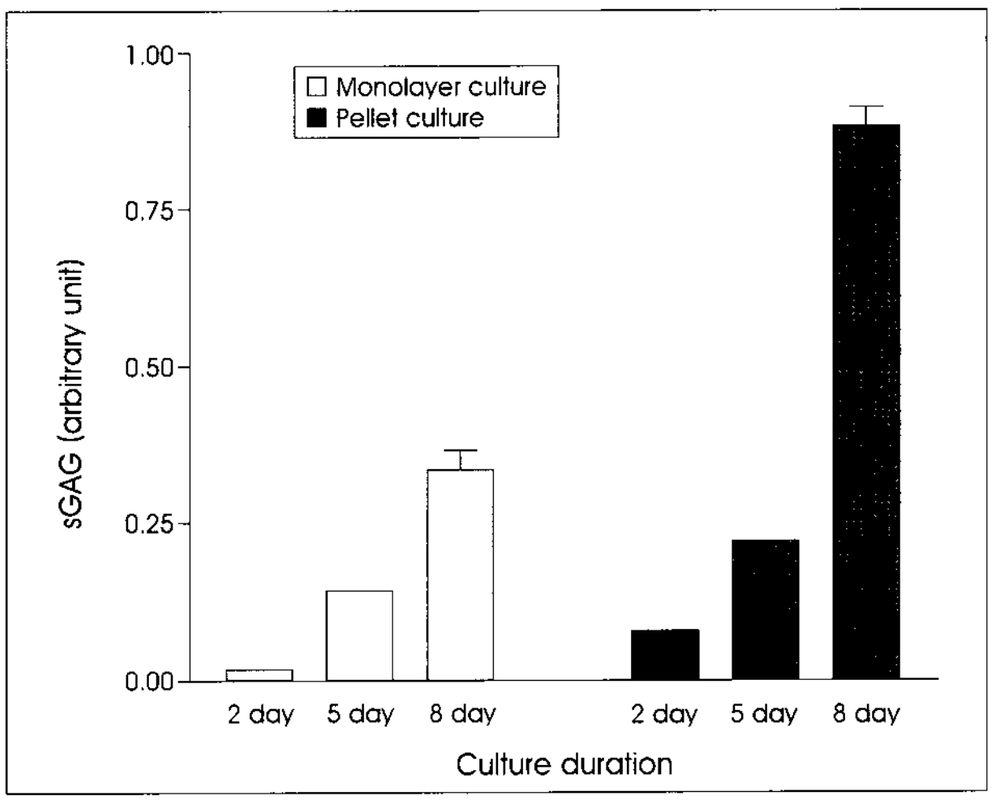

Fig. 1. Sulfated glycosaminoglycan (sGAG) production in monolayer and pellet cultures during cultivation. The sGAG level is proportional to cultural dulation in both cultures.

we estimated sGAG content from media in culture plate.

The sGAG production was increased to 767% at eighth day after culture compared to second day in monolayer culture system. In pellet culture sGAG content was elevated to 411% through 8 day culture compared to 2 day. The sGAG content of pellet culture was 11.9 folds larger than monolayer at second day after and 6.38 times larger at eighth day after culture (Fig. 1).

sGAG production in TGF-β1 treating condition of various concentrations in monolayer culture and in the condition of TGF-β1 treatment in both monolayer and pellet culture

To investigate the effect of TGF-β1 on sGAG synthesis of intervertebral disc cells in monolayer culture, we treated TGF-β1 of various concentrations (10-40 ng/ml) to monolayer cultured intervertebral disc cells. The sGAG level was incresed via TGF-β1 treatment of various concentrations. The production amount of TGF-β1 10 ng/ml treated group was increased to 460% more than control. The sGAG was produced most plentifully at TGF-β1 20 ng/ml (Fig. 2).

To compare sGAG production rate of monolayer culture with pellet culture, we treated TGF-β1 (20 ng/ml) to pellet cultured disc cells.

The sGAG production was increased by TGF-β1 treatment in both monolayer and pellet cultures (Fig. 3). TGF-β1 (20 ng/ml) treatment group produced 560% more elevated sGAG than control in monolayer culture. In pellet culture sGAG was increased to 20% by TGF-β1. Monolayer culture showed more high susceptibility to TGF-β1 than pellet culture.

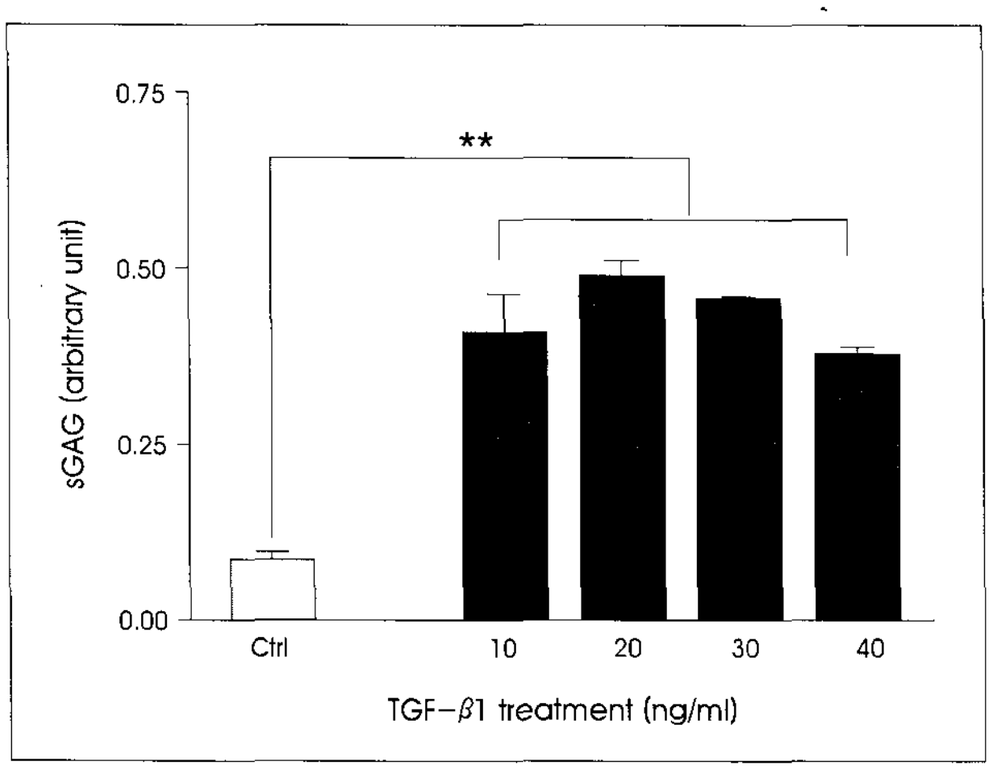

Fig. 2. Sulfated glycosaminoglycan (sGAG) production in transforming growth factor-β (TGF-β)1 treating condition of various concentrations. [monolayer culture] The sGAG level is increased via TGF-β1 treatment of various concentrations, however there is no statistically meaningful difference between TGF-β1 treated groups (** : $p<0.005$).

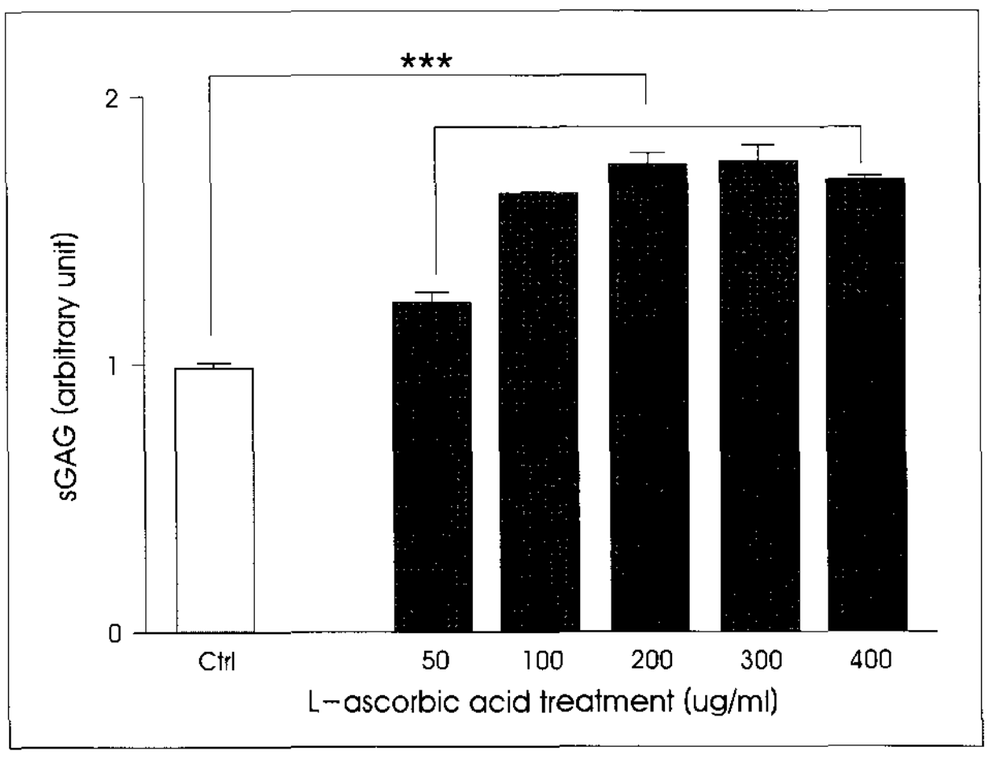

Fig. 4. Sulfated glycosaminoglycan (sGAG) production in the condition of treating L-ascorbic acid of various concentrations. [monolayer culture] The sGAG production level is increased from 50 to 200 ug/ml sequentially. (*** : $p<0.0001$).

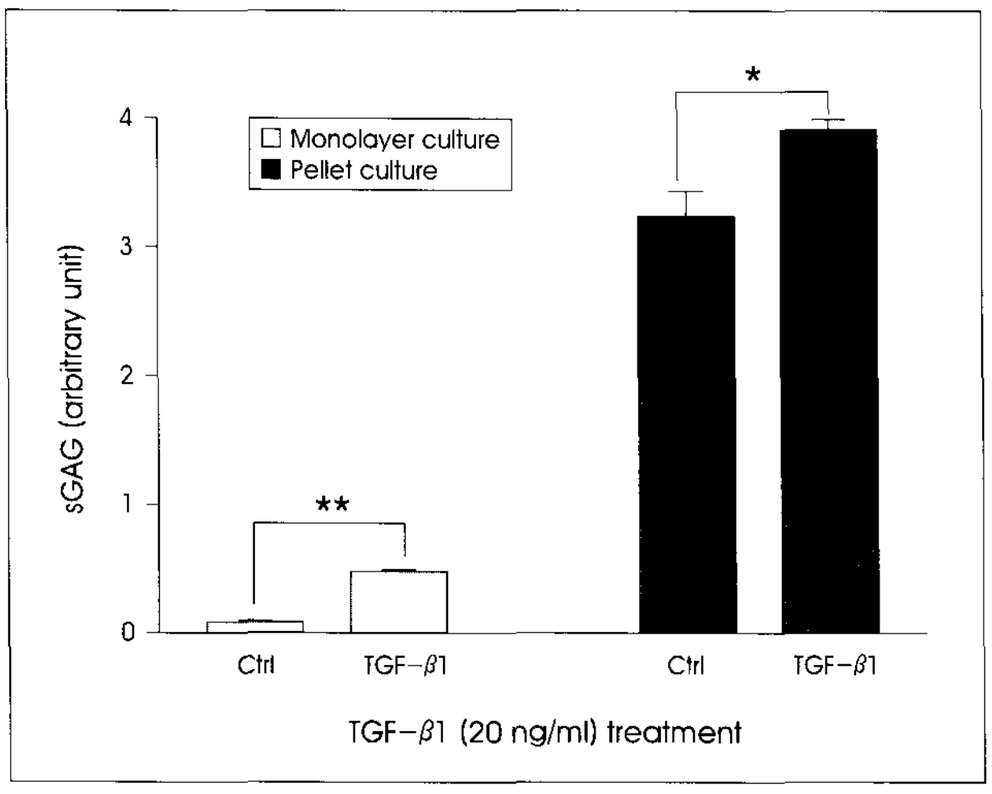

Fig. 3. Sulfated glycosaminoglycan (sGAG) production in the condition of transforming growth factor-β (TGF-β)1 treatment in monolayer and pellet cultures. The sGAG production is increased by TGF-β1 treatment in both cultures. The response rate to TGF-β1 is higher in monolayer culture than pellet culture. (** : $p<0.005$, * : $p<0.05$).

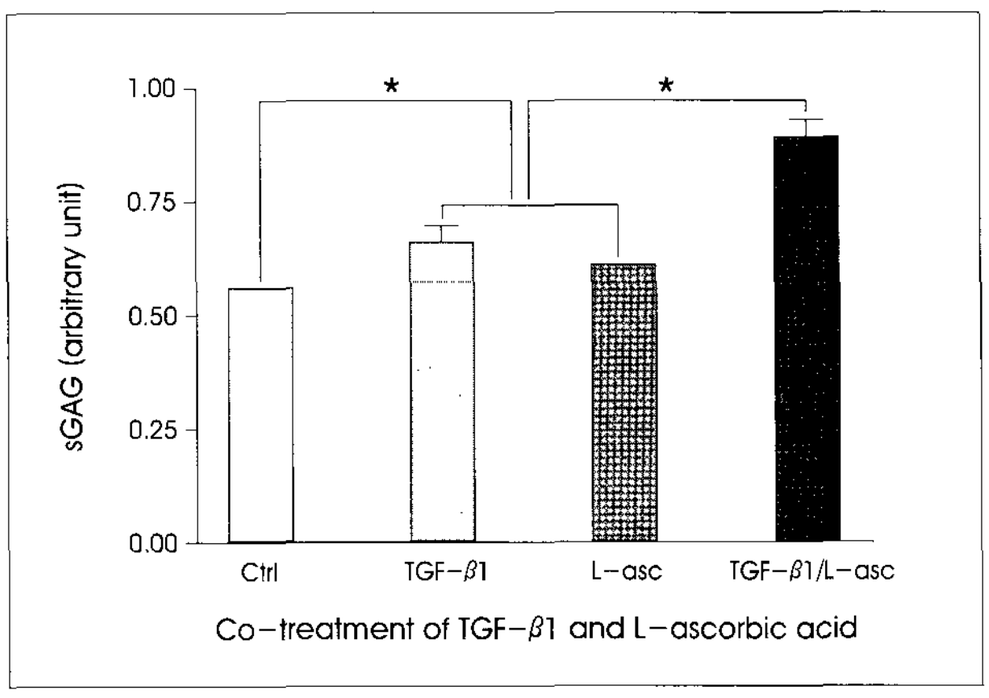

Fig. 5. Sulfated glycosaminoglycan (sGAG) production in the condition of co-treatment of transforming growth factor-β (TGF-β)1 and L-ascorbate. [monolayer culture] The sGAG production is more elevated by the co-treatment of TGF-β1 and L-ascorbic acid than respective treatment. (* : $p<0.05$).

sGAG production in the condition of treating L-ascorbic acid of various concentrations in monolayer culture and in the condition of co-treatment of TGF-β1 and L-ascorbate in monolayer culture

In order to examine the role of L-ascorbic acid on sGAG production, we treated L-ascorbic acid of serial concentrations (50-400 ug/ml) to intervertebral disc cells for two days. As the result, we confirmed the fact that the sGAG production level was increased by L-ascorbic acid treatment through all range of concentrations. Namely, it was elevated from 50 to 200 ug/ml sequentially, however it was not changed at concentration above 200 ug/ml (Fig. 4). We investigated the synergistic effect of L-ascorbate and TGF-β1 on sGAG synthesis in intervertebral disc cells. Therefore, we verified that the treatment of TGF-β1 (20 ng/ml) or L-ascorbate (200 ug/ml) increased sGAG amount to 18.5% and 9.5% respectively compared to control group, furthermore when TGF-β1 and L-ascorbate were treated together, sGAG production rate was elevated to 59.6% (Fig. 5).

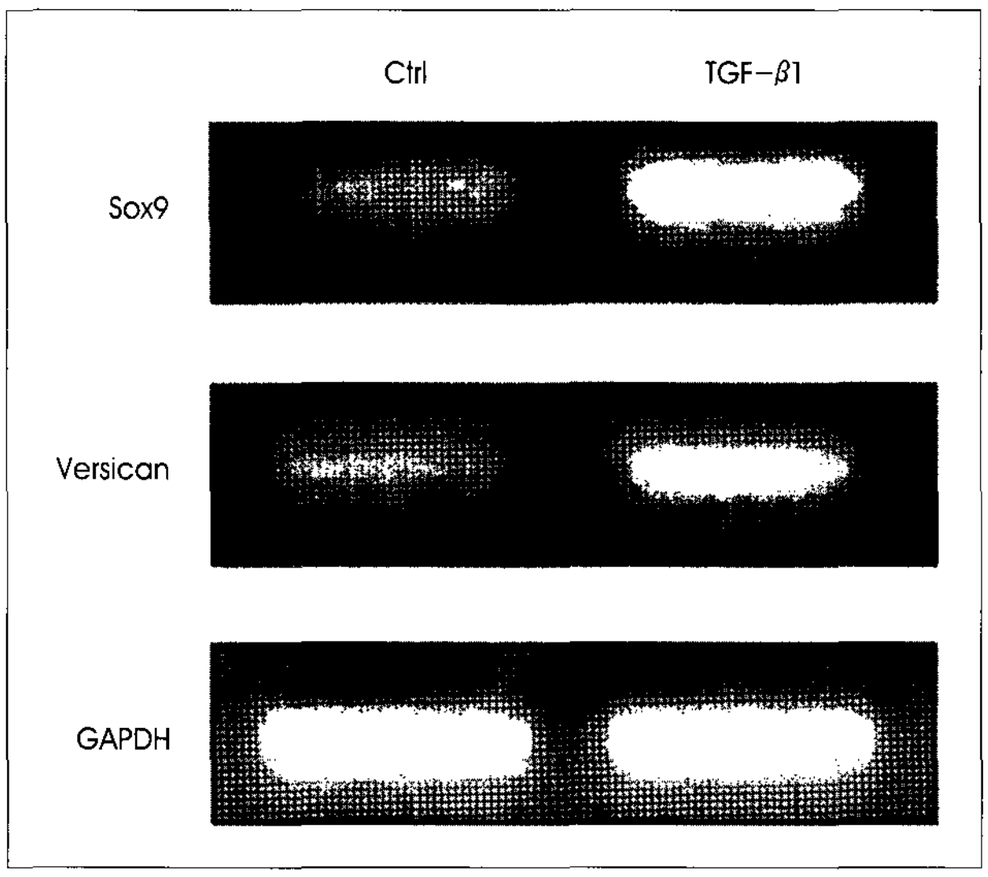

Fig. 6. mRNA expressions of Sox9, Versican, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) after transforming growth factor-β (TGF-β)1 treatment. The mRNA levels of Sox9 and versican by TGF-β1 are more increased than control.

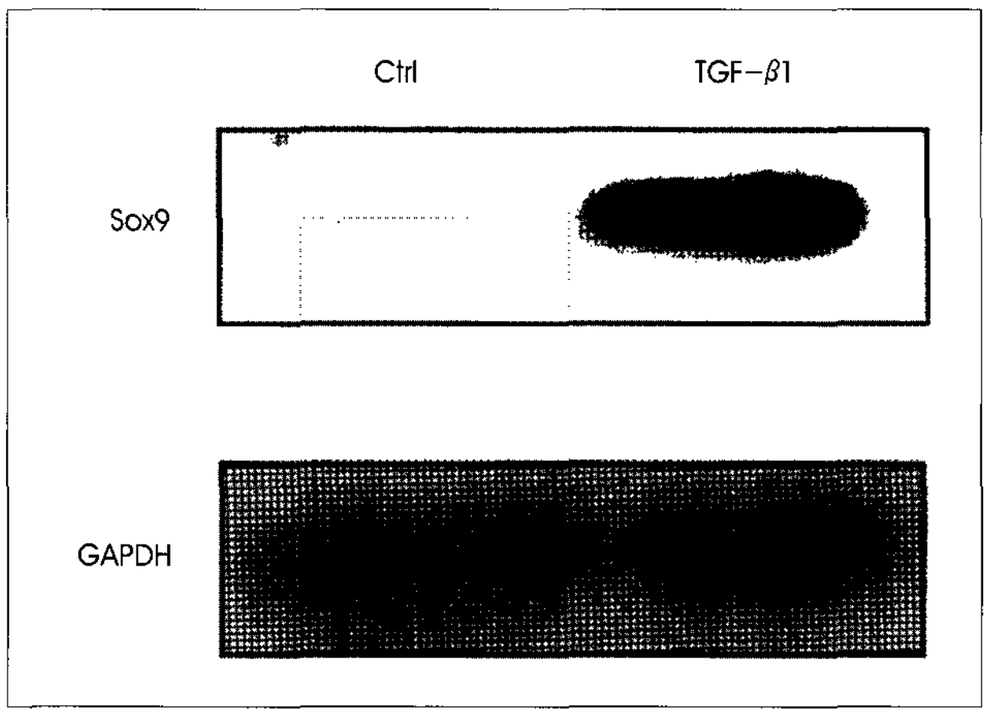

Fig. 7. Sox9 protein expression after Transforming growth factor-β (TGF-β)1 treatment. Sox9 protein level is increased remarkably by TGF-β1 treatment.

Elevation of Sox9 and versican mRNA levels through TGF-β1 treatment

We administered semi-quantitative RT-PCR to validate the effect of TGF-β1 on Sox9 and versican transcription. The mRNA expression rates of Sox9 and versican treated with TGF-β1 (20 ng/ml) were elevated to 70.4% and 28.7% in each compared to control (Fig. 6).

Increment of Sox9 protein expression by TGF-β1 treatment

Sox9 protein level was increased to 480% by TGF-β1 (10 ng/ml) treatment for two days (Fig. 7). Generally, it was reported that Sox9 protein was rarely expressed in monolayer cultured intervertebral disc cells.

DISCUSSION

The possible curing methods for degenerated intervertebral disc were known that direct injection of recombinant growth factor such as insulin-like growth factor, or TGF-β1, gene therapy, and cell therapy such as mature disc cells, or stem cells[18].

It was reported that DNA contents of pellet culture system was larger than monolayer during culture duration in response for TGF-β1 treatment[6]. We confirmed that the sGAG production rate was consistently increased during growing period (8 days) in both culture systems. Pellet culture produced sGAG much more than monolayer in whole culture duration.

TGF-β might play roles in several biological reactions such as repairing of intervertebral tissues, inducing of neovascularity into herniated disc tissues and proteolytic process of absorbing herniated disc[7]. In addition, TGF-β1 (10 ng/ml) substantially increased proteoglycan synthesis by ovine disc cells in bead culture, and decreased total matrix metalloproteinase (MMP) activity to 11.5% of control cultures[11]. Also, bone morphogenetic protein-2 (BMP-2), a member of the TGF-β family is known to induce chondrocyte differentiation and maintain articular chondrocyte phenotypes in long-term culture. Therefore, the treatment of human recombinant BMP-2 to intervertebral disc cells increased sGAG synthesis, and Type II collagen, aggrecan, Sox9, and osteocalcin gene expression[18].

Sequential treatment (10-40 ng/ml) of TGF-β1 increased sGAG production to 5.57-fold compared to control in

monolayer culture. Although sGAG contents were elevated in both culture systems, response degree for TGF-β1 was higher in monolayer than pellet culture in this study.

Supplement of sodium ascorbic acid (1.0 mM) stimulated collagen synthesis markedly in 3T6 fibroblasts and primary cultured chondrocytes, or the collagen production was increased with culture time[5].

In one study, L-ascorbate showed no effect on the synthesis of proteoglycans, but increased those associated with the cell/matrix layer. Furthermore, when L-ascorbate was added to the culture along with TGF-β1, the percentage of proteoglycans associated with the cell/matrix layer increased from 25.8 +/- 1.0 to 41.0 +/- 0.5%[14]. In our study, L-ascorbic acid increased sGAG larger than control in various concentrations (50-400 ng/ml), and the sGAG production was more elevated by the co-treatment of TGF-β1 and L-ascorbic acid than respective treatment.

Until now researches for influence of several substances on intervertebral disc were done seperately in general, but the synergistic effect of TGF-β1 and L-ascorbate that were known to have a role on degenerated intervertebral disc tissue or cells was elucidated in this investigation.

It was known that Sox9 is a member of the Sox family of transcription factors and regulate both chondrogenesis and sex determination[9], or Sox9 promotes cartilage-specific gene expression through cooperation with peroxisome proliferators-activated receptor γ coactivator 1α (PGC-1α) and therefore stimulates chondrogenesis[4]. Sox9 protein localization in healthy Grade I disc was greater than Grade II-IV, and a significant negative correlation was seen between the percentage of cells positive for Sox9 localization and age[2]. Sox9 mRNA expression was increased after stimulation of TGF-β3 in L6 rat myoblastic cell line[8].

In recent report, Sox9 expression during chondrogenesis might be under direct control of TGF-β/BMP activity, and that factors downstream of TGF-β/BMP pathways, such as Smads and TAK1 (TGF-β activated kinase), control chondrogenesis-specific enhancers of the Sox9 gene[3].

In this study, TGF-β1 increased protein level as well as mRNA expression of Sox9 very highly, and moreover the mRNA level of versican that is a large aggrecating proteoglycan mainly distributed in nucleus pulposus was also increased by TGF-β1 treatment. Consequently, we suggest that TGF-β1 promotes sGAG production and other proteoglycan synthesis through stimulating Sox9 mRNA and protein expressions sequentially. Direct investigation on relationship or regulation between TGF-β and Sox9 in intervertebral disc cells is being sparsely done at present, therefore, our study result may provide a important meaning in this viewpoint.

In future, it is necessary to consider correlation between Sox9, L-ascorbic acid and other cytokines such as bone morphogenetic protein-2 (BMP-2) or osteogenic protein-1 (OP-1).

CONCLUSION

It is suggested that TGF-β1 would increase sulfated glycosaminoglycan (sGAG) and other proteoglycans such as versican by elevating Sox9 mRNA and protein expressions in order.

L-ascorbic acid increased sGAG contents highly in intervertebral disc cells, and from this result it can be speculated that L-ascorbic acid may play a positive role for the regeneration of chondrocytes and cartilaginous tissue. In this study, it is considered that the examination of relation between TGF-β1, Sox9, and proteoglycan may provide an important clue for the regeneration of chondrocytes and cure for degenerated intervertebral disease.

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