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Expression of Transforming Growth Factor and Basic Fibroblast Growth Factor and Core Protein of Proteoglycan in Human Vertebral Cartilaginous Endplate of Adolescent Idiopathic Scoliosis

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Study Design. To compare the expression of cytokines and core protein of proteoglycan in the scoliotic concave and convex cartilaginous endplate using immunohistochemical staining.

Objectives. To define the possible role of transforming growth factor beta 1 (TGFβ1), basic fibroblast growth factor (bFGF), and core protein of proteoglycan in the development of adolescent idiopathic scoliosis.

Summary of Background Data. Changes in the endplate composition have been implicated as possible etiologic factors in the pathogenesis of adolescent idiopathic scoliosis. Cytokines have exclusive effects on cartilage. Thus comparing the expression of the cytokines and matrix on the convex and concave sides of scoliotic endplate tissues may help to understand the role of endplate tissues in the induction and/or progression of idiopathic scoliosis.

Methods. The convex and concave half of cartilage endplate was collected at the apex and end vertebrae from 12 patients. The expression of TGFβ1, bFGF, and core protein on both sides was examined with the immunohistochemistry method, and results were analyzed with the image analysis system.

Results. TGFβ1, bFGF, and core protein of proteoglycan were all expressed in the cytoplasm of chondrocytes in the cartilaginous endplate. The area density and quantity density of TGFβ1 and bFGF on the concave side are expressed in an even significantly higher level than that on the convex side ($P \geq 0.05$). The expression of the core protein of proteoglycan on the convex side is higher than that on the concave side, the difference is not significant ($P > 0.05$).

Conclusion. There was a significantly higher expression of TGFβ1 and bFGF, although a lower expression of the core protein on the concave side, which suggests a possible etiological factor or a secondary change in the development of adolescent idiopathic scoliosis.

Key words: scoliosis, vertebral growth plate, cartilaginous endplate, immunohistochemistry, transforming growth factor, basic fibroblast growth factor, core protein.
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Adolescent idiopathic scoliosis (AIS) is a complex three-dimensional anomaly of the spine involving lateral deviations in the frontal plane, modifications of the sagittal profile, spinal torsion, and transverse plane deformations. Thus it has become a source of medical concern in the orthopedic practice. AIS involves complex spinal intrinsic deformations such as the wedging of vertebral bodies and intervertebral discs.[1] Several hypotheses have been suggested in the literature as possible etiologies of idiopathic scoliosis. A variety of factors have been brought to our attention, such as deviating growth pattern, neuromuscular changes, abnormalities of connective tissues, and asymmetric growth, but none of them has been clearly implicated.[2–4] Although idiopathic scoliosis is regarded as a multifactorial disorder, hereditary has also been implicated as an important component.[5,6] Therefore it is difficult to determine whether an observed etiologic process is a primary or secondary etiologic factor in the development of scoliosis. However, longitudinal vertical growth occurs primarily through the endplate, and the neurocentral synchondrosis has also been recognized as the major contributor to the growth of the posterior vertebral body.[7] Little consideration has been given to the study of cartilage endplate tissues in scoliosis. The research by Roberts *et al*[8] showed that there were low levels of proteoglycan on endplates from scoliotic tissues and of water content compared with those from an age-matched control tissue. It is a general understanding that the endplate plays an important role in biomechanical integrity and disc nutrition. The cartilaginous endplate is believed to decrease in thickness, then tear and disappear with age,[9–11] so that degeneration of the endplate may lead to an accelerated degenerative process, but the underlying change that the scoliosis endplate experiences remains vague to us.

Proteoglycans are macromolecules comprising at least 1 glycosaminoglycan chain covalently attached to a core protein that plays an important function in the growth plate. In recent years there has been an increasing accumulation of knowledge of the cytokines on cartilaginous tissues, mainly of articular cartilage. The basic fibroblast

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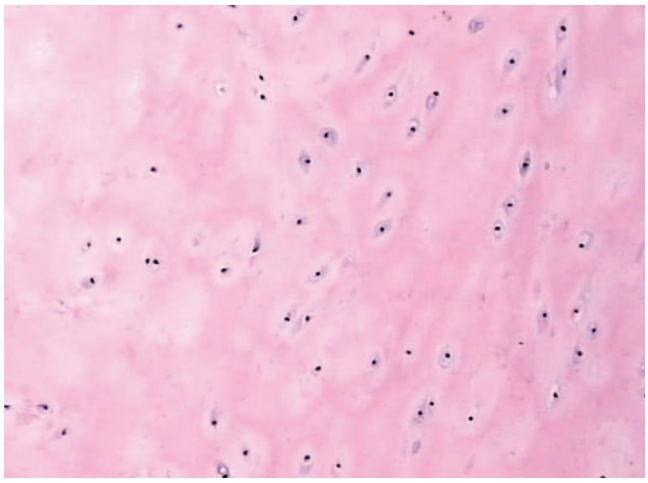

Figure 1. Cartilaginous endplate was confirmed with histopathology.

growth factor (bFGF) and transforming growth factor (TGF) can augment neovascularization and endothelial cell proliferation in vivo. In vivo and in vitro studies of humans and animals have detected the expression of these two cytokines and core proteins in articular cartilage and intervertebral disc, both in normal and pathologic conditions.[12–16] But the cytokines, such as the growth factor have not been studied in patients with idiopathic scoliosis.[17] Because immunohistochemistry can acquire qualitative analysis of the cytokines and the core protein, it may help to identify the expression location and the change of the cytokines and core protein observed in scoliotic endplate. This work aims to study the expression location, to quantitate changes of the cytokines and core protein in the cartilaginous endplates of AIS, and to probe into the possible role of the cytokines and core protein in the development of AIS.

■ Materials and Methods

Harvesting and Sampling. Ninety six vertebral cartilaginous endplates were harvested from 12 AIS patients of 12 to 20 years old (average 14.9), of whom there were 4 boys and 8 girls. The Cobb's angle of curve averages at 65.1° (from 43–102°). The curve is classified according to the Lenke classification system,[18] including 4 cases of Lenke type 1, 3 type 3, 2 type 4, and 3 type 5. All of the patients underwent the anterior fusion or release, and the endplates were obtained intraoperatively. The endplates were dissected and retrieved from the apex, the upper and lower end of the curve, and then were further separated into two groups: one in which the endplates were obtained from the concave side and the other in which they were obtained from the convex side. Sample tissues were confirmed by the histopathology (Figure 1). To ensure the satisfactory experimental results, it should be noted that the endplate samples were obtained from the concave side of the disc as far from the midline of the vertebral body as possible. Thereafter specimens were rinsed with saline, some of which were fixed in 10% neutral formaldehyde, and the remainder of which were then frozen at −20°C for other further analysis in the future.

Immunolocalization. For the purpose of immunohistochemistry, the tissues were fixed in 10% neutral formaldehyde, embedded in paraffin, and cut into 5-μm-thick sections. After these sections were deparaffinized in xylene and a graded series of ethanol., immunostaining was then carried out with the use of the streptavidin/biotin immunoperoxidase method (Ultra-Sensitive S-P kit, Maixin-Bio Corp., Fuzhou, China). Before immunohistochemical staining, these sections were immersed in 3% hydrogen peroxide to quench the endogenous peroxidase activity and then incubated with phosphate-buffered saline (PBS) containing 1% bovine serum albumin to reduce the nonspecific background staining, so that antigen retrieval step could be then taken. The two methods of antigen retrieval are illustrated as follows: 1) enzymatic (α-chymotrypsin for core protein) and 2) heat-mediated (microwave for TGFβ1 and bFGF). During the antigen retrieval step, the sections were rinsed with PBS and later incubated with one of the specific primary antibodies, accompanied by appropriate dilution, at room temperature for 10 minutes. After being handled with PBS, the sections were then incubated with biotinylated rabbit antihuman IgG, washed with PBS, and further incubated with peroxidase-labeled streptavidin for 10 minutes. It turned out that they were reacted with 3,3′-diaminobenzidine as the chromogen and counterstained with hematoxylin. Control slides were incubated in nonimmune rabbit serum or no primary antibody and processed in the same way as the experimental sections.

Antibodies. Quantitation of immunohistochemistry uses affinity-purified rabbit anti-TGFβ1 and rabbit anti-bFGF (Maixin-Bio Corp.) with no cross-reactivity to other TGFβ

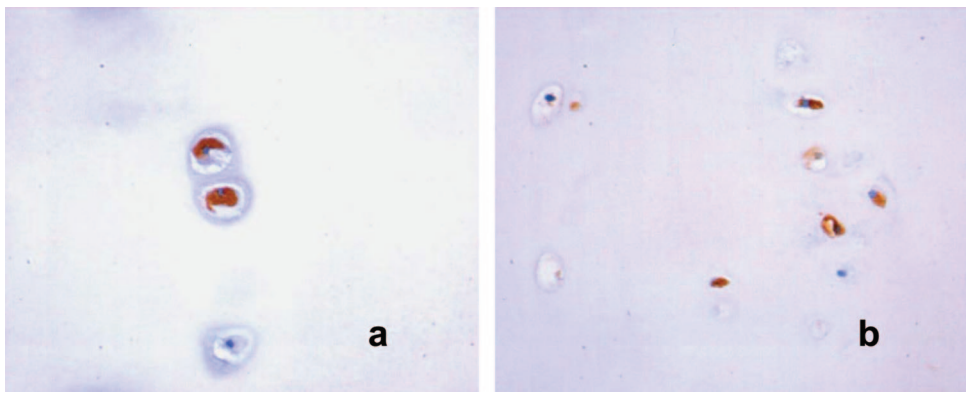

Figure 2. Expression of TGFβ1. **a**, Convex side. **b**, Concave side (×40).

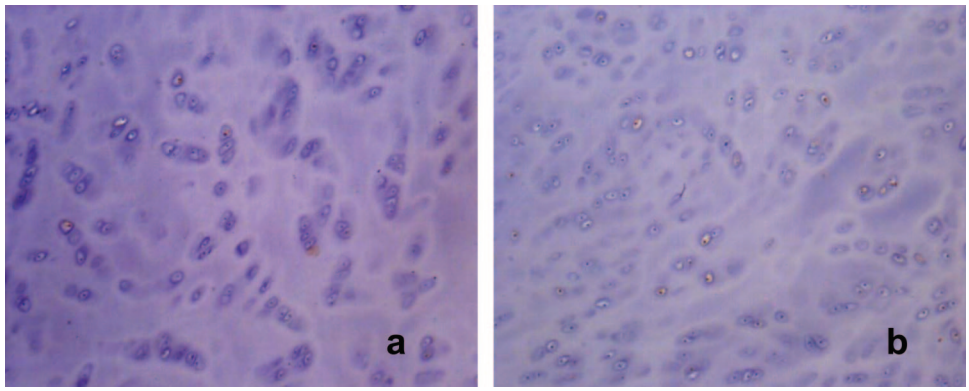

Figure 3. Expression of bFGF. **a**, Convex side. **b**, Concave side (×10).

and fibroblast growth factor isoforms, respectively, and adopts the rat anticore protein [ab2501, high molecular mass material derived from the Engelbreth-Holm-Swarm tumor matrix containing laminin, entactin, and HSPG; Abcam, Cambridge, UK].

■ Results

Microscopic Evaluation

Each of the endplates on the convex and concave sides was first stained with hematoxylin and eosin. The histological observation showed that the cartilaginous endplate were degenerated to some extent, some of which were calcificated. Moreover the extent of degeneration was correlated with the degree of severity of the curve.

Evaluation of Immunohistochemical Results

The cytoplasmic brown indicated a positive reaction. All parameters were semiquantitatively evaluated in terms of two distinct variables: the area density and number density of the proportion of cells staining within the given area and the intensity of staining in a given cell over and above the background. The pathologic imaging system of Bei Hang University (China) was used to measure the average area density and number density from five random visual fields (μm). The analytic results of quartiles (median) were recorded according to endplate sample regions. The significance of the concave and convex curves and the expression on each parameter were determined with nonparametric Wilcoxon rank test, and the results were analyzed with SPSS 10.0 software.

For apex and end vertebrae, the area density and quantity density of expression of TGFβ1 and bFGF in endplate on concave side were significantly higher than those on convex side ($P \leq 0.05$), whereas the core protein of proteoglycan on convex side is relatively higher than that on concave side, but the difference is not significant ($P > 0.05$; Figures 2–4; Tables 1–6).

■ Discussion

The etiology of AIS is yet unknown. Some argue that it is mechanically provoked, others have put forth the possibility that melatonin plays an important etiologic role.[19–23] Those advocating a mechanical cause point to the Hueter-Volkmann law, which states that growth is retarded by mechanical compression and accelerated by distraction or reduced compression of the growth plate relative to normal values. The role of vertebral cartilage endplates has not gotten much attention from researchers. Some studies have revealed early or erratic calcification of the vertebral endplates associated with the deformity. From this positive study, the endplate degeneration and calcification is associated with the degree of severity of the curve in the histological observation, which also implicates that the endplate plays some role in idiopathic scoliosis. Duance *et al*[24] have characterized the apparent differences in collagen crosslinking in scoliosis endplate versus normal endplate. Roberts *et al*[8] analyzed endplate

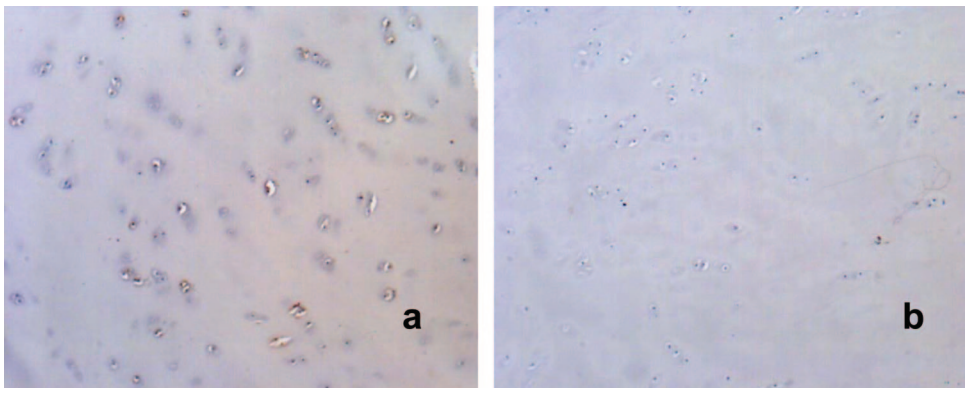

Figure 4. Expression of core protein. **a**, Convex side. **b**, Concave side (×10).

Table 1. The Area Density of TGFβ_1 in Immunohistochemistry M(Q_R)

	Convex Side	Concave Side	*P*
Apex	0.001480 (0.001710)*	0.001860 (0.004205)†	0.017
End	0.000505 (0.000965)*	0.002695 (0.005315)†	0.001

*$P > 0.05$.
†$P > 0.05$.

Table 3. The Area Density of bFGF in Immunohistochemistry M(Q_R)

	Convex Side	Concave Side	*P*
Apex	0.000685 (0.002135)*	0.002065 (0.003073)†	0.026
End	0.000495 (0.001530)*	0.002455 (0.003595)†	0.01

*$P > 0.05$.
†$P > 0.05$.

tissue and demonstrated that the proteoglycan content was considerably lower in endplates from scoliotic samples.

Proteoglycans play a major role in water retention and are important in maintaining the function of the cartilaginous endplate. They are macromolecules consisting of a variety of core proteins covalently attached to one or several polysaccharide chains of the glycosaminoglycan type (heparan sulfate, heparin, chondroitin sulfate, dermatan sulfate, or keratan sulfate). At least two forms of basement membrane heparan sulfate proteoglycan (HSPG) have been identified: one with a large core protein (>400 kD) and the other with a small core protein (30 kD). The ab2501 is used to recognize domain IV of the core protein of the large heparan sulfate proteoglycan or perlecan, the former of which is a glycosaminoglycan of repeating disaccharide subunits, comprising glucosamine and glucuronic/iduronic acid, covalently linked to a core protein backbone. A number of studies have shown that the growth plate contains heparan sulfate proteoglycans, and several lines of evidence indicate that these proteoglycans play an important function in the growth plate.[25–27] This study is the first immunohistochemical localization of heparan sulfate proteoglycans within the cartilaginous endplate of AIS. One domain of HSPG core protein was identified specially in this study. Expression of HSPG will directly influence the covalent formation of proteoglycan macromolecules, implicating that HSPG may play an important role in the function of cartilaginous endplate, but this needs to be elucidated further.

The study indicates that the proteoglycan responsible for the osmotic properties of the nucleus is aggrecan, the same molecule responsible for the compression resistance of articular cartilage. Aggrecan possesses a large core protein with over 2000 amino acids. From results of in vitro studies, it was concluded that mechanical load was necessary for maintaining cartilage integrity but that excess loading could cause damage and disturbed metabolism, leading eventually to accelerated degeneration.[28,29] Besides these, this study also shows that expression of the core protein at the concave curve is lower than that on the convex side.

The current study first identifies distinct differences of TGFβ1 and bFGF between the endplate tissues on the convex and concave sides of AIS. Before discussing these differences in details, we should point out again that the endplate samples were obtained from the concave side of the disc as far from the midline of vertebral body as possible and also that the end vertebrae is not exactly the real end vertebrae of the curve because of the limited operation.

The bFGF belongs to a highly conserved family of at least 10 closely related peptides, and TGFβ1 is a member of the TGF-β superfamily, which is composed of more than two dozen polypeptides. Both have widespread effects on cellular proliferation, angiogenesis, migration, immune response, wound healing, epithelial cell growth, embryonic skeletal development, and epithelial-mesenchymal interactions. In addition, fibroblast growth factors modulate the expression of numerous growth factors, transcription factors, and extracellular matrix components.[30–33] In vivo introduction of Ad/TGF-β1 into the rabbit nucleus pulposus increased nearly 6-fold as much as the increase of proteoglycan synthesis in matrix synthesis of the intervertebral disc.[34] Therefore their expression may influence the matrix synthesis of cartilaginous endplate directly or indirectly.

Restricted by ethical reasons, we cannot use normal children to do a comparative study. It is important to recall that the scoliotic tissue we analyzed mainly represents the convex and concave side of the entire scoliotic tissue (endplates), in which the tissue is experiencing tension or compression. As widely known, there is vertebral wedging deformity (on the concave side) in AIS. High expression of TGFβ1 and bFGF on the concave side may contribute to promoting matrix syntheses and prevent-

Table 2. The No. Density of TGFβ_1 in Immunohistochemistry M(Q_R)

	Convex Side	Concave Side	*P*
Apex	0.000137 (0.000182)*	0.000393 (0.000687)†	0.001
End	0.000046 (0.000086)*	0.000263 (0.000537)†	0.001

*$P = 0.015$.
†$P > 0.05$.

Table 4. The No. Density of bFGF in Immunohistochemistry M(Q_R)

	Convex Side	Concave Side	*P*
Apex	0.000035 (0.000090)*	0.000100 (0.00029)†	0.016
End	0.000020 (0.000068)*	0.000150 (0.00023)†	0.015

*$P > 0.05$.
†$P > 0.05$.

Table 5. The Area Density of Core Protein in Immunohistochemistry M (Q_R)

	Convex Side	Concave Side
Apex	0.000850 (0.001290)*	0.000750 (0.001253)*
End	0.000920 (0.001195)†	0.000310 (0.00152)†

*$P > 0.05$.
†$P > 0.05$.

Table 6. The No. Density of Core Protein in Immunohistochemistry M(Q_R)

	Convex Side	Concave Side
Apex	0.000121 (0.000235)*	0.000127 (0.000198)*
End	0.000104 (0.000202)†	0.000047 (0.000162)†

*$P > 0.05$.
†$P > 0.05$.

ing the wedging change. However, it seems that it is far from enough to resist this change. More research work is required to make it clear.

■ Conclusion

There are structural changes of vertebral body in AIS. And the concave side of the curve sustained compressive pressure. The high expression of TGFβ1 and bFGF at the endplate of the concave side may contribute to promoting matrix syntheses and to preventing the cartilaginous endplate degeneration, but the low expression of the core protein on the concave side implicates the malfunction of cartilaginous cells, which cannot be restored and maintained. But all of the changes may be secondary to the development of AIS.

■ Key Points

- This study investigated the changes of TGFβ1 and bFGF and the core protein of proteoglycan in the scoliotic vertebral cartilaginous endplates using immunohistochemical staining.
- The expression of TGFβ1 and bFGF on the concave curve is even significantly higher than that on the convex curve.
- The expression of the core protein of proteoglycan on the convex curve is higher than that on the concave curve, but the difference is not significant.
- Although these changes may be secondary to the development of AIS rather than etiological, such findings may suggest that the vertebral wedge change might be reversed by regulating the syntheses of proteoglycan, TGFβ1, and bFGF.

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