Remodeling of the Vertebral Body in Hereditary Lordoscoliotic Rabbits
Revealed by In Situ Hybridization

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Key Words: Scoliosis, Hereditary Lordoscoliotic Rabbit, In situ hybridization, Remodeling

ABSTRACT

The etiology of human idiopathic scoliosis remains still unknown, although several etiologic factors and many animal models of scoliosis have been reported. Kin previously reported radiological and histological findings of spinal deformities in the Hereditary Lordoscoliotic Rabbit (LSR), which develops thoracic lordosis or lordoscoliosis during growth. We investigated biological activities of bone cells of the LSR vertebrae by in situ hybridization. Digoxigenin labeled in situ hybridization probes of mRNA of osteopontin, type I collagen and bone morphogenetic protein (BMP)-2 were prepared. Hybridization was carried out on the sagittal section of lordotic or lordoscoliotic thoracic vertebra in the LSR. In the apical vertebral body and spinous process of the lordotic thoracic spine of LSR during developmental age, stronger positive signals for type I collagen and BMP-2 mRNA were detected in osteoblasts in the endosteum of the ventral portion than those in the dorsal portion of the vertebra, which indicates osteogenesis. In the apical vertebral body of the lordotic thoracic spine, positive signals for osteopontin mRNA were detected in osteoclasts of the Howship's lacunae and osteocytes around these pits in dorsal endosteal portion, which indicates bone resorption. No such signals were detected in the ventral endosteal portion of the same vertebral body. The vertebral bones of the LSR showed a specific mode of remodeling response to the force producing lordosis.

Introduction

The etiology of human idiopathic scoliosis still remains controversial. Several etiologic factors, such as genetic factor(1,2,3,4,5,6,7), hormonal factors(8), neuromuscular disease(9,10,11), subtle spinal growth abnormalities(12,13,14) have been reported. Many animal models (15, 16, 17) of scoliosis have also been reported. Kin (18) previously reported radiological and histological findings of the Hereditary Lordoscoliotic Rabbit (LSR), which develops thoracic lordotic or lordoscoliotic spinal deformity like a human idiopathic scoliosis, and the deformity appear during growth. To find the unknown etiology of the spinal deformity in LSR, we investigated biological activities of bone cells of the LSR vertebral body by in situ hybridization.
Materials and Methods

**LSR**

LSR is a type of Japanese White Rabbit which was incidentally found by a breeder of Rabbiton Institute (Hyogo, Japan) in 1982 (Fig.1). Since that time, crossbreeding has been continued. In LSR, spinal deformities are not recognized at birth, but appear at 4-6 weeks of age and develop until 16 weeks. Most of the rabbits demonstrated lordosis, which was seen at the T7-10 levels (44 degrees measured by Cobb’s method [19] at maximum), and was often accompanied by slight scoliosis (40 degrees at maximum) (Fig.2). No spinal anomalies were observed but severe deformities sometimes impaired their gait and ability to eat. The incidence of the deformities ranged from 50 to 60%, and no gender differences were observed.

**Tissue Preparation**

LSR were sacrificed at 6-14 weeks of age under intra venous general anesthesia and the thoracic spines were harvested with surrounding soft tissue and fixed for 5 days at 4°C in 4% freshly made paraformaldehyde (PFA) in 0.1M phosphate buffer. Then specimens were dehydrated in ethanol, cleared in chloroform, and decalcified with Morse’s solution (10% sodium citrate and 22.5% formic acid) until the specimen became soft (it took 3 to 4 weeks). Decalcified specimens were dehydrate and embedded in paraffin. Sagittal sections of 6 μm thick were cut and mounted on 3-aminopropyl-triethoxysilane-coated slides (20).

**Probe Preparation**

Bone samples of the skull, spine, ribs and femurs dissected from the fetus of Japanese White Rabbits 1 week before birth under intra venous general anesthesia were frozen in liquid nitrogen. Messenger RNA was extracted from the specimen using the Micro-Fast Track mRNA isolation kit (Invitrogen Corp., San Diego, CA), according to the manufacturer’s instructions. Complementary DNA (cDNA) was made so that mRNA was reverse transcribed in 20 μl reaction mixture containing reverse transcriptase and random primer. Thereafter, 1 μl of cDNA was amplified in 25 μl polymerase chain reaction (PCR) mixture containing 0.125U Taq DNA polymerase and 12.5pmol of each primer for type I collagen, osteopontin (Osp) and bone morphogenetic protein-2 (BMP-2) by PCR. Oligonucleotides for PCR were as follows: type I collagen, 5’-CAAGGGAGAACGTGGTTACC-3’ (5’sense;312-331) and 5’-TTCTTTCCGGGA GCCCTCAGG-3’ (3’antisense;934-963); and Osp, 5’-CACCCGAGCTATGTCC-3’ (5’sense;251-270) and 5’-GGCTCGATGGCTAGCTTGC-3’ (3’antisense;922-941); and BMP-2, 5’-ATGCCAAGTCCTGCTAGGG-3’ (5’sense;395-414) and 5’-GATCGGCTAATCCTTGACA TG-3’ (3’antisense;1100-1129). PCR conditions were as follows a total of 30 cycles was performed with the Astec DNA thermal cycler (Astec, Fukuoka, Japan) at 94°C for 0.5 minutes, 55°C for 1 minute, 72°C for 1 minute, and then at 72°C for 5 minutes at the end of the procedure. PCR products were subcloned into multicloning sites of the plasmid, and analyzed with the ABI Model 373A DNA Sequencer using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer Corporation) according to the manufacturer’s instructions. The sequence results were identical to those of the rabbit type I collagen (GenBank accession no.D49399), BMP-2
(GenBank accession no. D30751, X56848) and Osp (GenBank accession no. D11411) previously reported (21). Digoxigenin (DIG)-labeled single-strand antisense and sense complementary RNA probes were prepared using the DIG RNA labeling kit (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany) according to the manufacturer’s instructions (20).

**In situ hybridization**

In situ hybridization was carried out according to the manufacturer’s protocol (Boehringer Mannheim Yamanouchi, Tokyo, Japan) with minor modifications. Sections were deparaffinized, fixed in 4% PFA in 0.1M PB (pH 7.4), pretreated with HCl to inactivate endogenous alkaline phosphatase, and acetylated with acetic anhydrate. Hybridization was carried out at 50°C for 16h using DIG labeled probes at a concentration of approximately 0.5 µg/ml in hybridization buffer and was washed with Rnase A (10 µg/ml) at 37°C for 30 minutes. Hybridized probes were detected using a nucleic acid detection kit (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany) according to the manufacturer’s instructions.

Controls hybridized with the sense probe did not show positive signals.

**Results**

**Histological findings**

The thoracic spine of 4-week-old LSR has no deformity (Fig.3). In the apical vertebra and intervertebral disc in the lordotic thoracic spine of an 8-week-old LSR, the main histological findings were a deviation of the nucleus pulposus and extension in ventral and compression in the dorsal portion of the annulus fibrosus (Fig.4). No marked abnormality is observed in the epiphysis or in the growth plate of the vertebral body. H.E. stain (x 3)

vb: vertebral body  sc: spinal cord

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Fig.3  Sagittal section of the normal thoracic vertebra of LSR of 4 weeks of age. H.E. stain (x 3)  vb: vertebral body  sc: spinal cord

Fig.4 (a) Sagittal section of the normal thoracic vertebra of LSR of 10 weeks of age.
(b)(c) Sagittal section of the apical thoracic lordotic vertebra of LSR of 10 weeks of age. There is deviation of the nucleus pulposus and extension in ventral and compression in the dorsal portion of the annulus fibrosus. No marked abnormality is observed in the epiphysis or in the growth plate of the vertebral body. H.E. stain (x 3)

vb: vertebral body  sc: spinal cord  np: nucleus pulposus
abnormality was observed in the epiphysis or in the growth plate of the vertebral body.

In the apical vertebra of a 12-week-old LSR, proliferation of columnar cartilage composed of a vertebral growth plate was found on the ventral side. On the dorsal side, narrowing of the vertebral growth plate and proliferation of marginal epiphysial cartilage cells were found.

In the apical vertebra of a 24-week-old LSR, a wedging deformity in the epiphysis was found. The intervertebral disc was degenerated and the nucleus pulposus was destroyed.

Fig.5 Type I collagen in situ hybridization.
Sagittal section of the apical thoracic lordotic vertebra of LSR of 10 weeks of age.
(a) Arrows indicate markedly strong positive signals for type I collagen mRNA. (x 3)
(b) Arrows indicate markedly strong positive signals for type I collagen mRNA in osteoblasts in the endosteum at the ventral side of the vertebral body. (x 50)
(c) Arrows indicate positive signals for type I collagen mRNA in osteoblasts in the extraosteum at the dorsal side of vertebral body. (x 50)
Sagittal section of the normal thoracic vertebra of LSR of 10 weeks of age.
(d) Arrows indicate slightly positive signals for type I collagen mRNA in osteoblasts in the endosteum at the ventral side of the vertebral body. (x 50)
(e) Positive signals for type I collagen mRNA are not seen at the dorsal portion of the vertebral body. (x 50)
v:ventral side  d:dorsal side  cr:cranial side  ca:caudal side  sc:spinal canal
In situ hybridization

In the apical vertebral body and spinous process in the lordotic thoracic spine of LSR during growing age, stronger positive signals for type I collagen mRNA were detected in osteoblasts in the endosteum of the ventral than those in the dorsal portion of the vertebra (Fig. 5). In the vertebra without deformity, no differences of positive signals between the ventral and dorsal portion were observed. Positive signals for BMP-2 mRNA showed a similar pattern as that observed in type I collagen mRNA pattern (Fig. 6).

In the apical vertebral body of the lordotic thoracic spine, positive signals for Osp mRNA were detected in osteoclasts in Howship’s lacunae and osteocytes around these pits in dorsal endosteal portion. No such signals were detected in the ventral endosteal portion of the same vertebral body (Fig. 7). In the vertebral body without deformity, there were no differences in the Osp mRNA expression pattern between ventral and dorsal portion.

Discussion

To investigate the etiology of idiopathic scoliosis, several scoliotic animal models have been developed, including paravertebral muscle excision (22), division of the intercostal nerves (22), resection of the ribs (23), resection of the costotransvers joints (24), rib elongation (25), rib shortening (26). Recent report showed that the scoliosis which develops in chickens after pinealectomy was similar to human idiopathic scoliosis, and thus may be a useful model of idiopathic scoliosis (17). Moreover surgical tethering of the spinous apophysis and transverse apophysis on the same side of the spine produced scoliosis with characteristics similar to those of human idiopathic scoliosis (27). However these scoliotic animal models can not show deformity without surgery, therefore these are models of secondary spinal deformity. On the other hand the spinal deformities observed in LSR were similar to those of human idiopathic scoliosis; no spinal anomaly, no deformity at birth, the deformities of the spine appears at 4-6 weeks of age and progresses without surgery, and deformities include lordosis or lordoscoliosis with...
rotation from middle to lower thoracic levels (18). In the LSR with severe lordosis, rabbits cannot eat or reproduce, so they have to rotate their spine. This posture may enforce their lordosis into scoliosis. Therefore LSR is an useful animal model for studying etiology of human idiopathic scoliosis.

Concerning histological findings observed in present study, in the early stage of the deformity, main changes included a deviation in the nucleus pulposus of the intervertebral disc and extension in the ventral portion and compression in the dorsal region of the annulus fibrosus. In the late stage, proliferation of columnar cartilage composed of the vertebral growth plate was found on the ventral side. On the dorsal side, narrowing of the vertebral growth plate and proliferation of marginal epiphyseal cartilage cells were found. Finally, wedging deformity in the epiphysis was found, the intervertebral disc was degenerated and the nucleus pulposus was destroyed. These findings were considered to be secondary changes resulting from progressive local lordosis of the thoracic spine.

Dubousset et al. (28) found that scoliosis routinely developed in pinealectomized chickens, and scoliosis was caused from decreasing melatonin production. Sobajima et al. (29) reported that serum melatonin levels in LSR were significant higher than those of Japanese white rabbit. And they suggest that causes of spinal deformities in the LSR may be the result of the contribution of melatonin receptors as well as that of altered serum melatonin levels in the LSR. Although further studies will be required to investigate the expression of melatonin receptor in other tissues of the LSR as well as to delineate the role of melatonin in the pathogenesis of idiopathic scoliosis.

Type I collagen is a main component of connective tissues of the skin, tendon and bone. In present study, strong positive signals for type I collagen mRNA were detected in osteoblasts of the endosteum of the ventral portion of the
lordotic thoracic vertebral body and spinous process, indicating osteogenesis. BMP-2 is one of the most potent protein of osteogenesis and has been shown to induce bone formation successfully at heterotopic locations. Again in present study BMP-2 mRNA showed a similar pattern to type I collagen mRNA, indicating osteogenesis. Osp mRNA-positive osteoclasts in Howship's lacunae and osteocytes around the pits were detected in the dorsal endostial portion of the same vertebral body, indicating bone resorption(30). These bone cells activity indicates the remodeling for distraction force to the anterior side and compression force to the posterior side in the thoracic lordotic vertebral body. The vertebral bones showed a specific mode of remodeling response to the force producing lordosis.

Asymmetrical loading of the spine is one possible etiology of idiopathic scoliosis. Carpintero et al.(27) demonstrated that lordoscoliosis could be induced in a rabbit by surgical tethering of the spinous apophysis and transverse apophysis. Our results suggest that growth imbalance which may exist between the anterior component and the posterior component with muscle and ligament, enforced developing lordosis. This findings suggest that asymmetrical loading of the spine can be an etiology of idiopathic scoliosis. Whether asymmetrical loading is the cause of idiopathic scoliosis or the result of spinal deformity, is still unclear. Because the etiology of spinal deformity in LSR may be multifactorial same as human idiopathic scoliosis, further studies is necessary to know the etiology of spinal deformity of LSR.

Acknowledgments The authors thank Shintaro Nomura, PhD (Department of Pathology, Medical School of Osaka University), Akira Tsutsumi, MD(Department of Surgical Pathology, Osaka Medical College) for technical assistance and advice.

References


Received December 14, 2005
Accepted January 5, 2006