Immunohistochemical Observations on the Initial Disorders of the Epiphyseal Growth Plate in Rats Induced by High Dose of Vitamin A

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ABSTRACT. The initial disorders of the epiphyseal growth plate cartilage were immunohistochemically examined in the proximal tibia of rats administered a high dose of vitamin A. Male Wistar rats were given 100,000 IU/100 g body weight/day of vitamin A for administration periods of 1 to 5 days (Day 1 to 5) from 4 weeks after birth or were given deionized water and used as control. They were sacrificed after 5-bromo-2'-deoxyuridine (BrdU) injection on Day 1 to Day 5 to remove the tibiae. The tibiae were processed for immunohistochemical examinations using antibodies against type I, II, X collagens and BrdU. BrdU-incorporated chondrocytes and type X collagen-negative area were reduced since Day 2 and type X collagen-positive area was reduced since Day 4. The cartilage matrix partially lost type II collagen and deposited type I collagen in the epiphyseal growth plate near the periosteum on Day 5. These findings suggest that a high dose of vitamin A initially disturbed the differentiation from resting to proliferating chondrocytes, subsequently inhibited the differentiation from proliferating to hypertrophic chondrocytes, caused the chondrocytes to deviate from the process of normal differentiation, and finally resulted in the deformation of the epiphyseal growth plate.—KEY WORDS: 5-bromo-2'-deoxyuridine (BrdU), epiphyseal growth plate, type I collagen, type X collagen, vitamin A.


Bovine Hyena disease is regarded as a disorder of skeletal development. It occasionally develops in the calves which were given a high dose of vitamin AD3E premix during suckling period. Takaki and co-workers experimentally demonstrated in calves that high doses of vitamin A premix induced suppression of the growth of hind limb bones to develop Hyena disease at 1 year after administration [15, 16]. Morphologically, the reduction in thickness and the focal disappearance of the epiphyseal growth plates were detected in the distal femur and proximal tibia [16]. Similar disorders caused by high doses of vitamin A were previously reported in various mammals [4–7], although initial disorders of the epiphyseal growth plate caused by high doses of vitamin A still remain unclear. Recently, several studies showed that some derivatives of vitamin A were important factors to regulate proliferation and differentiation of chondrocytes [1, 3, 8, 9], and suggested that high doses of vitamin A initially affected the proliferation and differentiation of chondrocytes in the epiphyseal growth plates. In the present study, therefore, the proliferation and differentiation of chondrocytes were examined immunohistochemically to investigate the initial disorders of the epiphyseal growth plate caused by a high dose of vitamin A.

MATERIALS AND METHODS

Animals and experiments: Wistar rats born in our laboratory were bred under conditions of 23 ± 2°C, with humidity of 55 ± 10% and 12:12 hr light-dark cycle (lightening from 06:00 to 18:00). Twenty five male rats aged 4 weeks, weighing 80–100 g, were given orally 100,000 IU/100 g body weight/day of vitamin A suspended in deionized water (VA rats) for administration periods of 1 to 5 days (Day 1 to 5). Similarly, another 25 male rats were given deionized water and used as control. Five VA rats as well as control rats, were sacrificed on each day from Day 1 to 5 at 6 hr after the administration. To label proliferating chondrocytes, 10 mg/100 g body weight of 5-bromo-2'-deoxyuridine (BrdU, Sigma Chemical, St. Louis, U.S.A.) was injected intraperitoneally at 1 hr before sacrifice.

Tissue preparation: The animals were perfused cardiacly with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 under ether anesthesia. Proximal parts of tibiae were removed, cut into halves longitudinally and fixed in the same solution at 4°C overnight. They were decalcified in 10% EDTA in 0.01 M phosphate buffer pH 7.4 at 4°C for 1 week. After dehydration through a graded series of ethanol solution at 4°C, they were embedded in paraffin and sectioned at 5 μm. The sections were stained by hematoxylin and eosin (H.E.) or processed for immunohistochemistry.

Immunohistochemistry: The immunohistochemistry for collagens was performed according to Mizoguchi et al. [13]. Briefly, after blocking of endogenous peroxidase, the deparaffinized sections were treated with 2.5% hyaluronidase from bovine testis (Wako Pure Chemical Industries, Co., Ltd., Osaka, Japan) containing 0.025% Triton X 100 in 0.01 M phosphate buffered saline pH 7.4 (PBS) for 60 min at 37°C. Then, the sections were treated with swine serum (DAKO A/S, Glostrup, Denmark), and incubated with an rabbit antiserum against rat type I collagen.
(1:5,000), bovine type II collagen (1:500) or rat type X collagen (1:800) (LSL, Co., Ltd., Tokyo, Japan) at 4°C overnight. After treatment with swine anti-rabbit immunoglobulin (DAKO A/S, Glostrup, Denmark), the sections were incubated in a streptavidin-biotin-peroxidase complex (sABC; DAKO A/S, Glostrup, Denmark). To visualize the antigen-antibody reaction, the sections were treated with 0.02% diaminobenzidine tetrahydrochloride and 0.005% H2O2. Finally, the sections were counterstained by hematoxylin.

The immunohistochemistry for BrdU was performed according to Hume and Keat [10]. Briefly, after blocking of endogenous peroxidase, the deparaffinized sections were treated with 0.4% pepsin in 0.1 N HCl for 40 min at 37°C followed by 1 N HCl for 30 min at room temperature. The sections were then treated with rabbit serum (DAKO A/S, Glostrup, Denmark), and incubated in anti-BrdU mouse monoclonal antibody (1:50) (DAKO A/S, Glostrup, Denmark) at 4°C overnight. After treatment with rabbit anti-mouse immunoglobulin (DAKO A/S, Glostrup, Denmark), the antigen-antibody reaction was visualized as described above.

Statistical analysis: To evaluate proliferating chondrocytes, BrdU-incorporated chondrocytes were counted in 5 areas in one section selected at random from each animal. Each area included the resting zone, proliferating zone and hypertrophic zone. The BrdU-incorporating index was obtained by dividing the number of BrdU-incorporated chondrocytes by the total number of chondrocytes in 5 areas and expressed as a percentage.

The epiphyseal growth plate was divided into 10 parts along its width in the sections immunostained for type X collagen. One section was selected at random from each animal. The thickness of the whole epiphyseal growth plate, type X collagen-positive area and type X collagen-negative area were measured at the center of each of these 10 parts across the epiphyseal growth plate and described as a mean of 10 parts in each animal.

Statistical differences between VA rats and control rats on each day were evaluated by the Student’s t-test.

RESULTS

In control rats, the epiphyseal growth plate of the proximal tibia consisted of the resting zone and chondrocyte columnar zone, the latter divided into the proliferating zone and the hypertrophic zone. In VA rats, the columnar arrangements of chondrocytes became unclear in the proliferating zone since Day 3. In addition, the chondrocytes with hypertrophic appearance almost disappeared on Day 5. The thickness of epiphyseal growth plates of proximal tibiae in VA rats became significantly thinner than that of control rats since Day 3 and reduced to about 40% of that of control rats on Day 5 (Figs. 1 and 2).

The deposition of type X collagen was detected in the cartilage matrix in the hypertrophic zone and within the bony trabeculae in control rats. In VA rats, the thickness of type X collagen-negative area in the epiphyseal growth plate

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Fig. 1. Epiphyseal growth plates of proximal tibiae in VA rats on Day 1 (a), Day 3 (b) and Day 5 (c). The thickness of epiphyseal growth plate is reduced since Day 3 (b and c). (a) Clear columnar arrangements of chondrocytes are observed on Day 1. (b) Columnar arrangements of chondrocytes are reduced in the proliferating zone on Day 3. (c) Chondrocytes with hypertrophic appearance are markedly reduced on Day 5. H.E. × 150.
became significantly thinner than that of control rats since Day 2 and reduced to about 50% of that of control rats on Day 5 (Figs. 3 and 4). The thickness of type X collagen-positive area in the epiphyseal growth plates became significantly thinner than that of control rats since Day 4 and the deposition of type X collagen partially disappeared on Day 5 (Figs. 3 and 4).

BrdU incorporation was mainly detected in chondrocytes in the proliferating zone in control rats. In VA rats, the BrdU-incorporating index was significantly decreased since Day 2 and BrdU-incorporated chondrocytes almost disappeared since Day 4 (Figs. 5 and 6).

The deposition of type II collagen was detected in the cartilage matrix throughout the epiphyseal growth plates and within bony trabeculae in control rats (Fig. 7a). In all VA rats, the deposition of type II collagen partially disappeared in the epiphyseal growth plate near the periosteum on Day 5 (Fig. 7b).

The expression of type I collagen was observed in osteoblasts and osteocytes, and the deposition of type I collagen was observed in the osteoid, but not in the cartilage matrix of the epiphyseal growth plates in control rats (Fig. 8a). In all VA rats, the deposition of type I collagen was detected mainly in the cartilage matrix where type II collagen disappeared on Day 5 (Fig. 8b). In these areas, neither osteoblasts nor osteocytes were detected.

**DISCUSSION**

In the normal endochondral ossification, chondrocytes differentiate from resting cells, via proliferating cells, to hypertrophic cells in the epiphyseal growth plate [2]. The cartilage matrix also undergoes the phenotypical alterations

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**Fig. 2.** Changes in the thickness of epiphyseal growth plates of proximal tibiae in control and VA rats during the administration period. Values are mean ± SD. **:** Significantly different from the corresponding control (**: p<0.001).
During this process, in the resting and proliferating zone, the cartilage matrix contains a large amount of type II collagen, but not type X collagen. In the hypertrophic zone, the cartilage matrix shows the deposition of type X collagen in addition to type II collagen and is calcified [12].

In the present study, a high dose of vitamin A gradually reduced the epiphyseal growth plates during the administration period as previously reported [4–7]. During such process, the initial decrease was observed in the thickness of type X collagen-negative area corresponding to resting and proliferating zones, and the number of BrdU-incorporated chondrocytes corresponding to proliferating chondrocytes. Thereafter occurred the decrease in the thickness of type X-positive area corresponding to the hypertrophic zone. These findings suggested that a high dose of vitamin A initially disturbed the differentiation from resting to proliferating chondrocytes, subsequently disturbed the differentiation from proliferating to hypertrophic chondrocytes, and finally resulted in the deformation of columnar arrangement and the reduction in the thickness of the epiphyseal growth plates.

The reduction of hypertrophic zone was also suggested in previous studies on bovine Hyena disease and hypervitaminosis A [15, 16]. However, in vitro studies...
using chick chondrocytes demonstrated that retinoic acid, a derivative of vitamin A, promoted the expression of phenotypes of hypertrophic chondrocytes, such as the expression of type X collagen, high alkaline phosphatase and matrix mineralization \[11, 14\]. This contradiction may be settled by an assumption that the direct effects of retinoic acid were modified by the interactions with other types of cells such as osteoblasts, osteoclasts or vascular endothelial cells, or systemic factors \textit{in vivo}.

In the present study, the changes in the cartilage matrix phenotypes were observed on Day 5 as the disappearance of type II collagen deposition and the appearance of type I collagen. The type I collagen-positive areas did not correspond to the osteoid or bone tissue invading the cartilage matrix, because neither osteoblasts nor osteocytes were detected there. Type I collagen is known as one of major components of bone matrix, and not detected in the cartilage matrix of epiphyseal growth plates in the normal condition. However, Cancedda and co-workers demonstrated that retinoic acid reduced the synthesis of type II collagen and induced the expression of type I collagen in the chick chondrocytes \textit{in vitro} \[3\]. They suggested that the expression of type I collagen and disappearance of type II collagen represented the differentiation of chondrocytes to

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**Fig. 7.** Immunohistochemical staining of type II collagen in the epiphyseal growth plates of proximal tibiae in a control rat (a) and a VA rat (b) on Day 5. (a) Type II collagen is deposited in the cartilage matrix throughout the epiphyseal growth plate in a control rat. (b) Type II collagen-positive area is partially disappeared near the periosteum in a VA rat. × 150.

**Fig. 8.** Immunohistochemical staining of type I collagen in the epiphyseal growth plates of proximal tibiae in a control rat (a) and a VA rat (b) on Day 5. (a) Type I collagen is detected in the osteoid (arrowhead) and osteoblasts (arrow) in a control rat. (b) Type I collagen is deposited in the cartilage matrix in a VA rat. × 150.
the osteoblast-like stage. Their suggestion agrees to our present results that a high dose of vitamin A caused the chondrocytes to deviate from the process of normal differentiation and to express the osteoblast-like phenotypes.

REFERENCES


