Local Disappearance of Epiphyseal Growth Plates in Rats with Hypervitaminosis A

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ABSTRACT. Epiphyseal growth plates of proximal tibiae in rats with high doses of vitamin A (V-A) were observed. Of 4 groups, each consisting of 5 rats, three groups were given V-A at doses, IU/100 g by body weight/day, of 50,000, 100,000, and 150,000, respectively. The other group rats were given no V-A (control). Rats were administered V-A for the 5 days from 4 weeks after birth and sacrificed at 12 weeks after birth. Three rats of the 150,000 IU group died during the period of observation. The decalcified sections were stained with hematoxylin-eosin or toluidine blue. In the ground sections, microradiography, backscattered electron imaging, and energy-dispersive X-ray microanalysis were performed. These observations suggest that the local disappearance of epiphyseal growth plates under high doses of V-A goes in the order of the increased doses through the process of (1) calcified cartilage areae appearing in the resting cell zone, (2) some of the calcified areae extending in the growth plate towards the diaphysial side, (3) bone tissue replacing the calcified areae, and (4) the local disappearing of the growth plate. Such a local disappearance may be formed in the stressed proximal regions of tibiae. — KEY WORDS: calcified cartilage, epiphyseal growth plate, hypervitaminosis A, local disappearance, sulfur content.

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Bovine hyena disease is regarded as a disorder of skeltal development, localized mainly in the pelvic limbs of young calves. A virus may cause it [7], and high doses of vitamin AD_3E premix may be related to it [10, 21]. Some investigators [7, 10, 21] reported that the pelvic limbs of calves with hyena disease showed a deformation and a local disappearance of epiphyseal growth plates. Recently, high doses of vitamin A as well as vitamin AD_3E premix have caused hyena disease in young calves [15–17].

When high doses of vitamin A are given to mammals, such as pets and domestic animals, the growth of limb bones is interfered [3, 5, 8, 15–17, 22]; and the growth plates of hyaline cartilage were variously deformed [2, 11, 13, 15–17, 20, 22], and some parts of the growth plates occasionally disappeared [2–6, 15, 16, 22, 23]. Histologically, eosinophilic cartilage which appeared in the growth plates was undergoing necrosis and some areae of the region were destroyed [2], whereas blood vessels invaded the growth plates from the epiphyseal side [11]. Clark [3] also observed such invading by blood vessels and illustrated that, in discontinuous plates, V-shaped cartilage remnants on the diaphysial side bridged the gap between the broken ends. Similar to such observations, some areae of lost growth plates were substituted for bony trabeculae that had calcified cartilage spicules in the center [23]. Hence, it is likely that the growth plates exposed to high doses of vitamin A disappeared from the epiphyseal side rather than from the diaphysial, but the process of partial disappearance in growth plates remains to be explained.

From their previous studies on the local disappearance of growth plates [2–6, 10, 15, 16, 21–23], the local regions are likely to be apart from the center of growth plates. Such regions may be related to physical stress in limb bones [14]. In this study, we observed initial changes and local disappearance both occurring in proximal growth plates of tibiae in rats with hypervitaminosis A followed by the increased doses.

MATERIALS AND METHODS

Animals: The animals used in this study were similar to those in section one of the doctoral thesis by Takaki, who studied the etiology of bovine hyena disease [15]. Three experimental groups (I, II, and III) of Wistar male rats, each consisting of 5 rats, were given orally vitamin A, respectively, at 50,000 (I), 100,000 (II), and 150,000 IU/100 g by body weight/day (III) for the 5 days from 4 weeks after birth. Another group of 5 rats was given no vitamin A (control). After vitamin A administration, all rats were kept on ordinary solid diet and water. In group II and III rats, their disordered walking was already seen from about one week after administration (about 6 weeks after birth), although group I rats had not shown it. All rats were bred until the growth by body weight stabilized 12 weeks after birth and then sacrificed [15], except for 3 rats in group III, which died on 6 and 10 days after administration. Tibiae removed from the rats were fixed in 10% neutral formalin. Proximal tibiae were used for the following procedures.

Observations: Half the samples were decalcified with formic acid. After embedded in paraffin, serial 5-μm-thick sagittal sections were stained with hematoxylin and eosin (H-E) or with 0.05% toluidine blue in a 0.01-M citric buffered solution. The remaining samples were embedded without decalcification in epoxide resin (Epo-Mix Epoxide; Buehler, Evanston, IL, U.S.A.) and then cut sagittally with a diamond wheel. One tibia provided several slices, which were then ground with grindstones down to 100 μm in thickness and contact microradiographs were taken with a soft X-ray device (SRO-M50; Sofron, Tokyo, Japan). Some ground slices were polished with 5- and 0.3-μm alumina on

polishing cloths and observed after carbon coating by backscattered electron (BSE) imaging in a scanning electron microscope (SEM: S-2500CX; Hitachi, Tokyo, Japan) operated at an accelerating voltage of 20 kV.

Elemental analysis: Elements in specimens were analyzed qualitatively and quantitatively by energy-dispersive X-ray (EDX) microanalysis in a S-2500CX SEM equipped with an EDX detection system (Delta-4; Kevex, Foster City, CA, U.S.A.) under conditions of an accelerating voltage of 15 kV and a specimen irradiation current of 1×10^{-7} mA. For the quantitative elemental analysis of Ca, P, and S, the standard samples of fluoroapatite and strontium sulfate were prepared, and ZAF corrections were applied [12].

RESULTS

Control: The epiphyseal growth plates of long bones in control (normal) rats consist of a resting cell zone and a columnar chondrocyte zone that is divided into a proliferating, a hypertropic, and a calcifying zone as shown in Fig. 1. The cartilage matrix of the calcifying zone and the spicules of calcified cartilage in the centers of primary bony trabeculae are low basophilic (Fig. 1a) and high metachromasic areae (Fig. 1b).

Hypervitaminosis A: In rats given high doses of vitamin A, the growth plates of proximal tibiae showed basically continuous changes in the order of group I, II, and III rats, though the degree of damage varied in the same group rats. Figures 2 and 3 show initial changes occurring in the proximal tibiae of group I rats. Eosinophilic bands occupied transversely the resting cell and proliferating zones (Fig. 2a), or eosinophilic small areae were scattered in the proliferating zone (Fig. 3a). Such eosinophilic cartilage bands and areae were not present on the cranial and caudal sides of growth plates in group I rats, but, in group II and III rats, they extended towards both sides. These eosinophilic cartilage bands or areae were observed in all experimental rats (Table 1). However, no eosinophilic cartilage was found in control rats.

When stained with toluidine blue, the calcifying zone and the calcified cartilage spicules of primary bony trabeculae in experimental rats were as highly metachromatic as those in control rats; moreover, high metachromatic cartilage areae were scattered in the resting cell zone (Figs. 2b, 3b). In H-E preparation, low basophilic cartilage areae were also seen there (Figs. 2a, 3a). High metachromatic and low basophilic areae in the resting cell zone tended to widen and to increase in the order of group I, II, and III rats, although the areae were relatively rarely present in control rats. One or a few high metachromatic and low basophilic cartilage areae extending in growth plates on the way from the epiphyseal side towards the diaphysial or throughout the plates longitudinally were found in 2 rats of group I and in 3 rats of group II (Fig. 3, Table 1). Bone tissue with blood vessels also extended in the plate (Fig. 3a). In the local regions of growth plates, the columnar cell-arrangement was disordered, and primary bony trabeculae with thick cartilage spicules were present. Such

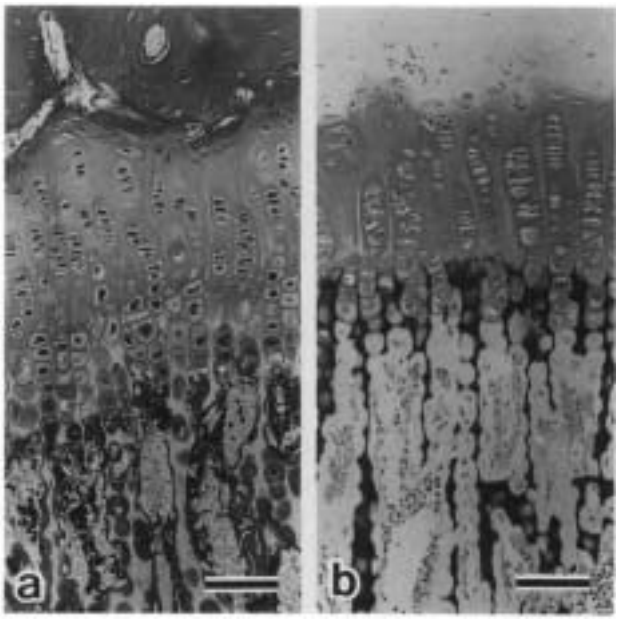

Fig. 1. Decalcified sections of proximal tibiae in control rats. (a) Hematoxylin and eosin (H-E) staining. (b) Toluidine blue staining. Cartilage matrix in the calcifying zone and the spicules of calcified cartilage in primary bony trabeculae are low basophilic (a) and high metachromatic areae (b). a, b Bars=100 μm.

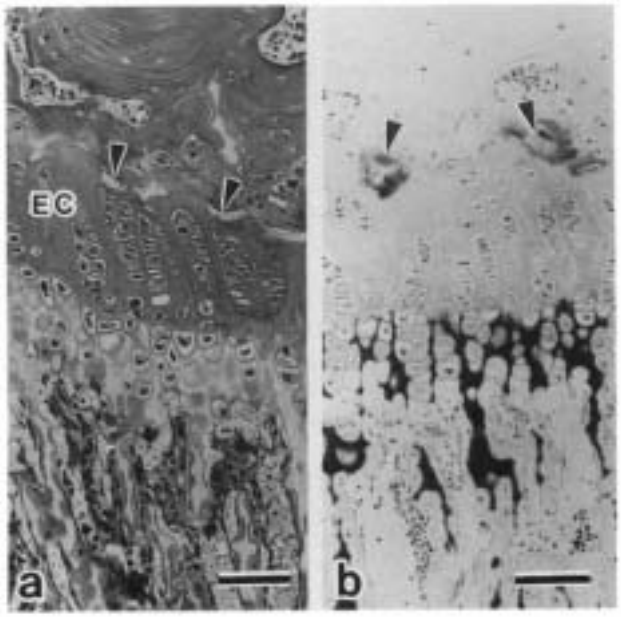

Fig. 2. Decalcified sections of proximal tibiae in rats of group I. (a) H-E staining. (b) Toluidine blue staining. The arrowheads indicate low basophilic (a) and high metachromatic catilage areae (b) in the resting cell zone. The transverse band of eosinophilic cartilage (EC) is seen in the growth plate (a). a, b Bars=100 μm.

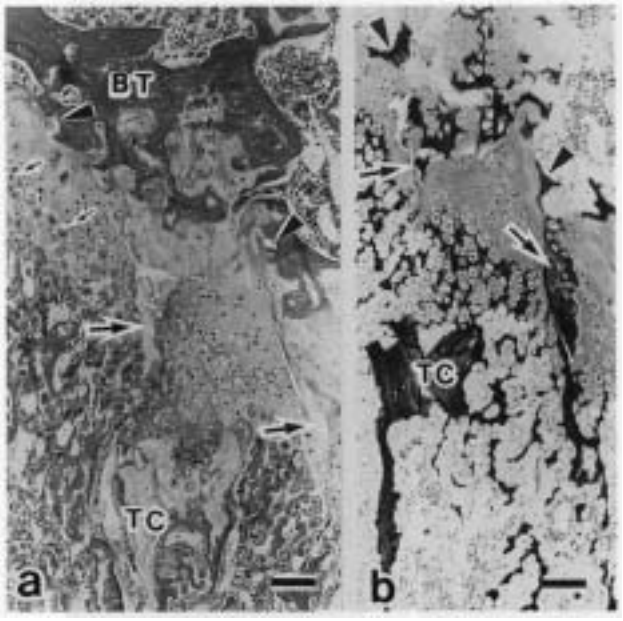

Fig. 3. Decalcified sections of proximal tibia in a rat of group I. (a) H-E staining. (b) Toluidine blue staining. In Fig. 3a, two low basophilic areae (large arrows) are locally present in the growth plate and eosinophilic small areae (smaller arrows) are scattered. In Fig. 3b, two high metachromatic areae (large arrows) are clearly seen. The arrowheads indicate low basophilic (a) and high metachromatic areae (b) in the resting cell zone (see Fig. 2). TC: thick cartilage spicule. BT: bone tissue. a, b Bars=100 μm.

Table 1. Frequencies of eosinophilc cartilage, high-metachromatic or calcified cartilage, bone tissue, and complete or incomplete local disappearance in epiphyseal growth plates of proximal tibiae in control and experimental rats (groups I to III)

Group (*n*)	Eosinophilic cartilage[a)]	Calcified cartilage[b)]	Bone tissue	Local disappearance
Control (5)	0/5	0/5	0/5	0/5
I (5)	5/5	2/5	2/5	0/5
II (5)	5/5	3/5	5/5	2/5
III (2[c)])	2/2	2/2	2/2	2/2

a) Excluding the resting cell zone. b) Excluding the resting cell and calcifying zones. c) The other 3 rats died during the period of observation.

regions were seen on the cranial side rather than on the caudal, although one rat of group II had two calcified regions in the growth plate: the center and the cranial side. Figure 4a shows a microradiograph of a rat in group II. Part of the growth plate near the patella was calcified from the epiphyseal side (Fig. 4b).

Backscattered electron (BSE) imaging: Figures 5 and 6 show BSE images obtained. A higher BSE signal means higher calcification. In control rats (Fig. 5), the spicules of hypercalcified cartilage in bony trabeculae partially contained fine calcospherulites measuring up to about 1 μm in diameter (Fig. 5a, b), and areae of relatively low

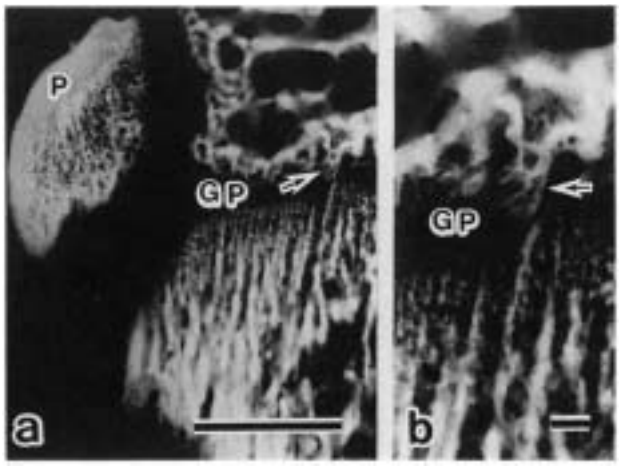

Fig. 4. Microradiographs of proximal tibia in a rat of group II. (a) At a low magnification. (b) At a higher magnification of a part of Fig. 4a. The arrows indicate a calcified tissue penetrating the growth plate (GP). P: patella. a Bar=1 mm. b Bar=100 μm.

calcification were scattered on the epiphyseal side of growth plates (Fig. 5a, c). Bone tissue containing osteocyte lacunae with canaliculi was less mineralized than calcified cartilage (Fig. 5b, c). In experimental rats (Fig. 6), the cartilage spicules contained variously sized calcospherulites (Fig. 6a, b). On the epiphyseal side of growth plates or the resting cell zone (Fig. 6a, c) and from the epiphyseal side towards the diaphysial (Fig. 6a, d), hypercalcified areae containing variously sized calcospherulites ranging from about 1 to 2 μm in diameter were identified as calcified cartilage similar to the spicules of calcified cartilage (Figs. 5b, 6b). These calcified cartilage areae of experimental rats contained larger calcospherulites than those of control rats.

Local disappearance: In 2 rats of group II and in 2 surviving rats of group III, the growth plates contained one area of local disappearance in part near the cranial side (Table 1), although in one rat of group II, the growth plate disappeared at two locations near the cranial and caudal sides. In Fig. 7, bone tissue in the epiphyseal side extended in the growth plates, and was connected with the calcified fibrous cartilage on the diaphysial side. Chondrocytes arranged disorderly and eosinophilic areae were present, but endochondral ossification still occurred. In one rat of group III, a large area of local disappearance was observed (Fig. 8a). A few thick bony trabeculae under the disappeared plate area were retained. Endochondral ossification towards the diaphysis had completely stopped. The non-calcified cartilage plate was enclosed with layers of hypercalcified cartilage and bone tissue (Fig. 8b).

Elemental analysis: By EDX microanalysis, C, O, Ca, P, S, Mg, and K elements were detected from growth plates and primary bony trabeculae. Table 2 is the concentrations of Ca, P, and S in control and experimental rats. Differences between data were analyzed statistically by means of Student's *t*-tests ($p<0.01$). Non-calcified cartilage of growth plates in experimental rats showed a lower amount of S

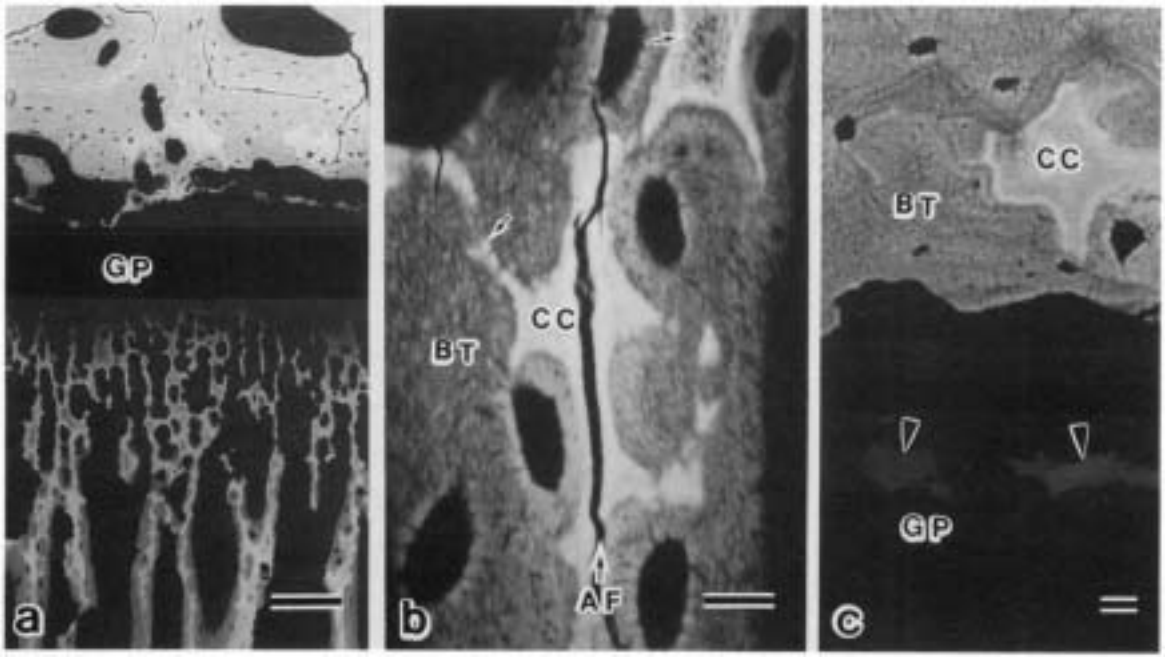

Fig. 5. Backscattered electron (BSE) images of proximal tibia in a control rat. The polished surface of a ground section. (a) At a low magnification. (b) Primary bony trabecula with a cartilage spicule (CC). (c) Epiphyseal side of the growth plate (GP) containing hypocalcified areae (arrowheads). (b, c) parts of Fig. 5a. BT: bone tissue containing cell lacunae with the canaliculi. AF (large arrow): artificial crack. Smaller arrows: calcospherulites. a Bar=100 μm. b, c Bars=10 μm.

than that in control rats and the content was similar to that of bone tissue, but there were no significant differences in the S concentration among calcified cartilage of control and experimental rats and non-calcified cartilage of control rats. The Ca and P concentrations of calcified cartilage were higher than those of bone tissue. Small amounts of Mg and K, as trace elements, tended to be higher in calcified cartilage and bone tissue than in non-calcified cartilage.

DISCUSSION

Eosinophilic epiphyseal cartilage was undergoing necrosis in dogs with hypervitaminosis A and some areae of the region were destroyed [2]. In this study, we found eosinophilic cartilage containing a small amount of sulfur (S) in the epiphyseal growth plates of proximal tibiae in all experimental rats, but no destroyed areae were found. Eosinophilic necrosis following the cartilage destruction [2] may be caused by the administration of higher doses of vitamin A for a long period to dogs compared with our experimental rats.

When rabbits were given high doses of vitamin A, metachromasia and basophilia seldom occurred in articular and epiphyseal cartilage, and preformed chondroitin sulfate or S element was lost [13, 20]. Calcified cartilage was occasionally present on the epiphyseal side of growth plates in normal rats [1]. Under hypervitaminosis A, we found that high metachromatic and hypercalcified cartilage areae in the resting cell zone locally extended in the growth plates on the way from the epiphyseal side or throughout the plates longitudinally, but the hypercalcified cartilage contained higher S content than the non-calcified cartilage which was probably eosinophilic. Such data may differ from the above-mentioned reports [13, 20].

High metachromatic and low basophilic cartilage in decalcified sections will correspond to the hypercalcified cartilage in the BSE images of undecalcified sections. We believe that the calcified cartilage does not overlap with the eosinophilic cartilage, because the calcified cartilage, similar to non-calcified growth plates in control rats, contained a relatively large amount of S, but non-calcified cartilage in experimental rats, like eosinophilic cartilage, contained a significantly smaller amount of S. The volume of high metachromatic and calcified cartilage in growth plates was remarkably larger in experimental rats than in controls. In addition, the calcospherulites of calcified cartilage in experimental rats showed larger diameters than those of trabecula spicules in control rats. Hence, some parts of the growth plates, but not the eosinophilic areae, may calcify rapidly from the resting cell zone under hypervitaminosis A [9].

Hypervitaminosis A causes chondrocytes in growth plates to de-differentiate in *in-vitro* study [18]. De-differentiated chondrocytes are probably inactive and cartilage matrix becomes eosinophilic rather than basophilic, similarly to those in the resting cell zone. Moreover, hypervitaminosis A causes chondrocytes to mature and cartilage matrix to calcify in *in-vitro* study [9], similarly to those in the calcifying zone. However, we do not know the reason why basophilic, high metachromatic, and calcified cartilage

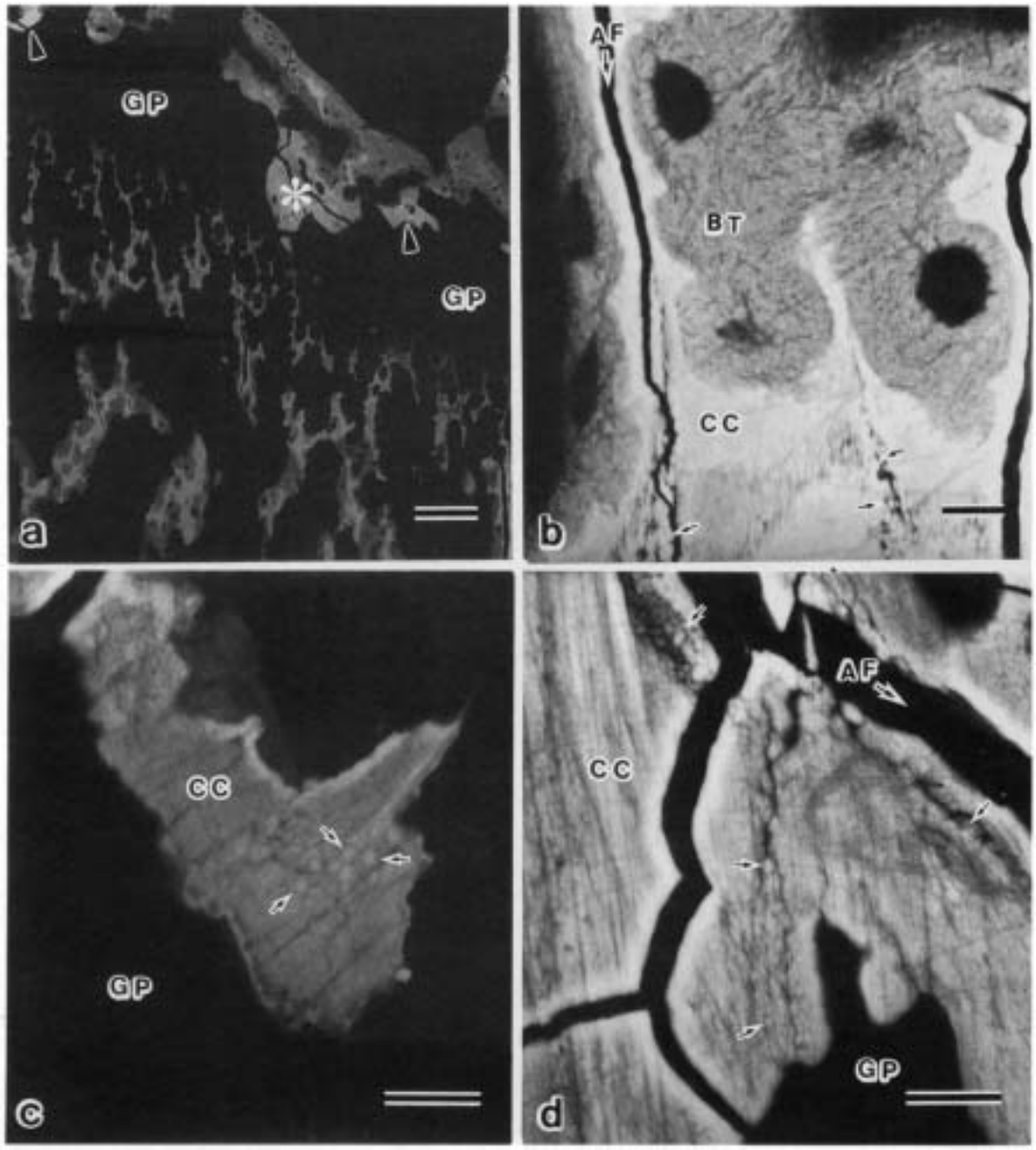

Fig. 6. Backscattered electron (BSE) images of proximal tibiae in rats of group I. The polished surfaces of ground sections. (a) At a low magnification. (b) Primary bony trabecula with a thick cartilage spicule. (c) Epiphyseal side of the growth plate (GP) or the resting cell zone. (d) Part of the calcified cartilage (CC) extending in the growth plate (* in a). The arrowheads in Fig. 6a indicate calcified cartilage in the epiphyseal side. BT, AF (large arrow), and smaller arrow: see Fig. 5. a Bar=100 μm. b-d Bars=10 μm.

(containing a relatively large amount of S) that appears in the local regions of growth plates coexist together with eosinophilic cartilage (containing a smaller amount of S) in the entire plates in *in-situ* study, though the correlation between retinoic acid, a derivative of vitamin A, and parathyroid hormone in cultured chondrocyte differentiation was reported [19].

From the order of the increased doses of vitamin A, growth plates under hypervitaminosis A may start calcification in mesensitive response to physical stress added to the proximal regions of tibiae compared with a normal diet. Calcified cartilage will be gradually replaced by bone

tissue with blood vessels from the epiphyseal side. Smith [14] reported that fibrous cartilage was formed and calcified in the stressed region of the growth plates. We also found fibrous calcified cartilage in regions that formed a local disappearance. In rats with higher doses of vitamin A, the areae of local disappearance followed by bone absorption completely penetrate the growth plates. Non-calcified cartilage plates will be enclosed with layers of calcified cartilage and bone tissue, and stop endochondral ossification [6]. Thick bony trabeculae under the region of local disappearance may physically support the remaining cartilage plate [23].

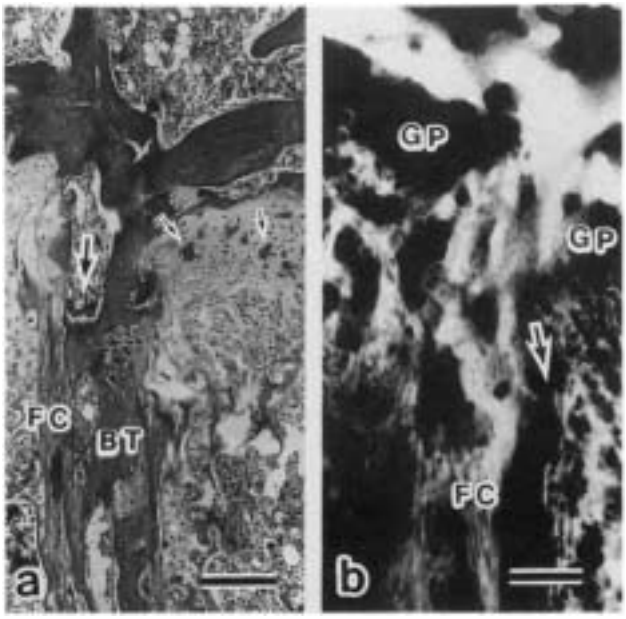

Fig. 7. Right and left proximal tibiae in a rat of group II. (a) H-E staining of a decalcified section. (b) Microradiography of a ground section. The large arrows indicate incomplete areae of local disappearance followed by bone tissue (BT) formation in the growth plate (GP). FC: fibrous cartilage. Smaller arrows: eosinophilic small areae in the growth plate. a, b Bars=100 μm.

In conclusion, we strongly suggest that the local disappearance of epiphyseal growth plates in rats under hypervitaminosis A starts from the calcification of the resting cell zone. Some calcified cartilage areae in the stressed proximal regions of tibiae may extend in growth plates towards the diaphysial side. The areae of local disappearance will be formed by osteoclasts after the calcified areae are replaced with bone tissue.

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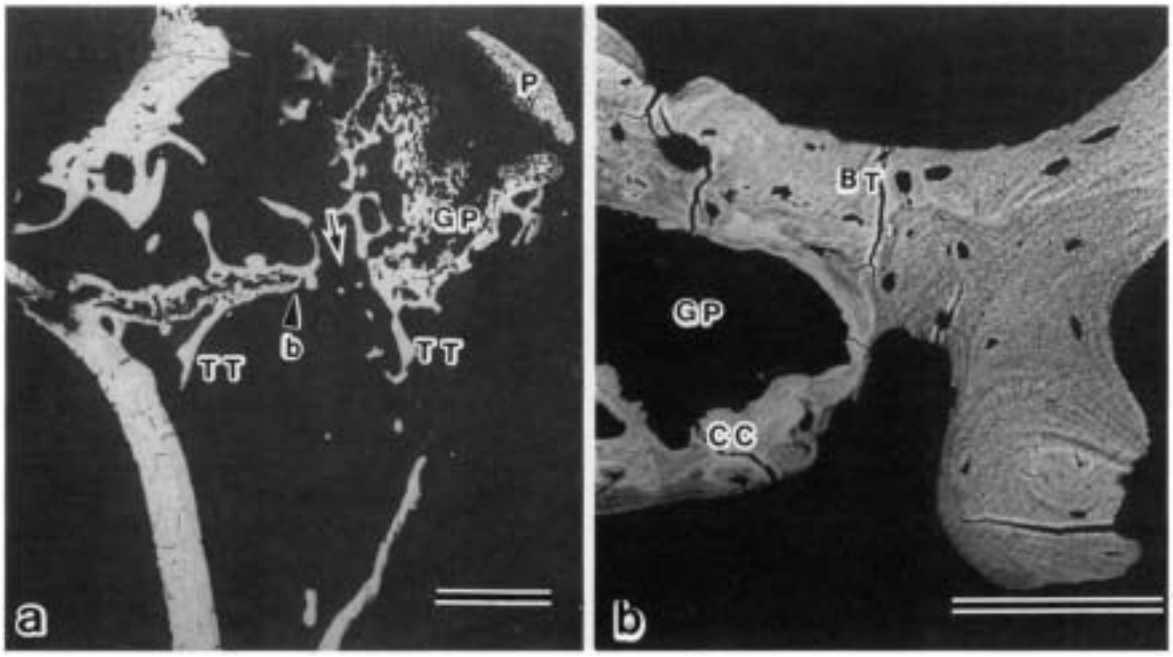

Fig. 8. Backscattered electron (BSE) images of proximal tibia in a rat of group III. The polished surface of a ground section. (a) At a low magnification. (b) Part of the growth plate (GP; arrowhead b in a) enclosed with the layers of calcified cartilage (CC) and bone tissue (BT). The arrow indicates a large area of local disappearance in the growth plate. TT: thick bony trabecula. P: patella. a Bar=1 mm. b Bar=100 μm.

Table 2. Concentrations of Ca, P, and sulfur (S) in non-calcified and calcified cartilage and bone tissue of proximal tibiae in control and experimental rats (groups I and II). Energy-dispersive X-ray (EDX) microanalysis. Compared with Figs. 5 and 6.

Epiphyseal growth plate		Ca	P	S	*n*
Control rat	Non-calcified cartilage	0.25 ± 0.07[a)]	0.04 ± 0.02	0.45 ± 0.08*	12
Experimental rat	Non-calcified cartilage	0.15 ± 0.04	0.05 ± 0.06	0.26 ± 0.08	24
	Calcified cartilage[b)]	30.99 ± 0.62*	14.32 ± 0.49*	0.45 ± 0.07*	12
	Calcified cartilage[c)]	29.87 ± 1.42*	13.84 ± 0.87*	0.39 ± 0.06*	24
	Bone tissue[c)]	24.57 ± 1.89	11.91 ± 0.84	0.19 ± 0.04	24
Primary bony trabecula		Ca	P	S	*n*
Control rat	Calcified cartilage	29.40 ± 0.32*	13.30 ± 0.16*	0.38 ± 0.04*	8
	Bone tissue	22.27 ± 0.93	10.86 ± 0.54	0.18 ± 0.02	8
Experimental rat	Calcified cartilage	29.92 ± 1.03*	13.91 ± 0.48*	0.40 ± 0.05*	8
	Bone tissue	24.24 ± 1.10	11.41 ± 0.42	0.22 ± 0.04	8

a) Percentage by weight (Mean ± S.D.). b) Excluding the resting cell zone. c) In the resting cell zone. In each of Ca, P, and S, * $p<0.01$ versus others. Note: in S content, non-calcified cartilage of experimental rats is lower than other non-calcified and calcified cartilage.

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