

Histological Disorders Related to the Focal Disappearance of the Epiphyseal Growth Plate in Rats Induced by High Dose of Vitamin A

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ABSTRACT. The histological disorders related to the focal disappearance of the epiphyseal growth plate were examined histochemically in the proximal tibia of rats administered a high dose of vitamin A. Animals were given 100,000 IU/100 g body weight/day of vitamin A for 5 days from 4 weeks after birth (VA rats) or given deionized water as control and sacrificed on Day 12 and 19 of the experiment. Tibiae were examined by immunohistochemistry for type I, II and X collagens, lectin-histochemistry for *Helix pomatia* and backscattered electron imaging. On Day 12, the abnormally developed calcified cartilage matrix was detected within the epiphyseal growth plate in VA rats. The uncalcified cartilage matrix contained type I collagen but lacked type II collagen. In addition, the eroded regions accompanied with numerous osteoclasts and osteoblasts were detected in the epiphyseal growth plate. On day 19, eroded regions penetrated the epiphyseal growth plate to result in its focal disappearances with the eroded surfaces entirely covered with bone tissue in VA rats. These findings suggested that the cartilage matrix of the epiphyseal growth plate was abnormally calcified and showed the phenotypes like bone matrix. The eroded regions of the epiphyseal growth plate seemed to be caused by the invasion of osteoclasts into the altered cartilage matrix and might develop to the focal disappearances by the modeling or remodeling due to action of osteoclasts and osteoblasts.—**KEY WORDS:** calcification, epiphyseal growth plate, focal disappearance, osteoclast, vitamin A.

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High doses of vitamin A (VA) are known to cause several disorders in the skeletal development. One of such disorders is reported as the bovine Hyena disease in calves [19, 20]. The focal disappearance of the epiphyseal growth plate, i.e., premature closure of the epiphyseal growth plate, is regarded as the characteristic conformational feature in bovine Hyena disease and hypervitaminosis A [2–5, 18, 20]. In our previous study [11], we suggested that the high calcification of cartilage matrix in the epiphyseal growth plate was closely related to the focal disappearances of the epiphyseal growth plate, although the detailed process of the focal disappearance still remains unclear. In our recent study [17], we, therefore, experimentally administered a high dose of vitamin A to young rats to investigate the cellular and matrical alterations chronologically and demonstrated that initial disorders in the epiphyseal growth plate were attributed to the disturbance caused by a high dose of VA in the differentiation of chondrocytes.

In the present study, as the next step, we examined the succeeding histological disorders in the process of the focal disappearance of the epiphyseal growth plate cartilage histochemically in young rats administered a high dose of VA.

MATERIALS AND METHODS

Animals and experiments: Wistar rats born in our laboratory were bred under conditions as described in our previous report [17]. Ten male rats aged 4 weeks, weighing 80–100 g, were given orally 100,000 IU/100 g body weight/day of VA suspended in deionized water (VA rats) for 5 days. Similarly, another 10 male rats were given deionized water and used as control. Five VA rats as well as control rats were sacrificed on 7 days and 14 days after the end of the administration period (Day 12 and 19, respectively).

Tissue preparation: The animals were perfused cardiacly with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer at 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. A half of each tibia was decalcified in 10% EDTA in 0.01 M phosphate buffer at pH 7.4 at 4°C for 1 week. After dehydration through a graded series of ethanol at 4°C, decalcified tissues were embedded in paraffin and sectioned at 5 µm. The sections were stained by hematoxylin and eosin (HE) or processed for immunohistochemistry and lectin-histochemistry. The remaining half of each tibia was embedded without decalcification in methylmethacrylate and processed for backscattered electron (BSE) imaging.

Immunohistochemistry: The immunohistochemistry for collagens was performed according to Mizoguchi *et al.* [12]. Rabbit antisera against rat type I collagen, bovine type II collagen and rat type X collagen were purchased from LSL (Tokyo, Japan). The procedure of the immunostaining was the same as described in our previous report [17].

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Lectin-histochemistry: After the blocking of endogenous peroxidase, deparaffinized sections were treated with 1% bovine serum albumin in PBS. Then, the sections were incubated in biotinylated *Helix pomatia* (HPA, 1:100; EY Laboratories, San Mateo, Canada) for 1 hr at room temperature. The reaction products were visualized as described in our previous report [17].

Backscattered electron (BSE) imaging: Methylmethacrylate-embedded samples were cut sagittally with a diamond wheel. The sections were ground with grindstones up to 10 μm in thickness and then were polished with 5 and 0.3 μm alumina on polishing cloths. The sections were rinsed in tap water, dehydrated in 100% ethanol and coated with carbon. They were examined by BSE imaging with a Hitachi S-2500CX scanning electron microscope (S-2500CX SEM; Hitachi, Tokyo, Japan) at an accelerating voltage of 20 kV.

RESULTS

In control rats on Day 12 (Fig. 1A) and 19, the epiphyseal growth plate of proximal tibiae consisted of the resting, proliferating and hypertrophic zone. The proliferating and hypertrophic zone showed clear columnar arrangements of chondrocytes. In VA rats, the epiphyseal growth plates on Day 12 had an irregular thickness with several erosions. Columnar arrangements of chondrocytes were reduced and deformed (Fig. 1B and 2A). Calcification of cartilage matrix distinguished as deposition of basophilic granules was detected in several areas within the epiphyseal growth plates (Fig. 2A and B). Occasionally, chondrocytes in the remaining columnar arrangement of the proliferating zone were entirely surrounded by such calcified cartilage matrix (Fig. 2B). On Day 19, reduction of columnar arrangement

and one or two focal disappearances of the epiphyseal growth plate were detected in all VA rats. On the site of the focal disappearances, uncalcified cartilage matrix was enclosed with the layers of calcified cartilage matrix. In addition, the surfaces of the calcified cartilage matrix was covered with a thin layer of bone tissue (Fig. 3).

Results of BSE imaging are shown in Figs. 4 and 5. A higher BSE signal means a higher degree of calcification. In control rats, calcified cartilage matrix distinguished as accumulations of fine granular high BSE signals was detected around lacunae of chondrocytes in the lower hypertrophic zone and within the bony trabeculae. The calcified cartilage matrix was faint and thin in the hypertrophic zone and the intensity of BSE signals gradually increased during development to bony trabeculae. Bone tissue, which contained lacunae of osteocyte, was less calcified than calcified cartilage matrix (Fig. 4A-C). In VA rats on Day 12 and 19, calcified cartilage matrix was detected within the epiphyseal growth plates, as observed by HE staining. Such calcified cartilage matrix contained numerous large calcospherulites (Fig. 5A and B).

In control rats on Day 12 and 19, the deposition of type X collagen was detected in both uncalcified and calcified cartilage matrix of the hypertrophic zone and in calcified cartilage matrix within the primary bony trabeculae (Fig. 6A). In VA rats on Day 12 and 19, the deposition of type X collagen was not detected in neither uncalcified nor calcified cartilage matrix in the epiphyseal growth plate, but detected in the calcified cartilage matrix within the primary bony trabeculae (Fig. 6B).

In control rats on Day 12 (Fig. 7A) and 19, the deposition of type II collagen was detected in the cartilage matrix throughout the epiphyseal growth plate and within the primary bony trabeculae. In VA rats on Day 12, the

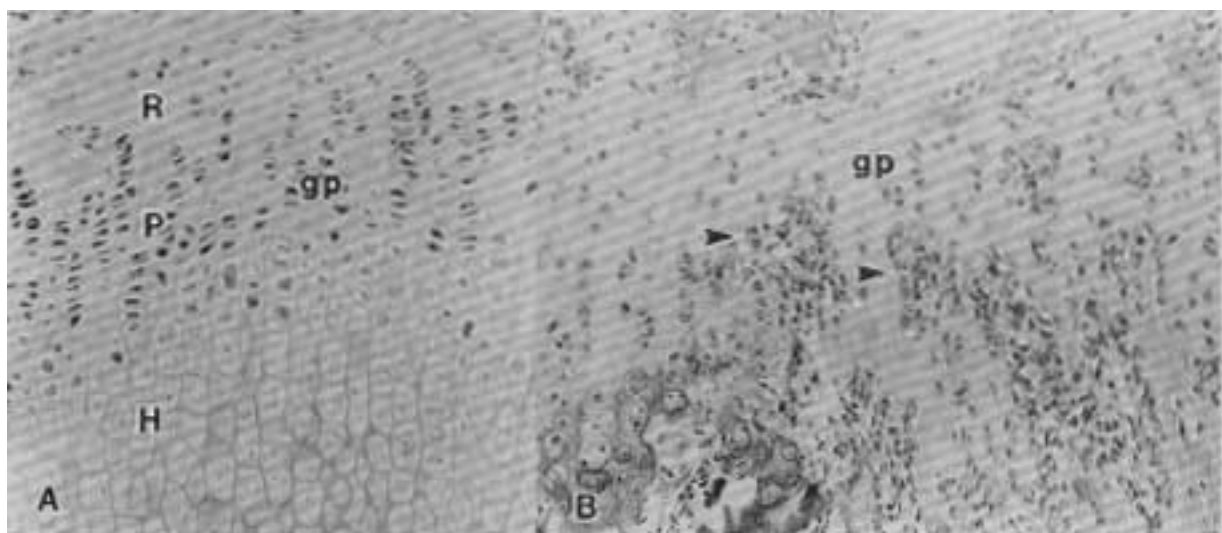


Fig. 1. Epiphyseal growth plates of proximal tibiae in a control rat (A) and a VA rat (B) on Day 12. A: The resting (R), proliferating (P) and hypertrophic zone (H) are clearly observed in a control rat. B: Partial erosions, focal holes (arrowheads) and deformed columnar arrangement of chondrocytes are observed in a VA rat. gp: epiphyseal growth plate. H.E. $\times 150$.

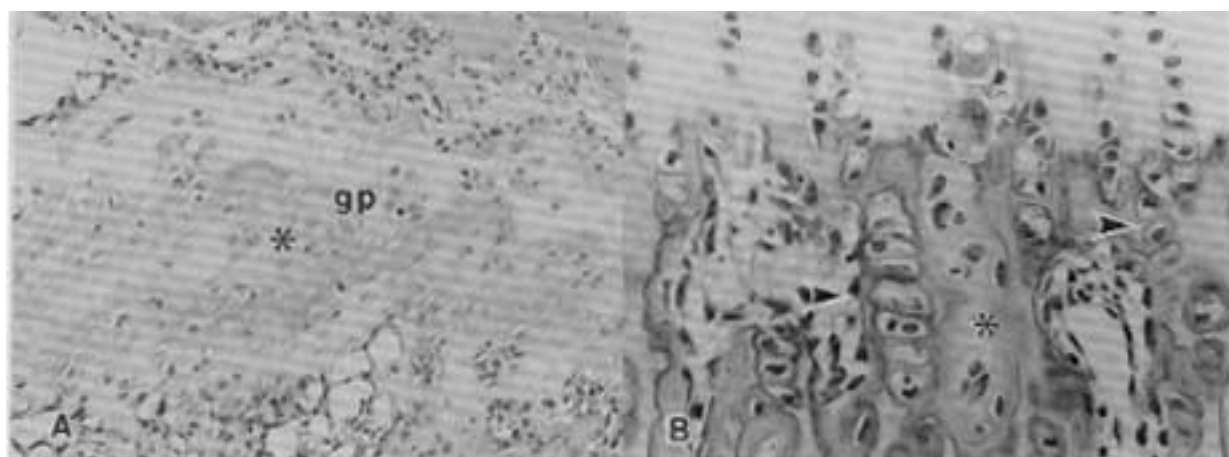


Fig. 2. Epiphyseal growth plates of proximal tibiae in VA rats on Day 12 (A and B). A: Calcified cartilage matrix (asterisk) is detected within the epiphyseal growth plate. B: Chondrocytes in the remaining columnar arrangement of the proliferating zone (arrowheads) are entirely surrounded by the calcified cartilage matrix (asterisk). gp: epiphyseal growth plate. H.E. A: $\times 150$, B: $\times 300$.

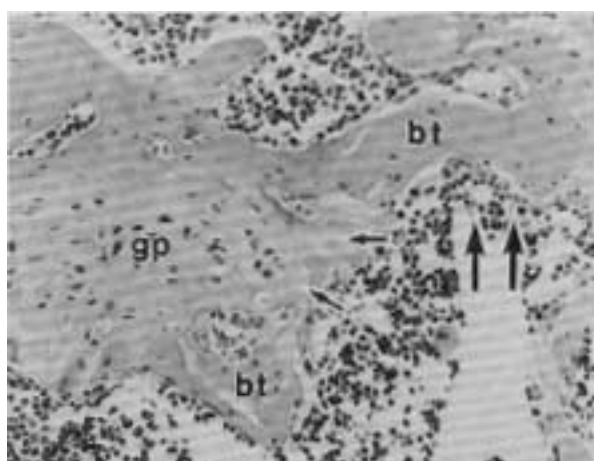


Fig. 3. Epiphyseal growth plate of proximal tibiae in a VA rat on Day 19. Reduction of chondrocytes in columnar arrangement and focal disappearance (large arrows) are observed. Uncalcified cartilage matrix is enclosed with the layers of calcified cartilage matrix (small arrows). The exposed surfaces of the calcified cartilage matrix are covered with a thin layer of bone tissue. bt: bone tissue. gp: epiphyseal growth plate. H.E. $\times 150$.

deposition of type II collagen was detected in the small areas of the uncalcified cartilage matrix at the epiphyseal side of the epiphyseal growth plate and in the calcified cartilage matrix within bony trabeculae, but not detected in the calcified cartilage matrix within the epiphyseal growth plates (Fig. 7B). In VA rats on Day 19, the deposition of type II collagen was detected throughout the cartilage matrix in the epiphyseal growth plate (Fig. 7C).

In control rats on Day 12 (Fig. 8A) and 19, the deposition of type I collagen was not detected in the cartilage matrix of the epiphyseal growth plate, but detected in the osteoid. In addition, osteoblasts were distinguished as type I collagen-

positive cells lining on the surfaces of bony trabeculae. In VA rats on Day 12, the deposition of type I collagen was detected in the most part of the uncalcified cartilage matrix in the epiphyseal growth plate. However, it was not detected in the calcified cartilage matrix within the epiphyseal growth plate. Osteoblasts and osteoid highly accumulated in the eroded regions in the epiphyseal growth plates (Fig. 8B). In VA rats on Day 19, the deposition of type I collagen was detected in a small area of the uncalcified cartilage matrix in the epiphyseal growth plate. A few osteoblasts and thin layers of osteoid were detected on the surfaces of bone tissue surrounding the epiphyseal growth plate (Fig. 8D).

In control rats on Day 12 (Fig. 9A) and 19, HPA-positive multinucleated cells, i.e., osteoclasts, adhered to calcified cartilage matrix at the chondro-osseous junction and primary bony trabeculae. In VA rats on Day 12, numerous osteoclasts adhered to the exposed surfaces of both uncalcified and calcified cartilage matrix at the eroded areas in the epiphyseal growth plate. In addition, some osteoclasts were detected within the epiphyseal growth plate (Fig. 9B and C). In VA rats on Day 19, osteoclasts occasionally adhered to the bone tissue surrounding the epiphyseal growth plate, but were not detected within the epiphyseal growth plate.

DISCUSSION

Cartilage matrix in the epiphyseal growth plate is calcified at the hypertrophic zone in the normal endochondral ossification. In the present study, the highly calcified cartilage matrix, which was distinguished as granular high BSE signals [11] or the basophilic granules by HE staining [14], was detected within the epiphyseal growth plate in VA rats on Day 12. Such calcified cartilage matrix contained large calcospherulites in VA rats, but did not contain them in the hypertrophic zone in control rats. These findings indicated that the calcification developed more

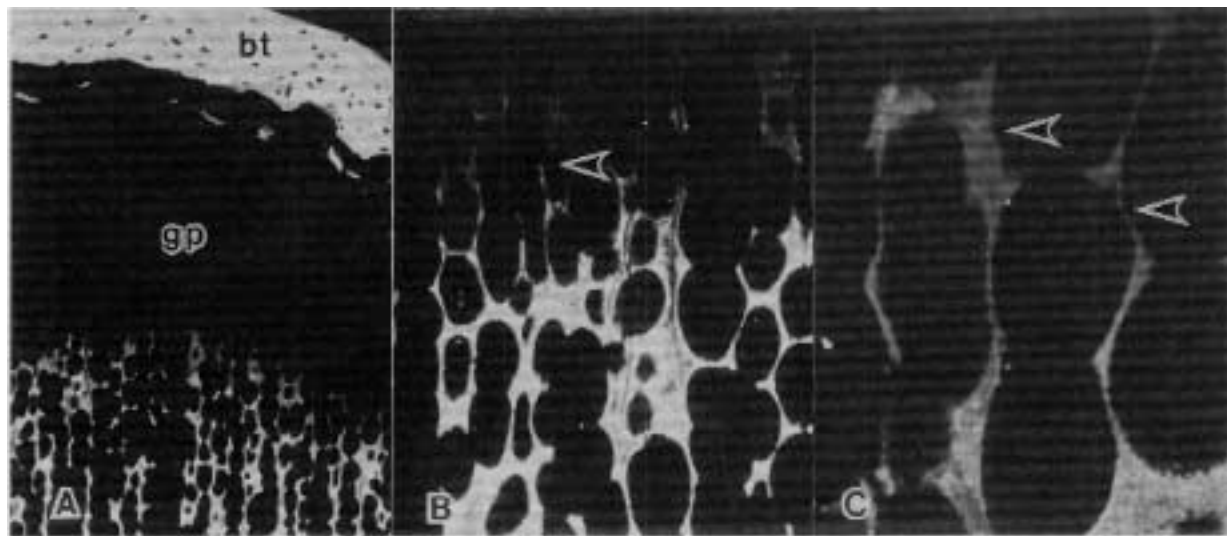


Fig. 4. Backscattered electron (BSE) images of epiphyseal growth plate of proximal tibiae in a control rat on Day 12 (A-C). A: Calcified cartilage matrix is detected around lacunae of chondrocytes in the lower hypertrophic zone and within the bony trabeculae. B and C: The calcified cartilage matrix is faint and thin (arrowheads) in the hypertrophic zone. bt: bone tissue. gp: epiphyseal growth plate. A: $\times 90$, B: $\times 450$, C: $\times 630$.

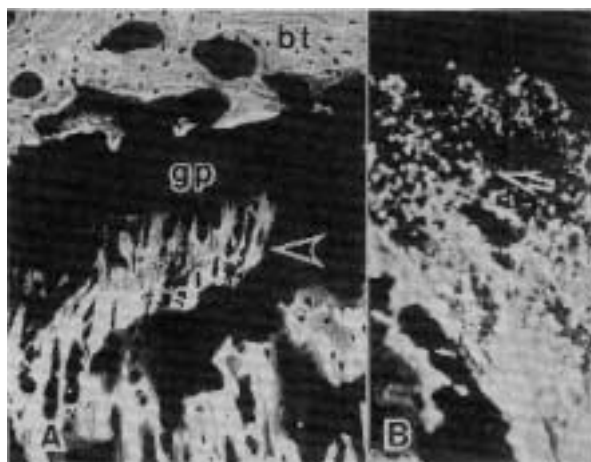


Fig. 5. Backscattered electron (BSE) images of epiphyseal growth plate of proximal tibiae in a VA rat on Day 12. A: Calcified cartilage matrix (arrowhead) is detected within the epiphyseal growth plate. B: The calcified cartilage matrix contains large calcospherulites (arrow). bt: bone tissue. gp: epiphyseal growth plate. A: $\times 90$, B: $\times 630$.

rapidly within the epiphyseal growth plate in VA rats than in the hypertrophic zone in control rats [8]. In the normal epiphyseal growth plate, the calcified cartilage matrix in the hypertrophic zone shows the deposition of type X collagen. However, the calcified cartilage matrix within the epiphyseal growth plate in VA rats on Day 12 showed neither the deposition of type X collagen nor the distribution of hypertrophic chondrocytes. In addition, some chondrocytes in columnar arrangement of the proliferating zone were occasionally detected within such calcified cartilage matrix.

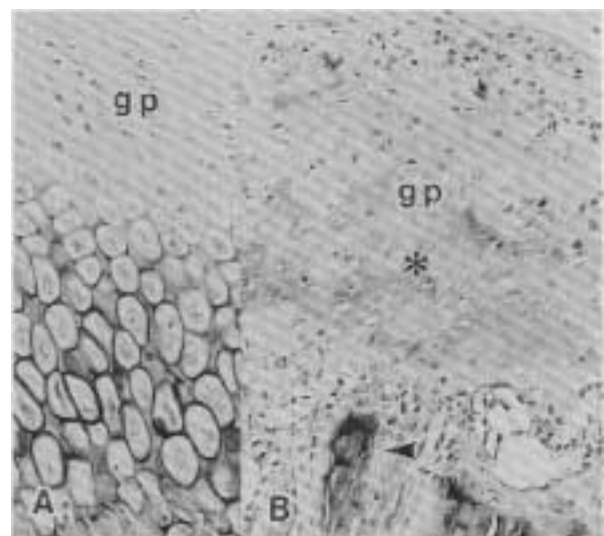


Fig. 6. Immunohistochemical staining of type X collagen in the epiphyseal growth plates of proximal tibiae in a control rat (A) and a VA rat (B) on Day 12. A: Type X collagen is detected in the cartilage matrix of the hypertrophic zone in a control rat. B: Type X collagen is not detected in neither uncalcified nor calcified cartilage matrix (asterisk) in the epiphyseal growth plate and is detected in calcified cartilage matrix within bony trabeculae (arrowhead) in a VA rat. gp: epiphyseal growth plate. $\times 150$.

These observations indicated that the calcification of the cartilage matrix in VA rats developed in the proliferating zone out of the process of calcification occurring in the normal hypertrophic zone.

Several previous reports demonstrated that retinoic acid,

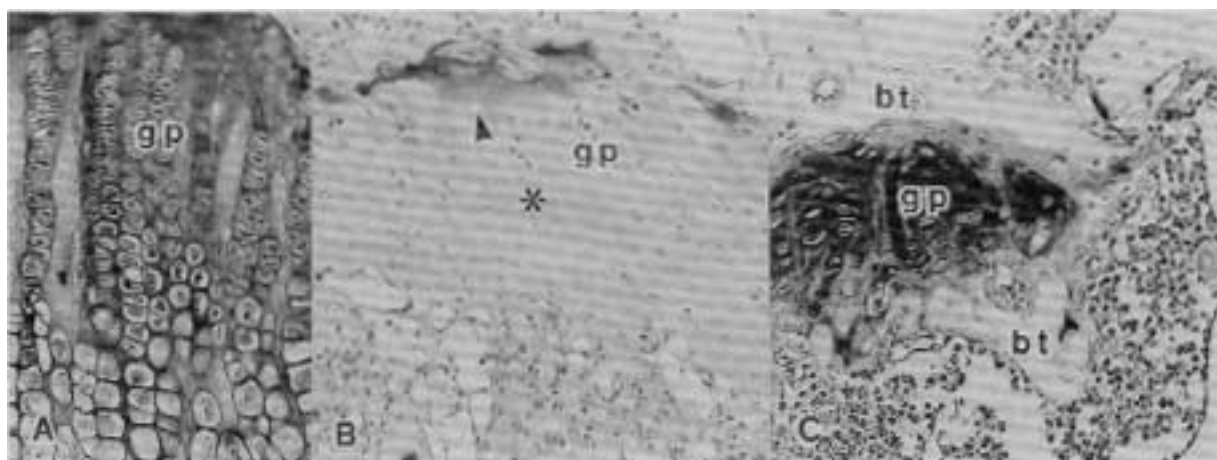


Fig. 7. Immunohistochemical staining of type II collagen in the epiphyseal growth plates of proximal tibiae in a control rat on Day 12 (A) and a VA rat on Day 12 (B) and Day 19 (C). A: Type II collagen is detected in the cartilage matrix throughout the epiphyseal growth plate in a control rat. B: Type II collagen is detected in the small areas of the uncalcified cartilage matrix at the epiphyseal side of the epiphyseal growth plate (arrowhead), but not in the calcified cartilage matrix (asterisk) within the epiphyseal growth plates in a VA rat on Day 12. C: Type II collagen is detected throughout the cartilage matrix in the epiphyseal growth plate in a VA rat on Day 19. bt: bone tissue. gp: epiphyseal growth plate. $\times 150$.

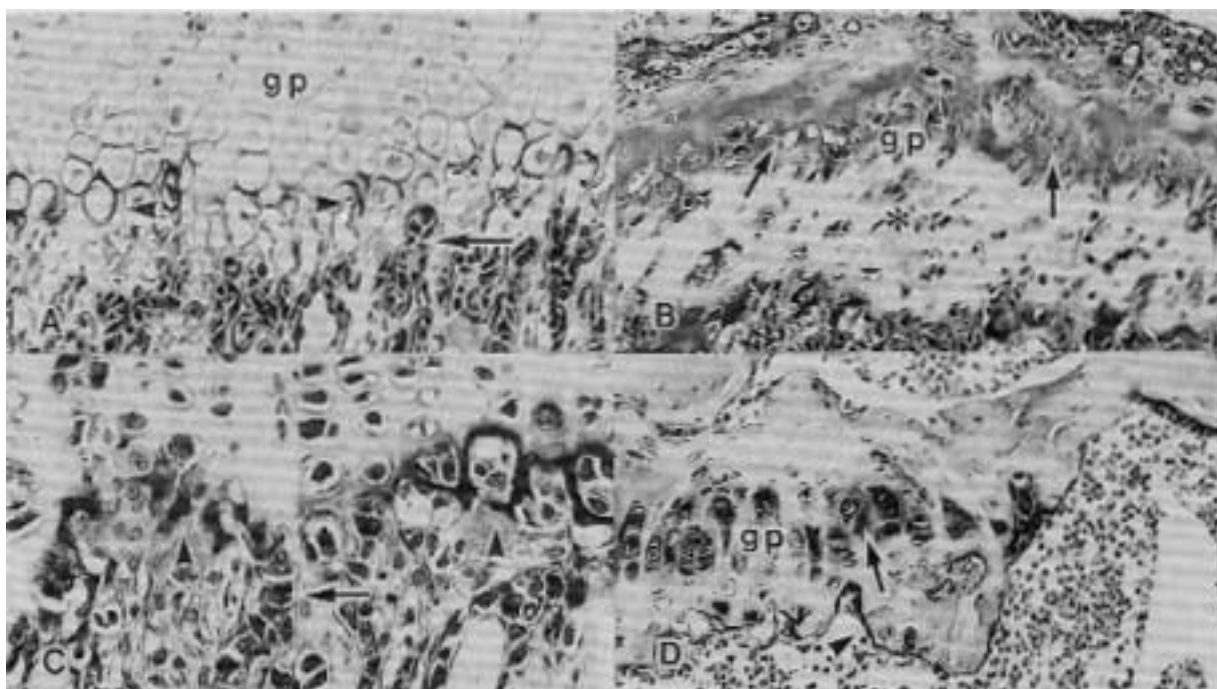


Fig. 8. Immunohistochemical staining of type I collagen in the epiphyseal growth plates of proximal tibiae in a control rat on Day 12 (A), VA rats on Day 12 (B and C) and Day 19 (D). A: Type I collagen is not detected in the cartilage matrix and detected in the osteoid (arrowheads) and osteoblasts (arrow) in a control rat. B: Type I collagen (arrows) is detected in the most part of the uncalcified cartilage matrix, but not in the calcified cartilage matrix (asterisk) in a VA rat on Day 12. C: Osteoblasts and osteoid highly accumulate in the eroded areas the cartilage matrix in a VA rat on Day 12. D: Type I collagen (arrow) is detected in small areas of the uncalcified cartilage matrix and thin layers of osteoid (arrowhead) are detected on the surfaces of bone tissue surrounding the epiphyseal growth plate in a VA rat on Day 19. bt: bone tissue. gp: epiphyseal growth plate. A and B: $\times 150$, C: $\times 300$, D: $\times 150$.

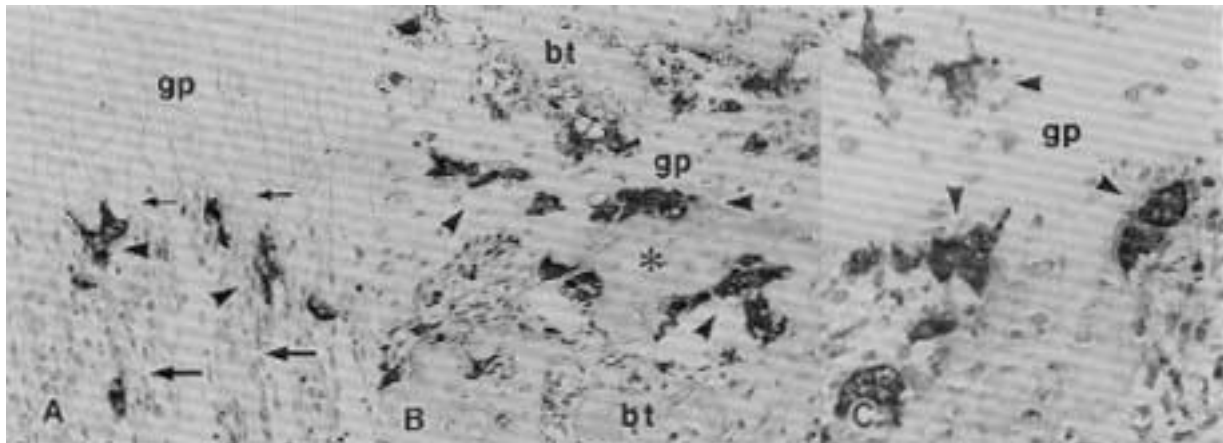


Fig. 9. Lectin-histochemistry of *Helix pomatia* (HPA) in the epiphyseal growth plates of proximal tibiae in a control rat (A) and VA rats (B and C) on Day 12. A: Osteoclasts (arrowheads), which are HPA-positive multinucleated cells, adhere to calcified cartilage matrix (small arrows) at the chondro-osseous junction and primary bony trabeculae (large arrows) in a control rat. B: Osteoclasts (arrowheads) are detected on the exposed surfaces of calcified cartilage matrix (asterisk) at the eroded areas and within the epiphyseal growth plate in a VA rat. C: Osteoclasts (arrowheads) adhere to uncalcified cartilage matrix in the epiphyseal growth plate in a VA rat. bt: bone tissue. gp: epiphyseal growth plate. A and B: $\times 150$, C: $\times 300$.

a derivative of VA, promoted the calcification of cartilage matrix. In *in vitro* study, Cancedda and co-workers reported that the calcification of cartilage matrix caused by retinoic acid occurred after the chondrocytes stopped the synthesis of type II collagen and initiated the synthesis of type I collagen [1]. We previously described that the same treatment of VA as employed in the present study caused the disappearance of type II collagen deposition and the appearance of type I collagen in uncalcified cartilage matrix in the proliferating zone at the end of the administration period [17]. Therefore, the calcification in the proliferating zone in VA rats on Day 12 seemed to occur after such phenotypical alterations in the cartilage matrix, in similar manner to that induced by retinoic acid *in vitro*.

In our previous study, we suggested that the chondrocytes in the epiphyseal growth plates in VA rats differentiated to the osteoblast-like cells and synthesized the cartilage matrix with the bone matrix phenotypes, because the cartilage matrix showed the disappearance of type II collagen and appearance of type I collagen [17]. In the present study, similar phenotypical alterations were detected in the most part of the uncalcified cartilage matrix in VA rats on Day 12. While in VA rats on Day 19, the deposition of type II collagen increased and the deposition of type I collagen decreased in the cartilage matrix as compared with those in VA rats on Day 12. From these observations, it was suggested that the effects of VA to chondrocytes in the epiphyseal growth plate were reduced and the differentiation of chondrocytes was more or less recovered on 14 days after the administration period for 5 days.

In the normal endochondral ossification, a modeling or remodeling process was observed at the chondro-osseous junctions. In such a process, osteoclasts absorbed the calcified cartilage matrix, and then osteoblasts formed bone tissues on the absorbed surface of the calcified cartilage

matrix [8–10, 16]. In this study, numerous HPA-positive multinucleated cells, which were proved to be osteoclasts [6], invaded the abnormal calcified cartilage matrix at the eroded areas in the epiphyseal growth plate in VA rats on Day 12. These findings indicated that osteoclasts absorbed the calcified cartilage matrix to result in the erosions of the epiphyseal growth plate. Normally, osteoclasts are known to be cells which absorb calcified tissues such as bone or calcified cartilage. However, in the present study, some osteoclasts were detected within the uncalcified cartilage matrix at the eroded areas in the epiphyseal growth plate in VA rats on Day 12. Recently, the adhesion between $\alpha 2\beta 1$ integrin on osteoclasts and Arg-Gly-Asp (RGD) site in type I collagen in the bone matrix was reported to play a important role in the absorption of bone matrix [7, 13]. Therefore, the cartilage matrix with the deposition of type I collagen may induce the invasion of osteoclasts to the uncalcified cartilage matrix in VA rats on Day 12. However, it was unclear whether these osteoclasts absorbed the uncalcified cartilage matrix, because induction of osteoclastic activity has been reported to require the adhesion of osteoclasts to the calcified matrix [15].

In the epiphyseal growth plate in VA rats on Day 12, osteoblasts and osteoid were detected on the surfaces of the eroded areas in accompany with osteoclasts. In addition, in VA rats on Day 19, the focal disappearances of the epiphyseal growth plate were observed and the exposed surfaces of the calcified cartilage matrix were covered with the bone tissue on the site of the focal disappearances. These findings indicated that a modeling or remodeling process, such as the absorption of the calcified cartilage matrix by osteoclasts and the formation of bone tissues by osteoblasts, were occurred within the epiphyseal growth plate in VA rats. Such a modeling or remodeling might further proceed, and finally bring about the focal

disappearances of the epiphyseal growth plate.

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