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Excessive mineralization with growth plate closure in rats on chronic warfarin treatment

(vitamin K deficiency/bone Gla protein/bone histomorphometry)

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ABSTRACT **Rats maintained for 8 months on a level of warfarin sufficient to decrease the vitamin K-dependent protein of bone (bone Gla protein) to 2% of normal have an excessive mineralization disorder characterized by complete fusion of the proximal tibial growth plate and cessation of longitudinal growth. The general features of this abnormality resemble the fetal warfarin syndrome in humans, a disorder also characterized by excessive mineralization of the growth plate. These excessive mineralization disorders may be caused by the decreased levels of bone Gla protein, a protein that potently inhibits mineralization *in vitro*.**

The metabolism of vitamin K presents several intriguing problems in vertebrate physiology and biochemistry. One of the most interesting of these is the role of vitamin K in systems other than blood coagulation. The classical approach to the analysis of vitamin K physiology has been the study of defects in vitamin K-deficient animals. Such studies have established that the first consequence of acute vitamin K deficiency is bleeding and death due to the synthesis of abnormal forms of blood coagulation factors such as prothrombin and factors VII, IX, and X. It is now known that the abnormality in these factors is the absence of γ-carboxyglutamate (Gla), a Ca^{2+} binding amino acid whose post-translational synthesis from glutamate requires vitamin K (1, 2).

Our primary interest over the past several years has been the function of bone Gla protein (BGP), a 49-residue protein of known structure (3, 4) which is numerically one of the 10 most abundant proteins in a typical vertebrate (5, 6). To analyze the function of this protein in bone metabolism we developed a simple protocol by which rats can be maintained on the vitamin K antagonist warfarin without problems due to bleeding (7). In previous studies we found that animals maintained from birth to 2 months of age on this protocol grow normally and have normal bone structure and mineralization in spite of bone levels of BGP which are only 2% of normal (7).

We report here the discovery of excessive mineralization with growth plate closure in the proximal tibia of rats maintained on warfarin from birth to 8 months of age.

MATERIALS AND METHODS

Maintenance of Animals on Warfarin. Simonsen albino rats (Sprague–Dawley-derived, Simonsen Laboratories, Gilroy, CA) were maintained from birth to 8 months of age on daily dosages of warfarin and vitamin K_1 as described (7). To facilitate comparisons, the 10 control rats were selected from litters born at the same time as experimental animals and received daily doses of saline and vitamin K_1 (7). Both experimental and control groups had seven male and three female rats. None of the 10 experimental rats chosen at birth for this study showed signs of bleeding or ill health over the 8 months of the experiment. As reported (7), the weight gain of experimental and control rats was identical up to 90 days of age. At 8 months of age the average weights were 402 g for the male and 237 g for the female experimental rats and 455 g for the male and 289 g for the female control rats. Serum calcium and phosphate levels were determined at sacrifice in experimental and control animals as described (7) and in neither instance was a significant difference noted.

The efficacy of the vitamin K-deficiency protocol was evaluated by measurement of BGP levels in formic acid demineralization extracts of whole ground tibias by radioimmunoassay procedures described elsewhere (7). The mean BGP level in the 10 experimental animals was 26.6 μg/g of bone and the mean in the 10 control animals was 1.46 mg/g.

Fluorescent Labeling. All rats received intraperitoneal injections of calcein (2,7-biscarboxymethyl-aminomethyl fluorescein; Sigma) at 30 mg/kg of body weight at 16 and 15 days before death and intravenous injections of tetracycline (Achromycin V; American Cyanamid, Pearl River, NY) at 15 mg/kg of body weight at 4 and 3 days before death.

Radiographs of Intact Tibia. Left tibias were removed at autopsy, cleaned of adhering tissue, and radiographed with a Hewlett–Packard model 4380N Flexitron system.

Histological Techniques. Right tibias were removed at autopsy, defleshed, trimmed with razor blades to expose the marrow cavities, and fixed in 70% alcohol. The proximal half of the tibia and a 6- to 8-mm portion of the tibial shaft immediately proximal to the tibiofibular junction were defatted and dehydrated in sequential changes of alcohol, acetone, and ether and then embedded in polystyrene resin (Plastic Fabrication and Supply, Salt Lake City, UT). Frontal sections of the proximal tibia and cross sections of the tibial shafts were cut at 280 μm with a precision saw. Selected sections were ground and polished at 100 μm thickness and microradiographed on Kodak spectroscopic plates 649-0 (Eastman Kodak) (8, 9). Bone sections were mounted on plastic microscope slides and then further ground to 30 μm for fluorescent label viewing by ultraviolet microscopy. Later, the 30-μm-thick bone sections were surface stained with 0.1% toluidine blue O and coverslipped for light microscopy.

Static and Dynamic Histomorphometry of the Proximal Tibial Growth Cartilage. Longitudinal bone growth, size of the degenerative or hypertrophic cartilage cells, growth plate thickness, and the fraction of the growth cartilage region with epiphyseal and metaphyseal mineralized tissue union were determined by light and ultraviolet microscopy by measuring distances between fluorescent labels, morphological features, and

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Abbreviation: BGP, bone Gla protein (vitamin K-dependent bone protein, osteocalcin).

volume determination (10–14). The rate of cartilage cell production was calculated by dividing the mean rate of longitudinal growth by the mean diameter of the degenerative cartilage cells measured along the longitudinal axis (12, 13).

Static Histomorphometry of the Proximal Tibial Epiphysis. The trabecular bone area, perimeter, fraction of bone, and surface-to-volume ratio were determined for longitudinal sections of the proximal tibial epiphysis. The cortex was excluded from the trabecular bone area by initiating measurements at a distance of two mean trabecular widths inside the cortical endosteal surface. Microradiographs were measured by using a quantitative television microscope (QTM; Imanco, Cambridge, England) coupled with an image editor and interfaced with a PDP-11 computer (14–16).

Static and Dynamic Histomorphometry of the Proximal Tibial Metaphysis. The trabecular bone area, perimeter, fraction of bone, and surface-to-volume ratio were also determined for longitudinal sections of the proximal tibial metaphysis. A metaphyseal area (about 2 × 2.8 mm) of the microradiographs of the proximal tibia was analyzed by using the same procedure as for the trabecular bone of the proximal tibial epiphysis. The mineral apposition rate (in micrometers per day) of the metaphyseal trabeculae (lower secondary spongiosa region only) was determined by measuring the distances between double labels by ultraviolet microscopy of 30-μm unstained and stained sections (11, 17, 18). Lastly, the occurrence of primary spongiosa was graded as present (+), partially present (±), or absent (0).

Static and Dynamic Histomorphometry of the Tibial Shaft. Total bone tissue area and total cortical bone area were measured from microradiographs of tibial shaft cross sections within 1,000 μm of the tibiofibular junction by using the quantitative television microscope and the image editor. The total area and the cortical bone of the tibial shaft were measured and the percentage of cortical bone in the tibial shaft or the ratio of cortical to total bone tissue was calculated. The volume-based bone formation rates at the periosteal and endosteal surfaces were determined by measuring the area between the two fluorescent labels or between the first fluorescent label and the respective mineralized bone surfaces (19, 20) by using a manual digitizer interfaced with an ultraviolet microscope and a small Hewlett–Packard computer.

Statistical Analysis of Data. The data were analyzed and the significance of the differences between experimental and control groups was determined by a 3-way analysis of variance which sorted out the variability due to differences between experimental and control groups, differences between animals, and differences between bone sections. The variability between animals was used to generate the error term for the determination of significance (21).

RESULTS

A typical radiograph of the left tibia from warfarin-treated and control rats is shown in Fig. 1. It is readily apparent that the growth plate of the experimental animal was more densely calcified. Microradiographs of sections from the right tibia of experimental and control animals show clearly that the growth plate of the experimental animal had fused whereas that of the control animal remained open (Fig. 2).

The battery of histomorphometric measurements made on the right proximal tibias demonstrated that all control animals had active longitudinal growth whereas all experimental animals

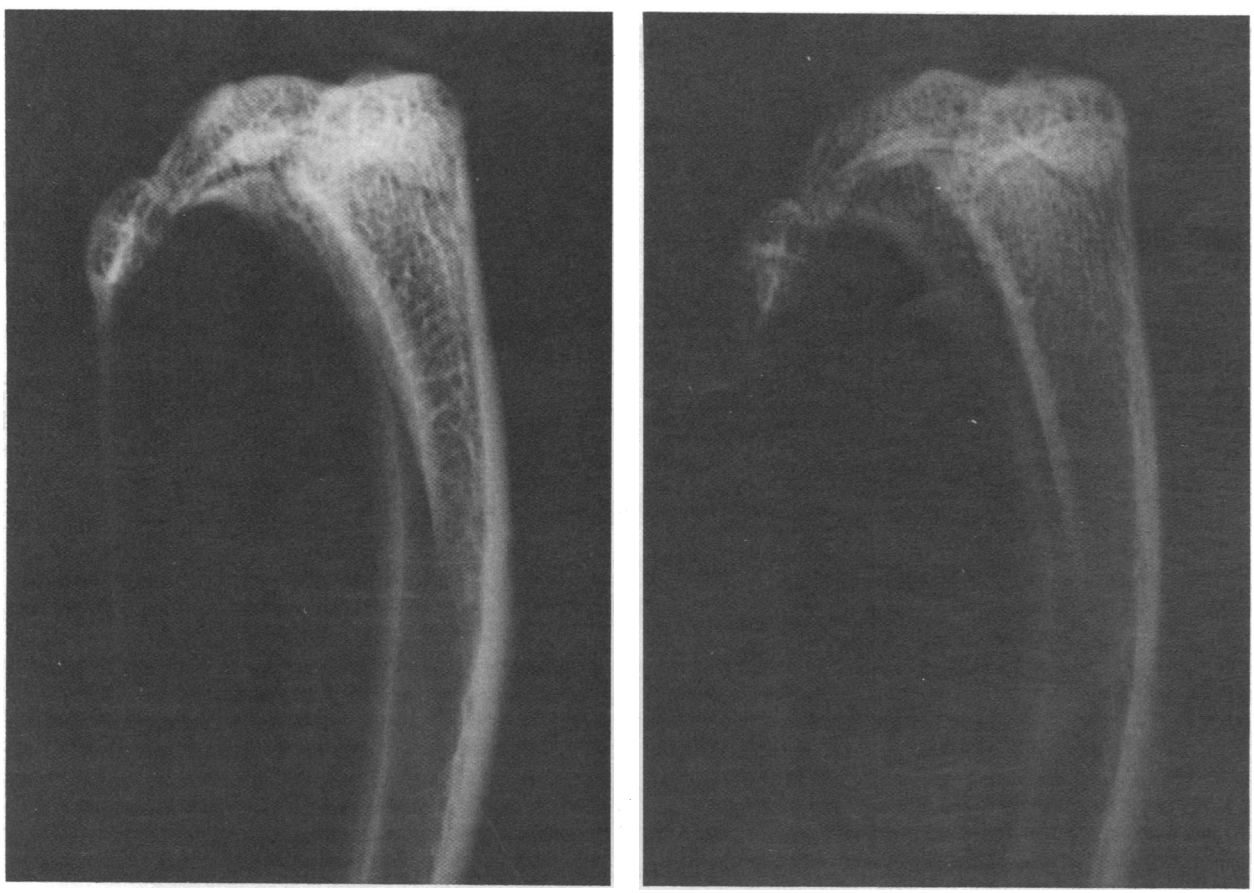

FIG. 1. Radiographs of representative left proximal tibia from rats maintained for 8 months on the warfarin (*Left*) or control (*Right*) protocol. (×5.)

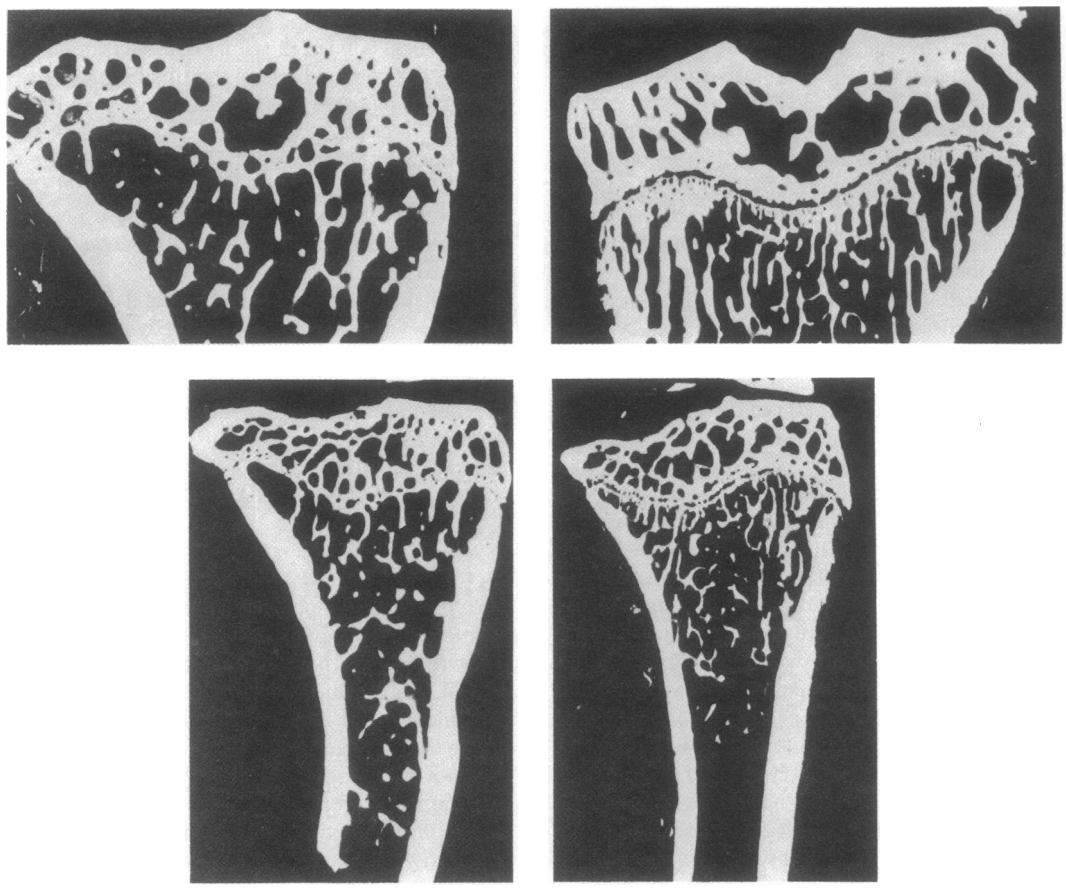

FIG. 2. Microradiographs of 100-μm-thick undecalcified frontal sections from the proximal tibia of warfarin-treated (*Left*) and control (*Right*) rats. Note the closed epiphysis and the bony union of epiphysis and metaphysis in the tibia from the warfarin-treated animals. (*Upper* ×10; *Lower*, ×5.)

had growth plate fusion with complete absence of longitudinal growth (Table 1; Fig. 3). As expected, overall tibial length was significantly decreased in the experimental animals.

Static bone tissue parameters of the proximal tibia also differed significantly between the experimental and control groups (Table 2). Bone mass was greater in the experimental group. It should be noted that the greater bone mass in the experimental animals also was apparent radiologically (Fig. 1). Although dynamic bone tissue parameters did not differ significantly between the experimental and control groups (Table 2), the statistical uncertainty in these measurements was high enough that it may be masking a difference sufficient to account for the greater bone mass in the experimental rats.

In a related experiment, rats were maintained from birth to 43 days of age on the same warfarin and control protocols used for the 8-month experiment. No differences could be detected in any histomorphometric parameters measured in the proximal tibias of the six experimental and six control animals.

DISCUSSION

The only skeletal disorder detected in chronically warfarin-treated rats is the complete fusion of the growth plate with associated cessation of all longitudinal growth. Growth plate clo-

Table 1. Parameters in the proximal tibia of control and warfarin-treated rats at 8 months of age

Measurement	$\bar{x} \pm$ SD: Control (n = 10)	$\bar{x} \pm$ SD: Warfarin-treated (n = 10)	P
Length of tibia, cm	4.16 ± 0.13*	3.86 ± 0.18*	<0.05
Width of growth cartilage, μm	111.5 ± 22.9†	0	
Rate of longitudinal growth, μm/day	9.69 ± 3.75	0	
Size of degenerative cell, μm	12.79 ± 1.60	0	
Rate of cartilage cell production, no./day	0.76 ± 0.25	0	
Fraction of growth cartilage with epiphyseal/metaphyseal union	0.16 ± 0.07†	0.81 ± 0.12†	<0.001
Occurrence of primary spongiosa‡	+ (n = 10)	0 (n = 5) ± (n = 5)	

* n = 7.
† n = 9.
‡ +, Present; ±, partially present; 0, absent.

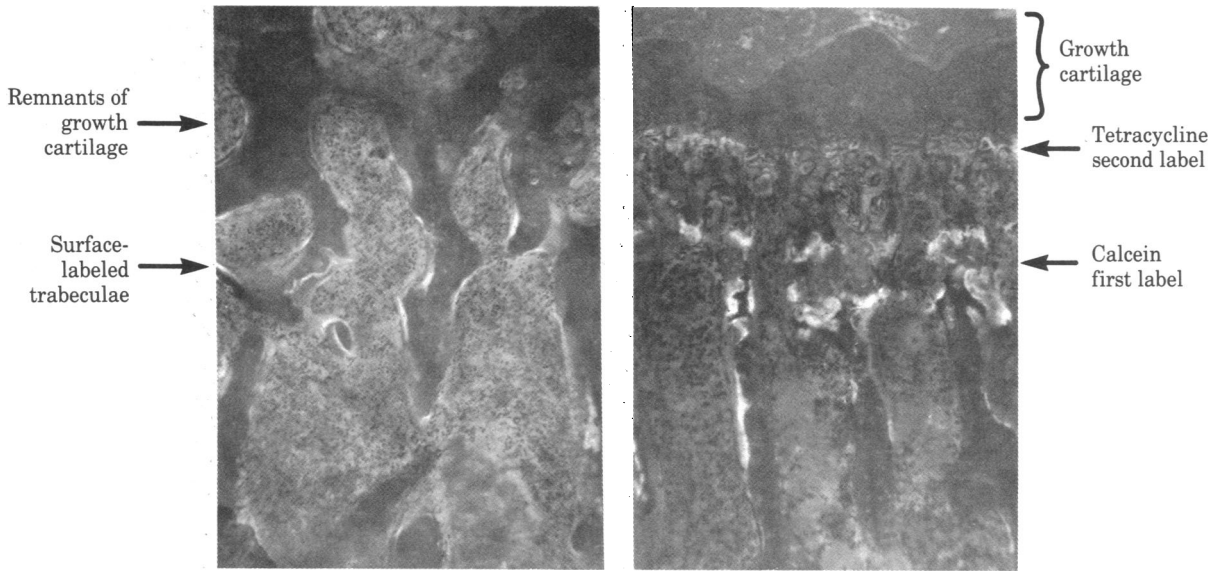

FIG. 3. Ultraviolet microphotographs of 30-μm-thick undecalcified frontal sections from the proximal tibia of warfarin-treated (*Left*) and control (*Right*) rats. Note the lack of fluorescent labeling in the metaphysis of the proximal tibial section from the warfarin-treated rat. (Surface stained with 0.1% toluidine blue O; ×130.)

sure was not seen in any control animal and, to our knowledge, growth plate closure never occurs in adult rats. We believe that this growth plate closure disorder in warfarin-treated rats reflects the impairment of a vitamin K-dependent system in bone rather than either sporadic bleeding due to defective synthesis of vitamin K-dependent coagulation factors in the liver or other toxic effects of warfarin on bone. Bleeding seems an unlikely explanation of growth plate closure because the experimental rats showed no evidence of hemorrhage at any time in the 8 months of warfarin treatment. The growth plate also is uniformly closed in both tibias of all experimental animals, a result that seems inconsistent with random occasional hemorrhagic events. Although we cannot rule out a toxic effect of warfarin on bone that is unrelated to its activity as a vitamin K antagonist, the fact that the warfarin protocol reduces bone levels of BGP to less than 2% of normal certainly demonstrates that the effect of the warfarin protocol on vitamin K-dependent systems in bone is of sufficient magnitude to provide an explanation for the excessive mineralization disorder observed here.

The growth plate closure abnormality seen here in chronically warfarin-treated rats bears a close resemblance to the fetal warfarin syndrome, a defect characterized by radiological stippling of the growth plate in children born to mothers who had received warfarin during weeks 8 to 11 of gestation (22). Although the fetal warfarin syndrome is characterized by islands of calcification within what normally is uncalcified growth plate rather than growth plate fusion, it seems reasonable that growth plate fusion could represent the eventual end point of the growth plate stippling defect were the same effective degree of vitamin K deficiency maintained over a longer period of fetal life. It should be noted that the fetal warfarin syndrome has been reported only with vitamin K antagonists and not with other

Table 2. Static and dynamic bone tissue parameters in proximal tibia of control and warfarin-treated rats at 8 months of age

Parameter	$\bar{x} \pm$ SD Control ($n = 10$)	Warfarin-treated ($n = 10$)	P
Static:			
Epiphyseal			
Bone area, mm^2	1.52 ± 0.46	2.02 ± 0.81*	<0.05
Perimeter, mm	27.46 ± 6.62	38.72 ± 10.69*	<0.001
% of bone	38.77 ± 9.98	47.14 ± 8.85*	<0.05
Surface/volume, mm^2/mm^3	23.94 ± 6.26	25.14 ± 4.26*	
Total area of tibial shaft, mm^2	6.77 ± 1.47	6.05 ± 1.17	
Cortical area of tibial shaft, mm^2	5.27 ± 0.78	4.79 ± 0.88	
Cortical area/total area, $\times 10^2$	74.75 ± 2.15	79.29 ± 2.51	<0.05
Dynamic:			
Mineral apposition rate of metaphyseal trabeculae, μm/day	0.76 ± 0.15	0.70 ± 0.19	
Cortical endosteal bone formation rate, $mm^2 \times 10^4$/day	3.40 ± 4.2	4.35 ± 4.60	
Periosteal bone formation rate, $mm^2 \times 10^3$/day	4.98 ± 2.83	2.64 ± 6.65	

* $n = 9$.

anticoagulants such as heparin, further supporting the view that growth plate defects are due to inhibition of a bone-specific vitamin K-dependent system rather than to bleeding.

It seems likely that the vitamin K-dependent bone system whose deficiency in warfarin-treated rats causes the growth plate closure disorder is BGP. BGP is the only vitamin K-dependent protein isolated from bone to date. BGP also strongly inhibits the precipitation of hydroxyapatite from supersaturated solutions of calcium and phosphate (6, 23) and so could be the means by which excessive mineralization of the growth plate is normally prevented *in vivo*. Finally, BGP synthesis is increased dramatically by the active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3 (24, 25). If BGP inhibits the formation of calcified tissue, as postulated to explain the growth plate defect, it could contribute to the action of this metabolite in mobilizing bone calcium by retarding ongoing mineralization.

If BGP inhibits the mineralization of calcified tissues as postulated, an excess of BGP could have the effect of reducing the normal level of mineralization. This raises the intriguing possibility that the 10- to 20-fold increase in serum BGP in anephric rats (26) and humans in renal failure (27) may cause the mineralization block seen in renal osteodystrophy.

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