Structural changes in blood vessels entering the growth plate during growth in rats

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Accepted: 2 August 1997

Summary. *We observed the structural changes in blood vessels entering the growth plates of the femur and tibia of rats during growth using scanning electron microscopy. The penetrating vessels had blind endings which were bulbous at a time when rats showed rapid skeletal development. With subsequent slowing of development, the density of the vessels decreased and the blind endings became short stumps. These changes were more prominent in the proximal femur than in the distal femur and proximal tibia. The present study indicates an intimate relation between endochondral ossification in the growth plate and the structure of the penetrating vessels.*

Résumé. *En utilisant la microscopie électronique à balayage les auteurs ont fait l'étude ultra-structurale des vaisseaux sanguins entrant dans les plaques de croissance des fémurs et des tibias chez des rats pendant la croissance. Les vaisseaux perforants avaient des boucles terminales de structure bulbeuse quand le développement squelettique des rats était intense. Avec le déclin du développement, la densité des vaisseaux a diminué avec des terminaisons en moignon. Ces changements ont été plus marqués au fémur proximal qu'au fémur distal et au tibia proximal. Cette étude montre un rapport intime entre l'ossification endochondrale des plaques de croissance et la structure des vaisseaux perforants.*

Introduction

The blood vessels entering the growth plate from the metaphysis play a vital role in the endochondral ossification of the growth plate [7, 19]. Investigation of the structure of the invading vessels can be expected to contribute not only to the elucidation of the mechanism of endochondral ossification but also to shed light on the pathology of congenital anomalies of the skeleton, metabolic diseases in children, traumatic lesions of the growth plate and various other pathological states associated with disorders of endochondral ossification. Trueta [17–19] and Ogden et al. [11] conducted studies with a light microscope on the vascular structure in the growth plate. Subsequently, a method for the preparation of vascular casts was established, making it possible to observe the vascular structure in three dimensions by scanning electron microscopy (SEM) [9, 15]. However, none of these studies went beyond the observation of a single growth plate or a single period of growth.

The purpose of the present study was to investigate by SEM the microscopic structure of vessels invading the growth plate of the femur and tibia in rats with reference to growth-related changes and variations among growth plates in different regions.

Materials and methods

A total of 40 male Wistar rats were raised in conventional rat cages at the Laboratory Animal Centre for Biomedical Research, Nagasaki University School of Medicine. After measurement of body weight, 10 rats each in 4, 8, 9 and 15-week age groups were subjected to the following procedures.

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Table 1. Changes of body weight and bone length during growth

Age (weeks)	4	6	9	15
Weight (g)	116±9	245±14	356±11	481±17
Length (mm)				
Femur	23±0.6	28±0.5	35±0.9	40±1.0
Tibia	27±1.1	33±0.5	40±0.7	45±0.9

Table 2. Changes of growth plate thickness during growth

Age (weeks)	4	6	9	15
Thickness (μm)				
Proximal femur	102±12.2	51±6.8	36±5.4	27±5.2
Distal femur	241±23.4	234±20.7	138±14.3	107±12.5
Proximal tibia	235±21.6	216±20.6	140±15.7	100±14.0

Fig. 1 a, b. SEM of vascular casts in the proximal tibia of a rat at 6 weeks of age. **a** The penetrating vessels run densely and in straight lines. $\times 80$ (*Bar* 100 μm). **b** The terminal vasculature shows vessels with blind terminals bulbous in shape, 10 to 20 μm in diameter and arranged in a cobblestone pattern. $\times 500$ (*Bar* 10 μm)

Fig. 2. SEM of the proximal tibia of a rat at 9 weeks of age. Knob-like projections of leaking resin are present on the terminal parts of the vessels. $\times 1000$ (Bar 10 μm)

Preparation of vascular casts

The abdominal aorta and inferior vena cava were exposed under ether anaesthesia, a catheter was inserted into the aorta in the direction of the tail, followed by perfusion with 10,000 units of heparin per 0.5 l of saline at a pressure of about 100 cm H₂O.

Then, with the inferior vena cava open for venous outflow, methyl methacrylate (Mercor, Dainihon Ink and Chemical Co Ltd, Tokyo) was infused via the abdominal aorta at a pressure of between 100 and 120 mmHg. After the methyl methacrylate hardened completely, the left and right femurs and tibiae were removed and fixed in 10% formalin.

Radiographs

Full-size soft X-rays (CMB2, Softex Co, Ltd) of the femora and tibiae were taken, and the lengths measured using a digitizer.

Preparation of SEM specimens

After radiographs were taken, the removed bones were demineralised in a mixture of hydrochloric and formic acid. The proximal femur, the medial half of the distal femur and the proximal tibia were then split longitudinally; one half of the bone was used for a SEM study and the other half for histology. After defatting in 80% ethanol, the epiphyseal nuclei were severed along the centre of the growth plate using a dissecting microscope. This procedure allowed the vascular casts to be removed from the epiphysis and clear observation of the structure of the vessels invading the growth plate from the metaphysis. The specimens were then fixed to thick paper with resin in such a way that the growth plate faced upward [8]. They were left to corrode in 20% NaOH for 48 hours at 50 °C and then washed in running water until only the vascular casts remained. After drying in air, the casts were mounted on sample holders and coated with gold. Finally, the casts were

examined by SEM (JSM-35S, JEOL) to determine the changes associated with the growth of the vessels invading the growth plate.

Preparation of histological specimens

For light microscopy, the specimens were embedded in paraffin and stained with hematoxylin and eosin, and measured with a digitizer to determine the thickness of the central portion of the growth plate.

The histological findings corresponding to the structure of the vascular casts observed by SEM were also investigated.

Results

Weight and bone length of the rats

The body weight of the rats increased by an average of 48 g per week from the age of 4 to 9 weeks (Table 1), but declined to 20.8 g per week thereafter. The growth of bones was also rapid from the age of 4 to 9 weeks, the tibiae increasing in length by an average of 2.6 mm per week during this period (Table 2).

After 9 weeks, the rate of growth decreased to 0.8 mm per week. The changes in the length of the femur were similar to those of the tibia.

SEM findings

The distal femur and proximal tibia showed similar findings during the period from 4 to 9 weeks of age. The invading vessels ran close together and in straight lines towards the growth plate. The terminal vasculature contained vessels with blind bul-

Fig. 3a, b. SEM of the proximal tibia of a rat at 15 weeks of age. **a** The invading vessels have become sparse. $\times 80$ (*Bar* 100 μm). **b** The vessel endings diminish from a bulbous shape to short stumps about 10 μm in diameter. $\times 500$ (*Bar* 10 μm)

bous endings, 10 μm to 20 μm in diameter and arranged in a cobblestone pattern (Fig. 1). Knobbly projections of leaking resin were observed on the terminal portion of the vessels (Fig. 2) [15].

At the age of 15 weeks, the invading vessels running toward the growth plate became sparse and the blind endings changed from a bulbous shape to short stumps of about 10 μm in size (Fig. 3). The leakage of resin also decreased in this period.

Up to the age of 9 weeks, the invading vessels running toward the growth plate in the proximal femur zigzagged and entwined with each other in a complex tangle, and the terminal vasculature showed the same bulbous blind terminals as those recognised in the distal femur and proximal tibia (Fig. 4). At 15 weeks of age, the terminal vasculature showed stump-like ends and the density of vessels was remarkably lower than that in the other growth plates (Fig. 5).

Fig. 4a, b. SEM of the proximal femur of a rat at 6 weeks of age. **a** The invading vessels zigzag and entwine with each other in a complex tangle. $\times 80$ (Bar 100 μm). **b** The structure of the terminal vasculature is similar to that in the distal femur and proximal tibia. $\times 500$ (Bar 10 μm)

Histological findings

The decrease in the thickness of the growth plate during growth was an average of 19.0 μm per week in the tibia from the age of 4 to 9 weeks (Table 2) and 6.7 μm a week thereafter. The decrease in the distal femur was similar to that in the tibia. The decrease in the thickness in the proximal femur was an average of 13.2 μm a week from the age of 4 to 9 weeks and 1.5 μm a week thereafter.

Histologically, the proliferating zone and hypertrophic zone of the growth plate showed regular, columnar arrangement of chondrocytes in all regions up to the age of 9 weeks (Fig. 6a, b).

At 15 weeks, the arrangement in the hypertrophic zone became disordered and the chondrocytes became smaller and fewer in number (Fig. 6c). These changes after the age of 9 weeks were particularly prominent in the proximal femur (Fig. 7).

Fig. 5. SEM of the proximal femur of a rat at 15 weeks of age. The stump-like vessel endings have become remarkably sparse. $\times 500$ (Bar 10 μm)

Fig. 6. Histological findings of the proximal tibial growth plate. **a** At 4 weeks of age. **b** At 9 weeks of age. **c** At 15 weeks of age. The chondrocytes in the proliferating zone and hypertrophic zone show regular, columnar arrangement up to 9 weeks of age. At 15 weeks, the chondrocytes in the hypertrophic zone are disordered and smaller in size. HE stain, $\times 100$

Fig. 7a–c. Histological findings of the growth plate of the proximal femur. The changes after the age of 9 weeks are more prominent than those in Figure 6. HE stain, $\times 200$

Discussion

The elucidation of the relation between the extent of endochondral ossification and the structure of vessels invading the growth plate is vital to an understanding of the mechanism of ossification of the growth plate. The present study revealed a difference in the structure of the penetrating vessels between the distal femur and proximal tibia where bone development is prominent, and the proximal femur where it is not as prominent. It was also noted that, in all the growth plates, the structure changed with age. In the deepest part of the hypertrophic zone, osteocytes appeared around the periphery of the invading vessels and the calcified cartilage matrix converted to bone tissue [18, 19].

It is thought that the invading vessels are involved mainly in the differentiation and proliferation of undifferentiated stem cells to osteoblasts and in the supply of nutrients for bone development [7].

Research has shown that the vessels in this area have a unique structure. Trueta [17, 18, 19] and Brighton et al. [2, 3] used light microscopy to observe the vascular casts of the invading vessels in cleared specimens and reported that after, or immediately before, reaching the deepest hypertrophic cells the vessels turn around and form loops. Hunter et al. [9] observed the vascular casts by SEM and reported the existence of terminal vasculature with stick-like blind terminals protruding from the loops. Referring to the structure of this terminal vasculature, Stanka et al. [15] stated that navel-like blind terminals with uneven surfaces predominate. Our study also revealed the presence of blind endings in the terminal vasculature. From 4 to 9 weeks of age, bulbous blind terminals, similar in size to hypertrophic chondrocytes, were arranged densely in a cobblestone pattern. After that, the terminal vasculature shows stump-like endings and the vessels were sparse. The changes during growth were more prominent

in the proximal femur than in the distal femur or proximal tibia. Moreover, the vessels prior to the terminal vasculature showed a tangled structure in the proximal femur, but ran in straight lines in the distal femur and proximal tibia. In the femur, 70% and 30% of bone development in the longitudinal direction occurs in the distal and proximal parts respectively [4, 12, 14]. In the tibia, the proportion is 40% and 60%. The regional difference in vascular structure probably reflects this disparity in extent of ossification. Indeed, the changes in the terminal vasculature were also related to the increase in the weight and bone length of the rats. During the period from 4 to 9 weeks of age when this increase is greatest, the blind ends of the terminal vessels were bulbous in shape and densely distributed, but when the increase declined the ends became stump-like and the distribution sparse. The changes in the structure of the vessels were also related to the decrease in the thickness of the growth plate. The period from 4 to 9 weeks of age corresponds to the time when the thickness of the growth plate shows a marked decrease, reflecting the promotion of vessels invading the growth plate. If the age of rats is applied to humans on the basis of reproductive age, the period from 4 to 9 weeks corresponds to the secondary sex character period when growth is greatest [10, 13]. It is likely, therefore, that the changes in structure associated with the development of the terminal vasculature reflect the disparity not only among different regions, but also in the speed of bone formation.

In the present study, we observed the microscopic structure of vessels invading the growth plate under physiological conditions. It has been reported that a decrease in the number of penetrating vessels and other abnormalities occur in pathological states such as in a rachitic rat where endochondral ossification is impaired [1, 5, 6, 16]. It is necessary now to clarify the abnormalities of vessels invading the growth plate and in that way to shed light on the various lesions arising from disorders of the growth plate.

Acknowledgements. This study was supported by a Japan Orthopaedic and Traumatological Foundation Inc Arkea Award no 0063.

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