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A staining method for bone canaliculi

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The modified Bodian method with protargol (silver protein) is ordinarily used to detect nerve fibers. With this technique, applied to decalcified rat bone sections, the bone canaliculi were clearly stained black with good contrast to the bone matrix in both lamellar and woven bone. In addition, the connections between the bone canaliculi and other canaliculi, osteoblasts, osteoclasts, and chondrocytes were easily detectable. We found that the bone canaliculi of woven bone were fewer in number and ran more irregularly than those of lamellar bone.

We believe that this staining method for bone canaliculi in decalcified bone is superior to previously reported methods and may be useful in studies on bone pathology.

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Bone canaliculi are largely undetectable in conventional sections of decalcified bone stained with hematoxylin-eosin (Kolliker 1853). Histologic studies of the details of bone canaliculi have advanced slowly, because it is time- and labor-consuming to produce ground bone tissue sections. Moreover, since ground sections are much thicker than those used for conventional histology, all of the cellular components are lost, making it difficult to clarify the relationship between bone canaliculi and cells. Electron microscopy has demonstrated that bone canaliculi contain the cytoplasmic processes of osteocytes (Holtrop and Weigner 1972, Pawlick 1975) and that they are interconnected by tight or gap junctions (Doty 1981, Shapiro 1988).

However, electron microscopic studies are not useful for clarifying the histologic or three-dimensional relationship of bone canaliculi to osteocytes, osteoblasts and chondrocytes during the ossification process, because it is only possible to target one or two cells. We recently discovered an excellent staining method for histologic detection of the fine structure of bone canaliculi using thin sections of decalcified bone tissues that were embedded in paraffin by ordinary methods.

Material and methods

The tibiae of normal young rats (Wistar, male, average body weight 150 g) were fixed with 10% buffered formalin immediately after resection. After 3 days to 1 week of fixation, the bones were decalcified in 20% buffered EDTA solution for 3 weeks at room temperature. The decalcified bones were trimmed and embedded with paraffin. Each section was sliced to a thickness of 4–6 μm and was mounted on glass slides coated with gelatin film, which prevents detachment of sections from the slides.

Staining was effected by a modified version of the Bodian method (Bodian 1936, 1937) that used protargol to demonstrate nerve fibers. After deparaffinization, the sections were rinsed with double-distilled water (DDW) at room temperature. The sections were then stained with 2% protargol (BDH Co., UK) solution containing copper balls (7.5 g to 40 mL staining solution) for 48 hours at 37 °C. The reduction of silver in the sections was accomplished with a solution containing hydroquinone and formalin (1 g hydroquinone, 5 mL conc. formalin and 100 mL DDW) for 30 minutes at 5–10 °C. Following this, the sections were rinsed 3 times with DDW at room temperature, then treated with a solution of 0.2% gold chloride for 50 minutes at 5–10 °C and rinsed 3 times with DDW at 5–10 °C. The color balance of the stained sections was modulated with 2% oxaluric acid solution for 60 minutes at 5–10 °C. Finally, after rinsing with DDW for 5 minutes, the sections were embedded in balsam after dehydration with alcohol.

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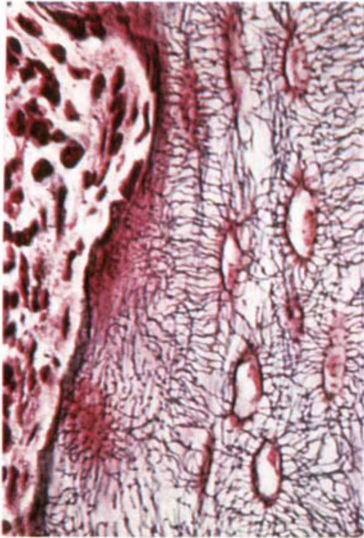
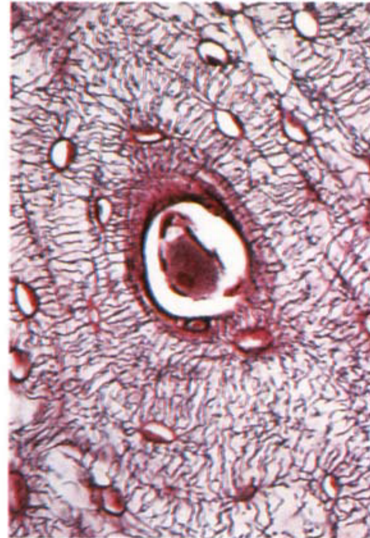
Figure 1. Both microphotographs show the histological stainability of bone canaliculi in decalcified cortical bone tissue obtained from normal rat tibia, using the staining method with protargol (×400). Since the bone canaliculi stain black and are in excellent contrast to the bone matrix in both sections, structural details are easy to detect, even at the terminal ends.

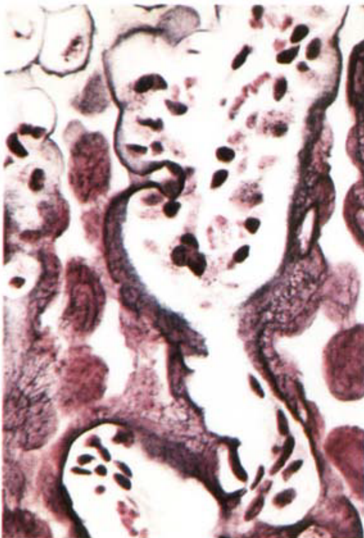


The section cut longitudinally to the long axis of the bone.

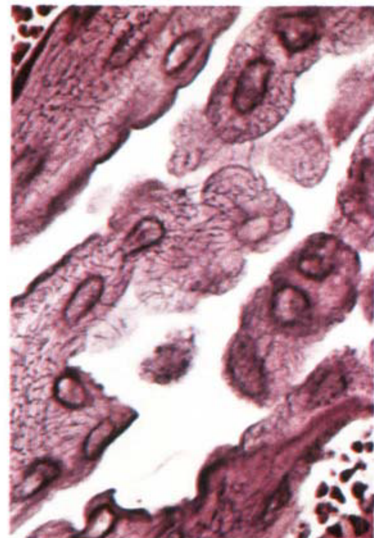


The cross-section.

Figure 2. The process of enchondral ossification observed at the junction between the growth plate and metaphyseal bone marrow in the proximal rat tibia



Histological findings at the junction between the growth plate (upper portion) and the metaphyseal bone marrow (lower portion) of decalcified bone tissue in the rat proximal tibia after staining with protargol (×400). Chondrocytes, osteoblasts, fibroblasts and hematopoietic cells are preserved intact and their nuclei are stained black with protargol. However, the cartilage matrix is unstained. Note that the woven bone osteocytes in the middle area have a very fine bone canaliculi structure in the osteoid matrix.



High magnification (×800) of woven bone at the metaphysis, showing that these canaliculi are fewer in number and run more irregularly than those of lamellar bone.

Results

Osteoblasts lining the cortex and hematopoietic cells in the bone marrow and the haversian canals were intactly preserved. The nuclei of all cells were conveniently stained black and the cytoplasm was stained pale red. Bone canaliculi ran densely and regularly towards the periosteum, haversian canals and bone marrow from the osteocytic lacuna, and they seemed to connect to other canaliculi or osteoblasts. A few of the canaliculi ran longitudinally to connect to other osteocytes. The bone canaliculi were slightly wavy or curly (Figure 1). The process of enchondral ossification observed at the junction between the growth plate and metaphyseal bone marrow were studied in the proximal tibia (Figure 2). Many osteoblasts were attached to the cartilage matrix, the edge of which was stained black. In the distal region, these osteoblasts differentiated to immature osteocytes surrounded by an osteoid matrix. They had a very fine structure of bone canaliculi that stained black in the matrix. Their bone canaliculi were fewer and were more irregularly distributed than those of osteocytes found in cortical bone.

Discussion

To stain bone canaliculi in decalcified bone sections, Schmorl (1934) introduced the thionin-picric acid method and Christie (1977) reported the nitric silver method. However, the stainability of bone canaliculi with both of these methods was not superior to that in ground sections. Recently, Shapiro (1988) found that the toluidine-blue staining of plastic-embedded bone tissue after fixation with glutaraldehyde and decalcification with EDTA was useful for the histological detection of bone canaliculi. However, only bone canaliculi in cortical bone were presented and they stated that it was difficult to detect canaliculi in woven bone.

Unlike these staining methods, our method using protargol provided excellent stainability of bone canaliculi in decalcified bone sections. Even in woven bone, a very fine structure of bone canaliculi can be detected in the osteoid matrix. Furthermore, soft tissue components such as osteoblasts, fibroblasts, osteoclasts and chondrocytes are preserved intact with this staining method. Therefore, the connection between bone canaliculi and cells is easily observed and a comparison with the findings of conventional histology using HE staining is possible. In this study, we found that the bone canaliculi of woven bone were fewer in number and ran more irregularly than those of lamellar bone. This finding may be important for distinguishing woven bone from lamellar bone, which is sometimes difficult to do in HE-stained sections.

To obtain constant stainability, it is essential to maintain a constant low temperature (5–10 °C) during the staining procedure. Furthermore, protargol should be selected with care, because the quality varies widely.

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