Changes in the Orientation of Collagen Fibers on the Superficial Layer of the Mouse Tibial Bone after Denervation: Scanning Electron Microscopic Observations

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Summary. This study was undertaken to evaluate the relationship between the mechanical stress loaded onto the bone and the orientation of collagen fibers formed by osteoblasts. The femoral, obturator, and sciatic nerves in the left posterior legs of 7-week-old mice were exposed and electrocoagulated to reduce the mechanical stress loaded onto the leg. Four weeks after operation, the tibial bones in the control and denervated legs were removed and observed by scanning electron microscopy (SEM) after NaOCl treatment. In the control right tibia, collagen fibers on the superficial bone matrix tended to be arranged parallel to the longitudinal axis of the bone. However, the arrangement of collagen fibers in the left tibia, which were immobilized for 4 weeks by denervation, was disorganized and ran in random directions. The findings suggest that the direction of collagen fibers in the bone changes in response to the mechanical stress loaded onto the bone, probably due to changes in the activity of osteoblasts in the denervated leg.

The orientation of bone structures such as bone lamellae, osteons and trabeculae is closely related to the direction of the mechanical stress loaded onto the bone (Bloom and Fawcett, 1986; Boyde and Riggs, 1990; Martin, 1991). For example, collagen fibers of bone lamellae in femurs and tibiae usually run parallel to the longitudinal axis of the bone shaft, since these bones are weighted to the direction of their longitudinal axes.

It has been well established that the bone matrix is formed by osteoblasts at the forming surface of bone (Jones et al., 1975; Bloom and Fawcett, 1986; Cormack, 1987; Marks and Popof, 1988). Using scanning electron microscopy (SEM), we have recently revealed that collagen fibers in the mouse tibial bone matrix are first formed along the processes of osteoblasts (Abe et al., 1998). This finding indicates that osteoblasts may influence the arrangement of collagen fibers in the forming bone in response to the direction of mechanical stress loaded onto the bone. In other words, the orientation of collagen fibers may change if the load to the bone is removed. However, little is known about the relationship between the arrangement of collagen fibers and mechanical stress loaded onto the bone. In the present study, we examined the arrangement of collagen fibers in the mouse tibial bone by removing the weight-load in the leg, using SEM after NaOCl treatment. To remove the weight stress, lower leg paralysis was induced by denervation.

MATERIAL AND METHODS

Animals and experimental procedures
Four adult female dd-mice (7 weeks of age) were used for this experiment. They were maintained in cages placed in a well-ventilated chamber, and food (a commercial pellet) and water were provided. Each animal was anesthetized by pentobarbital sodium (Nembutal; Dinabott) injected intraperitoneally at a dosage of 50 μg/g body weight, and surgically had their femoral, obturator and sciatic nerves supplying the left posterior legs cut by electroscissors. They were then maintained under the same condition mentioned above. Four weeks after operation, the mice were killed by an overdose of ether, and the left and right tibiae were removed from the bodies. These bones were freed of the periosteum with fine forceps and immersed in 5% sodium hypochlorite to remove any cells or organic materials on the bone surface. They were dehydrated in acetone, air-dried and attached onto aluminum stubs. All specimens were coated with platinum-palladium using an ion-coater...
(Hitachi, E-102) and examined with a scanning electron microscope (Hitachi, S-2500).

**Quantitative analysis**

Collagen fibers on the medial surface of each tibia were observed, since this surface is almost flat, showing no insertion of muscles or tendons. A nutrient foramen, an entry site of the nutrient artery seen in the medial surface of the bone, was used as a landmark in the present study (Fig. 1a). SEM photographs covering a rectangular area (900 x 350 μm) of the bone surface 900 μm distal from the nutrient foramen were obtained and used for composing montages at 600 times magnification. The area of each montage was divided into 126 squares of 50 x 50 μm. Since the neighboring collagen fibers tend to run parallel, their courses in each square were roughly determined by measuring the angles between the running directions of the fibers and the longitudinal axis of tibia (Fig. 1b). The angles measured, which were limited from 0 to 90 degrees, were classified into 9 groups at intervals of 10 degrees and the frequency distribution of the angles was obtained both in the control and denervated tibial bones.

**RESULTS**

**Superficial collagen fibers of the bone**

The sodium hypochlorite treatment removed cells and organic materials from the bone surface, but it preserved well the collagen fibers, which were embedded in the bone matrix (Fig. 2). In the control right tibia, superficial collagen fibers (bundles of collagen fibrils) of the bone matrix were 0.5-2.5 μm
thick, although many of them were more than 1.0 μm thick. These collagen fibers mostly ran in parallel with each other, forming clusters with the fibers coursing in the same direction, although they took a slightly tortuous path. Thus, the bone surface showed a mosaic pattern by differently oriented clusters of the fibers. This mosaic pattern in the control tibia, however, consisted of fiber clusters mostly running toward the longitudinal axis of the bone shaft. In the bone surface, numerous pores, in which osteocytes extended their processes, were seen between the collagen fibers, and the distance between two neighboring pores was 2–3 μm. Osteocytic lacunae in the bone matrix were scattered on the bone surface (Fig. 2a).

In the denervated tibia, collagen fibers on the bone surface were 0.3–1.5 μm thick, far thinner than those in the control tibia (Fig. 2b). These fiber bundles ran in various directions which showed no relationship to the longitudinal axis of the bone shaft.

Quantitative analysis of the directions of collagen fibers on the bone surface

To clarify any disturbance in the courses of the fiber bundles on the surface of the tibial bone after denervation, we measured angles between the direction of the bundles and the longitudinal axis of the bone shaft. The direction of the bundles was arbitrarily decided in a unit square of 50 × 50 μm on the bone surface. Each angle measured was classified into nine groups at intervals of ten degrees. In the control tibia, 51.7% of the total squares belonged to the class of 0–9°. Seventeen percent of the total squares were in the class of 10–19°, while only a few squares showed the class of over 80° (3.7%). Therefore, a majority of the collagen fibers ran toward the longitudinal axis of the bone, whereas those displaying a rectangular direction to the bone axis were few (Fig. 3a).

In the denervated tibiae, 27.9% of the total squares
belonged to the class of 0–10°, the ratio of which was much smaller than that of the control tibiae. Furthermore, the squares in the experimental group were more evenly distributed throughout the 9 classes than those in the control group; the collagen fibers showed no obvious tendency to run toward the longitudinal axis of the bone shaft (Fig. 3b).

DISCUSSION

Osteoblasts produce and secrete collagen molecules, which are formed into collagen fibrils in the bone matrix (Jones et al., 1975; Bloom and Fawcett, 1986; Cormack, 1987; Marks and Popoff, 1988). Previous studies have indicated that the orientation of collagen fibrils in the bone matrix is related to the direction of mechanical stress loaded onto the bone (Ascenzi and Bonucci, 1967, 1968; Boyde and Riggs, 1990). This well correlates with our present results showing that collagen fibers on the tibia tended to run parallel to the longitudinal axis of the bone.

As far as we know, our present paper is the first report demonstrating that the direction of collagen fibers becomes irregular on the bone surface of tibia in the leg immobilized by denervation. Although the weight bearing to the leg was reduced in the immobilized leg, the operated mice actively grew for four weeks after denervation. This indicates that changes in the direction of collagen fibrils on the bone surface are related to that in the mechanical stress loaded to the tibia, although the mechanisms of the arrangement of collagen fibers on the bone surface remain unknown. Our previous SEM studies have shown that osteoblasts extend numerous long processes along the collagen fibrils on the osteoid of the bone surfaces (Abe et al., 1998). This finding suggests that the processes of osteoblasts play an important role in the determination of the orientation of the collagen fibers.

The present study did not examine morphological alterations of osteoblasts on the bone surface of the tibia after denervation. However, considering that the arrangement of collagen fibers on the bone surface was highly disturbed 4 weeks after denervation, it seems likely that the alteration in the arrangement of collagen fibers could be attributed to changes in the activity of osteoblasts in the tibial bone of the immobilized legs. In addition, the bundles of collagen fibers became thinner on the operated side than those on the control side, suggesting that the activity of osteoblasts may also be involved in the regulation of the collagen fiber thickness in the forming bone.

In conclusion, our present results showing that the orientation of collagen fibers is highly disorganized on the bone surface of tibia in the immobilized leg after denervation suggest that the construction of bone structures is related to the mechanical stress. However, how the bone microstructure is organized remains unknown. Our study may provide a key for understanding the formation of bone microstructures relating to mechanical stress.
REFERENCES


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