Areal Distribution of Preferential Alignment of Biological Apatite (BAP) Crystallite on Cross-Section of Center of Femoral Diaphysis in Osteopetrotic (op/op) Mouse

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Osteoclasts are nearly non-existent in mutant osteopetrotic (op/op) mice due to defects in the expression of the macrophage colony-stimulating factor (M-CSF). Thus, areal distribution of the biological apatite (BAP) c-axis in a femoral cross-section of an op/op mouse was investigated using a microbeam X-ray diffraction system to understand the role of osteoclasts on formation of BAP preferential alignment.

The incident X-ray beam is focused to 20 µm in diameter. The diffraction analysis is performed at 20 µm displacement intervals from the periosteal surface along the radial axis of the anterior portion at a cross-section of the femoral diaphysis. The osteopetrotic (op/op) mouse and its normal littermate used as a control were 12 weeks old.

The lack of osteoclasts induces both abnormality of the skeletal system and calcification of the medullary cavity, which are typical features of osteopetrosis. Preferential alignment of the BAP c-axis in the osteopetrotic (op/op) mouse always shows a lower degree than that in the control mouse, regardless of the distance from the periosteum. Moreover, areal distributions of preferential alignment of the BAP c-axis on the femoral cross-section show quite different tendencies between the op/op and control mice. The preferential alignment of BAP gradually increases towards the periosteal surface in the op/op mouse due to the intramembranous ossification, while that in the control mouse is the lowest near the cortical envelope on the cross-section. This is because the decrease in the number of osteoclasts suppresses the normal modeling or remodeling, resulting in degradation of preferential alignment of the BAP c-axis as a bone quality parameter in the op/op mouse. [doi:10.2320/matertrans.48.337]

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1. Introduction

Bone is a composite material dominantly made up of biological apatite (BAP) as a mineral constituent and collagen as an organic matrix.¹⁾ The mineral component of bone has two primary functions: one is mechanical and the other is homeostatic. The BAP crystallites reinforce the organic matrix giving it strength and rigidity, while also serving as a repository for Ca, Mg, inorganic phosphate ions, etc. To perform their important role in bone, these BAP crystallites have a relatively narrow range of sizes (~10 nm), and the mineral properties, in general, differ depending on age, health and the presence of disease.²⁾ Understanding the structural and chemical transformations of BAP and the role of environmental constraints in determining the structure and composition of the ultimate bone mineral are all relevant biological and medical issues.³⁾

Recent research suggests that bone mineral density (BMD) is not a sufficient predictor of whole bone mechanical properties, while BMD is related to bone strength and fracture risk.^{4,5)} In untreated patients with the same BMD, fracture risk varies with age and perhaps with gender and race.⁶⁾ Therefore, the influences of still unidentified parameters controlling bone mechanical properties should be determined.⁷⁾

The additional factors, providing the so-called bone quality, are generally referred to as intrinsic bone material

parameters.^{8–11)} Among the various factors corresponding to bone quality, our research group has been focusing on the preferential orientation of the BAP crystallites, analyzed by the microbeam diffraction technique, because the preferential alignment of the BAP c-axis in various bones varies depending on the shape, stress distribution *in vivo*, and the related mechanical function.¹²⁾ The analysis of the BAP orientation also clarifies the behavior of bone regeneration by the tissue engineering technique, since the change in the BAP orientation does not accord with that in BMD during the regenerative process.^{13,14)} Moreover, it should be mentioned that some advanced and pioneering studies have been performed with respect to the orientation and arrangement of the bone mineral particles at different size scales by using transmission electron microscope analysis, X-ray diffraction analysis, small-angle X-ray scattering analysis, and neutron diffraction analysis.^{15–20)} Recently, the remarkable progress made in an analytical technique based on crystal diffraction has enabled researchers to examine the microstructure of bone tissues at the micrometer or nanometer level.^{1,21–23)}

Bone homeostasis is maintained dominantly by bone cells such as osteoclasts, osteoblasts and osteocytes.²⁴⁾ In mutant osteopetrotic (op/op) mice, osteoclasts rarely appear due to a defect in the expression of the macrophage colony-stimulating factor (M-CSF).^{25–28)} Accordingly, the purpose of this study is to clarify the role of osteoclasts in the formation of BAP preferential orientation during the bone modeling process, focusing on a small bone area relative to the bone's cortical thickness. Therefore, we investigated the areal distribution of the BAP c-axis at a cross-section of the

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Fig. 1 A schematic optical system of microbeam X-ray diffractometer composed of beam irradiating and receiving systems. The two-dimensional (2D) detector (HI-STAR, Bruker AXS) is a sensitive area detector and a true photon counter over a large area. The 2D diffraction information can be detected as Debye-Scherrer rings as shown in the left figure. The diffraction peak profile can be obtained from the Debye-Scherrer rings as a function of Bragg angle, 2θ .

femoral cortical bone of an osteopetrotic (op/op) mouse by using a microbeam X-ray diffraction system with an incident beam of $20\text{ }\mu\text{m}$ in diameter.

2. Experimental Procedure

B6C3Fe a/a-Csf1 [op/+]²⁵ mice were provided by the Jackson Laboratory (Bar Harbor, ME, USA). When the breeding pairs were mated, 12.5% homozygote recessive osteopetrotic (op/op, male) mice could be expected to appear. The op/op mice were distinguished by failure of incisor rupture and skull deformation at 10 days of age.²⁵⁾ The right femurs were obtained as bone specimens from an op/op mouse and a normal littermate, both 12 weeks old. All procedures were approved by the Graduate School of Engineering Animal Studies Committee, Osaka University.

The bone specimens were preserved in a 10% formalin neutral buffer solution at 4°C to avoid degradation of the organic matrix. The specimens were dehydrated in graded ethanol solutions (70–100%) and embedded in epoxy resin (Epo-ThinTM low viscosity epoxy; Buehler). Subsequently, the embedded bones were carefully cut perpendicular to the long bone axis at the center of the femoral diaphysis by a circular saw with a diamond wheel 0.15 mm thick and 75 mm in diameter. The analyzed cross-section of the bone specimen was abraded by silicon carbide paper of progressively finer grit (800, 1000 and 1200). The specimen surface was finally polished with a microcloth having a $0.05\text{ }\mu\text{m}$ aluminum suspension.

The X-ray diffraction analysis was conducted using the microbeam X-ray diffractometer system (D8 Discover with GADDS, Bruker AXS). The schematic figure of the experimental set-up for this microbeam X-ray diffraction is shown in Fig. 1. Cu-K α ($\lambda = 0.1542\text{ nm}$) radiation was generated at a tube voltage of 45 kV and a tube current of 110 mA (4.95 kW). The incident beam can be focused onto a beam spot of $20\text{ }\mu\text{m}$ in diameter using a mono-capillary collimator

with a beam divergence of 0.2° . The distance between the collimator and the specimen was fixed at 5 mm . A laser beam marks the focal spot on the specimen fixed on an XYZ stage in a quarter-circle Eulerian cradle. The specimen was swung in an appropriate angle range to obtain sufficient diffraction intensity along the bone's longitudinal axis: 13° – 20° for the ω axis. The scattered diffraction was registered by a 2-dimensional (2D) detector (HI-STAR, Bruker AXS), and the distance between the X-ray focus on the specimen and the detector was set at 15 cm . Thus, the angular resolution of the detector is $< 0.04^{\circ}$, and this setting enables the simultaneous detection of the diffraction data in a 2θ range of 36° . The detected radiation was counted for 2 ks to obtain an appropriate XRD profile. As a result, the irradiated area without the divergence of the incident beam was approximately $20\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$ on the specimen surface.

An isolated peak of (002) and overlapping peaks of (211), (112) and (300) were obtained from the diffraction planes at Bragg angles of 25.9° and 32.0° , respectively. Data were collected in a χ range of $\pm 7^{\circ}$, which is a similar deviation angle to that for the ω axis as shown in Fig. 1. The degree of preferential alignment of the BAp c-axis along the longitudinal direction was defined to be the relative integrated intensity ratio of the (002) diffraction peak to the overlapping diffraction peaks of (211), (112) and (300) from the XRD profiles.²⁹⁾

3. Results and Discussion

3.1 Change in appearance of op/op and control mice

The osteopetrotic (op/op) mouse is well known to be characterized by a generalized skeletal sclerosis, a small body, short length of long bones, domed skull, absence of incisors, etc. due to a severe reduction in the number of osteoclasts.^{25,26)} This mutation is associated with a total absence of the macrophage colony-stimulating factor (M-CSF).^{27,28)}

Fig. 2 Differences in body weight (a) and right femoral length (b) between the op/op and control mice used in this study, both aged 12 weeks.

Fig. 3 Center cross-section of right femoral diaphysis for the op/op ((a) and (b)) and control mice ((c) and (d)) used in this study. Regions in the anterior portion analyzed by microbeam X-ray diffraction are shown by the square areas in the high-magnification images for the op/op mouse (b) and for the control mouse (d). The squares in Figs. (a) and (c) correspond to those in Figs. (b) and (d), respectively.

Figure 2 shows the body weight and femoral length in the op/op and control mice (both 12 weeks, male) used in this study. The body weight is lower in the op/op mouse than in the control mouse. Skeletal growth in the op/op mouse is significantly slow compared with the control mouse. Thus, our op/op mouse exhibits the features of a typical op/op mouse accompanied by low weight and an abnormal skeletal system compared with the normal control mouse.

As for the op/op mouse, the lack of osteoclasts influences

not only the apparent condition but also the entire skeletal system. Figure 3 shows the overall appearances and the corresponding high-magnification images at the center cross-sections of the femoral diaphysis from the mice used in this study. Each position analyzed by microbeam X-ray diffraction with an incident beam of 20 μm in diameter, is also illustrated as a square area on the anterior of the cross-section in Figs. 3(b) and (d) for the op/op and the control mice, respectively.

Fig. 4 Variations in preferential alignment of the BAp c-axis along the longitudinal direction as a function of position along the radial axis in the center of the femoral diaphysis in op/op mouse (a) and control mouse (b), both aged 12 weeks. Position corresponds to the number as given in Figs. 3(b) and (d). The preferential alignment of the BAp c-axis is the relative integrated intensity ratio of the (002) diffraction peak to the overlapping diffraction peaks of (211), (112) and (300).

As seen clearly in Fig. 3(a), the cross-section of the femoral diaphysis in the op/op mouse possesses an unnecessary trabecular bone, which should be absorbed under normal bone conditions with osteoclasts as in the control mouse. The loss of marrow space and the subsequent skeletal sclerosis are typical features in an op/op mouse, while a small space for the bone marrow remains in the intertrabecular region of the op/op mouse for marrow hematopoiesis as shown in Fig. 3(a). The cortical portion analyzed is not clear because the cross-section of the femoral diaphysis in the op/op mouse shows an osteopetrotic pattern characterized by lack of a clear cortical envelope and an abundant amount of unabsorbed bone with marked narrowing of the medullary cavity, compared with the control mouse as reported by the other research group.²⁵⁾ Thus, the unabsorbed portion of bone between marks ⑦ and ⑬ in Fig. 3(b), which resembles a trabecular bone, was analyzed by microbeam X-ray diffraction.

In contrast, the cross-section of the femur in the control mouse shows an obvious cortical portion and no trabecular bone with sufficient marrow space as shown in Figs. 3(c) and (d).

3.2 Difference in areal distribution of BAp orientation on cross-section of femur between op/op and control mice

Figures 4(a) and (b) show the areal distribution of the preferential alignment of the BAp c-axis in the bone's longitudinal direction as a function of the position from the periosteal surface in the op/op and control mice, respectively. In this XRD system, the value of the orientation degree, which is the relative integrated intensity ratio of the (002) diffraction peak to the peaks of (211), (112) and (300) from the XRD profile, is 0.095 in randomly orientated apatite

(calcium hydroxyapatite) powders (NIST #2910). As shown in Figs. 3(b) and (d), the center of the incident beam was moved and irradiated at intervals of 20 μm from the periosteum. The preferential alignment of the BAp c-axis along the bone's longitudinal direction exhibits a conspicuous difference between the op/op and control mice.

Compared with the BAp orientation of the femur in the control mouse, which showed an appropriate high degree of BAp preferential alignment, a distinct change in BAp orientation as a function of position was found in the op/op mouse. In the op/op mouse, a quite low preferential alignment of BAp was observed at all positions on the cross-section of the femoral diaphysis along the longitudinal axis, as demonstrated previously.²⁹⁾ As shown in Fig. 4(a), the degree of preferential alignment of the BAp c-axis tends to increase toward the periosteal surface. Furthermore, no clear gap was found in the BAp orientation around the endosteum between the cortical portion and the residual bone portion in the marrow cavity, and there was a relatively low degree of BAp orientation here. In contrast, the preferential alignment of BAp exhibits a peak and tends to decrease toward the periosteum and endosteum, showing similar values near the cortical envelope as shown in Fig. 4(b). This result indicates that the deficit of osteoclasts greatly affects the areal distribution of the BAp orientation on the cross-section of the cortical bones between the periosteum and endosteum. The reason why clear and weak peaks of the preferential alignment appear in Figs. 4(a) and (b), respectively, is not clear yet.

Figure 5 schematically compares the distributions of the preferential alignment of BAp in the op/op and control mice. The position is normalized by the length of bone thickness between the periosteum and endosteum. This figure implies that the lack of osteoclasts changes the BAp orientation in the

Fig. 5 Distribution of preferential alignment of BAP c-axis along the longitudinal direction as a function of normalized position, which is normalized by cortical thickness between the periosteum and endosteum, in femurs of op/op and control mice, both aged 12 weeks. The preferential alignment of the BAP c-axis is the relative integrated intensity ratio of the (002) diffraction peak to the overlapping diffraction peaks of (211), (112) and (300).

portion near the endosteum, while there is no big difference near the periosteum. The residual bone with a low degree of BAP orientation, which forms in the early stages of bone growth, may suppress absorption and reconstruction into the appropriate nano-scale microstructure with preferential alignment of the BAP c-axis due to the lack of osteoclasts, resulting in low BAP orientation near the endosteum in the op/op mouse.

Since the intramembranous ossification, in contrast, induces modeling near the periosteum surface in the next stage of bone growth,³⁰⁾ the BAP orientation may increase toward the periosteum surface in the op/op mouse. Then the BAP orientation near the periosteum in the op/op mouse approaches a similar value as that in the control mouse. The slight difference in the BAP orientation near the periosteum may be related to the activity of osteoblasts, which produce new bone according to a recent report demonstrating that a lack of osteoclasts suppresses the activity of osteoblasts.³¹⁾

4. Conclusion

Areal distribution of preferential alignment of the BAP c-axis was evaluated as a bone quality parameter in osteopetrotic (op/op) and control mice aged 12 weeks by using a microbeam X-ray diffraction system. The following conclusions were reached:

- (1) Areal distribution of the preferential alignment of BAP could be continuously analyzed in a small bone surface area across the radial direction in the cortical and trabecular bones using the microbeam X-ray diffractometer with an incident beam of 20 μm in diameter.
- (2) The op/op mouse used in this study showed typical osteopetrosis conditions such as low body weight and an abnormal skeletal system compared with the control mouse.
- (3) The degree of the BAP c-axis orientation in the op/op mouse was always lower along the bone's longitudinal axis than that in the control mouse. The degree tended to increase toward the periosteal surface, and no clear gap in the BAP orientation appeared around the endosteum near the residual bone in the medullary

cavity. There was no large difference in the BAP orientation near the periosteum between the op/op and control mice due to the intramembranous ossification for bone modeling.

Finally, we concluded that the areal distribution of preferential alignment of BAP at the cross section of the cortical bone was strongly affected by the existence of osteoclasts. In other words, the decrease in the number of osteoclasts suppressed normal modeling or remodeling, resulting in a decline in bone quality, especially the preferential alignment of the BAP c-axis.

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