Two-Dimensional Quantitative Analysis of Preferential Alignment of BAp c-axis for Isolated Human Trabecular Bone Using Microbeam X-ray Diffractometer with a Transmission Optical System

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Two-dimensional quantitative analysis of microbeam X-ray diffraction (XRD) was performed using a transmission optical system to examine biological apatite (BAp) orientation in an isolated trabecula of a human fourth lumbar vertebral body. The incident X-ray beam is 20 μm in diameter, which is small enough for the isolated trabecula despite a slight beam divergence of 0.2°. Integrated intensities of (002) and (310) are obtained separately by different incident angles and detector positions. Distribution of the preferential orientation of the BAp c-axis is finally calculated quantitatively as an integrated intensity ratio of (002)/(310) in a plane containing the trabecular direction.

Preferential alignment of the BAp c-axis was finally determined to be perfectly parallel to the fiber direction in a rod-shaped trabecula, since accurate one-dimensional alignment is different from the alignment in the femoral cortical bone as a long bone that shows the local maximum of preferential alignment perpendicular to the longitudinal bone axis. For example, the integrated intensity ratio of (002)/(310) has a maximum value of 16 along the trabecular fiber and a minimum value of 0.09 in the perpendicular direction.

Using this method, the anisotropy of BAp orientation in the trabecular bone can be quantitatively evaluated in the plane including the trabecular fiber. Thus, we successfully obtained a methodology that two-dimensionally analyzes the distribution of the BAp c-axis along all axes within a plane in a bone specimen. [doi:10.2320/matertrans.48.343]

1. Introduction

Since trabecular bone shows much more metabolism activity than cortical bone, it acts as a reservoir to regulate the concentration of calcium and other mineral ions throughout the human body.1) During modeling or remodeling, on the other hand, the trabecular direction is selected along the stress line due to one functional adaptation of bone, as originally mentioned by Wolff.2) Many trabecular bone studies, which have been performed focusing on the patterns and distribution of trabecular fibers,3,4) have also been calculated to clarify stress distribution in cancellous bone.5,6)

At the nanoscale level, bone is a composite material mainly based on biological apatite (BAp) and collagen fibrils. BAp is an ionic crystal that crystallizes in an anisotropic hexagonal lattice in which the arrangement of ionic atoms is quite different along the crystallographic directions, for example, the a- and c-axes in the BAp crystal.7) The c-axes of BAp crystallites are basically distributed along the extending collagen fibrils in bones and form texture.8,9)

The orientation distribution of BAp in bone is crucial in terms of bone reinforcement.10) Nakano et al. showed that by using the microbeam X-ray diffraction technique mature cortical bones such as rabbit ulna, rabbit skull bone, and monkey dentulous mandible have unique preferential alignment and texture of the BAp c-axis depending on in vivo stress distribution.11) This indicates that normal original bone can exhibit an appropriate mechanical function by developing material anisotropy on the basis of the applied stress field. In contrast, the degree of BAp orientation in pathological and regenerated bones differs significantly along the representative bone axis from the original normal state.12–16)

Vertebral bones are frequently evaluated in diagnoses of bone diseases, mainly osteoporosis, which is a severe bone disorder in aging societies. Much attention has been paid to the trabecular bone in vertebrae because the trabecula is believed to sustain 30–50% of the vertebral strength.17) Moreover, the disease affects the trabecular bone more than the cortical bone.18) Many studies have focused on the relationship between trabecular architecture and mechanical properties, and actually, the notable contribution of trabecular architecture to the mechanical properties of cancellous bone has been demonstrated.19,20)

Previous reports have focused on BAp texture in cancellous bone.13,21–27) Bacon et al. found preferential alignment of BAp orientation in the portion composed of numerous trabeculae in vertebral bone by using a neutron diffraction technique.21,22) Both X-ray diffraction (XRD) and transmission electron techniques clarify the preferred orientation of the BAp c-axis in trabecular bone along the bone longitudinal direction in the pig distal metaphysis of femur due to the preferential arrangement of the BAp c-axis along each trabecular fiber.13) Jaschouz et al. impressively reported the preferential orientation of BAp crystal along the trabecula direction by recently conducting a microbeam X-ray technique onto individual trabecula from (002) pole figure analysis with a central focus on only the trabecular...
Scanning small-angle X-ray scattering (SAXS) studies revealed that the particle shape of BAp in trabeculae tends to be elongated along the trabecular fiber. Recent reports suggest that each trabecula has preferential alignment of BAp orientation in the fiber direction, but quantitative analysis for the degree of BAp c-axis orientation is needed in each trabecula between parallel and perpendicular to the fiber direction.

In this study, we performed 2D quantitative analysis of BAp orientation distribution in a plane with a trabecular fiber and then clarified the anisotropy of its distribution of BAp orientation parallel and perpendicular to the isolated trabecular fiber.

2. Materials and Methods

2.1 Bone specimen

A trabecula aligned approximately along the craniocaudal axis was selected from the central part of the fourth lumber vertebral body of a male cadaver (66-year-old) donated at Osaka City University Hospital. It has no evidence of metabolic bone disease. Analysis of this bone specimen was approved by the Ethics Committee. The bone specimen was fixed and kept in a 10% formalin neutral buffered solution to avoid infections and prevent denaturation of the organic matrix. The trabecular specimen was fixed on the x-y-z tables so that the fiber direction was exactly parallel to the y-z plane. Since the trabecular direction is always perpendicular to rotation axis $\chi$, we can analyze the two-dimensional distribution of the BAp c-axis along all axes within the y-z plane including the trabecular fiber. The short axis on the cross section of the trabecula was fixed to be parallel to rotation axis $\chi$. An online CCD camera and a laser beam were used for exact positioning of the trabecula. The center of the trabecula’s cross section was fixed to be the center of the rotation of the specimen and beam path.

We used two diffraction peaks of hexagonal-based BAp, (002) and (310), to analyze BAp orientation. The (002) and (310) diffraction peaks appear around Bragg angles of 25.9° and 39.8°, respectively, by Cu-Kα radiation. We defined the degree of orientation of the BAp c-axis as an integrated intensity ratio of the (002) diffraction to the (310) diffraction. The value of the orientation degree in randomly orientated apatite (calcium hydroxyapatite) powders (NIST #2910) is 0.6 in this XRD system.

To obtain diffraction information within the y-z plane including the trabecular axis, measurements for both (002) and (310) were performed independently (Fig. 2). Since normal direction to the (002) plane corresponds to the c-axis and is parallel to (310), two diffraction peaks of (002) and (310) are suitable to determine the BAp c-axis orientation. Incident angle ($\omega$) was selected as 13° for the (002) diffraction and 20° for the (310) diffraction to obtain accurate diffraction intensity along the normal direction to the
detectable diffraction lattice planes in each Bragg condition in a plane with the y-z plane. In other words, the accurate degree of BAp orientation for the (002) and (310) planes is detectable in all directions on the y-z plane including parallel and perpendicular directions along the trabecular fiber.

In this study, the specimen was swung along the ω axis within ±5° around the accurate Bragg angle on the y-z plane (8° ≤ ω ≤ 18° for (002) and 15° ≤ ω ≤ 25° for (310)) to compensate for the slight inclination of the trabecular fiber. The detector was placed at 2θ = 26° and 40° for (002) and (310), respectively. The specimen was rotated around the χ rotation axis to analyze the distribution of BAp orientation along the directions in the y-z plane. χ was rotated in steps of 20°, and measurements were done nine times to cover the 2D data around χ from 0 to 180° (Figure 2). Diffracted intensity was recorded for 3600 seconds at each χ position to obtain sufficient intensity for analysis.

The χ profile was calculated in steps of 1°, and the integrated intensities of (002) and (310) were obtained by subtraction of the background intensity on the side of (002) and (310) diffraction: 24.4° ≤ 2θ ≤ 24.9° and 26.9° ≤ 2θ ≤ 27.4° for (002) and 37.3° ≤ 2θ ≤ 38.3° for (310).

3. Results and Discussion

Figures 3(a) and (b) show examples of two-dimensional XRD patterns for analyzing (002) and (310) diffraction, respectively. Several reflections such as (002), (310), and overlapping diffraction peaks of (211), (112), (300), and (202) can be recognized in the figures. The (002) diffraction in Fig. 3(a) and the (310) diffraction in Fig. 3(b) can be obtained along the common axis normal to each corresponding diffraction lattice plane of the trabecula, as mentioned in Materials and Methods. In other words, each diffraction represents the orientation distribution along the directions within the y-z plane containing the trabecular fiber, independent of the rotation of the χ axis. The focused diffraction ring appeared through the center of the 2D detector because an appropriate detector position was selected as 2θ = 26° for (002) and 2θ = 40° for (310).

Figure 4 shows the appearance of the trabecula and the corresponding diffraction patterns. Clear arching of (002) reflection can be seen, and arching agrees well with trabecular axis. Intensity is shown in a pseudo-gray scale.

(002) and (310) plane.

In contrast, the distribution of the overlapping diffraction peaks of (211), (112), (300), and (202) along the χ axis seems more homogeneous than the (002) diffraction.

We quantitatively evaluated the integrated peak intensities of (002) and (310) as a function of χ from 0° to 180° in steps.

Fig. 3 Typical two-dimensional microbeam XRD patterns for (002) in (a) and for (310) in (b). Several reflections such as (002), (310), and overlapping diffraction peaks of (211), (112), (300), and (202) appear as a function of χ and 2θ axes. Intensity is shown in a pseudo-gray scale.

Fig. 4 Shape of analyzed trabecula and corresponding diffraction patterns. Clear arching of (002) reflection can be seen, and arching agrees well with trabecular axis. Intensity is shown in a pseudo-gray scale.
preferential orientation of BAp c-axis was present in the bone, but even weak alignment of BAp orientation was found along that direction other than the trabecular fiber because there is no local maximum of the intensity ratio of (002)/(310) (Fig. 6). This strongly suggests that rod-shaped trabecular fiber shows accurate one-dimensional preferential alignment of the BAp c-axis along the trabecular fiber, which is different from the femoral cortical bone as a long bone.

In this study, we separately measured (002) and (310) diffractions by adjusting the incident angle to the Bragg angle. This enabled us to analyze BAp orientation exactly in the directions between parallel and perpendicular to the trabecular fiber. Hence, the degree of BAp orientation is strongly reliable in the vertebral trabecula of the 66-year-old man. In cortical bone, it is reported that the preferential degree of BAp orientation varies depending on such factors as age, distribution and magnitude of in vivo stress, degree of disease progression, and fracture healing. These investigations were done using a comparably larger beam, which is sufficient for the cortical bone. However, since the size of the isolated trabecula is just a few hundred µm, a small beam is needed. We used an incident beam whose diameter was 20 µm with a beam divergence of 0.2°, which is small enough for individual trabeculae and quantitative mapping on a trabecula within the y-z plane. Analysis on the limited local area for observation, several µm², is also possible when using a synchrotron micro-focus X-ray beam with significant intensity, but the system is very ambitious and inconvenient. Therefore, the technique in this study conveniently allows verification of bone quality based on mechanical anisotropy to diagnose bone disease.

On the other hand, 2D quantitative analysis can be applied to cortical bone, which is needed to understand the 2D anisotropy of the BAp orientation in-plane. Skull bone, for example, has a unique two-dimensional BAp alignment on the skull surface, but preparing the bone specimen for quantitative analysis is troublesome. This technique is useful to analyze the distribution of the BAp c-axis two-dimensionally along all axes within a plane in a bone specimen.
4. Conclusion

Quantitative analysis of preferential alignment of the BAp c-axis for an isolated human trabecula was two-dimensionally performed using a microbeam X-ray diffractometer system with an incident beam 20 μm in diameter using a transmission optical system. The following conclusions were reached:

(1) An exact integrated intensity ratio of (002)/(310) for bone tissue can be obtained along in all directions within a plane by microbeam XRD with a small incident beam 20 μm in diameter. Thus, the anisotropy of BAp c-axis arrangement in a trabecula of human vertebra can be analyzed quantitatively in a plane containing directions parallel and perpendicular to the trabecular fiber.

(2) The rod-shaped trabecula exhibits an accurate one-dimensional alignment of BAp c-axis that is different from the femoral cortical bone as a long bone showing locally weak preferential alignment along a direction perpendicular to the longitudinal bone axis. The integrated intensity ratios of (002)/(310) are 16 and 0.09 in directions parallel and perpendicular to the trabecular fiber, respectively.

(3) The degree of BAp orientation as an integrated intensity ratio of (002)/(310) may be used to check bone quality and diagnose degree of bone disease, relating to mechanical function.

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