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Nanostructure and elastic modulus of single trabecula in bovine cancellous bone

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ABSTRACT

We aimed to investigate the elastic modulus of trabeculae using tensile tests and assess the effects of nanostructure at the hydroxyapatite (HAp) crystal scale on the elastic modulus. In the experiments, 18 trabeculae that were at least 3 mm in length in the proximal epiphysis of three adult bovine femurs were used. Tensile tests were conducted using a small tensile testing device coupled with microscopy under air-dried condition. The *c*-axis orientation of HAp crystals and the degree of orientation were measured by X-ray diffraction. To observe the deformation behavior of HAp crystals under tensile loading, the same tensile tests were conducted in X-ray diffraction measurements. The mineral content of specimens was evaluated using energy dispersive X-ray spectrometry. The elastic modulus of a single trabecula varied from 4.5 to 23.6 GPa, and the average was 11.5 ± 5.0 GPa. The *c*-axis of HAp crystals was aligned with the trabecular axis and the crystals were lineally deformed under tensile loading. The ratio of the HAp crystal strain to the tissue strain (strain ratio) had a significant correlation with the elastic modulus ($r=0.79$; $P<0.001$). However, the mineral content and the degree of orientation did not vary widely and did not correlate with the elastic modulus in this study. It suggests that the strain ratio may represent the nanostructure of a single trabecula and would determine the elastic modulus as well as mineral content and orientation.

1. Introduction

Mechanical properties such as elastic modulus, yield strain, and strength of the cancellous bone are essential to understand the changes in bone fracture risk with aging and/or osteoporosis and to evaluate the effects of osteoporotic medicines as well as bone volume fraction and bone mineral density. It is well known that the apparent elastic modulus of the cancellous bone is measured within the range of several hundred MPa to several GPa, and it is much lower than that of the cortical bone. The apparent mechanical properties of the cancellous bone and their relation with structural properties such as bone volume fraction, mineralization, architecture of trabecular networks, and shape of trabeculae in the cancellous bone have been investigated using uniaxial mechanical tests and micro-computed tomography (CT) scans with cubic or cylindrical specimens of the cancellous bone (e.g., Halgrin et al., 2012; Topoliński et al., 2011; Lievers et al., 2010; Perilli et al., 2007; Mittra et al., 2005). The mechanical properties of a single trabecula that constitutes trabecular networks and their nanostructure composed of hydroxyapatite (HAp) crystals and collagen fibrils are important factors in determining the mechanical properties of the cancellous bone. However, only a few studies have performed mechanical tests of a single trabecula because the mechanical tests of such small specimens are challenging, as reviewed by Carretta et al. (2013a) and Lucchinetti et al. (2000). Furthermore, the nanostructural effects on the mechanical properties of a single trabecula have not been investigated and elucidated, although various studies have been conducted on multiscale mechanical characterization in the cortical bone (e.g., Barkaoui et al., 2014; Yamada et al., 2013; Tadano and Giri, 2011; Feng and Jasiuk, 2011; Gibson et al., 2006; Hoc et al., 2006). Therefore, the present study focused on the mechanical properties of a single trabecula and on the nanostructural effects on its properties.

Tensile tests (Carretta et al., 2013b, 2013c; McNamara et al., 2006; Hernandez et al., 2005; Bini et al., 2002; Rho et al., 1993) and bending tests (Carretta et al., 2013b, 2013c; Hambli and Thurner, 2013; Lorenzetti et al., 2011; Szabó et al., 2011; Jungmann et al., 2011; Busse et al., 2009; Choi and Goldstein, 1992) of a single trabecula were conducted and some studies compared with finite element models (Carretta et al., 2013b, 2013c; McNamara et al., 2006; Hambli and Thurner, 2013; Lorenzetti et al., 2011; Jungmann et al., 2011) to investigate the elastic and post-yield mechanical properties. Nanoindentation tests were also conducted to obtain the elastic modulus and

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hardness (Tjhia et al., 2011; Smith et al., 2010; Wolfram et al., 2010; Brennan et al., 2009; Rho et al., 1999); however, the measured values did not necessarily correspond to those obtained in the testing of the whole trabecula. Tensile tests have some advantages over bending tests; for instance, mechanical anisotropy and the inhomogeneous shape of specimens do not severely affect the elastic modulus in the loading direction. In this study, we conducted tensile tests of the whole trabecula to measure the elastic modulus of a single trabecula.

HAp crystals have a hexagonal crystal structure. X-ray diffraction (XRD) is a promising tool for characterizing bone nanocomposites (Tadano and Giri, 2011). Using XRD techniques, it has been reported that on diaphyseal cortical bone, the *c*-axis of HAp crystals aligns along the bone axis, and that the degree of orientation is correlated with the elastic modulus in the bone axis (Yamada et al., 2013). Furthermore, many studies (Yamada et al., 2014, 2013, 2011a, 2011b; Yamada and Tadano, 2013, 2010; Giri et al., 2012, 2009; Dong et al., 2011; Stock et al., 2011; Tadano et al., 2008; Almer and Stock, 2007, 2005; Fujisaki and Tadano, 2007; Gupta et al., 2006; Fujisaki et al., 2006) have attempted to measure the deformation of HAp crystals in the cortical bone using XRD. For instance, Fujisaki and Tadano (2007) presented the relationship between bone tissue strain and HAp crystal strain under tensile loads *in vitro*. In addition, Almer and Stock (2007, 2005) investigated the strain and stress of the mineral phase under compressive loads *in vitro*. However, the orientation and deformation behavior of HAp crystals in a single trabecula have not been elucidated. As reported in a few studies, Rokita et al. (2005) noted the existence of preferential crystal orientation in the trabecular network of human vertebra using synchrotron X-rays. Akhtar et al. (2011, 2008) described the behavior of averaged HAp crystal strains in the cubic specimen of the cancellous bone under uniaxial compression using synchrotron X-rays. To better understand the nanostructure and its effects on the elastic modulus of a single trabecula, we investigated HAp crystal contents, orientation, and deformation behavior in a single trabecula and compared with the previous findings of the cortical bone.

Therefore, the present study aimed to investigate the nanostructural effects on the elastic modulus of a single trabecula, such as the mineral content and the HAp crystal orientation and deformation behavior in a single trabecula using XRD and energy dispersive X-ray spectrometry (EDS).

2. Materials and methods

2.1. Specimen preparation

In the experiments, 18 trabeculae that were at least 3 mm in length in the proximal epiphysis of three adult bovine femurs (2-year old) were used (Fig. 1). To measure the orientation of the longitudinal direction of a single trabecula (trabecular axis) in the femoral epiphyses, the trabecular networks in the epiphyses were observed using a microfocus X-ray CT instrument (inspeXio SMX-225CT, Shimadzu, Japan) before specimen extraction. The diaphysis of the femurs was scanned using a tube voltage of 160 kV, a tube current of 40 μA, and a voxel size of 0.398 mm/voxel. The centroid point of the perimeter line of the diaphysis in each cross-section was obtained from the CT images by using ImageJ software and the longitudinal direction of the diaphysis, which was defined as the bone axis, was calculated by approximating a straight line through those points, as shown in Fig. 1(c). Following this, the cancellous bone region was scanned at a voxel size of 0.146 mm/voxel. The trabecular orientation α was calculated as the angle between the trabecular axis and the bone axis, as shown in Fig. 1(a). After the CT scans, the specimens were collected and fixed to thin metal jigs using superglue, as shown in Fig. 1(b). Two small ink marks were placed on the surface of the ends of the specimens to serve as gauge points.

2.2. Trabecular morphology measured by μ-CT

To measure the cross-sectional area and the shape of a single trabecula, the specimens fixed to the jigs were scanned using a microfocus X-ray CT instrument (inspeXio SMX-90CT, Shimadzu, Japan) at a high resolution with a tube voltage of 90 kV, tube current of 110 μA, and voxel size of 0.011 mm/voxel. The average cross-sectional area (*A*), circularity (*Cir*), and aspect ratio (*AR*) in each specimen were calculated from the CT images using ImageJ software.

2.3. Elastic modulus measured using tensile test

The tensile tests were conducted using a small tensile testing device, which consisted of a linear stage (ALS-4011-G1M, Chuo Precision Industrial, Japan) with high resolution (2 μm) and repeatability (0.3 μm) and a load cell (LTS-1KA, Kyowa, Japan) with high repeatability (0.5% or less) and small hysteresis (within 0.5%), and viewed under a microscope (VH-5000, Keyence, Japan), as shown in Fig. 2. The tissue strain was measured from the distance between the gauge points using the microscope at 1 N intervals from 1 to 8 N. The tissue stress was calculated as the applied load divided by the average cross-sectional area *A*, and the elastic modulus *E* of a single trabecula was calculated. The tensile tests were conducted three times under the air-dried condition, and the average *E* was used for each specimen.

2.4. HAp crystal orientation measured by XRD

As shown in Fig. 3(a), the specimen was perpendicularly irradiated for 10 min with characteristic X-rays of Mo-Kα (λ=0.071 nm) using an X-ray diffractometer (Ultima IV, Rigaku, Japan) with a collimator of 0.5 mm diameter, at a tube voltage of 40 kV and a tube current of 40 mA. The measurements were conducted three times, and the average value was used for each specimen.

The incident X-rays were diffracted in lattice planes of HAp crystals in accordance with Bragg's equation. The traveling direction and the intensity of the diffracted X-rays depend on the direction and the number of the lattice planes, respectively. The XRD pattern of the specimen was detected using an X-ray imaging plate (IP) (BAS-SR 127 × 127 mm^2, Fujifilm, Japan) and was read using a scanner (R-axis DS3C, Rigaku, Japan). When the *c*-axis of the HAp crystals aligned along a specific direction in the specimen, the XRD pattern of the (002) plane, which was perpendicular to the *c*-axis, appeared as arcs. In the present study, the *c*-axis orientation of HAp crystals was defined as the direction of the highest intensity of diffracted X-rays from the (002) plane and the degree of *c*-axis orientation of the crystals with respect to the trabecular axis was calculated from Eq. (1) using the XRD pattern of the (002) plane (Yamada et al., 2013; Tadano and Giri, 2011).

$$\langle \cos^2\beta \rangle = \frac{\int_0^{2\pi} I(\beta)\cos^2\beta|\ \sin\ \beta|d\beta}{\int_0^{2\pi} I(\beta)|\ \sin\ \beta|d\beta} \quad (1)$$

Here, $I(\beta)$ is the intensity of the diffracted X-rays from the (002) plane at the azimuth angle β in the XRD pattern. If every crystal is completely oriented in the trabecular axis, $\langle \cos^2\beta \rangle$ is equal to 1.

2.5. HAp crystal deformation behavior measured by XRD

To measure the HAp crystal strains under the tensile test, the tensile tests were also conducted during the XRD measurements under the same air-dried condition.

The HAp crystal strain ε^H in the trabecular axis was calculated from the changes of the interplanar spacing *d* of the (002) plane in the direction, as in Eq. (2), where θ is the half of diffraction angle and the subscript 0 indicates the nonstrained condition.

$$\varepsilon^H = \frac{d-d_0}{d_0} = \frac{\sin\ \theta_0 - \sin\ \theta}{\sin\ \theta} \quad (2)$$

XRD measurements were performed at 1 N intervals from 1 to 8 N using the same tensile testing device attached to the X-ray diffractometer, as shown in Fig. 3 (b). The specimens were irradiated with X-rays using a collimator of 1 mm diameter, at tube voltage of 40 kV and a tube current of 40 mA. The XRD profiles were measured using a scintillation counter with 2θ value between 11.0° and 12.5°, which included the diffraction angle of the (002) plane of HAp crystals. The measurements were conducted three times at each loading condition, and the three XRD profiles were summed as a profile. The diffraction angle 2θ was defined as the angle at the peak position of the profile, and the peak position was determined by applying the full width at two-thirds maximum (FWTTM) method (Yamada et al., 2011a). The applied tissue strain was calculated from the tensile force obtained by the load cell, *A* measured by CT scan and *E* measured in the tensile test without XRD. The ratio of the HAp crystal strain to tissue strain (strain ratio), $\varepsilon^H/\varepsilon$, was calculated by linear approximation.

2.6. Mineral content evaluated by EDS

After the abovementioned measurements, the specimens were embedded in epoxy resin for 24 h and cut at the middle section using a low-speed diamond wheel saw (Model 650, South Bay Technology, USA). The transverse cross-section was ground using emery papers (up to #2000) and buffed using a buffing machine (Model 900, South Bay Technology, USA). The cross-section was observed using a low-vacuum scanning electron microscope (SEM) (JSM-6360LA, JEOL, Japan) at an accelerating voltage of 15 kV in low vacuum (30 Pa) without evaporation coating.