Alteration of Mechanical Properties of Remodeling Bone Adapted to Mechanical Stimuli*

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An ultra-microindenter was utilized for the evaluation of the local mechanical properties of bone tissue. The tangents of loading and unloading curve, A and D , were adopted as the parameters for the hardness and stiffness, respectively. An anisotropy due to the orientation of collagen fibers between sagittal and transverse planes of rabbit tibia was observed under the loading condition of 19.6 mN. When the indentation load was lowered, fragile properties of the hydroxyapatite could be evaluated. This technique was further used for the rat caudal vertebrae during mechanical adaptation in order to investigate in-plane distribution of the local mechanical property. Decrease of A and increase of $1/D$ were observed near the periosteum in the cortex, indicating that immature bone was formed and mineralized progressively. Additionally, higher A and lower $1/D$ under articular cartilage of the loaded rat suggested that the subchondral bone stiffened for it to adapt to the mechanical stimulation.

Key Words: Biomechanics, Hardness, Anisotropy, Ultra-Microindenter, Bone Tissue, Microhardness, Local Mechanical Properties, Mechanical Adaptation

1. Introduction

Bone tissue is remodeled by bone formation or resorption throughout life to adapt its mass and structure to functional demands. Failure of dynamic equilibrium in bone remodeling causes metabolic disorders such as osteoporosis. Several researchers found that connectivity and complexity of bone micro-architecture disappeared in senile osteoporosis, and that bone became fragile because its capability of matrix mineralization declined^{(1),(2)}. Accordingly, much interest has

been focused on understanding the micro-structural changes and the local alteration of mechanical properties in the disordered bone tissue.

Tensile test, compression test and bending test have been applied to the mechanical evaluation of industrial materials that are regarded as isotropic and homogeneous materials from a macroscopic viewpoint. These tests have been also used as conventional methods for biomechanical testing of bone, where whole long bone or machined bone block was tested^{(3),(4)}. Bone tissue, however, has a complex hierarchical structure composed of layers of collagen molecules and alternating layers of inorganic mineral-like components such as hydroxyapatite (OHAp). Mineralized collagen fibrils are, furthermore, arranged in complicated three-dimensional arrays. Due to the anisotropic and inhomogeneous nature of bone, macroscopic material tests would not be adequate to locally evaluate its mechanical properties.

Indentation test is a convenient method to determine the mechanical properties of small specimens. Recently, ultra-microindentation test has been shown as a promising method for evaluating local mechanical

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properties of thin films such as oxide film and ion-coated surface layer. The ultra-microindenter is, in principle, similar to a traditional hardness tester. A pyramidal diamond indenter is pressed onto the specimen, and gradually increasing load is applied. The load is then gradually reduced in the same manner after it has reached a maximal load below 10 mN. Mechanical characteristics are obtained from continuous measurement of the relationship between applied load and indentation depth in loading and unloading cycles, which differs from optical readings of the indentation area in a traditional hardness test. This technique reduces the depth of indentation below 1 μm , so that it is possible to determine the mechanical properties of a micro area.

Some studies that attempted to relate bone microstructure to its mechanical properties have utilized hardness tester for biomechanical testing⁽⁵⁾⁻⁽⁷⁾. Micro-Vickers hardness test or Knoop hardness test was performed for sectioned bone surface in these studies, where applied load was in the order of several hundred or thousand mN. Such large range of applied load seems to be insufficient to understand the mechanical properties of bone related to its microstructure. Thus the ultra-microindenter is a powerful tool for the mechanical property determination of bone; however there are few reports on this.

The purpose of this study is twofold. The first is to examine the usefulness of the ultra-microindenter for mechanical property determination by testing rabbit tibia specimens. The second is to evaluate the alteration of local mechanical properties of rat caudal vertebrae adapted to mechanical stimuli. We have already reported that rat caudal vertebrae developed trabecular architecture and deposited immature bone matrix over the periosteal surface when they were externally subjected to compressive stimulation^{(8),(9)}. To investigate the alteration of mechanical properties caused by adaptive remodeling, ultra-microindentation technique was applied to the vertebrae.

2. Materials and Methods

2.1 Ultra-microindentation test for rabbit tibia

The right tibia of a mature Japanese white rabbit weighing about 3.0 kg was used for the object. The bone was obtained shortly after sacrifice, and was frozen at -60°C . Slices of 0.6 mm thickness were prepared by using an Exakt precision diamond band saw (Exakt Medical Instruments Inc., Oklahoma City, OK) so that the specimens facing the transverse plane were sectioned in the 10 mm distal region from the tibiofibular junction. They were defined as transverse specimens. Additionally, specimens facing the sagittal plane, which were called sagittal specimens, were

Fig. 1 Preparation of specimens from rabbit tibia. The four quadrants in transverse specimen were tested to reveal the site dependence of mechanical properties. Anterior parts in transverse and sagittal specimens were provided to examine the dependence of detected mechanical properties on the penetrating load.

prepared at the distal region where transverse specimens were sectioned (Fig. 1). All slices were then polished with 2 000 grit abrasive paper under constant water irrigation. Finally, they were cleaned in a physiological saline bath prior to ultra-microindentation test.

Ultra-microindentation tests were conducted on a Shimadzu DUH-50 (Shimadzu Inc., Kyoto). The specimen was fixed on a stainless steel substrate with an adhesive, and was kept wet with saline solution during measurements. The penetrating load was applied at a rate of 0.24 mN/s until the selected maximal load. The indenter was then held for five seconds at this load before unloading was done at the same rate. Applied load and indentation depth were recorded every 10 ms during loading and unloading processes. In this study, a triangular base Berkovich pyramid indenter with an apex angle of 115° was used. First, both transverse and sagittal specimens of the anterior part were indented at various maximal loads in order to reveal the dependence of detected mechanical properties on the penetrating load. Maximal loads of 0.981, 1.96, 2.94, 3.92, 4.90, 6.86, 9.81 and 19.6 mN were selected on the middle site between periosteum and endosteum for each region and each load (Fig. 2). Second, indentations at the maximal loads of 0.981 and 9.81 mN were further carried out on the four quadrants (anterior, posterior, medial and lateral) of the transverse specimen in order to reveal the site dependence of the mechanical properties. Ten indentations at intervals of 50 μm were impressed as described above.

Fig. 2 Schematic positioning of the indentations for rabbit tibia. Ten indentations at intervals of $50\ \mu\text{m}$ were impressed for each region and each load.

Fig. 3 Schematic representation of applying mechanical load to the fifth caudal vertebrae (C5) of rats

2.2 Application of mechanical stimulation to rat caudal vertebrae

Three Sprague-Dawley female rats, 14 weeks of age, were divided as follows. (I) Axial-loaded. (II) Offset-loaded. (III) No load as control. Stainless steel pins with a diameter of 1.2 mm were inserted into the fourth and sixth caudal vertebrae (C4 and C6) under general anesthesia. The fifth caudal vertebrae (C5) of axial- and offset-loaded rats were subjected to a sinusoidal-wave-like compressive loading of 300 cycles per day. The maximum magnitude and the frequency of the stimuli were 10 N and 1.0 Hz, respectively. The pin in C4 was moved by 4 mm toward the ventral direction and was reciprocated on each plane for the offset-loaded rats, while pins were loaded normally on the same plane for the axial-loaded rats (Fig. 3). Mechanical stimulation was repeated daily for four weeks at 24-hour intervals. Pins were free on purpose to keep mechanical environment physiologic between episodes of loading.

Fig. 4 Preparation of specimens from rat caudal vertebrae. A 1-mm-thick specimen in median plane was sectioned with a diamond band saw.

Fig. 5 Schematic positioning of the indentations for the axial-loaded rat. Five indentations at $20\ \mu\text{m}$ intervals were made on the median and transverse surfaces, equally dividing the cortex into 11 regions.

2.3 Ultra-microindentation tests for rat caudal vertebrae

All rats were sacrificed by an overdose of pentobarbital sodium after the loading regime, and then each C5 was extracted. A 1-mm-thick specimen in the median plane was prepared for each rat with a diamond band saw (Fig. 4). Three specimens were polished and cleaned in the same manner as described above. To examine the usefulness of the ultra-micro-indenter for evaluating the in-plane distribution of local mechanical properties, cortical bone of axial-loaded rat was provided for the test. Five indentations at $20\ \mu\text{m}$ intervals were made on the median and transverse surfaces, equally dividing the distance from endosteum to periosteum into 11 regions (Fig. 5). Additionally, specimens from offset-loaded rat and control rat were examined in order to investigate the alteration of mechanical properties in externally loaded bone tissue. Six indentations at $20\ \mu\text{m}$ intervals

Fig. 6 Schematic positioning of the indentations on the median plane of offset-loaded rat and control rat. Six indentations at 20 μm intervals were impressed on nine defined regions (regions a~i).

Fig. 7 Typical indentation cycles obtained by indentation of bone tissue. The tangents of the loading/unloading curves over 50% of maximal load, A and D , were calculated. A and $1/D$ were adopted as evaluated values of hardness and elasticity in this study.

were impressed on the nine regions, which were defined for cortical bone (regions a, b, h and i) and subchondral bone (regions c, d, e, f and g) as described in Fig. 6. In these measurements, a load of 4.90 mN, a loading/unloading rate of 0.24 mN/s and a dwell time of five seconds were selected.

Statistical analysis was performed with the use of one factor analysis of variance (ANOVA). When ANOVA indicated a significant difference among the measured regions, the significance was determined by the Student's t -test. A confidence level of 95% ($p < 0.05$) was chosen for statistical significance.

3. Results

Figure 7 shows a typical loading/unloading cycle

Fig. 8 Relationship between d_{max} and $1/D$ in the sagittal plane for anterior part of rabbit tibia. (Error bars indicate standard deviation.)

obtained by indentation on bone tissue. The indentation depth was increased while the penetrating load was gradually applied. After the selected maximal load was achieved, bone deformed progressively during dwell time (Fig. 7, B-C). Recovery of elastic deformation was observed in the unloading process. The tangents of the loading/unloading curves over 50% of the maximal load, which were defined as A and D , were calculated from the relationship between the square of indentation depth d^2 and load F . Both experimental⁽¹⁰⁾ and analytical⁽¹¹⁾ studies on metal samples indicated that A and D correlated with hardness and Young's modulus, respectively. On the basis of these reports, A and $1/D$ were adopted as evaluated values of hardness and elasticity in the present study.

As for the indentations on the anterior part of rabbit tibia, $1/D$ in the sagittal plane increased when the maximal indentation depth d_{max} increased (Fig. 8). $1/D$ in the transverse plane, by contrast, reached a plateau at approximately $1.5 (\mu\text{m})^2/\text{N}$ (Fig. 9). The relationship between d_{max} and A had a same tendency in both sections; the values of A inverse-proportionally decreased with the increase of d_{max} (Figs. 10 and 11).

Figures 12 and 13 show the relationship between d_{max} and $1/D$ when the indentations of 0.981 and 9.81 mN were made on the four quadrants of the rabbit transverse specimen. $1/D$ had no site dependence at the load of 0.981 mN (Fig. 12). In case of 9.81 mN indentation, the medial part had a significantly higher value of $1/D$ than the posterior and lateral parts (Fig. 13). However, d_{max} had no significant difference among the four quadrants.

Regional dependence of $1/D$ and A in the cortex of axial-loaded vertebra is shown in Figs. 14 and 15,

Fig. 9 Relationship between d_{max} and $1/D$ in the transverse plane for anterior part of rabbit tibia. (Error bars indicate standard deviation.)

Fig. 12 $1/D$ of the four quadrants in the transverse specimen under a load of 0.981 mN. (Error bars indicate standard deviation.)

Fig. 10 Relationship between d_{max} and A in the sagittal plane for anterior part of rabbit tibia. (Error bars indicate standard deviation.)

Fig. 13 $1/D$ of the four quadrants in the transverse specimen under the a of 9.81 mN. (Error bars indicate standard deviation.)

Fig. 11 Relationship between d_{max} and A in the transverse plane for anterior part of rabbit tibia. (Error bars indicate standard deviation.)

Fig. 14 Regional dependence of $1/D$ on the median and transverse planes of the axial-loaded rat. (For the site number, see Fig. 5.)

Fig. 15 Regional dependence of A on the median and transverse planes of the axial-loaded rat. (For the site number, see Fig. 5.)

respectively. $1/D$ near the periosteal surface significantly rose in both median and transverse indentations (Fig. 14) while A declined (Fig. 15). These mechanical behaviors indicated that bone tissues near periosteum had good ability to recover from indented deformation; they had low elastic modulus and low hardness.

$1/D$ and A were compared between offset-loaded rat and control rat in Figs. 16 and 17. Significant decrease of $1/D$ and increase of A were observed in the subchondral bone of the offset-loaded rat.

4. Discussion

The precision of the evaluated mechanical property with the indentation test largely depends on the ratio of the indentation depth to specimen thickness. When the specimen is thinner than the region that was plastically deformed by indenter penetration, the obtained data would involve the property of substrate such as a sample table. Itokazu and Murakami suggested in their elastic-plastic analysis of a triangular pyramidal indentation that the extension of the plastic-deformed zone by indentation should be about 12 times of indentation depth⁽¹¹⁾. In our indentation test, the maximal indentation depths of approximately 10 nm and 300–500 nm were respectively observed at applied loads of 0.981 and 9.81 mN for rabbit tibiae; the necessary thickness of specimen would be at least 6 μm for setting the maximal load to 9.81 mN. According these considerations, the present specimens that we prepared have sufficient thickness to indent in the selected load range. A thin-sectioned sample of 8–20 μm is routinely prepared for optical microscopic observation and histomorphometric measurement in the studies of bone adaptive remodeling and osteopathy such as osteoporosis. The ultra-micro-indenter needs only submicron-ranged depth for inden-

Fig. 16 Comparison of $1/D$ between offset-loaded rat and control rat. (For the definition of site, see Fig. 6.)

Fig. 17 Comparison of A between offset-loaded rat and control rat. (For the definition of site, see Fig. 6.)

tation; this technique could be applied to conventional thin-sectioned samples. The ultra-micro-indentation test makes it possible to obtain local mechanical information, in addition to microscopic and histomorphometric examinations, from the same thin sections.

The major organic constituent of bone tissue is collagen fibrils (diameter of 2–10 μm), which are assembled from collagen filaments (diameter of 0.05–0.1 μm). The collagen fibrils are crystallized with OHAp (range of $(3-7) \times 20$ nm), resulting in higher hierarchical organization. When the maximal indentation depth d_{max} exceeded 0.3 μm in the examination of rabbit tibiae, $1/D$ in the sagittal specimen steadily climbed while $1/D$ in the transverse specimen plane leveled off (Figs. 8 and 9). In other words, anisotropic nature of bone was detected when the indentation depth exceeded 0.3 μm. Considering that an equilateral triangular indentation is produced by penetration of a pyramidal indenter, the indentation depth of

0.3 μm impressed a triangle that had an altitude of approximately 1.9 μm , which corresponds to the order of collagen fibril diameter. The obtained data would, therefore, reflect the elastic behavior and anisotropic orientation of collagen fibrils when the load is large enough to indent over 0.3 μm . The diaphyseal part of the long bone is considered to consist of collagen fibers arranged parallel to the bone axis. This fiber arrays would contribute to the anisotropic results shown in Figs. 8 and 9, implying that the longitudinal Young's modulus, which was examined in transverse specimens, was higher than the radial one.

When the indentation depth was less than 0.3 μm , $1/D$ showed a low value of the same tendency in sagittal and transverse specimens (Figs. 8 and 9), which indicated that the examined materials had a poor ability of elastic recovery. Bone tissue is considered to be a composite material between collagen fiber and OHAp crystal as described above. Collagen fiber has low elastic modulus and high tensile strength while OHAp crystal shows an inverse nature. The poor elastic recovery would result from the brittle behavior of OHAp crystal deposited among collagen fibers. Additionally, higher A was observed when the indentation depth was restricted to micro area (Figs. 10 and 11); the ultra-microindenter could detect materials with higher hardness, which corresponds to the property of OHAp.

As for the site dependence of the mechanical property, $1/D$ in medial part was significantly higher than that of posterior and lateral parts in rabbit transverse specimen under 9.81 mN indentation (Fig. 13). The maximal indentation depth d_{max} had no significant difference among four quadrants. Site differences in elasticity due to the orientation of collagen fibers would be detected by this examination.

Bone tissue principally has two different functions. One is to store mineral components such as calcium and phosphorus necessary for maintaining homeostasis of body fluid. The other is to support the musculoskeletal system mechanically. To fulfill these functions, bone restlessly remodels while composing a highly hierarchical structure. Katz et al. illustrated the hierarchical organization using four levels of structure as follows⁽¹²⁾. (1) Molecular level: organic components such as collagen, lipids and proteoglycans uniquely contain an inorganic mineral-like component, OHAp. (2) Ultrastructural level: small crystallites of OHAp appear in collagen fibrils with a highly organized geometrical arrangement. (3) Microstructural level: collagen-OHAp unit develops into higher level organizations such as woven, plexiform and haversian bone. (4) Macrostructural level: the size and shape of the whole bone are consid-

ered. Considering the size of the indentations and examined materials, our ultra-microindentation tests could detect bone mechanical properties and structures in the ultrastructural level.

We have already reported that the loaded vertebrae formed immature woven bone (fibrous bone) at the periosteal surface⁽¹³⁾. Bone would adapt to the alteration of mechanical environment by forming new bone, which had only low mechanical properties but could deposit rapidly. Woven bone appears to be a spinate organization that is fabricated by depositing OHAp on the bundles of irregularly oriented collagen fiber, namely osteoid. Rubin et al. demonstrated in their animal models that woven bone was remodeled into a mature lamellar construct once the initial mechanical demand was accommodated⁽¹⁴⁾. As can be seen in Figs. 14 and 15, ultra-microindentation tests for the axial-loaded rat revealed that the material near periosteum showed good elastic recovery and had low elastic modulus. The examinations at the load of 4.90 mN showed indentation depths from 0.09 - 0.19 μm for the caudal vertebrae; the penetration was restricted within the micro area. The obtained results would reflect the development of calcification during adaptive bone formation, in which the osteoid was progressively formed and calcified from intra-cortical bone to periosteal surface. Ultra-microindentation test was a useful tool for local evaluation of the bone mechanical property and its in-plane distribution.

The subchondral bone in the control rat was detected as a material that exhibited low elastic modulus and low hardness (Figs. 16 and 17). This region was developed into tissue with high hardness and poor elastic recovery by applying offset load daily. Higaki et al. inspected the denaturation of articular cartilage and bone tissue in early osteoarthritic (OA) rabbit models prepared by transecting articular ligament⁽¹⁵⁾. Non-physiological load that was caused by articular instability affected the cartilage, resulting in the stiffening of subchondral bone and the formation of osteophyte in OA rabbits. Our examinations seem to agree with their observations; subchondral bone in the loaded rat would be hardened with progressive calcification and would indicate the increased brittle behavior of OHAp.

5. Conclusion

Ultra-microindentation test is a useful technique for evaluating the local mechanical properties and microstructure of bone tissue. Brittle behavior of OHAp crystal can be detected under a small load so that the indentation depth is restricted to below 0.3 μm . On the other hand, elasticity and anisotropic orientation of collagen fiber are inspected when the

applied load is large enough for the indented area to be of the order of collagen fiber diameter. This technique further makes it possible to obtain local mechanical information from the thin-sectioned samples that are prepared for microscopic and histomorphometric examinations in the studies of bone adaptive remodeling and osteopathy.

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