Prefeferential Orientation of Collagen/Biological Apatite in Growing Rat Ulna under an Artificial Loading Condition

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Mechanical loading plays a key role in altering macroscopic and microscopic bone structure through functional adaptation; however, the anisotropic micro-organization of collagen and biological apatite (BAp) in adaptively created bone tissue is not well understood in spite of its importance in the mechanical function of bone. In this study, we artificially applied axial compressive loading (15 N at 2 Hz) to a growing rat ulna 10 min/day for 3 weeks to induce new bone under increased mechanical stimulus. Artificial loading induced marked increases in the structural properties of the loaded ulna; the cortical bone area of the mid-shaft was 43.3% larger in the loaded ulna than the contralateral control ulna. The newly formed bone was located mainly on the medial periosteal surface of the ulnar mid-shaft, which experienced the highest compressive strain. The present study firstly clarified that new bone induced by an artificial load showed preferential orientation of collagen and BAp c-axis along the ulnar long axis, which is similar to the pre-existing bone, although the degree of orientation and bone mineral density (BMD) were still impaired after loading for 3 weeks. This anisotropic organization of collagen and BAp crystals corresponded to that of osteocytes, implying that osteocytes are involved in the formation of anisotropic bone micro-organization which is important aspect of bone mechanical function regarding material properties. [doi:10.2320/matertrans.ME201314]

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1. Introduction

It is commonly accepted that bone adapts its mass and architecture according to the prevailing mechanical loading environment. The mechanisms involved in bone adaptation have been the object of considerable research over the past decades. To clarify the responses of bone to the mechanical environment, many loading and unloading models have been developed to modulate in vivo mechanical loads on bone.1-4) Most of these studies focused on the change in mass and/or geometry of bone along with the change in loading condition. Moderately increased mechanical stimuli induce bone formation,5,6) while unloaded bones experience bone absorption,7-10) to adjust the bone mechanical functions to the mechanical requirements. To our knowledge, however, the bone microstructure, including the crystallographic orientation of biological apatite (BAp) and collagen, induced by artificial loading is poorly defined in spite of its impact on mechanical function. The degree of the preferential BAp c-axis orientation along the longitudinal axis of the long bone, rather than bone mineral density (BMD), has been demonstrated to be a dominant determinant of Young’s modulus in the same direction.11)

The preferential orientation of BAp might be closely related to the applied load (stress) on bones. Nakano et al.12) have reported that BAp orientation varies depending on bone type, bone shape, and anatomical location in the body. In vivo stress conditions, especially principal stress,13) appear to affect the preferential BAp orientation. Demonstration of the formation of anisotropic BAp organization is noteworthy in bone biomechanics because BAp orientation effectively modifies bone mechanical functions. This may also contribute to clarification of the bone adaptive response through an alteration of bone microstructure, which has not been well documented. Moreover, osteocytes which inhabit in bone matrix are of interest because they alter their morphology and alignment in response to the stress applied on bone14) and are believed to be involved in the regulation of bone adaptive responses.15)

The aim of the present study was to clarify whether the preferential orientation of the BAp c-axis and related collagen fibers,16) which is similar to that of the pre-existing bone grown under physiological mechanical conditions, forms in the bone induced under artificial loading by utilizing a rat forelimb compressive loading model.

2. Materials and Methods

2.1 Animal

A skeletally immature male Sprague–Dawley rat (age, 7 weeks; body weight, 200 g) was purchased from Japan SLC, Inc. (Shizuoka, Japan), which shows rapid growth17) and unclosed ulnar epiphyseal line.18) The animal was housed in a cage with a 12 h/12 h light/dark cycle and provided with a standard diet and water ad libitum and acclimated for 1 week before experimentation.

2.2 In vivo loading of the ulna

In vivo ulna loading was carried out according to the method established by Torrance et al.19) The anesthetized rat was placed on a mat, and the right forelimb was held in rounded cups between the olecranon process of ulna and the flexed carpus (Fig. 1). The right forelimb was cyclically loaded in a compression mode with a peak force of 15 N at 2 Hz for 1200 cycles/day, 5 days/week, for 3 weeks using a micro-material testing machine (MMT-101N; Shimadzu, Kyoto, Japan). The load frequency of 2 Hz was used because it corresponds to the stride frequency during natural locomotion.20) The left forelimb received no treatment as a
Bone structural properties of cortical bone area (mm²), image, Tokyo, Japan) was used to quantitatively analyze the
12.45 µm. Tri under 53 kV and 60 µA radiation with a spatial resolution of
Kyoto, Japan). Images were taken at the ulnar mid-shaft
computed tomography (µCT) (SMX-100CT; Shimadzu,
2.3 Assessment of bone mass
The bone mass was measured by micro-focused X-ray
computed tomography (µCT) (SMX-100CT; Shimadzu, Kyoto, Japan). Images were taken at the ulnar mid-shaft under 53 kV and 60 µA radiation with a spatial resolution of 12.45 µm. Tri/3D-BON software (Ratoc System Engineering, Tokyo, Japan) was used to quantitatively analyze the bone structural properties of cortical bone area (mm²), periosteal circumference (mm), and second moment of inertia (I) (mm⁴) on the binalized images (Iₘ₋ₐ and Iₖ₋ₐ; subscripts M-L and C-C represent neutral axis for bending specified for the calculation). A 300-µm-thick region at the mid-shaft was used for calculations.

2.4 Assessment of volumetric bone mineral density
(BMD)
The volumetric BMD (mg/cm³) was measured at the ulnar mid-shaft by peripheral quantitative computed tomography (pQCT) (XCT Research SA+; Stratec Medizintechnik GmbH, Birkenfeld, Germany). The ulna was placed in a plastic tube filled with 10% formalin. After the performance of a scout view to enable scan localization, a cross-sectional scan was performed at the ulnar mid-shaft using a resolution of 70 × 70 × 460 µm.

2.5 Assessment of preferential orientation of BAp c-axis
and collagen
A 300-µm ulnar longitudinal section including the newly formed portion was prepared by sectioning in the mediolateral planes with a diamond circular saw (BS-300CP; Exakt Apparatebau, Norderstedt, Germany). The degree of preferential BAp c-axis orientation was quantitatively analyzed using a microbeam X-ray diffractometer (µXRD) system (R-Axis BQ; Rigaku, Tokyo, Japan) with a transmission optical system as previously described. Mo-Kα radiation was generated at a tube voltage of 50 kV and a tube current of 90 mA. The incident beam was focused on a beam spot 100 µm in diameter by a double-pinhole metal collimator and radiated vertically to the long bone axis on the anterior surface with an exposure time of 3600 s. Two peaks of specific crystallographic planes of BAp, (002) and (310), were used for analyzing the degree of the preferential BAp c-axis orientation as a relative ratio of (002) diffracted intensity to (310) intensity. The orientation degree of randomly oriented hydroxyapatite (NIST #2910: calcium hydroxyapatite) powder showed an intensity ratio of 0.8; therefore, a value over 0.8 indicates preferential BAp c-axis orientation in a specific direction. Measurements were carried out at 5 points, with 100-µm intervals, from the periosteal surface at the mid-shaft of the ulna.

After the thickness was reduced to 70 µm, the ulnar longitudinal section was observed using a polarized light microscope (BZ100; Olympus, Tokyo, Japan) with polarizer and analyzer in cross position.

2.6 Observation of osteocyte morphology and alignment
The morphology of osteocytes was observed with a confocal laser scanning microscope (CLSM) (FLX1000D-I9X81; Olympus, Tokyo, Japan) in both newly formed bone and pre-existing cortical bone. The above-mentioned longitudinal section of 70-µm thickness was stained with fluorescein isothiocyanate isomer I (FITC) (F7250; Sigma-Aldrich, St. Louis, Missouri, USA) according to a previously described method. A micrograph was captured using an UPlanSApo 60× oil immersion objective lens (numerical aperture = 1.35).

3. Results and Discussion
3.1 Loading-related adjustment in bone structural properties
Axial compressive loading (15 N) of the forelimb for 3 weeks apparently increased bone mass compared with the contralateral control ulna (Fig. 2, Table 1). The cortical bone area and the periosteal circumference of the mid-shaft were 43.3 and 13.8% greater, respectively, in the loaded ulna than control ulna.

Notably, the marked increase in bone formation induced by mechanical loading was mainly observed on the medial surface of the ulnar mid-shaft, principally on the periosteal surface, while there was very little osteogenic response on the lateral periosteal or endocortical surfaces (Fig. 2). Similarly, artificial loading did not induce evident new bone formation on cranial or caudal surfaces of the loaded ulna relative to the control ulna (Fig. 2). Due to the natural curvature of the ulna, an axial compressive force induced bending in the mediolateral direction, resulting in compression on the medial surface and tension on the lateral surface, with the ratio of compressive to tensile strain being around 1.5, whereas there was little strain at the caudal and cranial surfaces because the craniocaudal axis was the neutral axis for...
bending. Strain increases linearly with distance from the neutral axis; therefore, the highest strain was experienced on the periosteal surface. Because mechanically induced bone formation is distributed in accordance with strain magnitude, new bone formation was highest on the medial periosteal surface. Because mechanically induced bone formation is distributed in accordance with strain magnitude,5) new bone formation was highest on the medial periosteal surface, which experienced the highest compressive strain.20) These results are consistent with those of other studies showing that new bone is predominantly formed in response to compressive strains than tensile strains.29,30)

As shown in Table 1, second moment of inertia was markedly increased both across the medial–lateral (I_{M-L}) axis and caudal–cranial (I_{C-C}) axis in the loaded right ulna; I_{C-C} in particular showed nearly a 3-fold increase. The new bone formed on the medial periosteal surface largely contributed to this drastic increase in the I_{C-C}, indicating the great enhancement of resistance to bending induced by artificial loading. Bone adapted to the artificially increased load by effectively altering its macroscopic structure.

### 3.2 Loading-related change in bone material properties

#### 3.2.1 Loading-related change in BMD

The BMD of the newly formed bone (1002 mg/cm³) was somewhat lower than that of the pre-existing cortical bone (1246 mg/cm³), although the BMD of the newly formed bone was over 690 mg/cm³, which is usually regarded as the threshold of cortical bone. There were pores in the newly formed bone [Fig. 2(c)], suggesting that the bone induced by artificial loading was not fully consolidated and not a fully mature bone after loading for 3 weeks. Loading for more than approximately 2 months is required for the BMD of new bone to reach a normal level.29)

#### 3.2.2 Loading-related change in the preferential orientation of collagen and BAp c-axis

Another important aspect of bone material properties is the preferential collagen and BAp c-axis orientation, which is a more important determinant of Young’s modulus than BMD.11) Figure 3 shows a polarized microscopy image and representative microbeam X-ray diffraction patterns from the newly formed and pre-existing bones at the ulnar mid-shaft. Polarized microscopy revealed anisotropic collagen orientation along the bone axis in the new bone similar to that in the pre-existing bone, although the homogeneity of the orientation was slightly lower than that in the pre-existing bone. The two XRD patterns were similar, showing 002 arc and 310 arc along the vertical and horizontal directions, respectively. The vertical direction in the diffraction pattern corresponds to the longitudinal axis of the ulna; therefore, the preferential orientation of the BAp c-axis along the ulnar long axis was found both in the newly formed bone and the pre-existing bone.

Figure 4 shows the degree of the preferential orientation of the BAp c-axis [intensity ratio of (002)/(310)] along the long bone axis in the ulnar mid-shaft. The newly formed bone (points 1 and 2) showed high values for the degree of the preferential BAp orientation, which were much higher than the value of randomly oriented hydroxyapatite (NISt #2910: calcium hydroxyapatite) powder, 0.8. However, the degree of orientation in newly formed bone did not reach the levels found in pre-existing cortical bone (points 4 and 5). Taken together with the BMD result, the bone induced by artificial loading had not reached an equilibrium state at 3 weeks under the artificially increased mechanical stimulus; the new bone was still undergoing maturation at this time. To determine the equilibrium state with increased load, the loading period should be prolonged. During maturation, the degree of BAp
c-axis orientation along the bone axis possibly progresses along with mineralization according to the selective and anisotropic crystal growth proposed using a rat tibia mid-shaft. Takano et al. demonstrated that the anisotropy of acoustic velocity, corresponding to the anisotropy of elastic modulus of new bone, increased with time as BMD of the new bone increased, while the anisotropy in the demineralized bone (collagen matrix) did not change, possibly reflecting anisotropic crystal growth. The new bone induced in the present study could be a source of well-functioning bone with highly oriented BAp.

The micro-organization of the bone induced by artificial loading varies among literatures. In some cases, the new bone adaptively formed against increased load was reported to be a woven-type bone, which was poorly mineralized with disorganized microstructure, while in other cases, lamellar bone formation was reported. The micro-organization of the new bone varied partly depending on magnitude of in vivo strain and bone formation rate which are correlated to each other. According to the report by Turner et al., the disorganized collagen structure in the bone induced by artificial loading was seen even after the bone was densified. It is important to avoid the formation of woven bone possessing disorganized microstructure and impaired mechanical function because rat bone does not generally undergo remodeling to replace woven bone with lamellar bone. In the present study, the organization of collagen and BAp crystals was highly anisotropic; therefore, the loading condition might be optimal to finally form mature bone with well-organized microstructure and related mechanical functions. Further study will aim at clarifying the relationship between the amplitude of strain applied on the bone and resultant collagen/BAp orientation.

3.3 Shape and alignment of osteocytes in the new bone

It is generally well accepted that osteocytes, cells embedded within the mineralized matrix of bone, can sense in vivo stress (strain) and transmit chemical signals to control activation of osteoblasts and osteoclasts in response to the change of loading condition. The shape and alignment of osteocytes appear to change to effectively sense the mechanical stimuli. Osteocytes in load-bearing tibias and fibulas had a spindle shape and were aligned along the long axis corresponding to the principal loading direction, whereas those in less-loaded calvaria were more spherical. Thus, the shape and alignment of osteocytes can be an indicator of the stress applied on bone and osteocyte mechanosensing.

Figure 5 shows the CLSM image of the medial longitudinal section at the ulna mid-shaft. The osteocytes in the new bone were elongated and aligned along the principal loading direction, which corresponded to the direction of the bone long axis, similar to those in the pre-existing bone. Osteocytes appear to align themselves parallel to the principal loading direction, which reflects the preferential orientation of collagen and BAp c-axis, in response to mechanical loading. Thus, we believe that osteocytes not only mirror the orientation of collagen and BAp c-axis, but also play an important role in the formation of the anisotropic micro-organization composed of collagen and BAp crystals. Although it presently remains uncertain how osteocytes perceive mechanical loading and translate this into biochemical signals, the harmonized organization in the preferential orientation of collagen and BAp c-axis and the osteocyte long axis in response to mechanical loading during the bone formation process indicates that osteocytes can be regarded as an important factor in regulating bone microstructure, as well as bone mass and BMD, in the functional adaptation process of bone.
The findings in the present study are clinically important from a viewpoint of biomechanics because mechanical functions of bone change largely due to the alteration of the degree of preferential BAp orientation which forms in response to in vivo stress condition. In bone healing process, for example, in vivo artificial loading might be useful to enhance the formation of bone with preferential BAp orientation and the resultant mechanical integrity, which is usually not achievable even under the usage of tissue engineering techniques.\(^1\)\(^,\)\(^2\)\(^,\)\(^3\)

4. Conclusion

The present study first reported anisotropic micro-organization of collagen and BAp crystals as well as osteocytes along the principal loading direction in the bone induced by artificial loading. It is revealed that, in response to the increased load, additional bone with a preferential orientation of the collagen and BAp c-axis forms to adjust bone mechanical functions according to increased mechanical requirements. Bone with oriented collagen and BAp possesses superior mechanical integrity, which contributes to effective adaptation in terms of biomechanics.

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