Changes in bone microstructure and toughness during the healing process of long bones

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Abstract. It is of great importance to understand how bone defects regain the microstructure and mechanical function of bone and how the microstructure affects the mechanical function during the bone healing process. In the present study on long bone defects, we investigated the relationship between the recovery process of fracture toughness and biological apatite (BAP)/collagen (Col) alignment as an index of the bone microstructure to clarify the bone toughening mechanisms. A 5-mm defect introduced in the rabbit ulna was allowed to heal naturally and a three-point bending test was conducted on the regenerated site to assess bone toughness. The bone toughness was quite low at the early stage of bone regeneration but increased during the postoperative period. The change in toughness agreed well with the characteristics of the fracture surface morphology, which reflected the history of the crack propagation. SEM and microbeam X-ray diffraction analyses indicated that the toughness was dominated by the degree and orientation of the preferred BAP/Col alignment, i.e. bundles aligned perpendicular to the crack propagation clearly contributed to the bone toughening owing to extra energy consumption for resistance to crack propagation. In conclusion, regenerated bone improves fracture toughness by reconstructing the preferred BAP/Col alignment along the bone longitudinal axis during the healing process of long bones.

1. Introduction

Many techniques have been successfully developed to reconstruct bone defects. However, it remains less understood how bone defects regain the microstructure and mechanical function of bone. Bone mechanical functions vary depending on the microstructure of the bone tissue constituents, which are mainly collagen (Col) fibers and biological apatite (BAP) crystals. The microstructural characteristics, such as the BAP density, BAP crystallinity, Col cross-linking and BAP/Col texture, have been reported to affect some mechanical properties of bone [1, 2]. Among these parameters, the preferred BAP/Col alignment, as well as the BAP density, dominantly contributes to the material properties of regenerated bone tissue, such as the ultimate stress [3].

In the present study, we focused on bone toughness, which is one of the important mechanical aspects, to understand the fracture healing mechanism of bone. The purposes of the study were to analyze the recovery process of the preferred BAP/Col alignment and toughness of bone, and to investigate how the BAP/Col alignment affects the bone toughness.
2. Materials and Methods
A rabbit ulnar osteotomy model was prepared by introducing 5-mm long segmental defects in the right ulnar middiaphysis of mature male New Zealand White Rabbits. The defects were allowed to naturally regenerate for 4, 8 and 12 weeks (N=5 each), before the rabbits were sacrificed by an overdose of pentobarbital. The right ulnae were removed, wrapped in saline-soaked gauze and stored at -80°C until mechanical testing. Immediately after the ulna was thawed in saline at room temperature and separated from the neighboring intact radius, a three-point bending test (Model 5565; Instron) was conducted on the regenerated portion until fracture occurred. The bending force was applied on the medial surface at the center of the regenerated site at room temperature. The distance between the two supports (L) and the crosshead speed were 30 mm and 10 mm/min, respectively. An absorption energy to failure (U) corresponding to the area under the load-displacement curve was evaluated. Finally, toughness (u) was calculated using the following equation [4]:

\[ u = U \frac{3 y_{\text{max}}^2}{L I_x} \]  

where \( I_x \) is the cross-sectional moment of inertia and \( y_{\text{max}} \) is the maximum distance from the neutral axis for bending. \( I_x \) and \( y_{\text{max}} \) were measured in saline by peripheral quantitative computed tomography (pQCT) (XCT Research SA+; Stratec Medizintechnik) with a resolution of 0.08 × 0.08 × 0.46 mm³. Bone tissue was detected as pQCT values of >267 mg/cm³ [5]. Despite the irregular morphology of the regenerated portion, \( I_x \) and \( y_{\text{max}} \) were calculated at the center of the regenerated portion because the bending moment was largest at the loading point during the three-point bending test. The fracture surface was observed by a SEM (JSM-5600; JEOL). The BAp orientation, which is closely related to the arrangement of Col fibers [6], was analyzed on the fracture surface by microbeam X-ray diffractometry (µXRD) (D8 Discover with GADDS; Bruker AXS) with an incident beam of 20 µm in diameter. Data were represented as the mean ± standard deviation (SD). Statistical analyses were performed by one-way ANOVA and a two-tailed t-test. Values of \( P<0.05 \) were considered to be statistically significant.

3. Results and Discussion
At 4 weeks after surgery, the defects were already filled with newly formed bone tissue [3], and a bending force could be applied to the regenerated portion. Figure 1 shows typical load-displacement curves obtained by three-point bending tests. Normal bone showed significant deformation after the

![Figure 1. Typical load-displacement curves in three-point bending tests conducted on regenerated ulnae.](image1)

![Figure 2. Variations in bone toughness evaluated by load-displacement curves. *P<0.05 vs. the normal value.](image2)
peak load was reached. In contrast, the regenerated bone at 4 weeks fractured immediately after the peak load was reached. After 8 weeks, the fracture strain tended to become larger as the postoperative period increased. However, the absorption energy to failure, which was expressed as the area under the load-displacement curve, was significantly lower in the regenerated bone than in the normal bone throughout the experimental period (data not shown). Figure 2 shows the variations in toughness of the regenerated bone as a function of the postoperative period. The toughness continuously increased over time as evaluated by one-way ANOVA, but was still quite low compared with that of normal bone even at 12 weeks after the operation.

The toughness was closely related to both the initiation and propagation behaviors of cracks. Figure 3 shows SEM images of fracture surfaces. At 4 weeks, the fracture surface was flat and smooth, indicating low consumption of fracture energy during crack propagation. A cleavage-like surface pattern was observed in the early stage of bone regeneration (Figure 3(a), arrows). At 8 weeks, the fracture surface became rougher and a cleavage-like surface pattern was not seen. Osteons, concentric lamellar structures surrounding a blood vessel, were observable on the surface at 8 weeks (Figure 3(b), arrowheads), and have been reported to play a role in bone toughening [7]. The fracture surface at 12 weeks was very similar to that at 8 weeks (data not shown). Normal bone exhibited a rugged surface (Figure 3(c)), clearly indicating strong resistance to crack propagation and the related high energy consumption during crack propagation.

These macroscopic crack propagation behaviors reflect the structural characteristics of bone tissue at various scale levels [7, 8]. We focused on the correlation between the resistance to crack propagation and the orientation of the preferred BAp/Col alignment during bone regeneration. Figure 4 shows high-magnification SEM images of fracture surfaces of regenerated bone at 8 weeks. The microscopic arrangements of the Col bundles were clearly observed. The Col bundles in Figure 4(a) were parallel to the fracture surface, whereas the Col bundles in Figure 4(b) were somewhat perpendicular to the surface. These observations were confirmed by μXRD analysis using the diffraction peaks of inorganic BAp crystals because the c-axis of BAp crystals is nearly parallel to the Col direction [6]. Figure 5 shows the integrated intensity ratios of the (002) diffraction peak to the (310) peak taken on the surfaces corresponding to the images in Figures 4(a) and 4(b), and the intensity ratio for randomly oriented hydroxyapatite (HAp) powders. The μXRD measurements were obtained using a reflecting optical system, such that the BAp c-axis alignment along the normal direction to the fracture surface, i.e. almost parallel to the ulnar long axis, was analyzed. The parallel BAp/Col bundles (//) showed a significantly lower intensity ratio than the randomly oriented HAp powders, whereas the somewhat perpendicularly aligned BAp/Col bundles (\perp) showed a significantly higher intensity ratio than the randomly oriented HAp powders. These μXRD results are consistent with the surface patterns observed in Figure 4.

The SEM and μXRD analyses showed that the orientations of the BAp/Col alignment in the regenerated bone were not uniform on the fracture surface, although clusters of well-arranged
BAp/Col bundles could be seen locally. When Col bundles are perpendicular to the direction of crack propagation, the crack must propagate by cutting the Col bundles. The resultant crack bridged by the Col bundles causes extra energy consumption [8] and surely contributes to toughening of the regenerated bone. As bone healing proceeds, the bone microstructure is reconstructed toward that of normal bone by enhancing the degree of preferred alignment along the long bone axis [9]. This suggests that the regenerated bone improves the fracture toughness by reconstructing the preferred BAp/Col alignment along the bone longitudinal axis during the long bone healing process.

4. Conclusions
The toughness and preferred BAp/Col alignment of regenerated bone were investigated, and the following conclusions were reached:
1. The fracture surface was flat at the early stage of bone healing at 4 weeks, and became rougher during the postoperative period. As a result, the toughness of the regenerated bone gradually increased. However, it remained significantly lower than that of normal bone throughout the bone healing period.
2. The toughness was closely related to resistance to crack propagation and clearly seemed to reflect the degree and orientation of BAp/Col alignment, which can be quantitatively analyzed by µXRD.

Acknowledgments
This work was supported by funds from the "Priority Assistance of the Formation of Worldwide Renowned Centers of Research – The Global COE Program (Project: Center of Excellence for Advanced Structural and Functional Materials Design)" and a Grant-in-Aid for Scientific Research and Development from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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