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Trabecular packet-level lamellar density patterns differ by fracture status and bone formation rate in white females

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ABSTRACT

Spatial patterns of mineralization for human iliac crest cancellous bone were measured from images obtained by quantitative backscattered electron microscopy. Biopsies collected from vertebral fracture patients and healthy individuals with high or low bone formation rate (BFR_s) were examined (fracture/low BFR_s: $N=12$, fracture/high BFR_s: $N=10$, normal/low BFR_s: $N=12$, normal/high BFR_s: $N=15$). 20 by 20 pixel square areas or smaller were sampled from superficial and deep remodeling packets. Mean (Z_{mean}) and standard deviation (SD) of mineralization were measured, and coefficients of variation ($CV=SD/Z_{mean}$) were calculated. Fast Fourier transform analysis was used to quantify the distribution of the mineral in the packets. "FFT_ratio" was defined as the ratio magnitude of the principal spatial frequency to the average atomic number density. A higher FFT_ratio occurred in specimens with more pronounced alternating layers of light and dark as visible in the backscattered electron image, which was defined as lamellar patterning. Two-way ANOVA revealed that the coefficients of variation of mineralization for both superficial and deep packets were significantly lower in fracture patients than in normal individuals. However, the interaction between turnover rate and group (fracture/non-fracture) indicated that the difference in packet CV occurred among the low turnover individuals and not among those with high turnover. Mean mineralization levels and CV between deep and superficial packets were highly correlated. Regressions of packet CV of mineralization and FFT_ratio were highly significant ($p<0.001$) for all packets pooled and for packets divided by group (fracture/normal). However, analyses of packet CV and FFT_ratio by individual were variable (R^2 from 0.00338 to 0.700).
Packet-level mineralization variability may be associated with fracture toughness, and fracture patients had less variable packet-level mineralization. The result that the packet CV varied significantly between fracture and non-fracture individuals with low turnover suggests that for low turnover subjects without fracture, high variability in mineralization may have a protective effect. In high turnover patients, the accelerated turnover may prevent the lamellar variability from developing over time. Strong correlations between CV and Z_{mean} for both superficial and deep packets imply that newly formed bone is created similarly to older bone within an individual. Fourier transform results show that the mineralization variability found within packets is associated with lamellar patterning. Lamellar structure has been hypothesized to guide microcrack propagation in order to optimize bone strength and toughness. Osteoporotics with fracture had less pronounced lamellation than healthy normals and may be more prone to fracture.

Introduction

Average bone mineralization levels have been shown to significantly contribute to bone strength, stiffness, and fracture toughness [1]. Previous work from our laboratory demonstrated that patients with vertebral fracture had overall mean mineralization that was more widely distributed than that of normal controls [2]. However, little is known about mineralization levels or variability in normal or fracture groups at the packet level. It is believed that bone damage in the form of microcracks initiates within the bone matrix and is directed by the varying properties of lamellae [3,4]. Therefore, variations in matrix composition and lamellar organization may affect regional strength and stiffness and thus have significant effects on cancellous bone mechanics and fracture properties.

Lamellar structure, along with cement lines, is hypothesized to be effective in preventing propagation of microdamage, thus playing an

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important role in toughening bone [3]. Studies of composite materials have shown that material property and composition gradients can largely dictate the mechanism of failure under load [4–6]. This is particularly the case in natural materials whose fracture toughness properties greatly exceed that of any of the individual materials that make up the composite [6,7]. Since bone material properties are inextricably linked to the amount of mineral in the tissue, with increased mineral content causing stiffer, harder bone, it can be hypothesized that mineralization levels and variations in mineral content may be closely associated to the mechanical integrity of the tissue. Nanoindentation studies, in which material properties are probed at sub-micron resolution, have examined the variation in mechanical properties of osteonal bone [8–10] and cancellous bone [8,11,12]. However, the idea that mineral variation associated with lamellar patterning may be related to fracture susceptibility, to the extent of our knowledge, has not been explored.

Backscattered electron microscopy is a technique that can be used to quantitatively study the mineral content of bone samples [13,14]. It has been used to examine mineralization levels in patients with osteogenesis imperfecta [15] and osteoporosis [2], in addition to effects of aging [16,17] and bisphosphonate treatment [18,19]. The advantage of this method for measuring mineral content is the localization of mineral density at microscopic resolution. Thus it is a useful tool in analyzing mineralization levels as associated with lamellar structure. In the present work, we define lamellar patterning to be the alternating light and dark layers visible in a backscattered electron image. This layering pattern has been observed in many previous studies of bone using polarized light microscopy. The layers are consistent with those visible in electron microscopy, and thus indicate a morphological feature of bone structure. Regardless of the origins of the patterning, the underlying hypothesis is that the patterning is different between diseased patients and healthy controls.

Fourier transform analysis is a method by which the spatial patterning of an image can be quantified. It has been applied to studies of collagen distribution in histological sections of bone using an optical method, known as the optical Fourier transform [20]. In this study, the fast Fourier transform (FFT) algorithm is applied to digital backscattered images representing mineralization patterns in order to measure the degree of lamellar patterning within bone packets.

The objectives of this study were to 1) quantitatively compare tissue mineralization variability in packets at trabecular surfaces with older interior regions between vertebral fracture patients and healthy controls; 2) examine the relationships between mean packet mineralization levels and packet mineralization variability; and 3) determine the extent to which packet mineralization variability is related to the degree of lamellar patterning.

Methods

The bone samples and methods for mineralization measurement were previously described [2]. Briefly, backscattered electron microscopy was used to collect digital images of cancellous bone from iliac biopsies of normal ($N=27$) and vertebral fracture ($N=22$) white, female subjects. Specimens were selected based on high or low bone formation rate per bone surface (BFR/BS, or BFR_s), with 15 $\mu m^3/\mu m^2$/year as a threshold, within their respective groups (fracture/low BFR_s: $N=12$, fracture/high BFR_s: $N=10$, normal/low BFR_s: $N=12$, normal/high BFR_s: $N=15$). Mean and standard deviation of high BFR_s biopsies were 36.8 and 13.9, respectively. Mean and standard deviation of low BFR_s biopsies were 4.26 and 4.19, respectively.

Specimen preparation

The biopsies were embedded in poly(methyl methacrylate) (PMMA) blocks. The biopsy blocks were polished to a mirror finish with successively finer grades of carborundum paper and alumina polishing suspensions to a 0.05 μm particle size (Buehler Ltd., Lake Bluff, Illinois). The samples were glued onto a calibration standard block containing materials of known atomic number aluminum ($Z=13$), magnesium ($Z=12$), and Shapal-L aluminum nitride ($Z=9.12$) (Goodfellow Corporation, Berwyn, PA). Sample and calibration material surfaces were then made electrically conductive with gold palladium coatings to prevent charging effects.

Quantitative backscatter electron imaging

Backscattered electron images were taken at 300× magnification using a 30-kV excitation voltage. Five images, each of different trabeculae, were captured for each bone biopsy. Images of the calibration standard materials were captured periodically throughout each imaging session to account for signal drift due to the machine electronics. One operator collected all data.

Mineralization measurement

Mineralization levels were assessed by converting bone pixel grayscale levels to atomic number using a calibration relationship. The calibration curve is a least-squares regression line that relates the atomic numbers of the standard materials with corresponding grayscale levels obtained from backscattered images [13]. Mineralization levels can then be calculated since they have been shown to correlate strongly with atomic number, as validated by ash fraction studies [21,22]. Images of the standards were taken for each sample, thus the atomic number to grayscale calibration relationship was calculated for each biopsy block. R^2 values for the calibration relationships ranged from 0.97 to 1.0.

Trabecular packets are the basic remodeling units of trabecular bone. Packets were delineated by cement lines, clearly visible on all backscattered images. Packets with any portion extending to the bone surface were considered superficial. Only packets in the interior of trabeculae that were completely encapsulated by cement lines were categorized as deep packets.

Scion Image computer software (Scion Corp., Frederick, MD) was used for all image processing. 20×20 pixel areas were sampled within each packet of every image ($N=2040$). Pixels were 0.756 μm by 0.756 μm squares. Approximately 14% of packets (primarily deep packets) were too small to include a 20×20 pixel sample. For these, 15×15 pixel samples (~10% of all image samples), 10×10 pixel samples (~4%), or smaller (<1%) were collected.

Mean mineralization levels were calculated for trabecular packets deep within the interior of trabeculae (Z_{deep}) and superficial exterior packets ($Z_{superficial}$). Packet standard deviations of mineralization were also determined for each packet type (SD_{deep}, $SD_{superficial}$). The packet coefficients of variation (CV_{deep}, $CV_{superficial}$) of mineralization were calculated as packet standard deviation normalized by the respective packet mean ($CV_{deep}=SD_{deep}/Z_{deep}$, $CV_{superficial}=SD_{superficial}/Z_{superficial}$). Two-way analysis of variance with Tukey post-hoc analysis was used to examine effects of vertebral fracture and bone formation rate on CV_{deep} and $CV_{superficial}$. Relationships between Z and CV measures were examined with least-squares regression analysis.

One potential source of mineralization variability within packets exists in their lamellar organization. Backscattered electron images of trabeculae often show a distinct alternating dark/light patterning that persists within the cement line boundary of a packet (Fig. 1a). This striping pattern is characteristic of the lamellar structure of the region. In backscattered electron imaging, the pixel grayscale level is correlated with the mineral content of the region [22]. Thus it is hypothesized that the varying grayscale level intensities of adjacent lamellae indicate that the mineralization levels are alternating between low and high mineral contents. With the SEM settings used