INTRODUCTION

Bone is an exquisitely evolved biological material with a complex hierarchical structure. Despite bone’s importance to overall health and quality of life, there is a critical gap in our understanding of bone’s basic building blocks at the ultrastructural-level, and how those nanoscale characteristics change with disease. Type I collagen, the most abundant protein in the body, forms the structural scaffolding upon which bone is built. The need for accurate quantitative analytical methods to assess collagen’s nanoscale morphology and mechanical integrity with as little disruption to the tissue as possible has prompted us to study the collagen ultrastructure of bone in situ using atomic force microscopy (AFM). Samples imaged using AFM can remain intact and in their native state, implying that measured properties are characteristics of the sample rather than artifacts of processing or imaging. We hypothesized that using AFM, we could image and quantitatively analyze the morphology of collagen fibrils in fully intact and mineralized bone in situ to learn more about the normal nanoscale properties of this material.

Osteoporosis is a devastating disease which affects more than 75 million in the US, Europe and Japan. Clinically, osteoporosis is diagnosed exclusively using a measurement of bone mineral density (BMD). Most clinical and research experts would agree that BMD is not a sufficient metric, as osteoporotic fractures can occur in patients with normal BMD. Further, this method disregards the important role that collagen plays in bone health. We therefore further hypothesized that by analyzing collagen from sham-operated and estrogen-depleted sheep, we could distinguish between healthy and diseased bone. This important observation could have significant implications to understanding disease mechanism, and could provide an alternative diagnostic technique which may lead to earlier disease detection.

METHODS

Male C3H/He mice (murine) were sacrificed at 11 weeks of age (UCUCA protocol #8518). The right femur was harvested and the proximal and distal ends were removed leaving compact bone in the diaphysis. 5 year-old Columbia-Rambouillet sheep (ovine) were anesthetized and ovarietomized (OVX) or subjected to a sham surgery (ACUC #03-010A-02). After 2 years, the ewes were sacrificed, and beams approximately 2 mm x 2mm x 7 mm were removed from the mid-diaphysis of the left radius. Bones were mounted to a steel disk and a flat polished surface was created using a 3 µm diamond suspension and a 0.05 µm alumina suspension. To remove extracellular mineral, the surface of each bone was treated for up to 45 minutes using 0.5M EDTA at a pH of 8.0, then vigorously rinsed with ultrapure water and soaked at 4°C for at least 16 hours. Before imaging, the sample was briefly sonicated to remove surface bound mineral. Samples were imaged in air using a PicoPlus 5500 AFM. Images were acquired in tapping mode from at least 3 axial locations in each sample using a silicon cantilever (tip radius < 10 nm, line scan rates of 2 Hz or lower, 512 lines per frame). At each location, at least 10 fibrils were analyzed in 3.5 µm x 3.5 µm amplitude (error) images to investigate the D-periodic axial gap/overlap spacing. Two dimensional Fast Fourier Transforms (2D FFT) were performed on individual fibrils and the first harmonic peak was analyzed to determine the value of the D-periodic spacing (Figure 1).

To investigate disease-induced differences in fibril morphology, the D-periodic spacing was compared in sham versus OVX sheep. Values measured from an individual sample were pooled, yielding an average value for that sample. The values from sham samples (n=6) were then compared with those from OVX (n=5) using One Way ANOVA. To investigate differences in distributions of fibril morphology, a Kolmogorov-Smirnov (K-S) test was performed on the cumulative density functions (CDF) calculated from the kernel density of each distribution. In all cases, a value of p<0.05 was considered significant.

RESULTS

Measurements within each mouse bone were pooled to yield the mean fibril spacing for that bone. These mean values (n=5) ranged from 67.7 nm to 69.0 nm, with a mean axial spacing for all bones of 68.3 ± 2.4 nm (Figure 2a). The dashed horizontal line corresponds to the 67 nm value projected by Hodge and Fishman in bone, and represents the middle 50% of the data and the whiskers depict the data extremes. The diamond is the mean and the line within the box is the median of each group. Fibrils from sham sheep were similar in terms of mean (68.0 ± 2.6 nm) and distribution when compared with mouse fibrils (Figure 2a).

Further, comparison of sham to OVX showed striking differences. The mean from the OVX samples was 65.9 ± 3.1 nm and when compared with the sham samples by One-Way ANOVA, there was a significant effect of estrogen-depletion on fibril spacing (p=0.039). Further, different populations of fibrils existed within the groups (Figure 2b). The sham bones had a population of fibrils (11.5% of total) with spacings of 72 nm or greater which was almost completely absent from the OVX group (1.8%). Similarly, the OVX bones had a population of fibrils with spacings of 64 nm or less (28.0%) which was less prominent in the sham bones (7.1%). When compared using a K-S test, the CDF from the groups (inset in figure 2b) was significantly different (p<0.001) indicating a difference in the population distributions of the groups.

DISCUSSION

Our data indicate that normal bone contains a distribution of collagen fibril morphologies which can be related to disease state. That is, instead of a single spacing value for all fibrils, there is a distribution of spacings, a concept that is often overlooked in measurements of collagen. This distribution of morphologies is found in wild type and sham bone meaning that it is a fundamental property of normal tissue. We further demonstrated that by monitoring the distribution of collagen morphologies in bone of normal and estrogen-depleted sheep, we can detect disease-induced changes in bone at the nanoscale. The ability to measure fibril morphology and to distinguish between normal and diseased bone not only provides a powerful method to study nanoscale mechanisms of disease, but it also has important implications to the future diagnosis of disease in bone and perhaps other collagenous tissues. Ongoing research is being performed to understand how changes in mean spacing and distribution impact the static and dynamic mechanical properties of the bone material, observations which could help explain some of the variability in fracture risk associated with osteoporosis.

This work has provided new insight into the ultrastructure of bone as a material and the role of material structure in bone disease. The observations noted here promise a much-needed alternative to the current methods used to diagnose osteoporosis and other bone diseases.

ACKNOWLEDGEMENTS

The authors would like to thank Drs. Jennifer M. MacLeay and A. Simon Turner of Colorado State University, and Carola Pechey and Elizabeth Michels from Henry Ford Hospital for their contributions to this work.