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The 3D structure of the collagen fibril network in human trabecular bone: Relation to trabecular organization

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ABSTRACT

Trabecular bone is morphologically and functionally different from compact bone at the tissue level, but both are composed of lamellae at the micrometer-scale level. We present a three-dimensional study of the collagenous network of human trabecular lamellar bone from the proximal femur using the FIB-SEM serial surface view method. The results are compared to human compact lamellar bone of the femoral shaft, studied by the same method. Both demineralized trabecular and compact lamellar bone display the same overall structural organization, namely the presence of ordered and disordered materials and the confinement of the canalicular network to the disordered material. However, in trabecular bone lamellae a significant proportion of the ordered collagen fibril arrays is aligned with the long axis of the trabecula and, unlike in compact bone, is not related to the anatomical axis of the whole femur. The remaining ordered collagen fibrils are offset from the axis of a trabecula either by about 30° or 70°. Interestingly, at the tissue scale of millimeters, the most abundant angles between any two connected trabeculae — the inter-trabecular angles - center around 30° and 70°. This implies that within a framework of interconnected trabeculae the same lamellar structure will always have a significant component of the fibrils aligned with the long axes of connected trabeculae. This structural complementarity at different hierarchical levels presumably reflects an adaptation of trabecular bone to function.

Introduction

Trabecular bone is an intricate three-dimensional framework of struts and plates that is surrounded by a continuous layer of cortical bone [1]. The trabeculae are subject to intensive remodeling, and are particularly susceptible to deteriorating age-related changes [2]. An understanding of the structure–function relations of trabecular bone tissue, either in health or in disease, requires a detailed understanding of its hierarchical structure. Here we present information on the 3-D structure of human trabecular bone, and report an interesting correlation between the orientations of the collagen fibrils in trabecular bone matrix at one hierarchical level and the orientations of the trabeculae themselves at another hierarchical level. We do this by specifying a new parameter to characterize the 3-D fabric of the trabecular bone tissue — the inter-trabecular angle.

In the mature skeleton of humans, almost all bone is composed of lamellae [1,3,4]. The lamellae are just one structural feature within a complex hierarchy of structural features. Compact bone comprises two varieties of lamellar bone: circumferential lamellar bone and osteonal lamellar bone. Trabecular bone is also composed of lamellae [1,3,5]. The lamellae are however organized into so-called "lamellar packets" [3,6]. The lamellae of one packet are all aligned, but the lamellae of different packets have different orientations, with the more recently formed lamellar packets truncating the older ones at a low angle ca. 20 – 30° (Fig. 1). The result is that each trabecula has a patchwork-like texture composed of differently oriented lamellar packets (Fig. 1). All lamellae in one packet originate from one uninterrupted deposition event and are separated from their surroundings by cement lines [6]. As in compact bone, trabecular bone lamellae are about 6 μm thick and incorporate osteocytes in lacunae, regularly positioned a few tens of micrometers apart. The osteocytes are interconnected through canaliculi. Moreover, the transition areas between trabecular and compact bone display continuity of lamellar arrays [5,7]. Unlike remodeling within compact bone which does not usually change the overall bone morphology, remodeling of trabecular elements gradually sculpts a new trabecular surface and eventually leads to re-orientation and re-shaping of individual trabeculae, and ultimately the whole trabecular network [8–11].

The 3-D organization of circumferential and osteonal compact human bone was investigated using a dual beam microscope and the serial surface view (SSV) method. SSV involves sequentially removing thin layers (around 10 nm) from a sample surface using the focused ion beam (FIB), and then imaging the exposed surfaces using the electron

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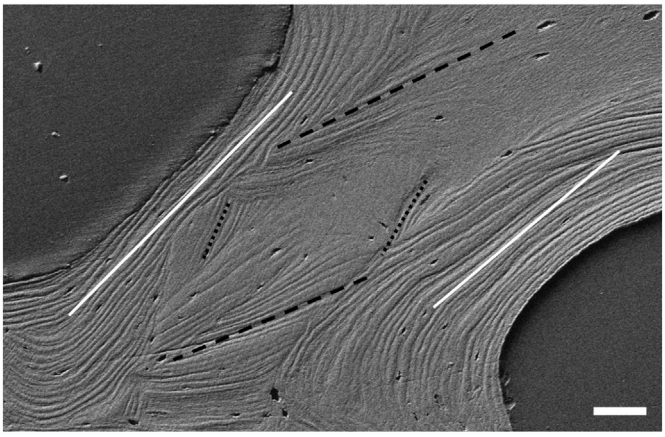

Fig. 1. Scanning electron microscope image of one dissected trabecula showing a mosaic of lamellar packets. Pairs of parallel lines highlight the orientation of lamellae: white solid — the newest lamellar packets, co-oriented with the trabecular axis; black dashed — truncated older lamellar packets, not aligned with the trabecular axis; black dotted — the most truncated and the oldest lamellar packets, not aligned with the trabecular axis. Scale bar 40 μm.

beam (SEM) [12]. The SSV method generates a 3-D stack of images with isometric nanometer-scale resolution in all directions in a volume of about 10 μm in all three orthogonal axes. An important observation of the 3-D study of compact bone was the identification of two different materials within the lamellar structure [13,14]. The predominant material (about 80% by volume) is composed of ordered arrays of collagen fibrils and the minor disordered material is composed of individual collagen fibrils with little or no orientation and abundant ground mass. Every repeating lamellar unit in the ordered material includes differently oriented ordered arrays of mineralized collagen fibrils. Within ordered arrays collagen fibrils are assembled into parallel bundles (rods) with rounded cross-sections and diameters of 2–3 μm. The disordered material is located between individual bundles and between differently aligned bundle arrays. The disordered material is not only a space-filler between the ordered arrays, but houses the whole lacuno-canalicular network of bone and thus may play an important role in mechano-sensing and mineral homeostasis [13,15]. No substantial difference was found in the 3-D structure of the matrices of circumferential and osteonal lamellae [13]. A significant advantage of the SSV method is that aspects of the structure can be quantified. Due to the isometric resolution of the SSV (a "cubic" voxel as a product of the 2D pixel and the slice thickness) and the repetitive and uniform structures of the collagen fibrils, the organization of the collagen network can be statistically analyzed by applying fast Fourier transform to the whole imaged volume and employing the frequency domain for tracing such structural trends as preferred orientation (direction) and extent of disorder (angular dispersion) [13,14].

Here we use SSV to study the 3D structure of the collagen network in human trabecular bone and address the question of whether or not trabecular lamellar bone architecture differs from the architecture of lamellae in compact bone. We also relate the structure at the lamellar level (micrometer range) to the structure of the trabecular network (millimeter range).

Materials and methods

Materials

We used cadaveric samples of proximal femora from two individuals: 20 year old female (F20, no macroscopic signs of bone pathology) and 63 year old male (M63, no ante-mortem fracture history). The bones were kept at −20 °C prior to analysis. The bones analyzed were obtained with complete ethical clearance.

Micro-CT

Marrow residues were removed using 3% sodium hypochlorite and then the specimens were fixed in 2% paraformaldehyde overnight on a rocking platform at 4 °C. Each proximal femur was dried with blotting paper, mounted on the micro-CT stage (Micro XCT-400, Zeiss X-ray microscopy, California USA) in the anatomical position and scanned at 40 kV and 200 μA with the pixel sixe 46.6 μm so that the metaphysis, the neck and the greater and lesser trochanters were in the field of view.

Analysis of the trabecular architecture in 3-D

We selected functionally and anatomically distinct areas in each CT-volume: the metaphysis and the neck. The cortical shell was digitally removed around the trabecular interior of each sub-volume. The trabecular interior was skeletonized, which means the replacement of each strut by a vector using Fiji software, plugin Skeletonize (http://fiji.sc/Skeletonize3D). The vector representation of the trabecular meshwork ignores the curvature of trabeculae and assumes that they are straight lines connected at nodes. The coordinates of the origin and the end of each vector (the nodes) were calculated using Fiji software plugin Analyze Skeleton (http://fiji.sc/AnalyzeSkeleton). The relationship between two vectors can be described by the dot product, which incorporates the cosine of the angle between them.

$$a \cdot b = \|a\|\|b\| \cos\alpha$$

$$\cos\alpha = (a_1 b_1 + a_2 b_2 + a_3 b_3) / \left(\sqrt{(a_1{}^2 + a_2{}^2 + a_3{}^2)} * \sqrt{(a_1{}^2 + a_2{}^2 + a_3{}^2)} \right)$$

Based on the coordinates of the nodes we calculated the angle between the pairs of connected vectors — the inter-trabecular angle.

A frequency distribution (histogram) of angles in each sub-volume (metaphysis and neck) was plotted and then separated into a minimal number of individual Gaussian peaks using PeakFit 4 (Jandel Co, USA) software. The quality of peak fitting was considered satisfactory when adjusted r^2 of the fit was better than 0.99.

Bone preparation for FIB-SEM SSV

Sections of 1–2 mm thick were cut from the samples F20 and M63 using a water-cooled rotary diamond saw (South Bay, USA). The sections were oriented parallel to the medial surface of the shaft (longitudinally) through the femoral neck basis. The sections were cleaned and defatted in acetone, and then demineralized by immersing in a solution of 5% ethylenediaminetetraacetic acid (EDTA), 2% paraformaldehyde (PFA) in cacodylate buffer, pH 7 on a rocking table for 72 h at room temperature. After demineralization individual trabeculae were dissected with a surgical blade and their anatomical orientation was recorded. We selected longitudinal trabeculae (oriented within ±15° with respect to the femoral longitudinal axis). These trabeculae are often the thickest and are most noticeable on sections or radiographs. For comparison of different trabecular orientations we also collected oblique trabeculae — those oriented at the highest angle to the femoral longitudinal axis and, therefore, to the first group of trabeculae. Note that the trabeculae of the proximal femur rarely connect at a 90° angle. Hence, we refrain from calling them "transverse" trabeculae.

Staining (for details see [13,14])

The samples were washed of residual EDTA using deionized water and pre-stained with Alcian Blue (5% in cacodylate buffer, pH 7) in order to stabilize bone proteoglycans [16], fixed again with 4% gluteraldehyde in cacodylate buffer, pH 7, and washed with deionized water. The staining was performed using the OTOTO protocol, also known as "conductive staining" [17,18]. This protocol uses a sequential application of osmium tetroxide (O) and thiocarbohydrazide (T). The