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Three-dimensional structure of minipig fibrolamellar bone: Adaptation to axial loading

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ABSTRACT

Fibrolamellar bone is transiently produced by large, fast growing mammals. The fibrolamellar bone unit is initially formed by elaboration of a network of blood vessels. This is followed by the deposition of a thin, porous and hypercalcified layer, then by the infilling of the vascular cavities by the sequential deposition of a relatively thick rapidly forming bone on both sides of the hypercalcified layer, and finally by lamellar bone. We investigated the 3D structure of the collagenous network of fibrolamellar bone from the femora of a young minipig using mainly the FIB–SEM dual beam microscope and the Serial Surface View method. This enabled us to identify the fibril orientation, the canalicular network organization and other structural motifs within each element of the fibrolamellar unit. The first formed primary hypercalcified layer (PHL) is composed of fibril arrays and multiple small pores, and appears to have an isotropic structure. The major bone component is deposited on both sides of the PHL, and is composed of collagen fibrils with a preferred orientation, mainly aligned parallel to the bone long axis. This bone component is therefore parallel-fibered bone and not woven bone. We also observed that the collagen fibers are organized into bundles. The lamellar bone has most of its collagen fibrils aligned with the bone long axis. This study therefore shows that the large majority of collagen fibrils in fibrolamellar bone are aligned with the bone long axis. This anisotropic structure therefore appears to be adapted to loading along the bone long axis.

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1. Introduction

Many large mammals that rapidly increase in size, initially form a special type of primary bone called fibrolamellar bone (Currey, 2002) (also known as 'wire-netting bone' (Brouwer, 1952), plexiform bone (Enlow and Brown, 1957; Martin et al., 1998) or laminar bone (Currey, 1960; Mori et al., 2003)). Fibrolamellar bone is a rapidly forming bone that results in the deposition of large amounts of new compact bone (Castanet et al., 2000; Francillon-Vieillot et al., 1990). As time progresses fibrolamellar bone is remodeled with secondary osteonal bone. Here we study the 3D structure of fibrolamellar bone with the aim of relating structure to function.

The formation of fibrolamellar bone begins with the elaboration of a network of blood vessels, followed by the deposition of a thin, porous and hypercalcified layer (Currey, 1960; Kerschnitzki et al., 2011a,b; Mason et al., 1995; Mori et al., 2007; Stover et al., 1992). This layer forms the substrate upon which the major component of fibrolamellar bone is deposited. The major component has been referred to as woven bone by some (Currey, 2002; Martin et al., 1998; Mori et al., 2007), or parallel-fibered by others (Currey, 1984) – two bone types with totally different structures. This fibrolamellar unit component together with the thin hypercalcified layer forms a reticulate network with large open spaces. These spaces are then filled in by lamellar bone to form what are referred to as primary osteons (Currey, 2002). Thus an individual fibrolamellar bone unit comprises a single thin primary hypercalcified layer, flanked on both sides by the major structural component and finally infilling lamellar bone. The fibrolamellar unit is schematically depicted in Fig. 1 and the components are numbered according to the temporal sequence in which they form. Fibrolamellar bone structure has been described in many different large mammals (ungulates), including cows and pigs (Brouwer, 1952; Dhem et al., 1976; Mori et al., 2005), sheep (Kerschnitzki et al., 2011b; Mori et al., 2005) and horses (Enlow and Brown, 1958; Mori et al., 2003; Stover et al., 1992).

The thin porous hypercalcified layer has been referred to as a "hypercalcified primer" by Dhem et al. (1976) based on its high opacity to X-rays. This layer has also been identified as a bright

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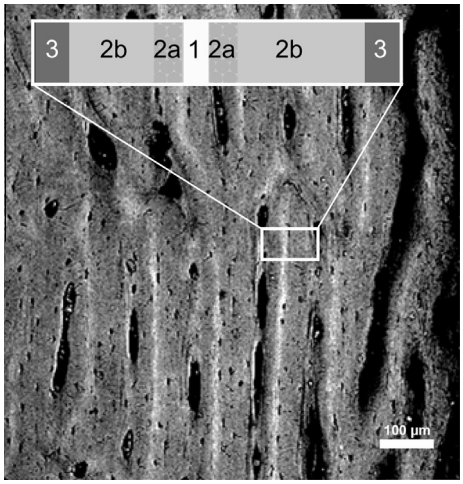

Fig.1. Schematic illustration of the 4 structural components of an individual fibrolamellar bone unit: (1) the central primary hypercalcified layer (PHL); (2) major structural component (shown below to be parallel-fibered bone where the inner part is organized into sheathed bundles (2a) and the outer part is organized into aligned fibril bundles (2b)); (3) lamellar bone. The bone growth is asymmetric on both sides of the PHL.

layer using a light microscope (Currey, 1960) and when observed by back scattered electron (BSE) imaging in the SEM (Kerschnitzki et al., 2011b; Mori et al., 2003). Others have referred to this layer as a "hypercalcified line" (Mori et al., 2007), or "woven bone" (Stover et al., 1992). This layer does have more mineral than the other components of the fibrolamellar unit and the Ca/P ratio is similar to the other components (Mori et al., 2007). TEM studies of the demineralized hypercalcified layer show collagen fibrils, as well as globules and rod-like structures comprising non-collagenous fibrils (Mori et al., 2007). The hypercalcified layer occasionally has large, closely packed, irregular and randomly oriented osteocyte lacunae and it has been proposed that this is the primary scaffold upon which additional bone forms (Kerschnitzki et al., 2011a; Mori et al., 2007). This layer is barely penetrated by canaliculi (Currey, 1960; Kerschnitzki et al., 2011a; Mori et al., 2007). Kerschnitzki et al also proposed that the formation of a highly oriented collagen matrix requires the presence of this unstructured substrate layer to direct the growth process (Kerschnitzki et al., 2011b). Here we refer to this layer as the "primary hypercalcified layer" (PHL).

We use the dual beam microscope (FIB–SEM) and the Serial Surface View method (SSV) to investigate the organization of the demineralized organic matrix of fibrolamellar bone in 3D following the studies of (Faingold et al., 2013; Reznikov et al., 2013, 2014). The method involves sequentially exposing new surfaces of an embedded sample using the focused beam of gallium ions, and imaging the exposed face with the electron beam (Heymann et al., 2006). In this way a stack of serial surface images with nanometric isotropic resolution in all three orthogonal directions is obtained. The lateral resolution and the slice thickness are both of the order of 10 nm and the field of view is around 10×10 microns. The preferred orientation and the angular dispersion of the collagen fibrils can be quantified using fast Fourier transform (FFT). Here we present the results of an SSV study of fibrolamellar bone from a young minipig femur.

2. Materials and methods

2.1. Bone preparation

Samples were obtained from the mid-diaphysis of 2 femora (right and left) of an 8 week old male minipig obtained from a commercial company (Harlan Biotech Israel Ltd. Rehovot, Israel). The femora were cleaned of soft tissue and periosteum using manual dissection. The bones were wrapped in cotton gauze soaked in double distilled water (DDW) to maintain a high humidity environment and were stored at −20 °C prior to analysis. The experiments were approved by the Institutional Animal Care and Use Committee at the Weizmann Institute of Science.

2.2. Scanning electron microscopy (SEM)

Transverse ring sections about 5 mm thick were cut from the mid-shaft of the femur using a water-cooled rotary diamond saw (Buehler IsoMet 1000 Precision saw, USA). The sections were ground with emery paper and then inspected by light microscopy (Nikon Eclipse E600-POL, USA) to identify areas of fibrolamellar bone close to the periosteal surface. Small bar-shaped specimens with dimensions of $1 \times 1 \times 5$ mm with the longest dimension parallel to the long axis of the bone were cut out of the ring sections in areas composed exclusively of fibrolamellar bone. The bar-shaped specimens were cleaned and defatted in acetone and then fractured into two fragments so that the fracture plane was oriented orthogonally to the femur longitudinal axis. The fragmented specimens were treated with 6% sodium hypochlorite for 2.5 min to remove organic residues, followed by sonication and thorough washing with double distilled water (DDW).

A few fractured specimens were partially demineralized in a solution of 5% ethylenediaminetetracetic acid (EDTA), 2% paraformaldehyde (PFA) in cacodylate buffer, pH 7, for 5 min at room temperature on a rocking table. After surface demineralization the samples were washed of residual EDTA using DDW, dehydrated in ethanol series and critical point dried (CPD) using a CPD-030 critical point dryer (Bal-Tec).

The fractured samples were mounted on an aluminum stub with carbon tape onto which a small aluminum cube was attached. The cube was used as a support to stabilize the elongated specimen. The samples were coated with a 15 nm thick layer of chromium by sputtering (EMITECH K575X, Quorum Technologies Ltd., UK). The CPD treated samples were carbon coated using an evaporator (Auto 306 Turbo, Edwards, United Kingdom). Samples were imaged in an Ultra 55 SEM (Carl Zeiss, NTS GmbH) using a secondary electron in-lens detector (2–5 kV).

Back-scattered SEM imaging was performed on transversally polished cross-sections. Transverse ring sections 1 mm thick were cut from the mid-shaft of the bone using a water-cooled rotary diamond saw (Buehler IsoMet 1000 Precision saw, USA). The sections were cleaned and defatted in acetone and ground with emery paper (P1200, P1500, P2500) and then a polishing cloth with 3 and 1 µm diamond suspension (Buehler Minimet 1000 Grinder-Polisher, USA). The transverse polished surfaces were photographed with a transmission light microscope (Nikon Eclipse E600-POL, USA). The samples were mounted using carbon tape on an aluminum stab and carbon coated by vacuum evaporation (Auto 306 Turbo, Edwards, United Kingdom). Samples were imaged in an XL30 environmental scanning electron microscope (ESEM) (FEI Company, USA) using a BSE detector at 15 kV and a working distance of 15 mm. Image brightness and contrast levels were adjusted by using Fiji/ImageJ (NIH, USA) and Adobe Photoshop.

2.3. Bone preparation for the FIB–SEM

The procedure used follows Reznikov et al. (2014). Transverse ring sections 1 mm thick were cut from the mid-shaft of the minipig femora and polished as described above for BSE imaging. In all sections the areas of fibrolamellar bone were located at the anterior–lateral periosteal region.

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