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Spatial heterogeneity in the canalicular density of the osteocyte network in human osteons

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ABSTRACT

Osteocytes interconnect with each other forming an intricate cell network within the mineralized bone matrix. One important function of the osteocyte network is the mechano-regulation of bone remodeling, where a possible mechanism includes the fluid flow through the porosity housing the cell network - the osteocyte lacuno-canalicular network (OLCN). In our study the OLCN in human osteons was three-dimensionally imaged with the aim to obtain a quantitative description of the canalicular density and spatial variations of this quantity within osteons. The topology of the OLCN was determined by first staining the bone samples with rhodamine, then imaging the OLCN with confocal laser scanning microscopy and finally using image analysis to obtain a skeletonized version of the network for further analysis. In total 49 osteons were studied from the femoral cortical bone of four different middle-aged healthy women. The mean canalicular density given as length of the canaliculi in a unit volume was 0.074 ± 0.015 μm/μm^3 (corresponding to 74 km/cm^3). No correlation was found between the canalicular density and neither the size of the osteon nor the volume fraction occupied by osteocyte lacunae. Within osteons the canalicular density varied substantially with larger regions without any network. On average the canalicular density decreases when moving from the Haversian canal outwards towards the cement line. We hypothesize that a decrease in accessible canaliculi with tissue age as a result of micropetrosis can reduce the local mechanosensitivity of the bone. Systematic future studies on age- and disease-related changes on the topology of the OLCN have to demonstrate the diagnostic potential of the presented characterization method.

1. Introduction

Although osteocytes are by far the most abundant bone cells, they attracted only recently the specific attention of bone researchers (Bonewald, 2011). One crucial reason for this late popularity is that osteocytes live buried in the mineralized matrix and, therefore, their activity is more difficult to observe than the activity of osteoblasts and osteoclasts. In living bone, osteocytes occupy lacunae of about 15 μm in their largest dimension and use a network of canaliculi of about 300 nm in diameter (Varga et al., 2015) to connect with each other via gap junctions. The high density of this network entails that the osteocyte lacuno-canalicular network (OLCN) contributes with >1% to the overall porosity of cortical bone (Cardoso et al., 2013), and its total surface in the human skeleton was estimated to be as large as 215 m^2 (Buenzli & Sims, 2015). Different functions have been attributed to the network of osteocytes. The osteocytes are known to be strongly mechano-sensitive cells (Kleinnulend et al., 1995; Klein-Nulend et al., 2013) with their cell processes more sensitive than the cell bodies (Adachi et al., 2009). The network of osteocytes is thought to control the structural adaptation of bone to mechanical needs (Schaffler et al., 2014; Lanyon et al., 1993). Different mechanisms have been proposed of how the mechanical stimulation is sensed by the network. The fluid flow hypothesis states that the mechanical loading squeezes bone fluid through the canaliculi creating shear forces on the cell processes of osteocytes (Han et al., 2004; Burger & Klein-Nulend, 1999). An alternative hypothesis proposes that microdamage results in the interruption of cell processes resulting in the death of the osteocyte (Verborgt et al., 2000). Besides mechanosensing, recently the role of the osteocyte network in mineral homeostasis has been extensively revisited (Qing &

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Bonewald, 2009). In a process called osteocytic osteolysis (Belanger, 1969; Teti & Zallone, 2009), osteocytes dissolve mineral from the surrounding matrix, thereby enlarging the lacunae (Qing & Bonewald, 2009) and influencing the mineral characteristics of the surrounding bone (Kerschnitzki et al., 2013).

This work focuses on the osteocyte lacuno-canalicular network in human osteons. Osteons are the result of cortical bone remodeling and constitute the basic building block of cortical bone (Parfitt, 1994). Most of the research on the OCLN in osteons concentrated on the osteocyte lacunae due to their larger spatial dimensions compared to the canaliculi. In particular, using synchrotron radiation tomography, the size and arrangement of osteocyte lacunae in osteons was measured in great detail (Hannah et al., 2010; Dong et al., 2014). It was reported that in human osteons the osteocyte density on average increased from $4 * 10^4/mm^3$ close to the Haversian canal to $9 * 10^4/mm^3$ at 80% of osteon radius. The nearest-neighbor distance between lacunae peaked at 23 μm (Hannah et al., 2010). Moreover, a framework has been established to quantify the osteocyte lacunae, in particular their shape and orientation (Mader et al., 2013).

However, for a proper assessment of the proposed functions of the osteocyte network mentioned above, the properties of the canalicular network seem even more essential. Different methods are nowadays available for a quantitative three-dimensional imaging of the OLCN (Schneider et al., 2010). The strength of synchrotron radiation based methods like phase nanotomography (Varga et al., 2015; Langer et al., 2012) and the combination of scanning electron microscopy (SEM) and focused ion beam (FIB) (Schneider et al., 2011) are a high spatial resolution. The method to combine staining and confocal laser scanning microscopy (Kerschnitzki et al., 2013; Tate et al., 2004; Ciani et al., 2009; Sugawara et al., 2005; Kerschnitzki et al., 2011) has the advantage to provide an image of the OCLN of large bone volumes. With this method, which is used in our study, the network can be analyzed over distances as large as a diameter of an osteon. Applied to the OCLN in sheep it was demonstrated that 80% of the bone matrix is within a distance of only 1.4 μm to the closest canaliculus (Kerschnitzki et al., 2013). Based on histological observations it was suggested that the integrity and three-dimensional organization of the osteocyte network change in disease states such as osteoporosis, osteoarthritis, and osteomalacia (Tate et al., 2004). Alterations in the osteocyte lacunar–canalicular microenvironment were reported with an increase of the effective canalicular size as a result of estrogen deficiency (Sharma et al., 2012).

For future investigations of changes in the OLCN linked to bone diseases, a first essential step constitutes in a quantitative description of the healthy state, which then can serve as a reference. Therefore, the aim of the current study is to quantify the architecture of the osteocyte canalicular network within osteons of healthy individuals. The main descriptor of the network is the canalicular length density of the network, i.e. the length of canaliculi present per bone volume measured in $\mu m/\mu m^3$. As a more concise term in the following only canalicular density is used, since length is the natural physical quantity of the network to be quantified per bone volume. For the functionality of the network, in particular the bone tissue permeability, the canalicular density is an essential parameter (Cardoso et al., 2013; Steck & Knothe-Tate, 2003; Verbruggen et al., 2012).

In this study special attention is dedicated on local variations of the network density within an osteon. Marotti and co-workers were first in addressing the question whether the density of the network changes in radial direction, i.e. the direction of bone apposition during remodeling, and found no statistical significant variation (Marotti et al., 1995). With the novel possibility to evaluate now the three-dimensional topology of the network we revisited this question. As the OLCN is imaged via a stain penetrating the network, only the accessible part of the network is imaged. Consequently, when canaliculae and/or lacunae get blocked by mineralized tissue – a process termed micropetrosis (Frost, 1960) – then this part of the network remains unstained. It has been reported that the amount of highly mineralized lacunar occlusions increases with age (Busse et al., 2010) and that osteoporosis and osteoarthritis can be responsible for a higher fraction of hypermineralized osteocyte lacunae (Carpentier et al., 2012). Since micropetrosis most likely has a strong impact on the mechanosensitivity of the bone tissue, a focus of our network evaluation was the detection of regions in the osteon which do not contain an accessible canalicular network.

2. Materials and methods

2.1. Samples

Osteons for the investigation of the OLCN were selected from transversal cross sections of the femoral midshaft from four different human cadavers. Directly after the necropsy bone samples were frozen and stored at −20 °C and transferred into 70% ethanol prior to further preparation. The sample preparation preserved a large part of the lateral region of the cortex covering the whole cortical thickness. Form such a sample transversal cuts of 0.5 mm thickness were then used for rhodamine staining. By polishing one of the surfaces using abrasive paper with grit designation P 1200 under wet conditions, the image quality could be improved due to removal of the rhodamine stained surface layer.

All individuals were female and middle-aged (age of death between 48 and 56 years) without any known bone-related metabolic disease. The cause of death was in all individuals related to cardiovascular diseases. Samples were provided by the Department of Forensic Medicine of the Medical University of Vienna in accordance with the ethic commission board of the institution (EK #: 1757/2013).

2.2. Staining and confocal imaging

Samples were placed in a phosphate buffered saline (PBS) solution with dissolved rhodamine-6G powder (0.02 %wt) for two days under constant movement. With the small size of the rhodamine molecule (roughly 1 nm * 1 nm * 0.4 nm (Lu & Penzkofer, 1986)) this time is sufficient that rhodamine can penetrate into all the accessible parts of the OLCN as tested by time dependent staining analyses and to attach to its mineralized surface. Using confocal laser scanning microscopy (CLSM) (Leica TCS SP5) the rhodamine fluorescence allows to image the canalicular network (Kerschnitzki et al., 2011), where the unembedded samples are not kept in the rhodamine solution during imaging. Rhodamine was excited with combined laser light of 488 nm and 514 nm and the fluorescence signal was then detected in the wavelength range of 550–650 nm (Fig. 1a). The microscope setting with a 100× oil-immersion objective (HCX PL APO CS 100.0 × 1.40 OIL) and a numerical aperture of 1.4 would result in a theoretical lateral resolution of about 280 nm. For the imaging the used voxel size was $(300\ nm)^3$. Imaged volumes were 155 μm * 155 μm * 40 μm, where the smallest value in axial direction is due to the limited transparency of the mineralized bone. During the measurement the decay of intensity of the fluorescence signal with increasing imaging depth was compensated by adapting the laser intensity and the voltage of the detecting photo multiplier tubes. For the imaging, in each of the four samples multiple osteons were selected over the whole width of the lateral femoral cortex. The selected osteons were middle-sized, had a roughly circular cross-section and were all alternating following standard terminology (Ascenzi & Bonucci, 1968). In total 49 osteons were investigated with at least 11 osteons in each sample (see Table 1 for number of investigated osteons in each sample).

2.3. Image analysis

As a first step in the analysis the stack of images from confocal microscopy (Fig. 1b) was binarized employing a customized adaptive thresholding algorithm. The used algorithm is based on difference of Gaussians (DoG) (Marr & Hildreth, 1980), a classical feature