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Osteocyte morphology in fibula and calvaria — Is there a role for mechanosensing?

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Abstract

Introduction: External mechanical forces on cells are known to influence cytoskeletal structure and thus cell shape. Mechanical loading in long bones is unidirectional along their long axes, whereas the calvariae are loaded at much lower amplitudes in different directions. We hypothesised that if osteocytes, the putative bone mechanosensors, can indeed sense matrix strains directly via their cytoskeleton, the 3D shape and the long axes of osteocytes in fibulae and calvariae will bear alignment to the different mechanical loading patterns in the two types of bone.
Materials and methods: We used confocal laser scanning microscopy and nano-computed tomography to quantitatively determine the 3D morphology and alignment of long axes of osteocytes and osteocyte lacunae *in situ*.
Results: Fibular osteocytes showed a relatively elongated morphology (ratio lengths 5.9:1.5:1), whereas calvarial osteocytes were relatively spherical (ratio lengths 2.1:1.3:1). Osteocyte lacunae in fibulae had higher unidirectional alignment than the osteocyte lacunae in calvariae as demonstrated by their degree of anisotropy (3.33 and 2.10, respectively). The long axes of osteocyte lacunae in fibulae were aligned parallel to the principle mechanical loading direction, whereas those of calvarial osteocyte lacunae were not aligned in any particular direction.
Conclusions: The anisotropy of osteocytes and their alignment to the local mechanical loading condition suggest that these cells are able to directly sense matrix strains due to external loading of bone. This reinforces the widely accepted role of osteocytes as mechanosensors, and suggests an additional mode of mechanosensing besides interstitial fluid flow. The relatively spherical morphology of calvarial osteocytes suggests that these cells are more mechanosensitive than fibular osteocytes, which provides a possible explanation of efficient physiological load bearing for the maintenance of calvarial bone despite its condition of relative mechanical disuse.

Keywords: Osteocyte 3D morphology; Fibula; Calvaria; Stress/strain; Mechanosensing

Introduction

The cellular response to mechanical forces is known to be inherently coupled to the internal organization of the cytoskeleton and connection to the surrounding cells and the extracellular matrix [1]. External mechanical forces on cells are known to influence cytoskeletal structure and thus cell shape [2]. In bone, it is widely believed that osteocytes residing within the bone matrix sense the external mechanical loads. Anatomically, the lacunae contain the osteocyte cell bodies from which long, actin-rich slender cell processes (50–60 per cell) radiate through the canaliculi to the surrounding osteocytes, to the cells lining the bone surface, and to the extraosseous space [3,4]. Osteocytes forming the intercellular network likely supervise the biomechanical regulation of bone mass and architecture by modulating the activity of osteoblasts and/or osteoclasts via the production of chemical signals [5,6]. How osteocytes sense external mechanical loads is unknown, however it has been suggested that mechanosensing by osteocytes occurs as a result of strain-derived flow of interstitial fluid through the lacuno-canalicular network [7]. Han et al. [8] hypothesised that the fluid flow-mediated excitation mechanism of osteocytes is

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caused by a unique strain amplification that results from the interaction of the pericellular matrix and the cell process cytoskeleton. This hypothesis is not extended to the osteocyte cell bodies as the fluid flow-mediated shear forces on the osteocyte cell bodies are too small to be sensed [9]. Hence osteocyte cell bodies have not been implicated in mechanosensation in bone.

Recently, we have shown that osteocytes are also sensitive to cytoskeleton-mediated localised mechanical stimulation, both at the cell body and the cell processes [10]. The skeletal system constitutes of bones with inherently distinct mechanical loading patterns e.g. load bearing long bones, such as fibulae, are predominantly loaded parallel to their longitudinal direction [11]. On the other hand, flat bones such as calvariae, are loaded with much lower amplitudes radially and/or tangentially due to intracranial pressure and/or mastication. Human calvaria experiences 200 microstrain (peak value), whereas human tibia experiences nearly 2100 microstrain (peak value) during extreme activities [12]. Reductions of tibial bone strain to less than 200 microstrain cause profound bone loss. Although human fibula is not the predominant load bearing bone, but it still transmits 1/6 of the total load on leg. Hence human fibula is estimated to be loaded nearly twice as the human skull bone [13]. Thus, if osteocytes can sense external mechanical loads via the matrix strains directly through their cytoskeleton, the shape and directional alignment of single osteocytes in fibulae and calvariae should differ and bear alignment parallel to the principle mechanical loading direction in these bones, which have different mechanical loading patterns.

Presently, there is little information about the three dimensional (3D) morphology of single osteocytes and lacunar spaces *in vivo*, because osteocytes are deeply embedded in the mineralised matrix and thus are difficult to visualise by microscopy. However, confocal laser scanning microscopy (CLSM) allows visualisation of single osteocytes within the bone matrix by using DAR 4 M AM. DAR 4 M AM is a rhodamine based chromophore, which fluoresces after reacting with intracellular nitric oxide [10,14]. Also the recently developed high-resolution nano-computed tomography (nano-CT) can be used to visualise single lacunae in bone specimens of up to 1 mm thickness. In this study, we applied CLSM and nano-CT techniques to quantitatively determine the 3D morphology and orientation of single viable osteocytes and lacunae *in situ*. This was investigated in two differently mechanically loaded adult mice bones; fibula, which is subjected to unidirectional load bearing and calvaria, which is subjected to multidirectional load bearing. This insight will shed light on the possible role of osteocyte cell body morphology in efficient direct mechanosensing of external loads in bone.

Materials and methods

Bone explant preparation

Whole calvariae and fibulae were aseptically isolated from 3–6 months old adult C57 B1/6 wild type mice. The periosteal surfaces of the bone explants were gently scraped to remove muscular attachments and periosteum for better imaging of the deeper embedded osteocytes. In fibular explants, bone marrow was flushed out thrice with 1 ml phosphate buffered saline. The bone was then dissected longitudinally through the marrow cavity and pieces of approximately 3 × 1 mm were prepared. In calvarial explants, the suture edges were trimmed and pieces of approximately 3 × 1 mm were prepared. The smaller width of bone pieces resulted in relatively flat sections, which aided in visualising more cells than the relatively wider and concave bone sections. The bone explants were then prepared for single osteocyte imaging and/or single lacunae imaging as described below.

Osteocyte staining for live cell imaging

For *in situ* online live cell imaging, osteocytes lying in the bone matrix were loaded with DAR-4 M AM chromophore (Calbiochem, Merck KGaA, Darmstadt, Germany) as described previously [10,14]. Briefly, bone explants were incubated with 10 μM DAR-4 M AM for 1–2 h at room temperature in Dulbecco's Modified Eagle's medium without phenol red (D-MEM; Gibco, Paisley, UK). Extracellular chromophore was then removed by washing twice the bone explants in D-MEM without phenol red.

Confocal laser scanning microscopy

Image acquisition

Confocal images of osteocytes were obtained by using BioRad MRC-1000 UV Leica confocal system attached to a Leica inverted microscope (Leica Microsystems, Wetzlar, Germany). A Leica 63× water immersion objective lens was used with a numerical aperture of 1.2 and a working distance of 170 μm. The fibular and calvarial bone explants were excited by using a krypton/argon laser at 545 nm and the emission was collected at 580 nm. Sequences of x–y optical slices were collected, separated by 0.25–0.50 μm on the z-axis. The osteocyte fluorescence intensity was deliberately saturated by the addition of 6 mM *S*-nitroso-*N*-acetylpenicillamine (SNAP; Sigma, St. Louis, MO, USA), an extracellular NO donor, in order to augment the signal-to-noise ratio, and to compensate for the reduced fluorescence intensity in the deeper parts of the bone explants.

Post acquisition image processing (deconvolution)

All images were processed by means of an interactive maximum likelihood estimation algorithm (Huygens II deconvolution software, Scientific Volume Imaging, The Netherlands, http://www.svi.nl) running on Octane and Origin 300 computers (SGI, Mountain View, CA, USA). Deconvolution of 'z-stacks' was done in order to achieve the very best resolution with the highest degree of statistical confidence [15]. This processing improves the quality of blurry or noisy images in such a way that the structures become sharper and clearer in an environment cleaned of noise, which in the end improves the quality of the 3D reconstruction to a large extent.