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Osteocyte calcium signaling response to bone matrix deformation

Taiji Adachi [a,b,*], Yuki Aonuma [a], Shin-ichi Ito [a], Mototsugu Tanaka [c], Masaki Hojo [a], Teruko Takano-Yamamoto [d], Hiroshi Kamioka [e]

[a] Department of Mechanical Engineering and Science, Graduate School of Engineering, Kyoto University, Sakyo, Kyoto 606-8501, Japan
[b] Computational Cell Biomechanics Team, VCAD System Research Program, RIKEN, Hirosawa, Wako 351-0198, Japan
[c] Department of Aeronautics, College of Engineering, Kanazawa Institute of Technology, Ougigaoka, Nonoichi, Ishikawa 921-8501, Japan
[d] Division of Orthodontics and Dentofacial Orthopedics, Graduate School of Dentistry, Tohoku University, Aoba, Sendai 980-8575, Japan
[e] Department of Orthodontics, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama 700-8525, Japan

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ABSTRACT

Osteocytes embedded in calcified bone matrix have been widely believed to play important roles in mechanosensing to achieve adaptive bone remodeling in a changing mechanical environment. *In vitro* studies have clarified several types of mechanical stimuli such as hydrostatic pressure, fluid shear stress, and direct deformation influence osteocyte functions. However, osteocyte response to mechanical stimuli in the bone matrix has not been clearly understood. In this study, we observed the osteocyte calcium signaling response to the quantitatively applied deformation in the bone matrix. A novel experimental system was developed to apply deformation to cultured bone tissue with osteocytes on a microscope stage. As a mechanical stimulus to the osteocytes in bone matrix, in-plane shear deformation was applied using a pair of glass microneedles to bone fragments, obtained from 13-day-old embryonic chick calvariae. Deformation of bone matrix and cells was quantitatively evaluated using an image correlation method by applying for differential interference contrast images of the matrix and fluorescent images of immunolabeled osteocytes, together with imaging of the cellular calcium transient using a ratiometric method. As a result, it was confirmed that the newly developed system enables us to apply deformation to bone matrix and osteocytes successfully under the microscope without significant focal plane shift or deviation from the observation view field. The system could be a basis for further development to investigate the mechanosensing mechanism of osteocytes in bone matrix through examination of various types of rapid biochemical signaling responses and intercellular communication induced by matrix deformation.

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1. Introduction

Osteocytes are differentiated from bone-forming osteoblasts and are embedded in bone matrix during calcification of the osteoid produced by the osteoblasts (Parfitt, 1994). In the calcified bone matrix, osteocytes have a characteristic morphology, constructing an intercellular network via their cellular processes (Kamioka et al., 2001). This distributed cellular network in bone matrix is considered to have important functions in sensing mechanical stimuli under dynamic loading and in transmitting signals to neighboring cells (Gu et al., 2007; Bonewald and Johnson, 2008). Therefore, it is widely believed that a mechanosensing mechanism exists in which osteocytes function as mechanosensors in bone tissue, underlying important processes for achieving bone functional adaptation by remodeling in response to a changing mechanical environment (Cowin et al., 1991; Weinbaum et al., 1994; Burger and Klein-Nulend, 1999; Wang et al., 2007; You et al., 2008).

Although osteocytes and their intercellular network spreading over the bone matrix imply a pivotal role in adaptive bone remodeling, their actual role in bone matrix is not yet well understood. This lack of knowledge can be attributed to the relative difficulty of direct observation and isolation of osteocytes compared to other bone cells such as osteoblasts and osteoclasts located on bone surfaces. After establishment of the isolation method (van der Plas and Nijweide, 1992) and the cell line (Kato et al., 1997, 2001), studies on osteocyte responses to biochemical and mechanical stimuli began to unfold. Several types of mechanical stimuli that might be generated in bone matrix, such as hydrostatic pressure, fluid flow-induced shear stress, and direct deformation, have been proposed mechanical stimuli that could affect osteocyte functions (Weinbaum et al., 1994; Klein-Nulend et al., 1995; Burger and Klein-Nulend, 1999; Miyauchi et al., 2000; You et al., 2000; Aonuma et al., 2007; Adachi et al., 2009).

* Corresponding author at: Department of Mechanical Engineering and Science, Graduate School of Engineering, Kyoto University, Sakyo, Kyoto 606-8501, Japan. Tel./fax: +81 75 753 5216.
E-mail address: adachi@me.kyoto-u.ac.jp (T. Adachi).

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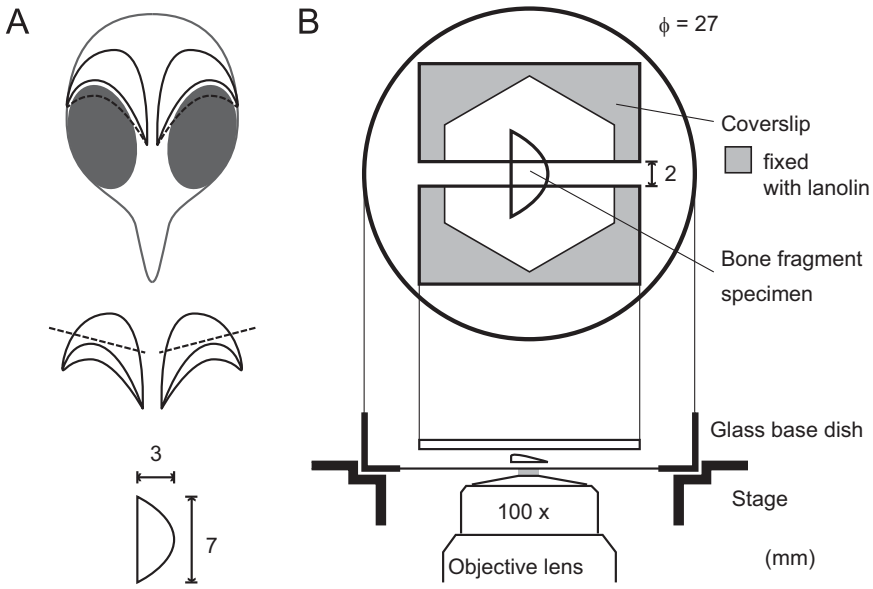

Fig. 1. Preparation and holding of the bone fragment specimen in the experimental system. (A) Two bone fragments were obtained from a 13-day-old embryonic chicken calvaria by cutting out flat semielliptical areas in the forehead region. (B) Each bone fragment was clipped between the bottom of the culture dish and two coverslips bonded with lanolin and set on a microscope stage. Clearance gap of 2 mm between the coverslips was made to access the bone fragment specimen for direct deformation using microneedles.

Osteocytes in the calcified bone matrix are in a unique mechanical environment very different from that of the osteoblasts and osteoclasts on bone surfaces. Given that osteocytes function to regulate bone metabolism by sensing mechanical stimuli induced by deformation of the bone tissue, it is preferable to investigate the mechanosensitivity of osteocytes in conditions as close to *in situ* as possible. Several approaches have been used to investigate the mechanosensitivity of osteocytes embedded in bone matrix. For example, an *ex vivo* experimental system using bone tissue (Takai et al., 2004; Mann et al., 2006) and a three-dimensional gel-embedded culture system (Kurata et al., 2006, 2007; Fukunaga et al., 2008) were employed to clearly demonstrate the osteocyte response to matrix deformation and damage, resulting in changes in viability, apoptosis, production of signaling molecules, and interaction with osteoblasts and osteoclasts. These studies also observed osteocyte behavior in bone matrix with fluorescent reagents as well as histochemical approaches. Therefore, time-course imaging of the cellular biochemical responses to mechanical stimuli and observation of matrix and cell deformation would be a challenging task to better understand the mechanosensing mechanism of osteocytes in bone matrix.

The aim of this study was to observe the calcium signaling response of the osteocytes in bone matrix under quantitatively applied deformation. We have developed a novel experimental system on a microscope stage that would enable us to apply mechanical deformation to bone tissue with live osteocytes and observed osteocyte response to the stimuli combined with an imaging technique. Direct deformation was applied to bone fragments obtained from chicken embryonic calvariae, and the deformation and calcium signaling response of single osteocytes in the bone matrix were observed.

2. Materials and methods

2.1. Preparation of bone fragments

Bone fragments were obtained from 13-day-old embryonic chicken calvariae with thickness of ~60 μm, within which osteocytes construct an intercellular network (Kamioka et al., 2001) that can be observed using transmitted light microscopy. The calvariae were trimmed to flat fragments, as shown in Fig. 1A, and shake-incubated with 1 mg/ml collagenase type I (Sigma-Aldrich) in a bone isolation buffer (Hefley, 1987) for 30 min at 37.5 °C to remove the periosteum on the bone surface together with cells such as osteoblasts. Subsequently, to allow for application of mechanical deformation, the fragments were incubated with 0.5 mM ethylenediamine tetraacetic acid (EDTA, Nakarai Tesque) in Dulbecco's phosphate buffered saline (DPBS) containing 0.1% bovine serum albumin (BSA, Sigma-Aldrich) for 15 min. The treated fragments were washed with DPBS, incubated for 9–24 h in α-MEM with 2% fetal bovine serum (FBS, Sigma-Aldrich) and antibiotics (ampicillin and streptomycin), and cultured at 37 °C, 100% relative humidity, and 5% CO_2 in air.

2.2. Immunolabeling of osteocyte membrane

Osteocyte membrane in the bone matrix was immunolabeled to trace its deformation associated with the matrix deformation. The bone fragments were incubated for 10 min with monoclonal antibody (MAb) OB7.3 (Nijweide and Mulder, 1986), a kind gift from Jenneke Klein-Nulend and Cornelis M. Semeins of ACTA-Vrije University, (diluted to 1:20 with α-MEM) that is specific to the transmembrane protein Phex of chicken osteocytes (Westbroek et al., 2002). After incubation, the fragments were washed with DPBS and loaded with Alexa Fluor 546 goat anti-mouse IgG as the secondary antibody.

2.3. Calcium indicators

To evaluate the intracellular calcium ion concentration $[Ca^{2+}]_i$ a ratiometric method (Grynkiewicz et al., 1985) was used in which the ratio of fluorescent intensities of two calcium indicators with different fluorescent characteristics were quantitatively evaluated. The calcium indicators, 5 μM Fluo-4 AM and 10 μM Fura Red AM (Invitrogen), were loaded into the cells with a solubilizing agent, 0.01% Cremophor EL (Sigma-Aldrich), for 15 min at 37 °C. Subsequently, the cells were washed with DPBS, and the culture medium was replaced with a CO_2-independent medium (Invitrogen) to stabilize pH and avoid drying.

2.4. Application of deformation to bone fragment

The bone fragment, within which osteocytes were fluorescently immunolabeled and loaded with calcium indicators, was held on a glass bottom dish ($\phi = 27$ mm, Iwaki) using two coverslips and lanolin, as illustrated in Fig. 1B.

As a mechanical stimulus to the osteocytes in bone matrix, shear deformation was applied to a bone fragment in the x–y plane, as schematically illustrated in Fig. 2, using a pair of glass microneedles with a tip diameter of approximately $\phi = 2$ μm. Both microneedles were attached to hydraulic micromanipulators (MM-188NE: controlled side; MC-35A: fixed side, Narishige). First, the bone matrix was indented by the tips of the microneedles (Fig. 2A). Then, with the position of

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