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The early mouse 3D osteocyte network in the presence and absence of mechanical loading

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ABSTRACT

Osteocytes are considered to act as mechanosensory cells in bone. They form a functional synctia in which their processes become interconnected to constitute a three-dimensional (3D) network. Previous studies reported that in mice, the two-dimensional osteocyte network becomes progressively more regular as they grow, although the key factors governing the arrangement of the osteocyte network during bone growth remain unknown. In this study, we characterized the 3D formation of the osteocyte network during bone growth. Morphological skeletal changes have been reported to occur in response to mechanical loading and unloading. In order to evaluate the effect of mechanical unloading on osteocyte network formation, we subjected newborn mice to sciatic neurectomy in order to immobilize their left hind limb as an unloading model. The osteocyte network was visualized by staining osteocyte cell bodies and processes with fluorescently labeled phalloidin. First, we compared the osteocyte network in the femora of embryonic and 6-week-old mice in order to understand the morphological changes that occur with normal growth and mechanical loading. In embryonic mice, the osteocyte network in the femur cortical bone displayed a random cell body distribution, non-directional orientation of cell processes, and irregularly shaped cells. In 6-week-old mice, the 3D network contained spindle-shaped osteocytes, which were arranged parallel to the longitudinal axis of the femur. In addition, more and longer cell processes radiated from each osteocyte. Second, we compared the cortical osteocyte networks of 6-week-old mice that had or had not undergone sciatic neurectomy in order to evaluate the effect of unloading on osteocyte network formation. The osteocyte network formation in both cortical bone and cancellous bone was affected by mechanical loading. However, there were differences in the extent of network formation between cortical bone and cancellous bone in response to mechanical loading with regard to the orientation, nuclear shape and branch formation.

Introduction

Osteocytes are derived from bone-forming osteoblasts that become embedded in the calcified bone matrix. In calcified bone, osteocytes display a characteristic morphology and form an intercellular network via their cellular processes [1–3]. Osteocytes in lacunae and canaliculi are surrounded by interstitial fluid. The interstitial fluid is required for maintenance of bone as it supplies nutrition to the cells and carries away waste matter and is also involved in mineral exchange [4]. In addition, the movement of the interstitial fluid is thought to act as a mechanical stimulus that triggers biological responses in osteocytes [5,6]. Therefore, the conservation of the osteocyte network is important for viability of bone and its adaptation to the mechanical loading.

The osteocyte network in bone displays a 3D structure composed of actin-rich osteocyte processes [7]. Therefore, we have developed a method for visualizing the osteocyte network by staining for actin filaments in the osteocyte processes with fluorescent phalloidin, and then examining the network in situ with confocal laser scanning microscopy (CLS) [3]. We have subsequently developed a method for the 3D morphometric analysis of osteocyte networks using CLS image stacks [8].

The key factors regulating osteocyte network formation are not well understood. Moreover, several reports have stated that different types of osteocyte networks can exist. We recently examined the 3D osteocyte network in chicks and mice, and detected differences among the species [9]. It has also been reported that several bone-specific diseases, such as osteopenia, osteoarthritis, and osteopetrosis, are accompanied by changes to the osteocyte network [10]. Furthermore, Vashishth et al. reported that in human bone, osteocyte density is inversely associated with the accumulation of microcracks with age [11]. Interestingly, Hirose et al. observed differences in the arrangement of canaliculi between 3-day-old mice and 12-week-old mice and found that the two-dimensional osteocyte network becomes progressively more regular as mice grow [12].

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Mechanical loading has been shown to markedly affect bone geometry and architecture. [13,14]. Therefore, we evaluated the effect of mechanical loading on osteocyte network formation during bone growth. We used our previously described technique to assess the morphological changes induced in the 3D osteocyte network by mechanical loading and unloading. We analyzed 3D image stacks of osteocyte networks in the cortical and cancellous bone of embryonic mice that had not been subjected to mechanical loading, and 6-week-old mice subjected to normal loading, and compared their features.

We further examined the differences in the 3D osteocyte network of femora that had or had not been subjected to immobilization by neurectomy. Sciatic neurectomy has been described previously as excellent model for studying the effect of mechanical unloading on bone [15–17]. Therefore, in this study, we employed sciatic neurectomized mice to examine the 3D morphological and morphometric changes that occur during osteocyte network formation in the presence or absence of mechanical loading.

Materials and methods

Animals

All animals were maintained at 20 °C under a 12 h light/12 h dark cycle, given food and water ad libitum, and allowed to move freely in the cage for the duration of the study. Two different study types were implemented to examine changes in the morphological characteristics of femoral osteocytes and the effect of sciatic neurectomy.

In the first experiment, female pregnant mice (C57BL/6J) were kept in individual cages. For the embryonic studies, 19-day-old embryonic (EB) mice were collected and femora were excised. To obtain adolescent specimens for comparison, 6-week-old (6W) mice were manually dissected to obtain femora free of soft tissues.

In the second experiments, female pregnant mice (C57BL/6J) were maintained in individual cages. At 5 days after parturition, the newborn mice underwent unilateral sciatic nerve section in order to examine the effect of immobilization on osteocyte network formation in the absence of mechanical loading. We used sham-operated mice as controls.

Cryoanesthesia was employed in order to reduce the amount of surgical stress suffered by the mice [18,19]. Each mouse was placed in the ice chamber for about 3 min and then removed and placed on an ice pack on the stage of a dissecting microscope for the duration of the surgery. We used latex sleeves and sheets of thin non-woven fabric to prevent mice's direct contact with the cold surface and the surgery was carried out using aseptic surgical technique. In the operation group, a small incision was made, and the left hind limb of each animal was subjected to sciatic nerve section. The surgical wound was closed with tissue adhesive (Histoacryl®, TissueSeal, MI). After the surgery, mice were placed on a warm surface to recover, and then returned to the cage containing their mother. We checked that the mice subjected to this procedure (NX mice) walked with a limp throughout the study. The left hind femoral bones of the NX mice were dissected and manually detached from the surrounding soft tissue after 6 weeks.

Preparation of bone specimens for CLS analysis

The obtained femora were washed with PHEM (60 mmol/L piperazine-*N*′,*N*′-bis [2-ethane-sulfonic acid], 25 mmol/L *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid], 10 mmol/L ethylene glycol-bis [2-amino-ethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid, and 2 mmol/L magnesium chloride, pH 6.9). The periosteal surfaces of the bone explants were scraped by hand to remove muscular attachments and the periosteum, and then the bone explants were fixed in 3% buffered paraformaldehyde for 2 days. After that, femora were decalcified with 5% ethylene diaminetetraacetic acid disodium (EDTA) in 0.1 M phosphate buffer (pH 7.4) at room temperature. This was performed for 1 day for 19-day-old EB mice and NX mice specimens, and 1 week for the 6W mice specimens. The specimens were submerged in 20% sucrose to adjust their osmotic pressure, frozen in OCT compound, and sagittally sectioned into 50-μm-thick specimens. Serial cryosections of the tissue were examined under confocal laser microscope, and the resultant images were used to reconstruct and analyze 3D images.

Fluorescence staining and confocal laser scanning images

Femoral specimens were permeabilized by incubation in 0.5% Triton X-100 in PBS for 10 min. Specimens were then rinsed and stained for 2 days at 4 °C with Texas red-X-conjugated phalloidin in PBS containing 1% BSA (1:100 dilution; excitation wavelength = 595 nm, emission wavelength = 615 nm; Molecular Probes Inc., Eugene, OR). After being rinsed with PBS, the samples were embedded in fluorescence mounting medium (Dako, Carpinteria, CA, USA) containing 1 mg/ml p-phenylenediamine dihydrochloride (Sigma, St. Louis, MO, USA), then viewed immediately. We stained internal actin filaments and not the cell membrane with phalloidin conjugated fluorescence.

Confocal optical sectioning was performed with an LSM510 CLS microscopy system (Carl Zeiss, Oberkochen, Germany) coupled to an upright microscope (Zeiss) with Plan Fluor objective (63×, N.A. = 1.4). The refractive index of the immersion media (Zeiss 518F) was 1.518. The theoretical *xy*- and *z*-axes resolutions were 0.263 and 0.604 μm, respectively. The images had a frame size of 146.2 × 146.2 μm and a color depth of 8-bit. Confocal images were taken with 0.3 μm step size and processed four times with Kalman averaging. The confocal images were processed for noise reduction with the Huggens software program (Bitplane AG, Zurich, Switzerland).

3D reconstruction and morphologic analysis of the osteocyte network

The 3D structure of each mouse's osteocyte network was reconstructed from CLS images of cortical femoral bone using the IMARIS software program (Bitplane AG, Zurich, Switzerland), as reported previously [8]. The images were shown as a Z-projected image of their confocal image stacks, and the Z-projection was generated from the sum of the confocal images. Due to the difficulties in determining the start and end points of osteocyte processes, all of the calculations were performed based on the 3D-reconstructed IMARIS images, which normally contained 3–6 osteocytes in one sample region. The number of processes radiating from each osteocyte was counted by rotating the 3D-reconstructed fluorescent model created by the software program. In order to know how much of the bone compartment volume is occupied by single osteocyte, the bone compartment volume, which included 3–6 osteocytes, was divided by the number of osteocytes within it and showed as bone volume/osteocyte. The point-to-point distances between the centers of the osteocytes were also calculated, since the IMARIS software program enables the recognition of the center of each osteocyte.

Osteocyte process length was measured using the NEURON TRACER software program (Bitplane AG, Zurich, Switzerland), as reported previously [8]. By mapping fluorescence-positive sites, we used this software to construct a dendritic model of the osteocyte network, and it calculated the total length of the osteocyte processes.

In addition, the surface area and volume of the osteocytes including their processes were quantified using the SURPASS software program (Bitplane AG, Zurich, Switzerland), as reported previously [8]. Volume and surface area data correction was necessary due to elongation of the *x*-, *y*-, and *z*-axes on the SURPASS software. Approximate volume and surface area correction values were calculated using the method described in our previous report [8]. In brief, using correction values obtained from 2.0 and 0.5 μm fluorescent beads, the volume and surface area of the osteocyte cell bodies were calculated. The volume correction values for the 0.5 and 2.0 μm beads were 0.30 and 0.41, respectively, and