Bone 74 (2015) 134–139

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

Intravital bone imaging by two-photon excitation microscopy to identify osteocytic osteolysis in vivo

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ARTICLE INFO

Article history:
Received 6 October 2014
Revised 13 January 2015
Accepted 21 January 2015
Available online 24 January 2015

Edited by Sakae Tanaka

Keywords:
Osteocytic lacunae
Two-photon microscopy
Intravital imaging
Osteocyte
Osteocytic osteolysis

ABSTRACT

Bone is a highly dynamic organ in which several cell types function cooperatively. Among these, osteocytes have recently emerged as an important regulator of bone homeostasis, although their mechanism of regulation is unclear. Here, intravital bone imaging by two-photon excitation microscopy allowed us to directly visualize 'osteocytic osteolysis', or resorption of bone in the lacuno-canalicular system. Osteocyte lacunae and the canalicular network in the cortex of murine tibiae were imaged by in vivo calcein staining, and local acidification in these structures was monitored using a topically applied pH sensor. We also demonstrated that sciatic neurectomy causes significant acidification around osteocytic lacunae and enlargement of lacuno-canalicular areas. These results provide strong evidence for osteocytic osteolysis, and demonstrate that two-photon intravital microscopy is useful for analysis of this phenomenon.

Introduction

Osteocytes are the most abundant cellular component of bone, comprising around 90–95% of all bone cells. These cells are 'entombed' within bone tissue, but seem to survive for extended periods, up to 25 years in humans [1]. Osteocytes are terminally differentiated osteoblasts, considered dormant until recent evidence demonstrated their critical role in endocrine regulation and bone homeostasis [2–5]. Osteocytes are connected to one another via a network of cytoplasmic projections [4,5], consisting of disk-shaped osteocytic lacunae (OL) and numerous dendritic processes (canaliculi) radiating therefrom.

Since Baud reported electron micrographic observations of osteocytes' roughly bordered lacunar walls in 1962 [6], the concept of bone resorption by osteocytes, so-called 'osteocytic osteolysis', has been proposed and reviewed [7–11], although these initial histological studies provided little definite evidence. On the contrary, OL enlargement has also been attributed to an artifact of specimen preparation [12]; isolated avian osteocytes fail to resorb bone in vitro [13]. Since similar changes in OL can also be found in younger osteocytes, enlargement could result from insufficient mineralization of the periosteocytic matrix [12,14]. In addition, the irregular, variable morphology of OL presents major challenges to examination of osteocytic osteolysis.

Nevertheless, recent circumstantial evidence supports the concept [15,16]. OL enlargement was detected by lactation [17] in the presence of sclerostin [18] or microgravity [19], suggesting active regulation of the OL space and osteolysis. However, no direct evidence has yet been presented.

Over the past few years we have established a system for visualizing the bone tissues of living animals 'intravitally' under completely intact conditions [20–25]. This novel system has unraveled mechanisms of migration and functions of bone-resorbing osteoclasts and their precursors in vivo. In this study, we exploited this new imaging technique to visualize and analyze the function of osteocytes in vivo.

Materials and methods

Intravital in vivo bone tissue imaging

Intravital microscopy of mouse tibiae was performed using protocols modified from a previous study [20,21]. Briefly, mice were anesthetized with isoflurane (2.0%, vaporized in 100% oxygen), and two-thirds of the length of the medial tibia was exposed by stripping the periosteum. Exposed cortical bone tissues were observed by two-photon excitation microscopy with a custom-made stereotactic holder. The imaging system was composed of a multiphoton microscope (SP5; Leica) driven

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http://dx.doi.org/10.1016/j.bone.2015.01.013
8756-3282/

by a laser (Mai-Tai HP Ti: Sapphire; Spectraphysics) tuned to 870 nm and an upright microscope (DM6000B; Leica) equipped with a 20× water immersion objective (HCX APO, N.A. 1.0; Leica). Fluorescent materials were detected through band pass emission filters at 430/80 nm (for second harmonic generation; SHG), at 500/50 nm (for calcein) and at 565/605 nm (for RhP-EF). Raw imaging data was processed using the Imaris software (Bitplane) with a Gaussian noise filter.

Bone labeling with calcein

Calcein (Sigma-Aldrich, 20 mg/kg, s.c.) was systemically administered the day before histological examination. Femurs were removed and frozen in chilled hexane (Wako, Osaka, Japan) on dry ice. Sections (10 μm thick) of non-decalcified femurs were subjected to bright-field and fluorescence microscopy. For intravital in vivo imaging using two-photon microscopy, calcein (20 mg/kg, s.c.) was injected 3 days before examination; injection the day before caused over-staining, preventing quantification (data not shown).

RhP-EF staining

RhP-EF (AcidiFluor™ ORANGE, Goryo Chemical (Hokkaido, Japan); 10 μM in PBS) was applied topically to the exposed bone surface for 20 min, followed by thorough washing with PBS before observation by intravital two-photon microscopy.

Image analysis

All images were captured at a 0.5-μm volume step size. The area of the largest OL from each sequential image was measured using Image J. Fluorescence intensity and length were measured using Leica TCS SP5 software. For standardization of image acquisition, OLs were selected only at depths of 15 to 25 μm beneath the bone surface. We chose OLs sufficiently labeled with calcein for analysis because we could not correctly detect the sizes of the OLs if staining deficits were present (data not shown). The areas of five randomly selected OLs were measured in each analysis, and five analyses were performed in each mouse. Fluorescent ratios were measured in three OLs per analysis. We obtained the image data exactly 20 μm below the exposed surface to detect the actual values of the fluorescent signals of RhP-EF in three randomly selected OLs per analysis.

Bone loss model with sciatic neurectomy

C57BL/6 mice at 9 weeks of age were obtained from CLEA Japan. All mice were bred and maintained under pathogen-free conditions in the animal facilities of Osaka University (Osaka, Japan). Ten-week-old female C57BL/6 mice were subjected to unilateral sciatic neurectomy (SNR) or sham surgery (day 0) under isoflurane-induced anesthesia. SNR consisted of resecting a 3- to 4-mm segment of the right sciatic nerve posterior to the hip joint [26,27]. For sham surgery, the same procedure was performed without resection.

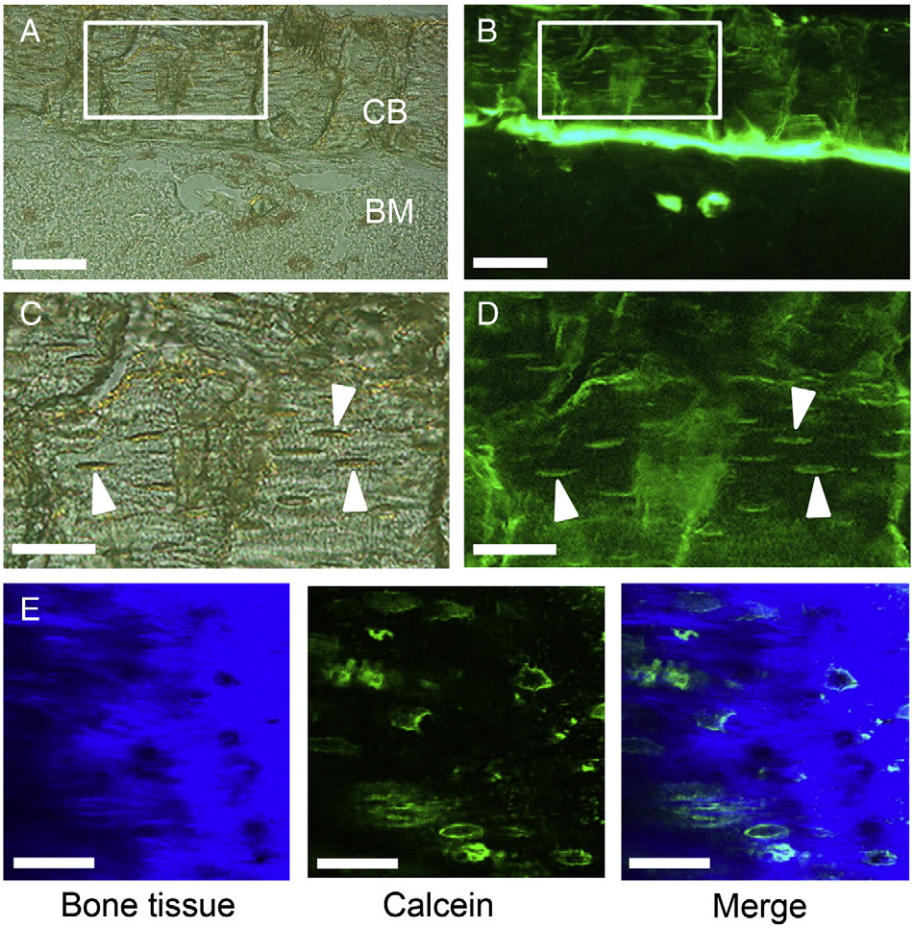

Fig. 1. Osteocytes incorporate calcein in the OL. Representative bright-field (A, C) and fluorescence microscopy (B, D) images of the middle femur 1 day following s.c. injection. (C, D) High magnification of the boxed areas in A and B, respectively. Arrowheads indicate OLs. (E) Intravital two-photon imaging of tibia cortex 3 days after calcein injection. Blue, SHG signal from collagen; green, calcein. CB: cortical bone, BM: bone marrow. Scale bars: (A, B) 100 μm; (C–E) 35 μm.