Review

Visualizing bone tissue in homeostatic and pathological conditions

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Abstract: The human body is comprised of hundreds of bones, which are constantly regenerated through the interactions of two cell types: osteoblasts and osteoclasts. Given the difficulty of analyzing their intravital dynamics, we have developed a system for intravital imaging of the bone marrow cavity using two-photon microscopy, to visualize the dynamic behaviors of living bone cells without sectioning. Combined with the newly developed chemical fluorescent probes to detect localized acidification caused by osteoclasts, we identified two distinct functional states of mature osteoclasts, i.e., “bone-resorptive” and “non-resorptive”. Here, we focus on the dynamics and functions of bone cells within the bone marrow cavity and discuss how this novel approach has been applied to evaluate the mechanisms of action of drugs currently in clinical use. We further introduce our recent study that identified arthritis-associated osteoclastogenic macrophages in inflamed synovium and revealed their differentiation trajectory into the pathological osteoclasts, which together represent to a new paradigm in bone research.

Keywords: inflammation, arthritis, osteoclast, macrophage, imaging

Introduction

Bone is constantly regenerated through a dynamic process called “bone remodeling”; 20–30% of the entire skeleton of the human body is renewed every year. The destruction of mineralized bone by osteoclasts is followed by the synthesis of the new bone by osteoblasts, maintaining the integrity of the skeleton and normal calcium levels in the body. In pathological osteolytic conditions, the reciprocal interplay between the bone and immune system also plays a major role in osteoclastogenesis.1)–3)

Over the past two decades, conventional methodologies such as histology, flow cytometry, cell culture, and real-time PCR analysis have identified a number of molecules, including cytokines and receptors, which control bone remodeling. However, cellular dynamics and interplay in the living body, especially within the bone marrow cavity, remain largely unexplored due to the challenges in applying experimental approaches to examine deep tissues. The advent of an intravital imaging system using multi-photon microscopy, combined with the increasing variety of fluorescent reporter mouse strains and fluorescent probes, has permitted direct recording of the behaviors of osteoclasts, osteoblasts, and immune cells in the bone marrow in four dimensions at subcellular resolution. This approach facilitates elucidation of cellular dynamics in both homeostatic and pathological conditions, enabling direct observation of complex biological phenomena in situ.

Here, we focus on state-of-the-art methods for intravital imaging of the bone marrow cavity and discuss how advances in imaging methods in living mice have contributed to our understanding of the dynamic bone regenerative process of bone remodeling. Furthermore, we introduce our recent findings, including the identification of pathological osteoclast precursor (OP) macrophages in inflamed joints, termed arthritis-associated osteoclastogenic...
macrophages (AtoMs), and discuss future perspectives in bone biology using this novel imaging technique.

**Intravital two-photon imaging of bone tissue**

Bone marrow is covered by one of the hardest tissues in the body – bone. Therefore, it is technically difficult to visualize the dynamic behavior of bone cells inside the bone marrow cavities of living animals. Conventional methods, including histochemistry and micro-computed tomography (micro-CT), have been applied in bone research to analyze cell morphology, three-dimensional bone architecture, and molecular expression patterns in individual cells. However, information regarding vital cells and cellular interactions in conditions with intact blood flow circulation is lost using these methods. Using near-infrared lasers in multi-photon microscopy, deeper tissue penetration can be achieved to visualize the cellular dynamics of organs *in vivo*, and we have previously established a multi-photon imaging system to visualize living bone tissue.

Two-photon microscopy has several advantages compared with single-photon confocal microscopy. First, upon excitation, multiple photons are absorbed simultaneously only in the region of the focal plane, thus restricting the excitation area and permitting bright, high-resolution images of the region of interest. Second, photobleaching and phototoxicity can be minimized by excitation using a laser operating at near-infrared wavelength, which is essential to obtain long-term time-lapse imaging data. Third, deeper penetration into tissue (100–1,000 µm) can be achieved by light at near-infrared wavelengths compared with confocal microscopy, which yields data to a depth <100 µm. Fourth, multi-photon microscopy can take advantage of a nonlinear optical process called second harmonic generation (SHG). When two photons with the same frequency interact with a coordinated material, a photon with exactly half the wavelength of the excitation photons is generated without exogenous fluorescent labeling. Because multi-photon excitation uses near-infrared lasers (800–1,000 nm), the visible spectrum of SHG can be observed (400–500 nm) to detect collagen fibers and bone matrix. Therefore, two-photon microscopy affords efficient light detection, reduces photobleaching, penetrates deeper into tissues, and detects SHG, which makes it an important tool for intravital imaging of deep tissues. However, multi-photon microscopy should not entirely replace confocal microscopy. In tissues with depth <50 µm, confocal microscopy can record multicolor images with high spatial and temporal resolutions. In fact, the combination of single-photon and two-photon images allows simultaneous acquisition of a wide variety of fluorescence signals, together with SHG signals for the detection of fibrous tissues in thin sections (Fig. 1).

Bone marrow is surrounded by crystallized calcium phosphate of the bone matrix, which can scatter excitation light. Therefore, we selected parietal bones of mice with a thickness of approximately 100 µM, which is sufficient to permit penetration of the near-infrared excitation laser into the cavity. Intravital two-photon imaging of the skull bone allowed visualization of the behavior of a variety of cells in bone marrow cavities, and is useful for evaluating the effects of novel drugs targeting skeletal diseases.

Furthermore, two-photon excitation phosphorescence lifetime measurements are used for quantitative oxygen determination *in vivo*. This system revealed that local oxygen tension (pO2) of the bone marrow is heterogeneous, with the lowest pO2 in the deep peri-sinusoidal regions (1.3%), and the endosteal region is less hypoxic.
Multi-photon imaging of the migration of osteoclast precursors

Osteoclasts are multinucleated cells with unique bone-destroying properties that are derived from the monocyte/macrophage lineage cells. However, the means by which OPs migrate to the endosteum in vivo remains unknown. In a previous study using two-photon imaging of skull bone tissue, we found that a blood-enriched mediator of lipid metabolism, sphingosine-1-phosphate (S1P), controlled the migratory behavior of OPs.6),7) S1P is a bioactive sphingolipid metabolite that regulates various biological activities through five receptors, designated as S1PR1 to S1PR5.11–15) OPs were labeled with enhanced green fluorescent protein (EGFP) in knock-in mice expressing EGFP under the control of the CX3CR1 promoter (Fig. 2A), and these precursors were stimulated to move into blood vessels by S1P stimulation in vivo.6),7) Although S1PR1 promotes cell movement toward higher S1P concentrations in the circulatory fluids, S1PR2 negatively regulates S1PR1 responses and S1PR2 antagonists mobilize OPs into the bloodstream.6),7) Together, the local S1P concentration and two cognate receptors for S1P, S1PR1 and S1PR2, regulate the number of OPs on the bone surface by bidirectional exchange with the circulation.

Another study showed that active vitamin D analogs regulate the migratory behavior of OPs by inhibiting S1PR2 expression.9) Injection of vitamin D significantly inhibited the expression of S1PR2 in OPs in the bone marrow, and two-photon microscopy revealed that the motility of these cells was significantly increased when treated with active vitamin D derivatives. Thus, analysis of the migratory behavior of OPs on the bone surface can shed light on previously unknown mechanisms of conventionally used medications.

Multi-photon imaging of mature osteoclasts

Osteoclasts are unique bone-resorbing multinucleated cells that are generated through the fusion of mononuclear precursor cells. Upon stimulation with macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB (RANKL), monocytc cells can form osteoclast-like cells in vitro, containing up to 100 nuclei. However, it remains unclear whether such extraordinarily gigantic cells indeed exist in vivo, and how mature osteoclasts resorb bone in living bone tissue. To answer these questions, we applied intravital two-photon microscopy to visualize mature osteoclasts in situ.

Mature osteoclasts form a tight attachment zone, called the “sealing zone”, via interactions between integrin αvβ3 on the osteoclast membrane and bone matrix components.36) Among the multiple vacuolar type H+-ATPases (V-ATPases) expressed along the ruffled border membrane,17) the α3 isoform

![Fig. 2. Visualization of CX₃CR1⁺ osteoclast precursors (OPs) and mature osteoclasts in the bone marrow. (A) Image of the calvaria of CX₃CR1-enhanced green fluorescent protein (EGFP) transgenic mouse taken by two-photon microscopy. Blood vessels were stained by intravenous injection of dextran-conjugated Texas Red (red). Scale bar: 50 µm. The maximum intensity projections (MIPs) of two-dimensional image stacks of vertical calvarial slices are shown. (B) Image of the calvaria of an a3-GFP knock-in mouse taken using two-photon microscopy. Mature osteoclasts were a3-GFP⁺ (green). Scale bar: 50 µm. Second harmonic fluorescence generated from two-photon excitation of collagen fibers defined the bone matrix (blue). The maximum intensity projections (MIPs) of two-dimensional image stacks of vertical calvarial slices are shown. (C) Representative intravital multi-photon image of the bone marrow of a heterozygous TRAP-tdTomato transgenic mouse treated with a pH-sensing fluorescent probe. Mature osteoclasts expressing TRAP-tdTomato signals (red), fluorescent signals from high H⁺ concentration (green), and SHG defining the bone matrix. Scale bar: 50 µm.](image-url)
of the a-subunit is preferentially and abundantly expressed in fully differentiated osteoclasts.\textsuperscript{16,17}) To fluorescently label these osteoclasts, we generated knock-in mice expressing a fusion protein of the a3 subunit and GFP under the control of the a3 subunit promoter (a3-GFP knock-in mice) (Fig. 2B).\textsuperscript{8,18,19})

We next generated pH-sensing chemical fluorescent probes capable of detecting an acidic region caused by bone-resorbing osteoclasts on the bone surface \textit{in vivo} (Fig. 2C). These probes were composed of boron-dipyrromethene (BODIPY) dye and a bisphosphonate group. BODIPY dyes are widely used fluorescent dyes because of their large molar absorption coefficients, environmental stability, and environment-independent fluorescence quantum yields.\textsuperscript{20}) The bisphosphonate group forms a tight bond with the bone matrix to facilitate delivery of the probe to the bone surface.\textsuperscript{21}) As a result, the probe detects the area of bone resorption by osteoclasts and emits a green fluorescent signal, which can be visualized by multi-photon microscopy. Intravital imaging of mature osteoclasts and bone-resorbing lesions led to identification of two distinct functional states of osteoclasts, \textit{i.e.}, bone-resorbing static (R) osteoclasts and non-resorbing moving (N) osteoclasts.\textsuperscript{8}) The compositions of these two populations were altered by injection of recombinant RANKL, an essential osteoclastogenic cytokine in both homeostatic and arthritic conditions,\textsuperscript{20–23}) indicating that RANKL is involved not only in osteoclast differentiation but also in controlling the bone-resorptive function of osteoclasts.\textsuperscript{8,24,25})

\textbf{Imaging the crosstalk between osteoclasts and osteoblasts}

Bone homeostasis is maintained by complex communications between bone-resorbing osteoclasts and bone-forming osteoblasts, in a process called bone remodeling. However, their spatiotemporal relationships and interactions \textit{in situ} remain elusive. Therefore, we generated transgenic reporter mice expressing a red fluorescent protein, tdTomato, in the cytosol of osteoclasts (TRAP-tdTomato mice)\textsuperscript{8}) and enhanced cyan fluorescent protein (ECFP) in the cytosol of osteoblasts (Col2.3-ECFP mice)\textsuperscript{26}) to visualize the reciprocal interplay between osteoclasts and osteoblasts. Then, we crossed TRAP-tdTomato mice with Col2.3-ECFP mice to generate TRAP-tdTomato/Col2.3-ECFP double transgenic mice and visualized the coupling interactions between osteoclasts and osteoblasts \textit{in vivo} using multi-photon microscopy (Fig. 3). Osteoblasts and osteoclasts showed separate distributions in physiological conditions, a degree of some direct cell–cell contact was observed in the boundary areas, and osteoclasts making contact with osteoblasts projected synapse-like structures toward the osteoblasts. Application of a pH-sensing fluorescence probe revealed that osteoclasts actively resorb bone when they are not in contact with osteoblasts, whereas osteoclasts in contact with osteoblasts do not show bone resorption, indicating that the bone resorption capacity of osteoclasts is exquisitely regulated by osteoblasts in a contact-dependent manner.

Another study also visualized osteoblasts within the bone marrow cavity using Col2.3-GFP/CFP reporter mice. The dynamic interactions between human T-cell acute lymphoblastic leukemia cells and the bone marrow microenvironment, including osteoblasts and nestin\textsuperscript{+} cells, were observed using intravital microscopy.\textsuperscript{27})

\textbf{Differentiation trajectory of arthritic osteoclast precursors in inflamed synovium}

Osteoclasts play key roles not only in maintaining skeletal homeostasis in the bone marrow, but also in pathological arthritic bone erosion in patients with rheumatoid arthritis (RA), which takes place where
the inflamed synovium invades the articular bone. Despite extensive studies, precise analysis of OPs in the hypertrophied synovium (called “pannus”) has not been performed because of technical difficulties in purely isolating the tiny synovial tissues on the so-called “bare area”, where the synovium is attached to the bone. In addition, empirical evidence of the origin of pathological osteoclasts forming on the pannus-bone interface is still lacking.

To address these issues, we developed an original protocol to isolate the inflamed synovium on the bare area, by removing the patella and quadriceps femoris muscles together under a stereoscopic microscope. Flow cytometric plots of the pannus were distinct from those of other organs, and identified CX3CR1hi-Ly6ChiF4/80I-A+/I-E+ macrophages, which we designated as AtoM, as the OP-containing population in the arthritic joints. A bone marrow chimeric model and parabiosis model together showed that AtoMs and pathological osteoclasts in the pannus-bone interface are derived exclusively from bone marrow-derived cells and not from synovium-dwelling macrophages. AtoMs showed substantial potential to form osteoclasts upon simultaneous stimulation with RANKL and tumor necrosis factor (TNF) (Fig. 4), and single-cell RNA-Seq analysis further indicated that about 10% of AtoMs differentiated into osteoclasts in the pannus microenvironment.

Inhibition of FoxM1, a transcription factor that is reported to play key roles in carcinogenesis by rendering tumor cells more invasive, suppressed the osteoclastogenesis of AtoMs. These findings underscored the need for studies to delineate the mechanism of pathological bone destruction in situ within the pannus-bone interface. Therefore, we are currently engaged in the development of an intravitral imaging system of the inflamed synovium using multi-photon microscopy, which will contribute to a better understanding of the dynamic interactions of bone and the immune system in the joint microenvironment.

Conclusion

Over the past two decades, substantial progress in clarifying the mechanisms involved in interactions among bone cells has led to the successful translation of research into therapeutic approaches in a number of bone diseases, such as osteoporosis and RA. However, the functional properties of living bone and immune cells within the bone marrow cavity and joint microenvironment remain largely unclear. Intravital multi-photon imaging of fluorescently labeled cells together with functional probes, such as pH sensors, provides unbiased spatiotemporal information on the complex biological phenomena occurring in living animals. In addition, new fluo-
resent probes and optogenetic techniques such as photoconvertible fluorescent proteins (Kaede and KikGR), cell-cycle indicators (Fucci), and caspase-3 indicators (SCAT3.1), are consistently being developed. The next frontier may involve the incorporation of novel visualization tools into multi-photon imaging to better understand the sophisticated biological processes involved in bone metabolism, hematopoiesis, bone marrow metastasis of tumors, and arthritic osteolysis.

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Profile

Masaru Ishii was born in Osaka in 1973 and graduated from the Osaka University Medical School in 1998. After working as a physician specialized in rheumatology and internal medicine, he studied at the National Institutes of Health as a Human Frontier Science Program fellow (2006–2008), as a laboratory head in Osaka University Immunology Frontier Research Center (2008–2013), and then appointed as a Professor of the Department of Immunology and Cell Biology, Graduate School of Medicine, Osaka University in 2013. The bulk of his studies have so far elucidated cellular dynamics in live tissues and organs in bone and immune systems using a cutting-edge intravital imaging technique, which he has originally developed. His research focus currently covers diverse research topics where cells are dynamically moving, such as immune cell migration in inflammatory sites and cancer invasion/metastases. He has authored over 100 publications in this field and has received several prestigious awards: the Young Investigator Award, Japanese Medical Association (2013), JSPS Prize (2014), JSBMR Ogata Prize (2018), and Setsuro Ebashi Prize from the The Japanese Pharmacological Society (2019). He serves as the Vice President of the Japanese Society for Bone and Mineral Research, and also serves as a Trustee of the Japanese College of Rheumatology and the Japanese Society of Inflammation and Regeneration.