osteoblasts. Osteoclast-lineage cells have also been shown to change the expression levels of chemokines and chemokine receptors after stimulation by RANKL. These chemokines and their receptors probably regulate the migration of the precursors not only onto the bone surface but also to other precursors for fusion in an autocrine/paracrine manner. RANKL induces the expression of C-C chemokines such as CCL2 (or monocyte chemoattractant protein-1, MCP-1) (8;11;13), CCL3 (or macrophage inflammatory protein-1α, MIP-1α) (9;11;26), CCL5 (or regulated on activation, normal T cell expressed and secreted, RANTES) (8;11), and CCL9 (or MIP-1γ) (10;26), as well as C-X-C chemokines such as CXCL2 (or MIP-2α) (11) and CXCL10 (or interferon-γ-inducible 10-kDa protein, IP-10) (11;12). In addition, the chemokine receptors CCR1 (7;8;10;26), CCR2 (7;8;13), CCR3 (10), and CXCR1 (26) are reported to be induced by RANKL. During osteoclastogenesis, some chemokines (for example, CCL3, CCL4, CCL5, CXCL2, and CXCL10) and receptors (such as CCR2 and CX3CR1) are downregulated (5-7;11;12). Presumably, after the cells mature and arrive at their destinations, these chemoattractants have served their function and are no longer needed. Table 1 summarizes the chemokines and their receptors, which are reported to be involved in the migration of osteoclast precursors.

In addition to protein chemokines, we have clarified that sphingosine-1-phosphate (S1P), a lipid mediator enriched in blood, regulates the migration of osteoclast precursors. S1P is synthesized in most cells, but is irreversibly degraded by intracellular S1P lyase or dephosphorylated by S1P phosphatase. Therefore, the levels of S1P in most tissues, including bone marrow, are relatively low. On the other hand, its concentration in the blood is extremely high. In addition, S1P is an amphiphilic molecule that cannot be expelled easily across membranes. In this way, a S1P gradient between the blood and tissues is stably maintained. S1P transmits signals through GPCRs, as do chemokines. Mammals possess five types of S1P receptors, $S1P_1$ to $S1P_5$, and macrophage-monocyte lineage cells express $S1P_1$ and $S1P_2$ (27-29). $S1P_1$ is coupled primarily to PTx-sensitive $G_{i/o}$ proteins, and $S1P_2$ is coupled to $G_{12/13}$, and G_s. These differences account for the different biological effects of $S1P_1$ and $S1P_2$, which have opposite effects on osteoclast precursor migration. Expression levels of $S1P_1$ are reduced by RANKL stimulation, dependent on NF-κB, not NF-AT. Osteoclast precursors show chemoattracting responses to a S1P gradient *in vitro*, which is blocked by PTx. In addition, S1P treatment of osteoclast precursors induced an increase in the active form of Rac (GTP-Rac), suggesting that Rac and Gαi are involved in the $S1P_1$ chemotactic signaling pathway. Additionally, $S1P_1$ agonists promote the recirculation of osteoclast precursors and ameliorate ovariectomy-induced bone loss (14). On the other hand, $S1P_2$ has a binding affinity for S1P that differs from that of $S1P_1$. A higher concentration of S1P is required to activate $S1P_2$, which induced negative chemotactic responses to a S1P gradient and causes the cells to move out of the bloodstream into the bone marrow cavity (unpublished observation).

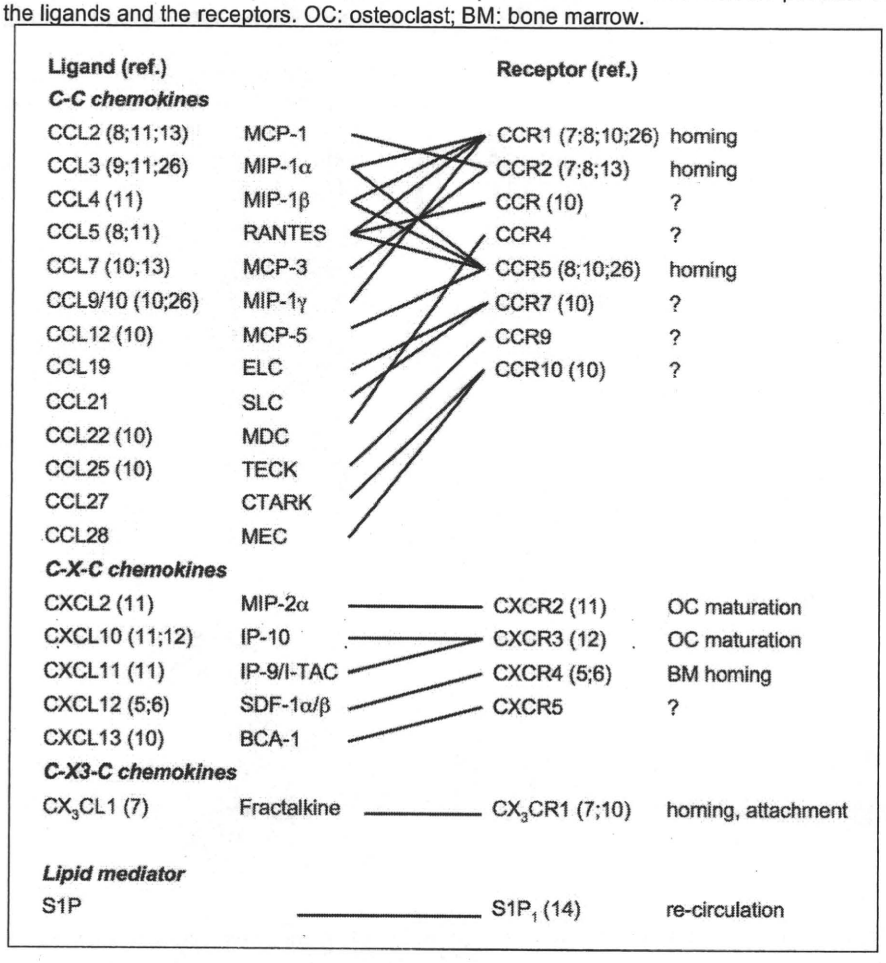
Seeing Is Believing

Typically, chemotaxis has been assayed using several *in vitro* systems, including transmigration assays using Transwell filters or a Boyden chamber (30). These methods are convenient for determining quantity and are highly reproducible. However, these *in vitro* assay systems may not accurately reflect *in vivo* cellular behavior.

Recent technological progress in fluorescence microscopy, especially two-photon excitation-based laser microscopy, has enabled the visualization of dynamic cell behavior deep inside intact living organs (23;24). With two-photon microscopy, we have observed osteoclast migration by visualizing murine bone marrow in real-time in a living body (14). There are limitations to visualizing the deep tissue of bone, because the crystallized calcium phosphate in the bone matrix scatters both visible and infrared light. However, we have developed

IBMS BoneKEy. 2010 August;7(8):279-286
http://www.bonekey-ibms.org/cgi/content/full/ibmske;7/8/279
doi: 10.1138/20100459

Table 1. Chemoattractants and repellents for osteoclast precursors. The lines indicate possible interactions between the ligands and the receptors. OC: osteoclast; BM: bone marrow.



a novel intravital imaging system for visualizing the living bone marrow cavity with high spatiotemporal resolution. We chose the skull of a mouse as the observation site because it is about 100 μm thick, which is within the range of two-photon microscopy (31). Monocytes present in the bone marrow cavity, including osteoclast precursors, are generally stationary. However, a subset of these cells becomes motile shortly after the intravenous application of SEW2871, a selective S1P1 agonist, with some of the mobilized cells entering the blood circulation. Thus, S1P1 agonists promote the recirculation of osteoclast precursor monocytes from the bone surface into the blood, thereby repressing osteoclastogenesis (14;15).

Intravital imaging is making a great contribution to visualizing these animated processes *in vivo*. It provides spatiotemporal information in a living body, which cannot be procured by other methods. This approach has revealed active features of both physiological bone homeostasis and pathological bone destruction. Nevertheless, intravital microscopy imaging has several limitations. First, two-photon microscopy has a penetration depth of up to 200 μm in hard tissues, and thus deeper tissues cannot be observed. Given this resolution limitation, the technique is applicable only in small animal models such as mice and rats, and not in humans. Second, owing to the wide scattering of light on the skin, it is necessary to exteriorize the target organ, and it is difficult to observe tubular bones. To

IBMS BoneKEy. 2010 August;7(8):279-286
http://www.bonekey-ibms.org/cgi/content/full/ibmske;7/8/279
doi: 10.1138/20100459

overcome these limitations, technical innovations in fluorescent probes and optical systems are needed, including improved emission light and resolution.

In the future, in addition to its use in viewing morphology and motion, intravital imaging will be applied to functional analyses. This will be possible by using new photoresponsive fluorescent proteins that change fluorescence upon absorbing light energy of specific wavelengths, *e.g.*, photoactivation (acquiring fluorescence) and photoconversion (changing the wavelength of the emitted light) (32;33), and light-sensing devices such as photo-activating GPCRs (34;35).

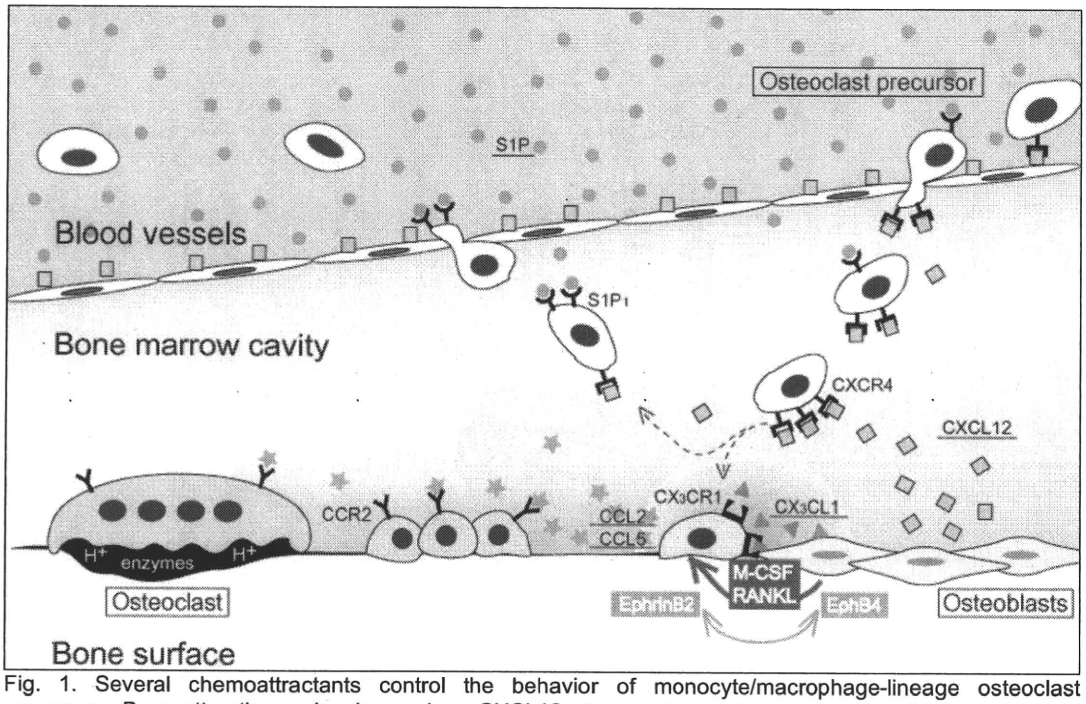


Fig. 1. Several chemoattractants control the behavior of monocyte/macrophage-lineage osteoclast precursors. Bone-attraction molecules such as CXCL12 attract osteoclast precursors into the bone marrow cavity from the bloodstream. Then, bone-attachment inducers such as CX3CL1 recruit and attach the precursors to the bone surface, where they resorb bone. Finally, paracrine effectors such as CCL2 and CCL5 cause the precursor cells to fuse with each other. Circular-attraction molecules such as S1P drive the cells out of the bone marrow cavity and into the bloodstream. To maintain bone homeostasis, these processes regulate the number of osteoblastic stromal cell-derived osteoclast precursors on the bone surface that are available for stimulation by M-CSF, RANKL, or Eph.

Conclusion

Osteoclastogenesis can be considered to occur in three steps: 1) recruitment of precursors; 2) cell fusion; and 3) bone resorption. Of these, cell recruitment is the most dynamic step and the most dependent on the microenvironment of the bone marrow cavity. The results achieved so far are summarized in Fig. 1. Briefly, the regulation of monocyte-lineage osteoclast precursor migration is critical for the development of osteoclasts and the maintenance of bone homeostasis. Several chemokines recruit osteoclast precursors to sites of resorption, and cause them to fuse with each other, and other circular-attraction molecules such as S1P drive osteoclast precursors out of the bone marrow cavity. Given the importance of temporospatial information in elucidating these processes, intravital imaging has made a huge contribution. For example, this new technique has revealed that several

chemoattractants act in concert to shepherd osteoclast precursors to appropriate sites. Controlling the recruitment and migration of osteoclast precursors can be a promising new therapeutic target for bone diseases. In addition, intravital imaging will afford new opportunities for studying both the physiology and pathology of bone.

Conflict of Interest: None reported.

Peer Review: This article has been peer-reviewed.

References

1. Harada S, Rodan GA. Control of osteoblast function and regulation of bone mass. *Nature.* 2003 May 15;423(6937):349-55.

2. Teitelbaum SL, Ross FP. Genetic regulation of osteoclast development and function. *Nat Rev Genet.* 2003 Aug;4(8):638-49.

3. Wada T, Nakashima T, Hiroshi N, Penninger JM. RANKL-RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med.* 2006 Jan;12(1):17-25.

4. Henriksen K, Neutzsky-Wulff AV, Bonewald LF, Karsdal MA. Local communication on and within bone controls bone remodeling. *Bone.* 2009 Jun;44(6):1026-33.

5. Yu X, Huang Y, Collin-Osdoby P, Osdoby P. Stromal cell-derived factor-1 (SDF-1) recruits osteoclast precursors by inducing chemotaxis, matrix metalloproteinase-9 (MMP-9) activity, and collagen transmigration. *J Bone Miner Res.* 2003 Aug;18(8):1404-18.

6. Wright LM, Maloney W, Yu X, Kindle L, Collin-Osdoby P, Osdoby P. Stromal cell-derived factor-1 binding to its chemokine receptor CXCR4 on precursor cells promotes the chemotactic recruitment, development and survival of human osteoclasts. *Bone.* 2005 May;36(5):840-53.

7. Koizumi K, Saitoh Y, Minami T, Takeno N, Tsuneyama K, Miyahara T, Nakayama T, Sakurai H, Takano Y, Nishimura M, Imai T, Yoshie O, Saiki I. Role of CX3CL1/fractalkine in osteoclast differentiation and bone resorption. *J Immunol.* 2009 Dec 15;183(12):7825-31.

8. Kim MS, Day CJ, Morrison NA. MCP-1 is induced by receptor activator of nuclear factor-kappaB ligand, promotes human osteoclast fusion, and rescues granulocyte macrophage colony-stimulating factor suppression of osteoclast formation. *J Biol Chem.* 2005 Apr 22;280(16):16163-9.

9. Choi SJ, Cruz JC, Craig F, Chung H, Devlin RD, Roodman GD, Alsina M. Macrophage inflammatory protein 1-alpha is a potential osteoclast stimulatory factor in multiple myeloma. *Blood.* 2000 Jul 15;96(2):671-5.

10. Lean JM, Murphy C, Fuller K, Chambers TJ. CCL9/MIP-1gamma and its receptor CCR1 are the major chemokine ligand/receptor species expressed by osteoclasts. *J Cell Biochem.* 2002;87(4):386-93.

11. Ha J, Choi HS, Lee Y, Kwon HJ, Song YW, Kim HH. CXC chemokine ligand 2 induced by receptor activator of NF-kappaB ligand enhances osteoclastogenesis. *J Immunol.* 2010 May 1;184(9):4717-24.

12. Kwak HB, Ha H, Kim HN, Lee JH, Kim HS, Lee S, Kim HM, Kim JY, Kim HH, Song YW, Lee ZH. Reciprocal cross-talk between RANKL and interferon-gamma-inducible protein 10 is responsible for bone-erosive experimental arthritis. *Arthritis Rheum.* 2008 May;58(5):1332-42.

13. Binder NB, Niederreiter B, Hoffmann O, Stange R, Pap T, Stulnig TM, Mach M, Erben RG, Smolen JS, Redlich K. Estrogen-dependent and C-C chemokine receptor-2-dependent pathways determine osteoclast behavior

in osteoporosis. *Nat Med.* 2009 Apr;15(4):417-24.

14. Ishii M, Egen JG, Klauschen F, Meier-Schellersheim M, Saeki Y, Vacher J, Proia RL, Germain RN. Sphingosine-1-phosphate mobilizes osteoclast precursors and regulates bone homeostasis. *Nature.* 2009 Mar 26;458(7237):524-8.

15. Germain RN, Bajénoff M, Castellino F, Chieppa M, Egen JG, Huang AY, Ishii M, Koo LY, Qi H. Making friends in out-of-the-way places: how cells of the immune system get together and how they conduct their business as revealed by intravital imaging. *Immunol Rev.* 2008 Feb;221:163-81.

16. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell.* 1998 Apr 17;93(2):165-76.

17. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. Osteoclast differentiation factor is a ligand for osteo-protegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A.* 1998 Mar 31;95(7):3597-602.

18. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature.* 1999 Jan 28;397(6717):315-23.

19. Zhao C, Irie N, Takada Y, Shimoda K, Miyamoto T, Nishiwaki T, Suda T, Matsuo K. Bidirectional ephrinB2-EphB4 signaling controls bone homeostasis. *Cell Metab.* 2006 Aug;4(2):111-21.

20. McClung MR, Lewiecki EM, Cohen SB, Bolognese MA, Woodson GC, Moffett AH, Peacock M, Miller PD, Lederman SN, Chesnut CH, Lain D, Kivitz AJ, Holloway DL, Zhang C, Peterson MC, Bekker PJ; AMG 162 Bone Loss Study Group. Denosumab in postmenopausal women with low bone mineral density. *N Engl J Med.* 2006 Feb 23;354(8):821-31.

21. Cummings SR, San Martin J, McClung MR, Siris ES, Eastell R, Reid IR, Delmas P, Zoog HB, Austin M, Wang A, Kutilek S, Adami S, Zanchetta J, Libanati C, Siddhanti S, Christiansen C; FREEDOM Trial. Denosumab for prevention of fractures in postmenopausal women with osteoporosis. *N Engl J Med.* 2009 Aug 20;361(8):756-65.

22. Mizoguchi T, Muto A, Udagawa N, Arai A, Yamashita T, Hosoya A, Ninomiya T, Nakamura H, Yamamoto Y, Kinugawa S, Nakamura M, Nakamichi Y, Kobayashi Y, Nagasawa S, Oda K, Tanaka H, Tagaya M, Penninger JM, Ito M, Takahashi N. Identification of cell cycle-arrested quiescent osteoclast precursors in vivo. *J Cell Biol.* 2009 Feb 23;184(4):541-54.

23. Cahalan MD, Parker I, Wei SH, Miller MJ. Two-photon tissue imaging: seeing the immune system in a fresh light. *Nat Rev Immunol.* 2002 Nov;2(11):872-80.

24. Germain RN, Miller MJ, Dustin ML, Nussenzweig MC. Dynamic imaging of the immune system: progress, pitfalls and promise. *Nat Rev Immunol.* 2006 Jul;6(7):497-507.

25. Rot A, von Andrian UH. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune

cells. *Annu Rev Immunol*. 2004;22:891-928.

26. Ishida N, Hayashi K, Hattori A, Yogo K, Kimura T, Takeya T. CCR1 acts downstream of NFAT2 in osteoclastogenesis and enhances cell migration. *J Bone Miner Res*. 2006 Jan;21(1):48-57.

27. Rosen H, Goetzl EJ. Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. *Nat Rev Immunol*. 2005 Jul;5(7):560-70.

28. Rivera J, Proia RL, Olivera A. The alliance of sphingosine-1-phosphate and its receptors in immunity. *Nat Rev Immunol*. 2008 Oct;8(10):753-63.

29. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu Rev Immunol*. 2005;23:127-59.

30. Boyden S. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J Exp Med*. 1962 Mar 1;115:453-66.

31. Cavanagh LL, Bonasio R, Mazo IB, Halin C, Cheng G, van der Velden AW, Cariappa A, Chase C, Russell P, Starnbach MN, Koni PA, Pillai S, Weninger W, von Andrian UH. Activation of bone marrow-resident memory T cells by circulating, antigen-bearing dendritic cells. *Nat Immunol*. 2005 Oct;6(10):1029-37.

32. Lippincott-Schwartz J, Patterson GH. Development and use of fluorescent protein markers in living cells. *Science*. 2003 Apr 4;300(5616):87-91.

33. Remington SJ. Fluorescent proteins: maturation, photochemistry and photophysics. *Curr Opin Struct Biol*. 2006 Dec;16(6):714-21.

34. Wu YI, Frey D, Lungu OI, Jaehrig A, Schlichting I, Kuhlman B, Hahn KM. A genetically encoded photoactivatable Rac controls the motility of living cells. *Nature*. 2009 Sep 3;461(7260):104-8.

35. Airan RD, Thompson KR, Fenno LE, Bernstein H, Deisseroth K. Temporally precise in vivo control of intracellular signalling. *Nature*. 2009 Apr 23;458(7241):1025-9.

Intravital two-photon imaging: a versatile tool for dissecting the immune system

Taeko Ishii, Masaru Ishii

Laboratory of Biological Imaging, WPI-Immunology Frontier Research Center, Osaka University, Osaka, Japan

Correspondence to
Dr Masaru Ishii, Laboratory of Biological Imaging, WPI-Immunology Frontier Research Center, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan;
mishii@ifrec.osaka-u.ac.jp

Accepted 8 August 2010

ABSTRACT

During the past decade, multi-photon or 'two-photon' excitation microscopy has launched a new era in the field of biological imaging. The near-infrared excitation laser for two-photon microscopy can penetrate thicker specimens, enabling the visualisation of living cell behaviour deep within tissues and organs without thin sectioning. The minimised photobleaching and toxicity enables the visualisation of live and intact specimens for extended periods. In this brief review, recent findings in intravital two-photon imaging for the physiology and pathology of the immune system are discussed. The immune system configures highly dynamic networks, where many cell types actively travel throughout the body and interact with each other in specific areas. Hence, real-time intravital imaging may be a powerful tool for dissecting the mechanisms of this dynamic system.

The most unique characteristic of the immune system is its highly dynamic nature. A variety of cell types, such as lymphocytes, macrophages and dendritic cells (DCs), are continuously circulating throughout the body, migrating through the peripheral tissues and interacting with each other in their respective niches. Conventional methodologies in immunology, such as flow cytometry, cell or tissue culture, biochemistry and histology, have brought tremendous achievement within this field, although the dynamics of immune cells in an entire animal remain less clear.

Technological progress of fluorescence microscopy has enabled us to visualise the intact biological phenomenon that has been uninvestigated. Among the advancements, the recent emergence and prevalence of two-photon, excitation-based, laser microscopy has revolutionised the research field, such that the dynamic behaviour of cells deep inside living organs can be visualised and analysed.

ADVANTAGES OF TWO-PHOTON IMAGING

Here we briefly describe the advantages of the two-photon microscopy compared with conventional (single-photon) confocal microscopy.[1–4] In confocal microscopy, upon excitation, a fluorophore molecule absorbs energy from a single photon and thereafter releases the energy as an emission photon. In contrast, in two-photon excitation, a fluorophore absorbs two photons simultaneously. Such an event rarely occurs, and can only occur in areas of high photon density. Based on this principle, two-photon microscopy can spatially confine the excitation area to the focal point of an objective lens, which concentrates photons into a very small area. The spatiotemporally restricted excitation provides many advantages over confocal microscopy for biological imaging. First, bright and high-resolution images can be obtained of regions deep inside tissues and organs. Because near-infrared lasers for two-photon excitation can penetrate deeper with less absorption or scattering than visible or UV light as used with confocal microscopy, objects can be visualised at a depth of 100–1000 μm with two-photon microscopy, whereas areas <100 μm are accessed with confocal microscopy. This capacity is especially useful for dissecting live tissues and organs. A broader range of tissue can be seen using conventional microscopy if the specimen is fixed and thin-sectioned, but the cells in the section are dead and not moving. To visualise the cells moving in live specimens, the areas to be analysed are sometimes lying deep inside. In such cases, two-photon excitation microscopy enables one to see the inside from the surface without fixation or thin-sectioning. In addition, excitation with near-infrared lasers can minimise photobleaching, the destruction of fluorophores and phototoxicity-induced tissue damage, which is beneficial for live imaging over an extended period of time.

Another advantage of two-photon microscopy is the non-linear optical effects such as second-harmonic generation.[4 5] Owing to the intensity of the laser passing through a highly polarised material, second-harmonic emission at precisely half the wavelength of the original light is generated. Using near-infrared lasers for two-photon excitation, the second-harmonic emission is in the range of the visible wavelength. Because many intrinsic biological structures, including collagen fibre, muscle, brain, cornea and bone, induce this kind of effect, these structures can be visualised without labelling them with exogenous probes.

Two-photon excited in vivo real-time imaging has revolutionised biology. In the following sections, we summarise findings in the field of immunology, focusing on the physiology and pathology of the immune system.

IMAGING OF THE IMMUNE SYSTEM

Lymphoid tissues

One of the first applications of two-photon imaging of the immune system was an explanted lymph node.[6] It was reported that naive T cells showed higher mobility than B cells in an intact lymph node, and that their speed was as rapid as 25 μm/min. These results challenged the previous belief that T cells were immobile without antigen stimulation. Further observations have modified the 'random-walk model' of T cells to a model of 'organised migration', probably because unstained objects such as other cells, stroma and the reticular network could not be detected.[3 7 8]

Real-time observations have also shown that T cells change their migration behaviour upon contact with antigen-presenting cells.[7 8] At the induction of priming, when T cells encounter activated antigen-presenting DCs during their rapid movement in a lymph node, they make stable complexes that last for several hours at least. Subsequently, the T cells become motile again. On the other hand, during tolerance induction, T cells have much shorter sustained contact with DCs.

Conventionally, it has been assumed that B-cell proliferation occurs only in the germinal centre and that activated T cells, with decreased expression of C–C chemokine receptor 7 (CCR7) and increased expression of C–X–C chemokine receptor 5 (CXCR5), migrate towards B-cell follicles to help promote antibody production. Interestingly, real-time imaging has shown that B cells upregulate CCR7 expression and migrate to the boundary of the follicle.[7 9]

Intravital imaging has disclosed these dynamic interactions, but two important questions remain: What contributes to the differences between priming and tolerance? What regulates the differences in interaction times?

Thymus

In thymic organ cultures, two-photon microscopy has shown interactions between thymocyte and stromal cells during positive and negative selection.[10] The immature CD4 CD8 double-positive thymocytes localise in the outer cortex. During positive selection, they become $CD4CD8^-$ or $CD4^-CD8$ single-positive thymocytes, and migrate to the central medulla. Real-time imaging revealed that thymocytes were highly motile and that major histocompatibility complex (MHC) recognition by thymocytes was associated with both stable and dynamic contacts with thymic stromal cells. The different interaction patterns could be associated with different signals or could correspond to different stages of positive selection. After positive selection, the thymocyte population displayed rapid, directed migration toward the medulla.[10] Compared with thymocytes in the cortex, the medullary thymocytes migrated limitlessly and more rapidly, and made frequent and transient contacts with DCs. During negative selection, thymocytes migrated slowly and in a highly confined manner within zones of up to 30 μm in diameter.[11]

Bone marrow

There is an inherent limitation to visualising the deep tissue of bone, because both visible and infrared light are easily scattered by crystallised calcium phosphate in the bone matrix.[12–14] However, the mouse skull is only 100 μm thick, which is thin enough to be within the range of two-photon microscopy. A pilot study showed that central memory CD8 T cells were preferentially recruited to, and accumulated in, the bone marrow cavity and interacted with mature circulating DCs.[13 14]

We have developed a novel intravital two-photon imaging system for visualising the living bone marrow cavity with high spatiotemporal resolution (figure 1). Using this technique, we have demonstrated that osteoclast precursors migrate under the influence of several chemoattractants and chemorepellants.[8 15 16] The bone marrow is one of the critical developmental sites in the immune system, and bone tissue has a crucial role in the maintenance of its structure. Bone is an active organ, which is continuously remodelled and kept in equilibrium between resorption by osteoclasts and formation by osteoblasts. The disruption of this balance leads to several pathological states, such as osteoporosis, tumour-induced osteolysis and rheumatoid arthritis. In bone-resorptive conditions, osteoclasts are excessively activated and contribute to bone destruction. Osteoclasts are giant multinucleate cells that stem from the monocyte-macrophage lineage of precursor cells. It had remained to be determined where and how the precursors were recruited on the bone surface. Using real-time two-photon microscopy in vivo, we observed migration of osteoclast precursors between the bone marrow cavity and blood vessels, and found that this process depended on C–X–C chemokine ligand 12 (CXCL12), stromal cell-derived factor 1 (SDF-1) and a signalling sphingolipid, suphingosine-1-phospate.

Blood vessels

Following activation within the lymph nodes, immune cells can enter inflamed tissues through blood vessels.[7 17] Neutrophils are innate cell types that are first recruited to the inflamed sites. Intravital microscopy has observed tethering, rolling, crawling and invasion of these cells out of circulation and into the tissues in real time. This recruitment is controlled by several selectin

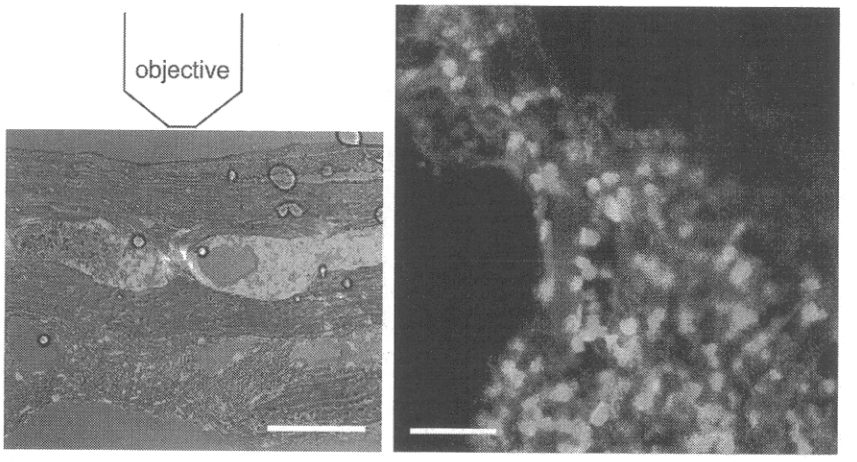


Figure 1 Intravital two-photon imaging of bone marrow. A vertical section of calvaria bone from lysozyme M promoter driven mice expressing enhanced green fluorescent protein (left panel) and intravital imaging of the bone surface using two-photon microscopy (right panel). Blood vessels were visualised by Texas Red-conjugated high molecular dextrans (70 kDa) injected intravenously. Scale bars represent 100 μm (left panel) and 30 μm (right panel), respectively.

adhesion molecules, such as P-selectin, E-selectin and L-selectin, as well as integrins. The paths of neutrophil emigration are still controversial. There are two possible routes: a paracellular route, in which neutrophils emigrate at cell–cell junctions, and a transcellular route, in which they emigrate through the endothelial cells.

In addition to neutrophils, monocytes and macrophages also circulate through the vascular system, crawling over the endothelial cell surface.[17] Their attachment depends on interactions between CX_3-chemokine receptor 1 (CX_3CR1) and CX_3-chemokine ligand 1 (CX_3CL1) and between lymphocyte function-associated antigen 1 (LFA1) and intercellular adhesion molecule 1 (ICAM1).

Autoimmune models

Antigen-specific pathogenic T cells have been visualised to migrate through the spinal cord in a murine encephalitis model, [1] EIA.[18] At this site, T cells can be highly motile and arrest antigens upon recognition in the same manner as seen in lymph nodes. In a type I diabetes model using NOD mice, the interaction between antigen-specific T cells and DCs was observed in a draining lymph node.[19] Islet antigen-specific $CD4CD25^-$ T helper cells (Th cells) and regulatory T cells (Treg cells) homed to similar areas of the lymph node and their movement patterns were indistinguishable from each other—that is, they both swarmed and arrested in the presence of antigens. No stable interaction between Th cells and Treg cells was seen, but Treg cells directly interacted with DCs and inhibited Th cell activation via DCs.

FUTURE CHALLENGES

The greatest strength of intravital imaging, the ability to obtain spatiotemporal information in a living body, is not feasible by other methods. This approach has revealed and continues to reveal dynamic features of the immune system including the physiological and pathological process. However, there are several limitations to two-photon microscopy imaging. First, although it has a more extensive penetration depth, it can only image up to 800–1000 μm in soft tissues such as the brain and up to 200 μm in hard tissues such as bone and therefore is not applicable to humans but rather only to small animal models such as mice and rats. Owing to the wide scattering of light by the skin, it is necessary to exteriorise the target organ, and there is a possibility that operative invasion and changes in oxygen concentration and humidity might influence cellular behaviour. To resolve these concerns, technical innovations in fluorochrome and optical systems are expected, including improvements to light emission and resolution.

In the future, intravital microscopy will be applied to both observation and functional analysis. Newly developed fluorescence tools, such as cell cycle indicators,[20] and light-sensing devices, such as light-induced G protein activators, are being introduced.[21] These new approaches are continuing to expand the capacity of in vivo imaging.

CONCLUSIONS

In the past decade, two-photon microscopy has expanded the horizon of intravital imaging. This new technique enables the visualisation of complicated systems of the living body, in which multiple cells are involved. Some technical limitations remain; however, it seems that the range of application is continually increasing.

Competing interests None

Provenance and peer review Not commissioned; externally peer reviewed. [3]

REFERENCES

1. **Denk W,** Strickler JH, Webb WW. Two-photon laser scanning fluorescence microscopy. *Science* 1990;**248**:73–6.
2. **Cahalan MD,** Parker I, Wei SH, *et al.* Two-photon tissue imaging: seeing the immune system in a fresh light. *Nat Rev Immunol* 2002;**2**:872–80.
3. **Germain RN,** Miller MJ, Dustin ML, *et al.* Dynamic imaging of the immune system: progress, pitfalls and promise. *Nat Rev Immunol* 2006;**6**:497–507.
4. **Wang BG,** König K, Halbhuber KJ. Two-photon microscopy of deep intravital tissues and its merits in clinical research. *J Microsc* 2010;**238**:1–20.
5. **Campagnola PJ,** Loew LM. Second-harmonic imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms. *Nat Biotechnol* 2003;**21**:1356–60.
6. **Miller MJ,** Wei SH, Parker I, *et al.* Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* 2002;**296**:1869–73.
7. **Garside P,** Brewer JM. Real-time imaging of the cellular interactions underlying tolerance, priming, and responses to infection. *Immunol Rev* 2008;**221**:130–46.
8. **Germain RN,** Bajénoff M, Castellino F, *et al.* Making friends in out-of-the-way places: how cells of the immune system get together and how they conduct their business as revealed by intravital imaging. *Immunol Rev* 2008;**221**:163–81.
9. **Schwickert TA,** Lindquist RL, Shakhar G, *et al.* ***In vivo*** imaging of germinal centres reveals a dynamic open structure. *Nature* 2007;**446**:83–7.
10. **Bousso P,** Bhakta NR, Lewis RS, *et al.* Dynamics of thymocyte-stromal cell interactions visualized by two-photon microscopy. *Science* 2002;**296**:1876–80.
11. **Le Borgne M,** Ladi E, Dzhagalov I, *et al.* The impact of negative selection on thymocyte migration in the medulla. *Nat Immunol* 2009;**10**:823–30.
12. **Sumen C,** Mempel TR, Mazo IB, *et al.* Intravital microscopy: visualizing immunity in context. *Immunity* 2004;**21**:315–29.
13. **Mazo IB,** Honczarenko M, Leung H, *et al.* Bone marrow is a major reservoir and site of recruitment for central memory CD8+ T cells. *Immunity* 2005;**22**:259–70.
14. **Cavanagh LL,** Bonasio R, Mazo IB, *et al.* Activation of bone marrow-resident memory T cells by circulating, antigen-bearing dendritic cells. *Nat Immunol* 2005;**6**:1029–37.
15. **Ishii M,** Egen JG, Klauschen F, *et al.* Sphingosine-1-phosphate mobilizes osteoclast precursors and regulates bone homeostasis. *Nature* 2009;**458**:524–8.
16. **Klauschen F,** Ishii M, Qi H, *et al.* Quantifying cellular interaction dynamics in 3D fluorescence microscopy data. *Nat Protoc* 2009;**4**:1305–11.
17. **Hickey MJ,** Kubes P. Intravascular immunity: the host-pathogen encounter in blood vessels. *Nat Rev Immunol* 2009;**9**:364–75.
18. **Kawakami N,** Nägerl UV, Odoardi F, *et al.* Live imaging of effector cell trafficking and autoantigen recognition within the unfolding autoimmune encephalomyelitis lesion. *J Exp Med* 2005;**201**:1805–14.
19. **Tang Q,** Adams JY, Tooley AJ, *et al.* Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nat Immunol* 2006;**7**:83–92.
20. **Sakaue-Sawano A,** Kurokawa H, Morimura T, *et al.* Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* 2008;**132**:487–98.
21. **Wu YI,** Frey D, Lungu OI, *et al.* A genetically encoded photoactivatable Rac controls the motility of living cells. *Nature* 2009;**461**:104–8.

DOI/10.1007/s10059-011-1010-x

Minireview

Molecules and Cells

The Role of Sphingosine 1-Phosphate in Migration of Osteoclast Precursors; an Application of Intravital Two-Photon Microscopy

Taeko Ishii, Yutaka Shimazu, Issei Nishiyama, Junichi Kikuta, and Masaru Ishii*

Sphingosine-1-phosphate (S1P), a biologically active lysophospholipid that is enriched in blood, controls the trafficking of osteoclast precursors between the circulation and bone marrow cavities via G protein-coupled receptors, S1PRs. While S1PR1 mediates chemoattraction toward S1P in bone marrow, where S1P concentration is low, S1PR2 mediates chemorepulsion in blood, where the S1P concentration is high. The regulation of precursor recruitment may represent a novel therapeutic strategy for controlling osteoclast-dependent bone remodeling. Through intravital multiphoton imaging of bone tissues, we reveal that the bidirectional function of S1P temporospatially regulates the migration of osteoclast precursors within intact bone tissues. Imaging technologies have enabled *in situ* visualization of the behaviors of several players in intact tissues. In addition, intravital microscopy has the potential to be more widely applied to functional analysis and intervention.

INTRODUCTION

Bone is a highly dynamic organ that is continuously turned over during growth, even in adults. During bone remodeling, homeostasis is regulated by the balance between bone formation by osteoblasts and bone resorption by osteoclasts (Harada et al., 2003; Teitelbaum et al., 2003). However, in pathological conditions such as osteoporosis, osteopetrosis, arthritic joint destruction, and bone metastasis, this equilibrium is disrupted. Since osteoclasts are excessively activated in osteolytic diseases, the inhibition of osteoclast function has been a major therapeutic strategy. Bisphosphonates, the most widely used group of anti-osteoporosis drugs, bind to hydroxyapatite, enter osteoclasts via endocytosis, and induce osteoclast apoptosis (Russell et al., 2007). Recently, the inactivation of osteoclasts, as opposed to their elimination, has generated interest as an alternative treatment strategy (Deal, 2009; Yasuda et al., 2005). One promising regulation point is the recruitment of osteoclast precursors. In addition to several chemokines that are known regulators of migration, including CXCL12 (Yu et al., 2003) and CX_3CL1 (Koizumi et al., 2009), we have shown that sphingosine 1-phosphate (S1P), a lysophospholipid abundant in the plasma, plays an important role as both a chemoattractant and a chemorepellent (Ishii et al., 2009; 2010). In this review, we summarize the bidirectional regulation of osteoclast precursor migration by S1P and briefly describe intravital bone imaging in living animals.

S1P and its receptors

S1P is a bioactive sphingolipid metabolite that regulates diverse biological functions including cell proliferation, motility, and survival (Cyster, 2005; Rivera et al., 2008; Rosen et al., 2005; 2007). Sphingolipids are essential plasma membrane constituents composed of a serine head group and one or two fatty acid tails. They are easily metabolized and converted to sphingosines, which are ATP-dependently phosphorylated by sphingosine kinases 1 and 2 (SPHK1 and SPHK2) in most cells, yielding S1P (Hannun et al., 2008). SPHKs, which are regulated by a variety of growth factors, hormones, and cytokines, control S1P's acute reactive generation and homeostasis in the circulation (Hannun et al., 2008). Immediately after its synthesis, free S1P is irreversibly degraded by intracellular S1P lyase or dephosphorylated by S1P phosphatases. As a result, the levels of S1P in most tissues, including bone marrow, are relatively low. In contrast, large amounts of S1P are continuously produced in the plasma, especially by erythrocytes, and the serum concentration of S1P is extremely high (several hundred nanomolar to low-micromolar range). Most S1P in the circulation is bound to high-density lipoprotein (HDL) and albumin, which serve as stable reservoirs and efficiently deliver S1P to epithelial cell-surface receptors (Argraves et al., 2008). In addition, because S1P is an amphiphilic molecule that cannot easily cross membranes, an S1P gradient between the blood and tissues is maintained.

S1P signals via five 7-transmembrane receptors or G protein-coupled receptors (GPCRs), S1PR1 to S1PR5, previously referred to as endothelial differentiation gene (Edg) receptors (Rivera et al., 2008; Rosen et al., 2007). Because of the different distribution of these receptors and their different coupling to signal-transducing G proteins, S1P shows a broad range of

Laboratory of Biological Imaging, WPI-Immunology Frontier Research Center, Osaka University, Osaka, Japan
*Correspondence: mishii@ifrec.osaka-u.ac.jp

Received January 13, 2011; accepted January 31, 2011; published online February 25, 2011

Keywords: cell dynamics, chemokine, chemotaxis, lipid mediator, live imaging

Table 1. S1P receptors and phenotypes of their genetic deletion

S1P Receptors	S1PR1	S1PR2	S1PR3	S1PR4	S1PR5
Coupling G proteins	$G_{i/O}$	G_i G_q G_s $G_{12/13}$	G_i G_q G_s $G_{12/13}$	G_i $G_{12/13}$	$G_{i/o}$ $G_{12/13}$
Distribution	Ubiquitous	Ubiquitous Highest expressed in embryonic brain Expressed high in adult heart and lung	Spleen, heart, lung, thymus, kidney, testis, brain, skeletal muscle	Thymus, spleen, lung, peripheral leukocytes	Brain, spleen, peripheral leukocytes
Phenotypes of gene deletion (mouse)	Embryonic lethal (e12.5-e14.5)	Vestibular defects Hearing loss Seizures (C57BL/6 only) Perinatal lethal (reduce litter size) Survivours show no phonotype	Disruption of alveolar epithelial junctions	Ddisorder of mega-karyocyte differentiation	Reduced number of NK cells
Biological function	Rac activation	Rho activation Vasoconstriction angiogenesis Wound healing	Cardioprotection by HDL		
References	Liu et al. (2000) Matloubian et al. (2004)	Kono et al. (2007) Serriere-Lanneau et al. (2007)	Nofer et al. (2004) Gon et al. (2005)	Golfier et al. (2010)	Walzer et al. (2007)

Cyster et al. (2005), Rivera et al. (2008), Rosen et al. (2005; 2007).

bioactivities (Table 1). S1PR1 is ubiquitously expressed and primarily coupled to PTX-sensitive $G_{i/o}$ proteins, whereas S1PR2 and S1PR3, whose distributions are more limited, are coupled to $G_{12/13}$ as well as G_q, G_s, and G_i. The expression of S1PR4 and S1PR5 is much lower than that of S1PR1, S1PR2, and S1PR3, and their functions remain to be elucidated. However, it has been reported that they are coupled to $G_{i/o}$ and $G_{12/13}$.

S1P receptors have key roles in the regulation of cellular motility. S1PR1 activates Rac through Gi and promotes cell migration and intercellular connection, whereas S1PR2 activates Rho signaling via $G_{12/13}$, thereby counteracting the effects of S1PR1 and inhibiting Rac activity (Takuwa, 2002). These differences account for the different biological functions of S1PR1 and S1PR2, which produce opposite effects on migration toward/against S1P gradients *in vitro* (Okamoto et al., 2000).

Osteoclast precursors and S1P

Osteoclasts are derived from macrophage/monocyte-lineage cells that express both S1PR1 and S1PR2 (Ishii et al., 2009). As described above, S1PR1 and S1PR2 have opposite effects on the migration of osteoclast precursors. Osteoclast precursors are chemoattracted to S1P *in vitro*, a response that is blocked by PTX. In addition, treatment with S1P increases osteoclast precursor levels of the active form of Rac (GTP-Rac), suggesting that Rac and Gi are involved in S1PR1 chemotactic signaling in osteoclast precursors. On the other hand, S1PR2 requires a higher concentration of S1P for activation and induces negative chemotactic responses, "chemorepulsion," to S1P gradients. S1PR2 activation causes cells to move from the bloodstream into bone marrow cavities (Ishii et al., 2010). As in leukocytes, the migration of osteoclast precursors is regulated by chemokines. Like the S1PRs, chemokine receptors are GPCRs and signal via Gi components. One of the best-known chemoattractants for osteoclast precursors is CXCL12 (also known as stromal derived factor-1), a CXCR4 ligand (Yu et al., 2003). CXCL12 is constitutively expressed at high levels by osteoblastic stromal cells and vascular endothelial cells in bone, whereas CXCR4 is expressed on a wide variety of cells types, including circulating monocytes and osteoclast precursors. CXCL12 has chemotactic effects on osteoclast precursors, which express large amounts of CXCR4.

Recently, another chemokine, CX_3CL1 (also known as fractalkine), which functions as a membrane-bound adhesion molecule, was shown to act as a chemoattractant after its cleavage by ADAM10 and ADM7. Expressed by osteoblastic stromal cells, it was reported to be involved in both the recruitment and attachment of osteoclast precursors (Koizumi et al., 2009). Expression of both chemokine receptors and S1PRs is reduced by RANKL stimulation, dependent on NF-κB, but not on NF-AT. Presumably, after cells mature and arrive at their ultimate destinations these chemoattractants are no longer needed.

Application of intravital imaging to the analysis of cell behavior in bone

To study the behavior of osteoclasts and their precursors *in vivo*, we developed a new intravital two-photon imaging system for use in the analysis of bone tissues (Fig. 1) (Ishii et al., 2009; 2010). Recent advances in microscope, laser, and fluorophore technology have made it possible to visualize living cells in intact organs and to analyze their mobility and interactions in a quantitative manner.

As calcium phosphate, the main structural component of the bone matrix, can scatter laser beams, it was difficult to access the deep interior of bone tissues, even using a near-infrared laser. We decided to use parietal bone in which the distance from the bone surface to the bone marrow cavity is 80-120 μm (within the appropriate range for two-photon microscopy). We

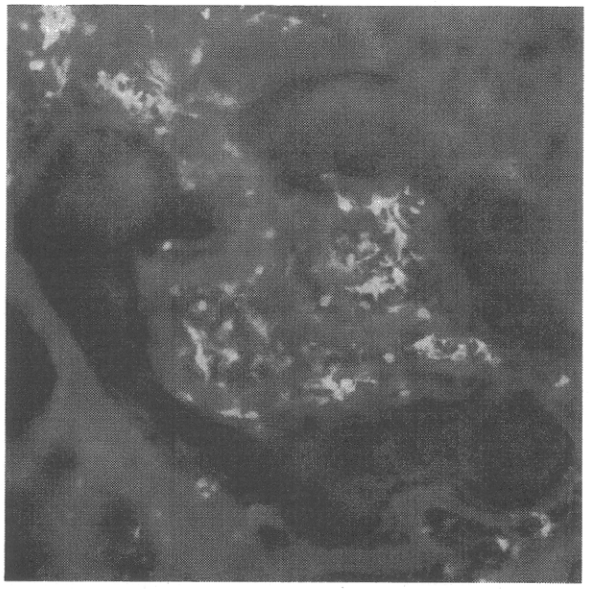


Fig. 1. Bone marrow structure visualized by intravital two-photon imaging. Murine skull bone tissues of heterozygous Cx_3CR1-EGFP knock-in mice. Collagen fibers in bone are detected by second-harmonic generation (in blue), and the microvasculature are visualized by intravenous injection of 70 kDa dextran-conjugated Texas Red. CX_3CR1-EGFP positive cells appear green in bone marrow cavity.

modified the method used in a pilot study, which revealed that central memory $CD8^+$ T cells were preferentially recruited to, and accumulated in, the bone marrow cavity and interacted with mature circulating dendritic cells (Cavanagh et al., 2005; Mazo et al., 2005).

Using this new intravital two-photon imaging method, we showed that S1P controls the migratory behavior of osteoclast precursors, dynamically regulating bone mineral homeostasis, and we identified a critical control point in osteoclastogenesis. While monocytoid cells containing osteoclast precursors (CSF1R-EGFP-positive or CX3CR1-EGFP-positive cells) were stationary at the steady state, osteoclast precursors were stimulated and moved into vessels when a potent S1PR1-specific agonist, SEW2871 (Wei et al., 2005), was injected intravenously.

To clarify the physiological significance of S1P-directed chemotaxis of osteoclast precursors in bone homeostasis, we examined osteoclast/monocyte-specific S1PR1-deficient ($S1PR1^{-/-}$) mice. [Global S1PR1 deficiency causes embryonic lethally at e12.5 to e14.5 due to defective blood vessel development (Liu et al., 2000)]. The attachment of osteoclast precursors to bone surfaces was significantly enhanced in $S1PR1^{-/-}$ animals compared with controls. $S1PR1^{-/-}$ osteoclasts precursors on bone surfaces subsequently develop into mature osteoclasts and absorb bone tissues. S1P-mediated chemotaxis of osteoclast precursors would thus be expected to contribute to their redistribution from bone tissues to blood vessels.

We also performed intravital two-photon imaging of bone tissues to define the role of S1PR2 *in vivo* (Ishii et al., 2010). We showed that certain osteoclast precursors (CX_3CR1-EGFP-positive cells) moved into the bloodstream when a potent S1PR2 antagonist, JTE013 (Osada et al., 2002), was injected intravenously. The effect of JTE013 was less pronounced than that of the S1PR1 agonist SEW2871. Furthermore, to clarify the physiological significance of $S1P^{-/-}$ chemotaxis of osteoclast precursors in bone homeostasis, we examined S1PR2-deficient ($S1PR2^{-/-}$) mice. Although $S1PR2^{-/-}$ mice suffer auditory impairment due to vessel defects in the inner ear, they survive and reproduce (Kono et al., 2007). Although bone resorption of osteoclasts was significantly lower in $S1PR2^{-/-}$ animals than in controls, *in vitro* osteoclast formation was not significantly affected. In a high-S1P environment such as the bloodstream, S1PR1 is activated and rapidly internalized, allowing S1PR2 to predominate. Osteoclast precursors enter the bone marrow as a result of chemorepulsion mediated by S1PR2, and other chemo-

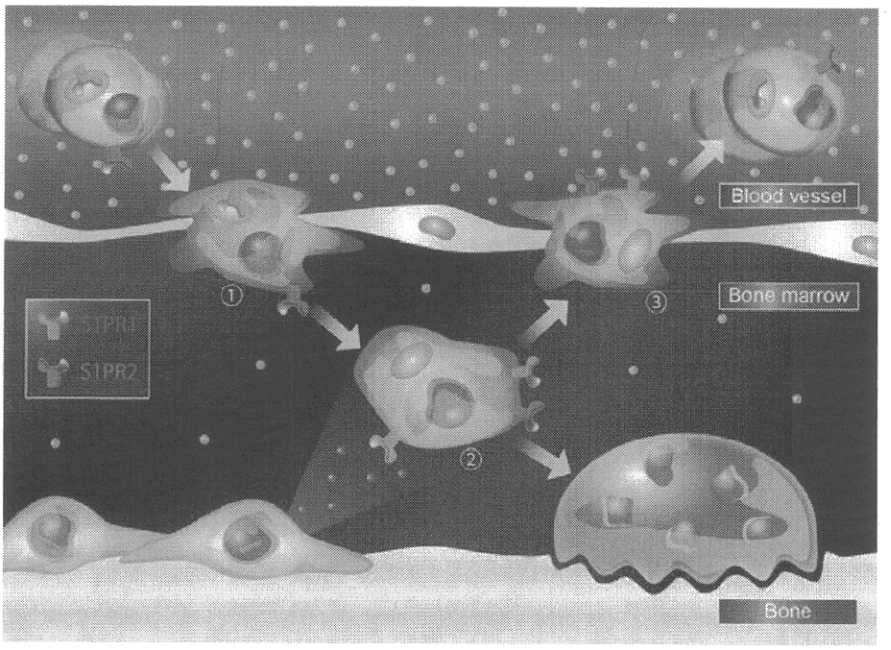


Fig. 2. A schematic model for S1P-mediated osteoclast precursors localization. The entry of osteoclast precursors from blood vessels where S1P is at high concentration, is initiated by chemorepulsion through S1PR2 (1). Once enter in bone marrow, osteoclast precursors migrate toward chemokines enriched in bone marrow cavity (2). On the other hand, their recirculation toward blood vessels is regulated by chemoattraction through S1PR1 (3).

kines attract them to bone surfaces. After they enter a low-S1P environment such as bone marrow, S1PR1 is transported back to the cell surface, and osteoclast precursors return from bone tissues to blood vessels as a result of chemotaxis to an S1P gradient.

The number of osteoclast precursors on bone surfaces is determined by the balance between the trafficking of osteoclast precursors to and from the circulation. These data provide evidence that S1P controls the migratory behavior of osteoclast precursors, dynamically regulating bone mineral homeostasis, and identify a critical control point in osteoclastogenesis. Based on our findings, we propose that regulation of the migratory behavior of osteoclast precursors controls osteoclast differentiation. This control mechanism is summarized in Fig. 2. This critical control point in osteoclastogenesis may represent an attractive target for new treatments for osteoporosis. We previously showed that treatment with FTY720, which is metabolized by SPHK2 to a compound that acts as an agonist for four of the five S1P receptors (not S1PR2) (Cyster, 2005; Matloubian et al., 2004), relieved ovariectomy-induced osteoporosis in mice by reducing the number of mature osteoclasts attached to bone surfaces (Ishii et al., 2009). The mechanism of action of S1P is completely different from that of conventional treatments such as bisphosphonates, which suppress mature osteoclasts. We anticipate that the regulation of osteoclast precursor migration may be a useful clinical strategy in the near future.

FTY720 is a reversible immunosuppressive agent approved as a treatment for multiple sclerosis in the United States. It induces lymphopenia by confining lymphocytes to lymphoid organs (Mandala et al., 2002). The precise mechanisms behind this phenomenon remain controversial, and it is necessary to determine how FTY720 produces the opposite effect on monocyte-macrophage cells in bone marrow (which are expelled into the circulation by FTY720).

Future directions for two-photon microscopy

Two-photon intravital imaging has revealed, and continues to reveal, dynamic features of physiological and pathological process. Its greatest strength is its ability to provide spatiotemporal information in living organisms, which cannot be achieved using other methods. However, current two-photon microscopy imaging techniques have several limitations. First, we cannot see everything in the visual fields in two-photon microscopy. Although fluorescence labeling and second-harmonic generation enable us to observe target cells and organs, the lack of a signal does never reflect an open field, as diverse structures and cellular components should be present. To avoid misinterpretation, we must interpret our observations with caution. Second, although two-photon microscopy has greater penetration depth than conventional confocal microscopy, its penetration depth is only 800-1000 μm in soft tissues (e.g., brain cortex) and 200 μm in hard tissues (e.g., bone). Because of these resolution limitations, it may only be applied to small animals, such as mice and rats. Moreover, due to the wide scattering of light by the skin, it is necessary that target organs should be exteriorized. It is possible that the necessary operative invasion and changes in oxygen concentration and humidity may influence cellular behavior. To resolve these problems, technical innovations in fluorochrome and optical systems, including improvements in light emission and amelioration of resolution problems (Ntziachristos, 2010), are needed.

Intravital microscopy has begun to be applied not only to observational studies, but also to functional analysis and interventions. Recently, several new fluorescence tools have been developed. These include cell-cycle indicators (Sakaue-Sawano et al., 2008) and light-sensing devices such as photoactivatable fluorescent proteins (Victora et al., 2010) and light-induced activators of G protein-coupled receptors (Airan et al., 2009).

CONCLUSION

As the recruitment of osteoclast precursors during osteoclastogenesis is dynamic and dependent on the microenvironment of the bone marrow cavity, temporospatial information is very important. Intravital imaging has made a huge contribution to improving our understanding of these processes. It enables us to visualize, temporospatially, complicated systems in living organisms. This new technique has revealed that S1P acts in concert with several chemoattractants to shepherd osteoclast precursors to appropriate sites. Controlling the recruitment and migration of osteoclast precursors represents a promising new therapeutic strategy for combating bone diseases. Although their limitations remain to be resolved, the range of applications for *in vivo* imaging techniques continues to expand.

REFERENCES

Airan, R.D., Thompson, K.R., Fenno, L.E., Bernstein, H., and Deisseroth, K. (2009). Temporally precise *in vivo* control of intracellular signalling. Nature *458*, 1025-1029.

Argraves, K.M., Gazzolo, P.J., Groh, E.M., Wilkerson, B.A., Matsuura, B.S., Twai, W.O., Hammad, S.M., and Argraves, W.S. (2008). High density lipoprotein-associated sphingosine 1-phosphate promotes endothelial barrier function. J. Biol. Chem. *283*, 25074-25081.

Cavanagh, L.L., Bonasio, R., Mazo, I.B., Halin, C., Cheng, G., van der Velden, A.W., Cariappa, A., Chase, C., Russell, P., Starnbach, M.N., et al. (2005). Activation of bone marrow-resident memory T cells by circulating antigen-bearing dendritic cells. Nat. Immunol. *6*, 1029-1037.

Cyster, J.G. (2005). Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu. Rev. Immunol. *23*, 127-159.

Deal, C. (2009). Future therapeutic targets in osteoporosis. Curr. Opin. Rheumatol. *4*, 380-385.

Golfier, S., Kondo, S., Schulze, T., Takeuchi, T., Vassileva, G., Achtman, A.H., Gräler, M.H., Abbondanzo, S.J., Wiekowski, M., Kremmer, E., et al. (2010). Shaping of terminal megakaryocyte differentiation and proplatelet development by sphingosine-1-phosphate receptor S1P4. FASEB J. *24*, 4701-4710.

Gon, Y., Wood, M.R., Kiosses, W.B., Jo, E., Sanna, M.G., Chun, J., and Rosen, H. (2005). S1P3 receptro-induced reorganization of epithelial tight junctions compromises lung barrier integritiy and is potentiated by TNF. Proc. Natl. Acad. Sci. USA *102*, 9270-9275.

Hannun, Y.A., and Obeid, L.M. (2008). Principles of bioactive lipid signaling: lessons from sphingolipids. Nat. Rev. Mol. Cell. Biol. *9*, 139-150.

Harada, S., and Rodan, G.A. (2003). Control of osteoblast function and regulation of bone mass. Nature *423*, 349-355.

Ishii, M., Egen, J.G., Klauschen, F., Meier-Schellersheim, M., Saeki, Y., Vacher, J., Proia, R.L., and Germain, R.N. (2009). Sphingosine-1-phosphate mobilizes osteoclast precursors and regulates bone homeostasis. Nature *458*, 524-528.

Ishii, M., Kikuta, J., Shimazu, Y., Meier-Schellersheim, M., and Germain, R.N. (2010). Chemorepulsion by blood S1P regulates osteoclast precursor mobilization and bone remodeling *in vivo*. J. Exp. Med. *207*, 2793-2798.

Koizumi, M., Saitoh, Y., Minami, T., Takeno, N., Tsuneyama, K., Miyahara, T., Nakayama, T., Sakurai, H., Takano, Y., Nishimura, M., et al. (2009). Role of CX3CL1/fractalkine in osteoclast differentiation and bone resorption. J. Immunol. *183*, 7825-7831.

Kono, M., Belyantseva, I.A., Skoura, A., Frolenkov, G.I., Starost, M.F., Dreier, J.L., Lidngton, D., Bolz, S.S., Friedman, T.B., Hla, T., et al. (2007). Deafness and stria vascularis defects in S1P2 receptor-null mice. J. Biol. Chem. *282*, 10690-10696.

Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C.X., Hobson, J.P., Rosenfeldt, H.M., Nava, V.E., Chae, S.S., Lee, M.J., et al.

(2000). Edg-1, the G-protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. J. Clin. Invest. *106*, 951-961.

Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G.J., Card, D., Keohane, C., et al. (2002). Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science *296*, 346-349.

Matloubian, M., Lo, C.G., Cinamon, G., Lesneski, M.J., Xu, Y., Brinkmann, V., Allende, M.L., Proia, R.L., and Cyster, J.G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature *6972*, 355-360.

Mazo, I.B., Honczarenko, M., Leung, H., Cavanagh, L.L., Bonaisio, R., Weninger, W., Engelke, K., Xia, L., McEver, R.P., Koni, P.A., et al. (2005). Bone marrow is a major reservoir and site of recruitment for central memory CD8+ T cells. Immunity *22*, 259-270.

Nofer, J.R., van der Giet, M., Tolle, M., Wolinska, I., von Wnuck Lipinski, K., Baba, H.A., Tietge, U.J., Godecke, A., Ishii, I., Kleuser, B., et al. (2004). HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. J. Clin. Invest. *113*, 569-581.

Ntziachristos, V. (2010). Going deeper than microscopy: the optical imaging frontier in biology. Nat. Methods *7*, 603-614.

Okamoto, H., Takuwa, N., Yokomizo, T., Sugimoto, N., Sakurada, S., Shigematsu, H., and Takuwa, Y. (2000). Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. Mol. Cell. Biol. *20*, 9247-9261.

Osada, M., Yatomi, Y., Ohmori, T., Ikeda, H., and Ozaki, Y. (2002). Enhancement of sphingosine 1-phosphate-induced migration of vascular endothelial cells and smooth muscle cells by an EDG-5 antagonist. Biochem. Biophys. Res. Commun. *299*, 483-487.

Rivera, J., Proia, R.L., and Olivera, A. (2008). The alliance of sphingosine-1-phosphate and its receptors in immunity. Nat. Rev. Immunol. *8*, 753-763.

Rosen, H., and Goetzl, E.J. (2005). Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. Nat. Rev. Immunol. *5*, 560-570.

Rosen, H., Sanna, M.G., Cahalan, S.M., and Gonzalez-Cabrera, P.J. (2007). Tipping the gatekeeper: S1P regulation of endothelial barrier function. Trends Immunol. *28*, 102-107.

Russell, R.G.G., Xia, Z., Dunford, J.E., Oppermann, U., Kwaasi, A., Hulley, P.A., Kavanagh, K.L., Triffitt, J.T., Lundy, M.W., Phipps, R.J., et al. (2007). Bisphosphonates. An update on mechanisms of action and how these relate to clinical efiicacy. Ann. N Y Acad. Sci. *1117*, 209-257.

Sakaue-Sawano, A., Kurokawa, H., Morimura, T., Hanyu, A., Hama, H., Osawa, H., Kashiwagi, S., Fukami, K, Miyata, T., Miyoshi, H., et al. (2008). Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. Cell *132*, 487-498.

Serrier-Lanneau, V., Teixeira-Clerc, F., Li, L., Schippers, M., de Wris, W., Julien, B., Tran-Van-Nhieu, J., Manin, S., Pelstra, K., Chun J., et al. (2007). The sphingosine 1-phosphate receptor S1P2 triggers hepatic wound healing. FASEB J. *21*, 2005-2013.

Takuwa, Y. (2002). Subtype-specific differential regulation of Rho family G proteins and cell migration by the Edg family sphingosine-1-phosphate receptors. Biochem. Biophys. Acta *1682*, 112-120.

Teitelbaum, S.L., Ross, F.P. (2003). Genetic regulation of osteoclast development and function. Nat. Rev. Genetic. *4*, 638-649

Victora, G.D., Schwichkert, T.A., Fooksman, D.R., Kamphorst, A.O., Meyer-Hermann, M., Dustin, M.L., and Nussenzweig, M.C. (2010). Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. Cell *143*, 592-605

Wei, S.H., Rosen, H., Matheu, M.P., Sanna, M.G., Wang, S.K., Jo, E., Wong, C.H., Parker, I., and Cahalan, M.D. (2005). Sphingosine 1-phospate type 1 receptor agonism inhibits transendothelial migration of medullary T cells to lymphatic sinuses. Nat. Immunol. *12*, 1228-1235.

Yasuda, Y., Kaleta, J., and Bromme, D. (2005). The role of cathepsins in osteoporosis and arthritis: rationale for the design of new therapeutics. Adv. Drug. Deliv. Rev. *57*, 973-993.

Yu, X., Huang, Y., Collin-Osdoby, P., and Osdoby, P. (2003). Stromal cell-derived factor-1 (SDF-1) recruits osteoclast precursors by inducing chemotaxis, matrix metalloproteinase-9 (MMP-9) acrivity, and collagen transmigration. J. Bone Miner. Res. *18*, 1404-1418.

LECTURE

骨組織の多光子励起顕微鏡によるライブイメージング

石井　優*

Intravital Multi-photon Imaging of Live Bone Tissues

Masaru Ishii

臨整外 45：324～329，2010

Key words：イメージング，多光子励起顕微鏡，破骨細胞，細胞ダイナミクス(動態)

破骨細胞は単球系細胞から分化して骨を吸収する特殊な細胞である．単球系破骨前駆細胞がいかにして骨表面に到達するか，その遊走がどう制御されているかは長い間不明であった．筆者は最近，多光子励起顕微鏡という，生体深部の観察が可能な特殊な顕微鏡を用いて，生きたままのマウス骨組織内を可視化することに成功し，破骨前駆細胞の遊走・接着が，脂質メディエーターの一種であるスフィンゴシン1リン酸や種々のケモカインによって動的に制御されていることを解明した．本稿ではこの研究成果に加え，われわれが開発した骨組織のライブイメージングの方法論や応用について概説する．

はじめに

硬い石灰質に囲まれた骨の中はこれまで，生きたままでの観察が極めて困難であると考えられていた．実際にこれまで骨や骨髄の研究では，固定して摘出した骨を，カルシウムキレート剤に1週間ほど漬け込んでカルシウムを取り除き(脱灰し)，切片にして観察していた．この従来法でも，骨組織内の細胞の「形態」や「分子発現」(免疫染色による)を解析することはできたが，決定的な情報が欠落していた．それは細胞の「動き」である．細胞の動きをみるためには，どうしても生きた細胞を生きた組織の中で観察する必要がある．特に骨髄腔のように，豊富な血管床による血流を保ったまま，そこで流出入する細胞の動きを捉えることが重要な場所では，「摘出して生かした」骨組織ではなく，「生きたままの個体中」での骨組織を観察する必要がある．

筆者は最近，「多光子励起顕微鏡」という，組織の奥深くまで観察できる顕微鏡を駆使して，マウスを生かしたままの状態で骨組織内を観察するイメージング法を世界に先駆けて開発した(図1)．この方法を用いると，骨のリモデリングに関わる破骨細胞や骨芽細胞，骨髄内で分化・成熟を遂げる単球・顆粒球・リンパ球，その他の間葉系細胞や血液幹細胞などの生きた動きを，リアルタイムで観察することができる．われわれは特に，骨を破壊・吸収する働きをもつ破骨細胞の動きと機能に注目して解析を行い，この前駆細胞の骨への遊走・位置決めが，種々のケモカインや脂質メディエーター(スフィンゴシン1リン酸)によって動的に調節されていることを明らかにした．本稿では，これらの研究成果の解説に加えて，骨組織内の2光子励起ライブイメージングの方法論や，その今後の応用と将来性について，実際の画像を紹介しながら概説する．

* 大阪大学免疫学フロンティア研究センター生体イメージング研究室〔〒565-0871 大阪府吹田市山田丘3-1〕Laboratory of Biological Imaging, Immunology, Frontier Research Center, Osaka University

0557-0433/10/¥500/論文/JCOPY

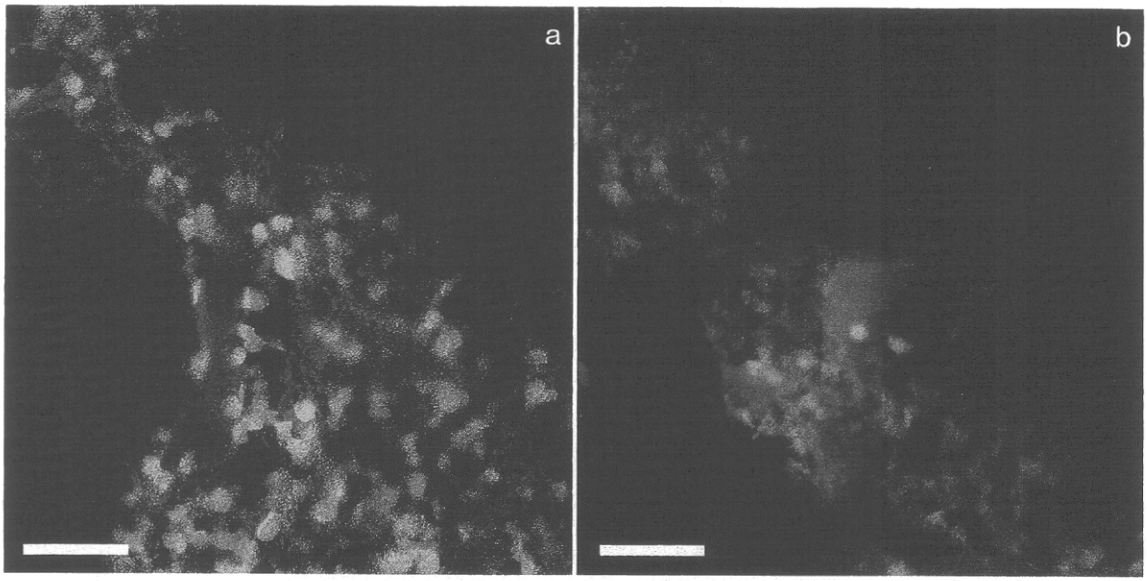


図1　骨組織の多光子励起ライブイメージング

好中球(a)または単球(b)がそれぞれ緑色蛍光(GFP)を発現している遺伝子改変マウスの骨髄腔の生体2光子励起顕微鏡による観察像．骨髄内の血管構造は，赤色蛍光(Texas Red)を結合させた高分子デキストランを静脈注射して可視化している．実際の実験ではこれを一定時間間隔で撮影し，動画を作成する(筆者HPを参照)．スケールバー：30 μm.

破骨細胞は「どこから来て，何者で，どこへ行くのか?」

骨組織は，古い骨を壊して吸収する「破骨細胞」と，骨を新生する「骨芽細胞」のバランスの取れた働きにより新陳代謝が繰り返されている．骨は，硬そうで一度できたら変わらない組織にみえるが，厳密にいえば昨日の骨と今日の骨は少し違う．加齢や炎症などによって破骨細胞の働きが異常に活性化し，破骨細胞と骨芽細胞の働きのバランスが崩れる骨吸収側に傾くと，骨粗鬆症や関節リウマチなど，いわゆる骨吸収性疾患の発症へとつながる．特に，関節リウマチでは，関節炎局所に活性化した破骨細胞が多数誘導され，骨破壊に関与している[18,19]．

骨芽細胞は骨内に恒常的に存在する間葉系由来の細胞であるが，破骨細胞は血液(単球)系の前駆細胞が骨表面に遊走してきて，その場所で最終分化を遂げて骨吸収能をもつ成熟破骨細胞となる．興味深いことに，破骨細胞と骨芽細胞は単純なライバル関係ではなく，その働きは相互に緊密に連関している．例えば，骨表面で破骨前駆細胞が成熟破骨細胞へと分化するために必須の因子であるRANKL(receptor activating NF-κB ligand)は，主に骨芽細胞が発現している[11,17]．このRANKLの発見をはじめとして，これまで国内外での精力的な研究の結果，破骨細胞の分化・成熟に関わる機構について多くの事実が解明されてきた．その一方で，長らく解決されていなかった重要な謎があった．それは「破骨細胞(およびその前駆細胞)はどうやって骨表面に到達するのか」である．

RANKLは骨組織以外の組織にも発現がみられるが，その他の組織では破骨細胞はみられない．またRANKLを欠損したマウスでは，成熟破骨細胞の形成が完全に阻害されているが，このマウスにrecombinantのRANKLを投与すると破骨細胞分化がみられる．このとき興味深いことに，成熟破骨細胞は骨表面にしかできない．これは，骨表面には(骨芽細胞による)RANKLの発現以外に，破骨前駆細胞を引き寄せて，成熟させるのに適した特別な環境(破骨細胞ニッチとも呼ぶべき)が存在することを強く示唆している．

筆者は，破骨前駆細胞がいかにして骨表面へ到達するのか，またその遊走・位置決めがどのように制御されているかについて解明するために，まず，種々のケモカインや脂質メディエーターについて，破骨前駆細胞を動かし得るかどうかを

in vitro の実験系でスクリーニングを行った．その結果，スフィンゴシン1リン酸をはじめとした，いくつかの候補分子を得た．しかしながら，次の研究段階として，「これらの候補分子が実際に *in vivo* で破骨前駆細胞を動かすのかどうか」を解決する必要があった．このため，多光子励起顕微鏡を用いて生きた骨組織内部を観察することに挑戦した．

骨組織の生体多光子励起顕微鏡観察

1. 多光子励起顕微鏡はなぜ生きた組織の観察に適しているか？

多光子励起(通常は“2”光子励起)顕微鏡では，通常の蛍光顕微鏡観察(共焦点レーザー顕微鏡も含む)で用いる励起光より小さいエネルギー(＝波長が長い)をもったレーザー光を，細かいパルス状に放出したものを励起光源に用いる．パルス状の光子はフォーカスで一点に集められ密度が高い状態となるため，焦点平面でのみ多光子励起(＝通常1光子励起)では光子1個で励起する蛍光分子を，複数の光子(2光子励起であれば2個分)で励起する現象が起こりうる[6]．このため，多(2)光子励起顕微鏡の特長として，以下が挙げられる[3,4]．

①高いz軸分解能：焦点平面のみでしか励起が起こらない〔その他のz軸平面では(通常の励起に必要な半分のエネルギーの)光子が当たっているものの励起には至らない〕．

②高い組織透過性：励起光として通常よりも波長の長い(2光子励起であれば，通常の2倍の波長)の近赤外光(通常は波長が780～1,050 nm)を用いるため，組織の深部まで励起光を到達させることができる〔※テレビのリモコン(赤外線)は障子を通過するが，紫外線は紙一枚で容易にカットできる〕．

固定した組織・臓器は，薄切してプレパラートにすれば，あらゆる断面を観察することができるが(物理的スライス)，生きた丸ごとの組織の内部を観察するには，多光子励起顕微鏡を用いて，深部組織でz軸平面を変えて観察することが有用である(光学的スライス)．このため，生組織の観察手段として，多光子励起顕微鏡の有用性が国内外で示されてきた．例えば，動物の脳や分泌腺など臓器・組織を摘出して，培養液中で生かしながら多光子励起観察されてきた(tissue-explant two-photon imaging)[7,15,16]．

2.「生体」多光子励起観察のメリット

免疫・血液系は，特に細胞の動きが重要なシステムである．好中球やリンパ球が全身をくまなく遊走し，免疫組織内の微小環境で会合し互いに相互作用を行うことにより，適切な機能が維持されている．この細胞遊走は時空間的に精緻にコントロールされており，各細胞が適切な場所に適切な時間に存在しなければ，機能を十分に発揮できない．このような免疫系における統率された細胞遊走システムは，神経系での固定した軸索システム(“hard-wired”)と比較して，“soft-wired”と形容される[8]．このsoft-wiredネットワークの解析のために，多光子励起観察をさらに一歩進めて，実験動物を生かしたままで顕微鏡に乗せて，注目する組織を観察する“intravital multi-photon microscopy(生体多光子励起顕微鏡観察)”の手法が，2002年頃から海外の複数の研究者によって開発された[1,12,14,20]．この方法論では，注目する組織のみならず，個体自体が生きており，全身の血流や代謝が完全にインタクトに保たれた状態で観察できるため，極めて情報量が多い．

骨組織内での破骨前駆細胞の遊走・位置決めを観察するために，われわれは骨内・骨髄腔の“intravital” imagingに取り組んだ[5,9,10]．この方法では，骨髄腔内を流れる豊富な血流が保たれているため，骨組織に定着している細胞の動きのみならず，血管から骨髄内へ細胞が流入したり，逆に血中へ還流していく様子を観察することができる．さらには，薬剤を尾静脈などから全身投与すると血流を通して速やかに観察部位に到達させることができる．このような長所から，われわれは骨のintravital imagingを行ったが，そもそも骨のように血流が豊富な組織は，取り出して生かしたまま観察することはかなり難しい．

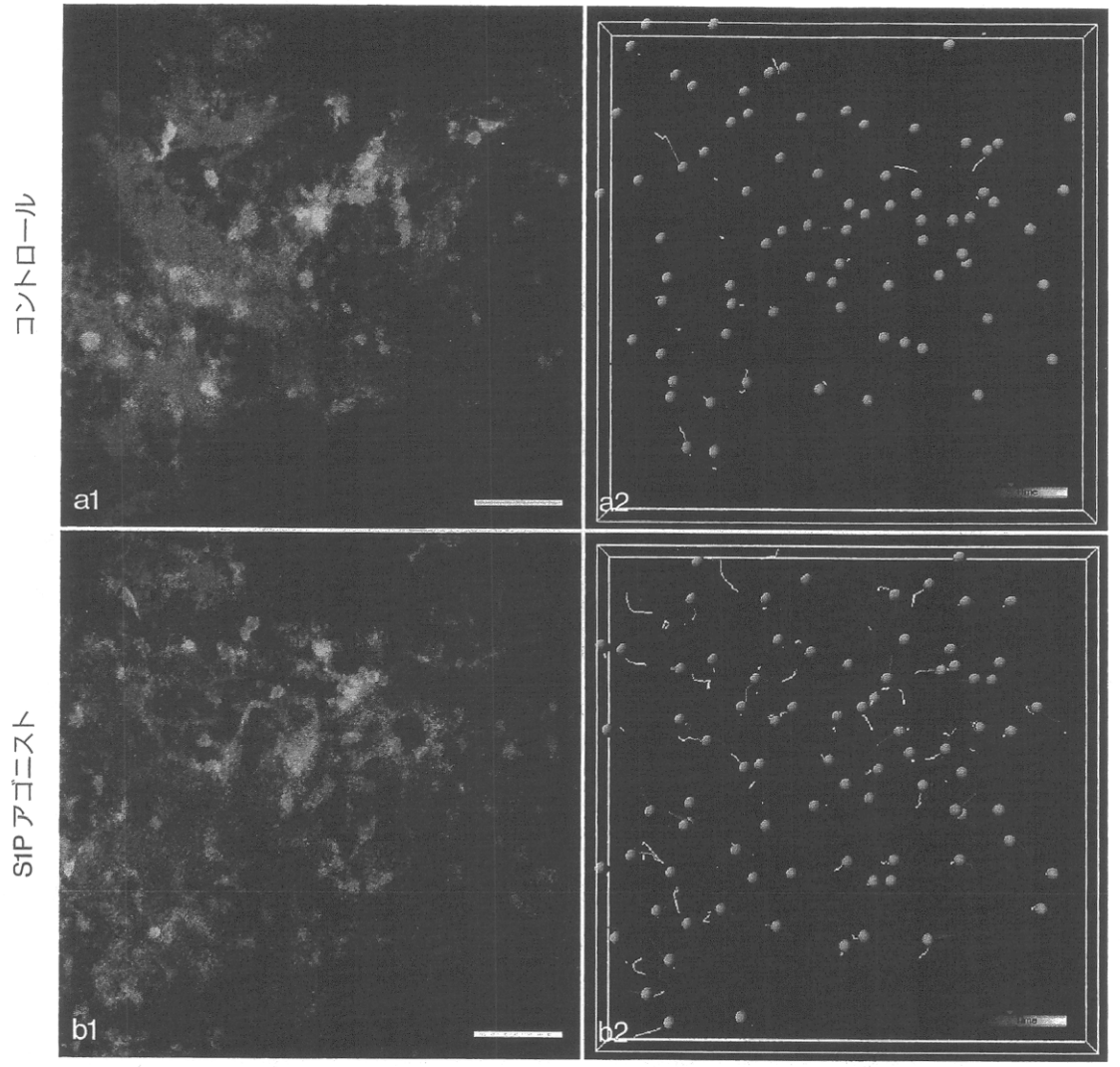


図2　ライブイメージングによる破骨前駆細胞の動態の解明

破骨前駆細胞を含む単球系細胞(CX_3CR1-$EGFP^+$)を緑色でラベルして，Texas Red を conjugate した高分子デキストラン(～70 kDa)を静注して血管構造を赤色でラベルして，それぞれ可視化している(a1，b1)．また，各細胞を球体に置き換え，軌道を書いて速度を計算している(a2，b2)．定常状態では単球系細胞はほとんど静止しているのに対し(a1，a2)，S1P アゴニストである SEW2871 を投与すると，急速に細胞の運動能が亢進し，血中へ還流していく様子が観察される(文献 9 より一部改変)．

生体多光子骨組織観察によってみえた，脂質メディエーター S1P による破骨前駆細胞の遊走と位置決めの制御

種々のケモカイン・脂質メディエーターを *in vitro* でスクリーニングした結果，破骨前駆細胞の遊走を刺激するいくつかの分子を得ていたが，中でもわれわれが注目したものは，現在リンパ球の遊走制御について重要な知見が得られているスフィンゴシン 1 リン酸(S1P)である[2,13]．S1P は主に赤血球や血小板によって作られるため血中に豊富に存在する．一方，組織には S1P を分解する S1P リアーゼが豊富に発現しており，一般に S1P は血中で高く，組織で低い濃度に保たれている．このため，S1P に対するケモタキシスは，基本的には細胞が組織から血中へ還流する際に作用すると考えられている．

われわれは，破骨前駆細胞が S1P に対する受容体($S1P_1$)を発現しており，*in vitro* で S1P に対して強いケモタキシスが惹起されることを見出し

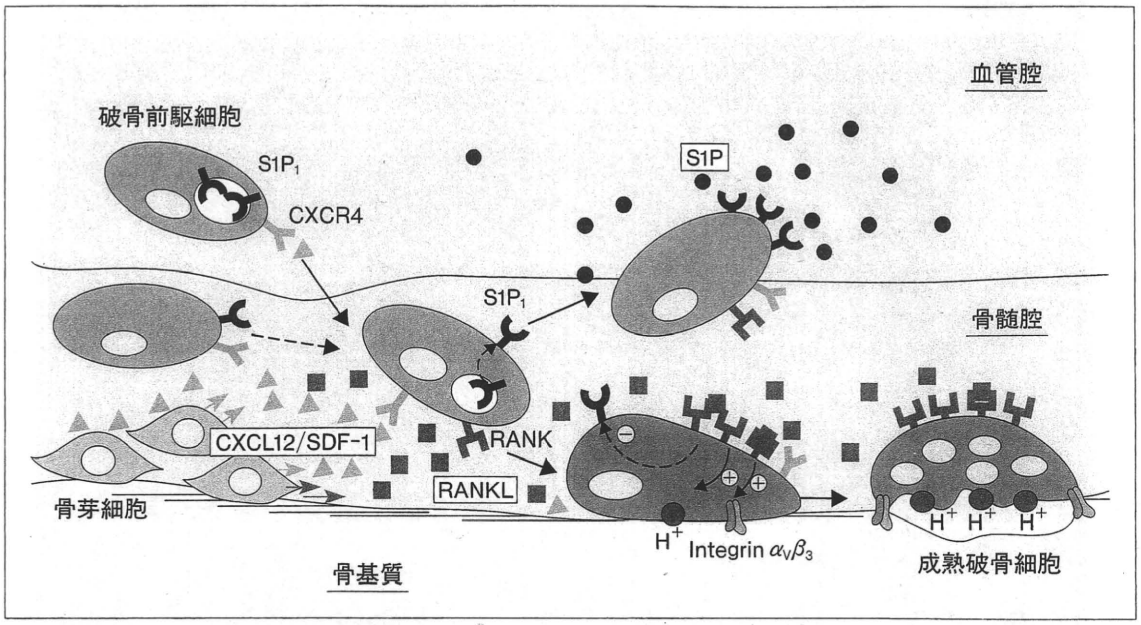


図3　破骨前駆細胞の遊走と位置決めの機構

単球系破骨前駆細胞は，骨髄内にあるケモカインCXCL12/SDF-1によって骨質内へ引き寄せられ，逆に血中のS1Pによって血管内へと再還流する．この流出入のバランスの上に骨表面に存在する破骨前駆細胞の数が決められており，一定数がRANKLの刺激を受け成熟破骨細胞へと分化する．

た．このS1Pに対する細胞遊走が*in vivo*でもみられるかどうかを確認するために，2光子励起顕微鏡を用いて骨組織内部の生体観察を行った[5,9]．骨組織に存在する破骨前駆細胞を含む単球系細胞(CX_3CR1-$EGFP^+$)は，定常状態ではほとんど動かなかったが，$S1P_1$受容体に対する強力なアゴニストであるSEW2871を経静脈的に投与すると，急速に動きが大きくなり，血管へと移行していく様子が観察された(図2，筆者のHP：http://bioimaging.ifrec.osaka-u.ac.jp/に動画が紹介されている)．これにより，*in vivo*の骨組織内でも，破骨細胞は確かにS1P受容体刺激に反応して遊走能が亢進することが証明された．

さらにわれわれは，この「S1Pに対する破骨前駆細胞の遊走」の生理意義を解明するために，破骨前駆細胞を含む単球系細胞($CD11b^+$)に特異的にS1P受容体($S1P_1$)を欠損させたマウスの解析を行った．$S1P_1$を欠損した破骨前駆細胞は骨組織にとどまりやすくなり，その結果として骨表面に接着する成熟破骨細胞の数が増加し，骨吸収側へと傾くことがわかった．S1Pの濃度が血中で高く，S1Pに対する遊走が一般に組織から血中への還流に寄与していることを考慮すると，以下の結論を得ることができる．

単球系の破骨前駆細胞は，血管から骨内部に流入するだけでなく，血中のS1Pに対して遊走することにより血中へ再還流するシステムが存在する．この流出入のバランスの上に骨表面に存在する前駆細胞数が決められており，一定数がRANKLの刺激を受け成熟する(図3)．これまで，RANKLなどの分化誘導因子やその下流にある転写制御が，破骨細胞研究の主要な課題であったが，この研究はその前の段階すなわち破骨前駆細胞が最終分化を遂げる場所(骨)へと遊走・位置決めを行うシステムが，破骨細胞分化・骨代謝の新たな制御点であるという新概念を提唱するものである．

今後の展開

1．破骨前駆細胞の遊走・位置決めを標的とした新しい創薬

この新しい調節点は，骨吸収疾患に対する創薬ターゲットとしても極めて魅力的である．われわれは骨粗鬆症のモデル動物を用いて，S1P受容体に対する強力なアゴニストの投与が，破骨前駆細胞を骨表面から引き剝がし血中へ再還流させ(結果として骨表面上の破骨細胞の数を減らし)，骨吸

収を抑制することを示した．この結果は，S1Pによる破骨前駆細胞の遊走制御が，治療標的としても有望なものであることを示している[9]．これは，ビスホスホネート製剤など成熟破骨細胞を標的とした従来の骨吸収抑制薬とは異なった作用機序を持っているので，併用による相乗効果も期待でき，今後の臨床応用が注目される．

2. 骨組織の生体多光子励起観察の応用

骨組織・骨髄は謎めいたブラックボックスである．破骨細胞や骨芽細胞による骨代謝制御の重要な場であるばかりでなく雑多な血液系・間葉系細胞が，所狭しと詰め込まれて，生存・分化・機能を行っている．多様な血液系細胞はそれぞれ決まった場所(ニッチ)に存在し，また互いに複雑な静的・動的ネットワークを形成している．しかもそれは余程重要なもののようで，硬くて頑丈な入れ物(骨皮質)で囲まれている．血液幹細胞の自己複製や血球分化など，骨髄機能の生理・病理には，いまだ不明な点が数多く残されているが，2光子励起顕微鏡による“非破壊検査”を用いた今後の解明が期待される．

文　献

1) Bousso P, Bhakta NR, Lewis RS, et al: Dynamics of thymocyte-stromal cell interactions visualized by two-photon microscopy. Science **296**: 18676-1880, 2002
2) Cyster JG: Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu Rev Immunol **23**: 127-159. 2005
3) Denk W, Strickler JM, Webb WW: Two-photon laser scanning fluorescence microscopy. Science **248**: 73-76, 1990
4) Denk W, Svoboda K: Photon upmanship: why multiphoton imaging is more than a gimmick. Neuron **18**: 351-357, 1997
5) Germain RN, Bajénoff M, Castellino F, et al: Making friends in out-of-the-way places: how cells of the immune system get together and how they conduct their business as revealed by intravital imaging. Immunol Rev **221**: 163-181, 2008
6) Goeppert-Mayer M: Über Elementarakte mit zwei Quantensprüngen. Ann Phys **9**: 273-295, 1931
7) Helmchen F, Denk W: Deep tissue two-photon microscopy. Nat Methods **2**: 932-940, 2005
8) Huang AY, Qi M, Germain RN: Illuminating the landscape of in vivo immunity: insights from dynamic in situ imaging of secondary lymphoid tissues. Immunity **21**: 331-339, 2004
9) Ishii M, Egen JG, Klauschen F, et al: Sphingosine-1-phosphate mobilizes osteoclast precursors and regulates bone homeostasis. Nature **458**: 524-528, 2009
10) Klauschen F, Ishii M, Qi H, et al: Quantifying cellular interaction dynamics in 3-D fluorescence microscopy data. Nat Methods **4**: 1305-1311, 2009
11) Lorenzo J, Horowitz M, Choi Y: Osteoimmunology: interactions of the bone and immune system. Endocr Rev **29**: 403-440, 2008
12) Miller MJ, Wei SM, Parker I, et al: Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. Science **296**: 1869-1873, 2002
13) Rosen H, Goetzl EJ: Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. Nat Rev Immunol **5**: 560-570, 2005
14) Stoll S, Delon J, Brotz TM et al: Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. Science. **296**: 1873-1876, 2002
15) Takahashi N, Kishimoto T, Nemoto T: Fusion pore dynamics and insulin granule exocytosis in the pancreatic islet. Science. **297**: 1349-1352, 2002
16) Takahashi N, Kasai H: Exocytic process analyzed with two-photon excitation imaging in endocrine pancreas. Endocr J **54**: 337-346, 2007
17) Takayanagi H: Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat Rev Immunol **7**: 292-304, 2007
18) Teitelbaum SL: Bone resorption by osteoclasts. Science **289**: 1504-1508, 2000
19) Teitelbaum SL, Ross FP: Genetic regulation of osteoclast development and function. Nat Rev Genet **4**: 638-649, 2003
20) von Andrian UH: Immunology: T cell activation in six dimensions. Science **296**: 1815-1817, 2002