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PERSPECTIVES

Control of Osteoclast Precursor Migration: A Novel Point of Control for Osteoclastogenesis and Bone Homeostasis

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Abstract

Osteoclasts are bone-resorbing, multinucleated giant cells that differentiate from mononuclear macrophage/monocyte-lineage hematopoietic precursors. They have critical roles not only in normal bone remodeling but also during pathogenesis of destructive bone disorders such as osteoporosis, rheumatoid arthritis, and cancers metastatic to bone. Many molecules, especially macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL), make significant contributions to osteoclast differentiation. However, the process of osteoclast precursor trafficking to and from the bone surface, where cell fusion occurs to form the fully differentiated multinucleated cells that mediate bone resorption, is less well-documented. Recent studies have shed light on the mechanisms involved and have demonstrated the vital participation of various chemokines such as CCL2, CCL5, CXCL12, and CX_3CL1, and lipid mediators such as sphingosine-1-phosphate (S1P). In addition, advances in imaging technologies, such as the development of intravital multiphoton microscopy, have enabled the *in situ* visualization of the behavior of osteoclasts and their precursors within intact bone tissue. This capability will be extremely useful for dissecting the mechanisms controlling the migration of these cells *in vivo*. In this *Perspective*, we review the latest knowledge in this new field of bone biology, with a focus on novel imaging methodology and its applications in this field. *IBMS BoneKEy*. 2010 August;7(8):279-286.

Introduction

Bone is a dynamically regulated tissue that continuously undergoes remodeling to maintain mineral homeostasis and structural robustness. The balance between bone resorption by osteoclasts and bone formation by osteoblasts is finely regulated (1-3), and several complex mechanisms maintain this equilibrium. The mechanism that has been investigated most extensively is the control of both osteoclast and osteoblast differentiation (1;2;4). Recently, the regulation of precursor cell recruitment has attracted attention (5-14). We have also investigated extensively the highly organized migration of osteoclast precursors between the bone marrow and blood vessels, in real-time using intravital multiphoton microscopy (14;15). In this review, we summarize recent findings regarding the recruitment of osteoclast precursor cells to the bone surface and briefly introduce *in vivo* imaging of bone.

What Are Osteoclasts? Where Do They Come From, and Where Are They Going?

Even in adults who have completed their growth, osseous tissue is continuously remodeled via bone resorption by osteoclasts and bone formation by osteoblasts, to maintain bone strength and electrolyte balance. In several pathological states, including osteoporosis, tumor-induced osteolysis, and rheumatoid arthritis, osteoclasts are activated excessively, and the balance between bone formation and resorption is disrupted. Consequently, the inhibition of osteoclast function is a major therapeutic target in these diseases (3).

The osteoclast is a unique multinucleated giant cell formed by the fusion of mononuclear precursors of the macrophage-

monocyte lineage. The differentiation of precursor cells into mature osteoclasts requires two important molecules: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) (16-18). In addition, although this is still controversial, it has been reported that many hormones, cytokines, and growth factors such as parathyroid hormone, estrogen, 1,25-dihydroxyvitamin D_3, and interleukin-6 can affect osteoclast differentiation by regulating the expression of M-CSF, RANKL, and osteoprotegerin, a non-signaling decoy receptor for RANKL (1-3). The major source of M-CSF, RANKL, and osteoprotegerin in bone tissue is osteoblastic lineage cells, or stromal cells, and the interaction between osteoclast and osteoblast lineages is critical in bone homeostasis (1-3). Signaling also occurs in the opposite direction. Stimulatory factors such as transforming growth factor-β, insulin-like growth factor-1, and cardiotrophin-1 are released from osteoclasts during bone resorption and stimulate bone formation by osteoblasts (4). In addition, EphrinB2 expressed by osteoclasts and EphB4 expressed by osteoblasts participate in bidirectional communication between osteoclast- and osteoblast-lineage cells (19).

The mechanism by which osteoclast precursors are recruited at the proper time to appropriate sites for differentiation is unclear. The bone marrow cavity and bloodstream contain monocyte-lineage precursor cells, which can differentiate into osteoclast precursor cells. These precursors are recruited to the surface of bone, where they differentiate into mature osteoclasts. Moreover, cell cycle-arrested quiescent osteoclast precursors on the bone surface can differentiate into mature osteoclasts upon exposure to several stimuli (22). Although the differentiation stage at which osteoclast precursor cells migrate to the bone surface is controversial, several cytokines were shown recently to be involved in their recruitment (5-13). In addition to *in vitro* migration assays, intravital two-photon imaging permits the observation of cell behavior *in vivo* in real-time and is a powerful tool for determining spatiotemporal control mechanisms (15;23;24). Below, we review recent findings from many groups, including ours, regarding the recruitment and release of osteoclast precursors.

Attractants and Repellents

Although the mechanism of osteoclast precursor recruitment remains elusive, several chemoattractants and chemorepellants have been shown to play critical roles in controlling the migration of monocyte-lineage precursor cells from blood vessels into the bone marrow cavity. As with leukocytes, the migration of osteoclast precursors is regulated mainly by short peptides (approximately 70-90 amino acids) known as chemokines. Chemokines have been classified into C, CC, CXC, and CX3C subfamilies, according to their structural cysteine motif. Chemokine receptors are G protein-coupled receptors (GPCRs, also known as seven-transmembrane receptors), and act specifically through pertussis toxin (PTx)-sensitive Gαi components. Although some chemokine-receptor pairs are exclusive, most receptors interact with multiple ligands, and most ligands interact with more than one receptor. This redundancy makes their regulation complex (25). The best-known chemoattractant is CXCL12 (or stromal derived factor-1, SDF-1), a CXCR4 ligand (5;6). CXCL12 is expressed constitutively at high levels within bone by osteoblastic stromal cells and vascular endothelial cells, while CCR4 is expressed on a wide variety of cells, including circulating monocytes and osteoclast precursors. CXCL12 has chemotactic effects on osteoclast precursors, which express high levels of CCR4 (5). Recently, another chemokine, CX_3CL1 (or fractalkine), which works as an adhesion molecule as a membrane-bound chemokine, and as a chemoattractant after being cleaved by ADAM10 and ADAM17. CX_3CL1, which is the only known member of the CX3C subfamily and expressed by osteoblastic stromal cells, was reported to be involved in both the recruitment and attachment of osteoclast precursors (7). These cytokines are engaged mainly in the interaction between osteoclasts and

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

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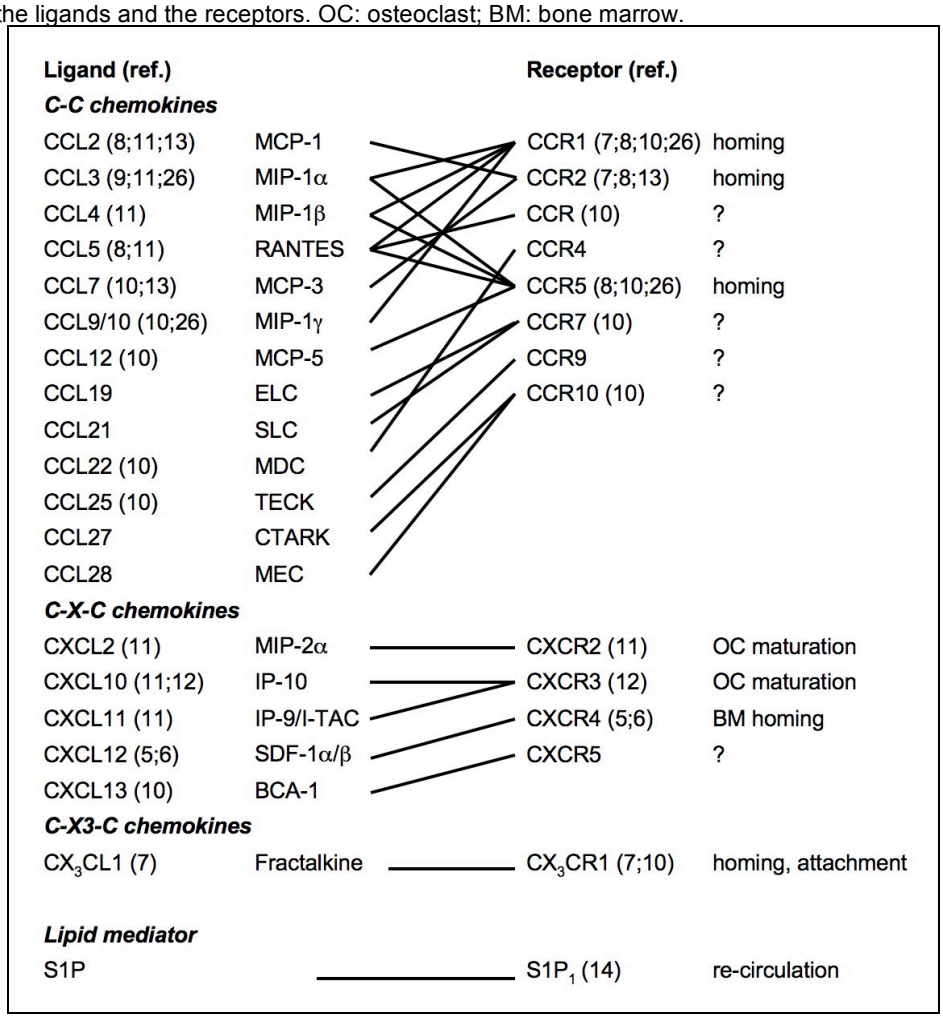
Table 1. Chemoattractants and repellents for osteoclast precursors. The lines indicate possible interactions between the ligands and the receptors. OC: osteoclast; BM: bone marrow.



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

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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).

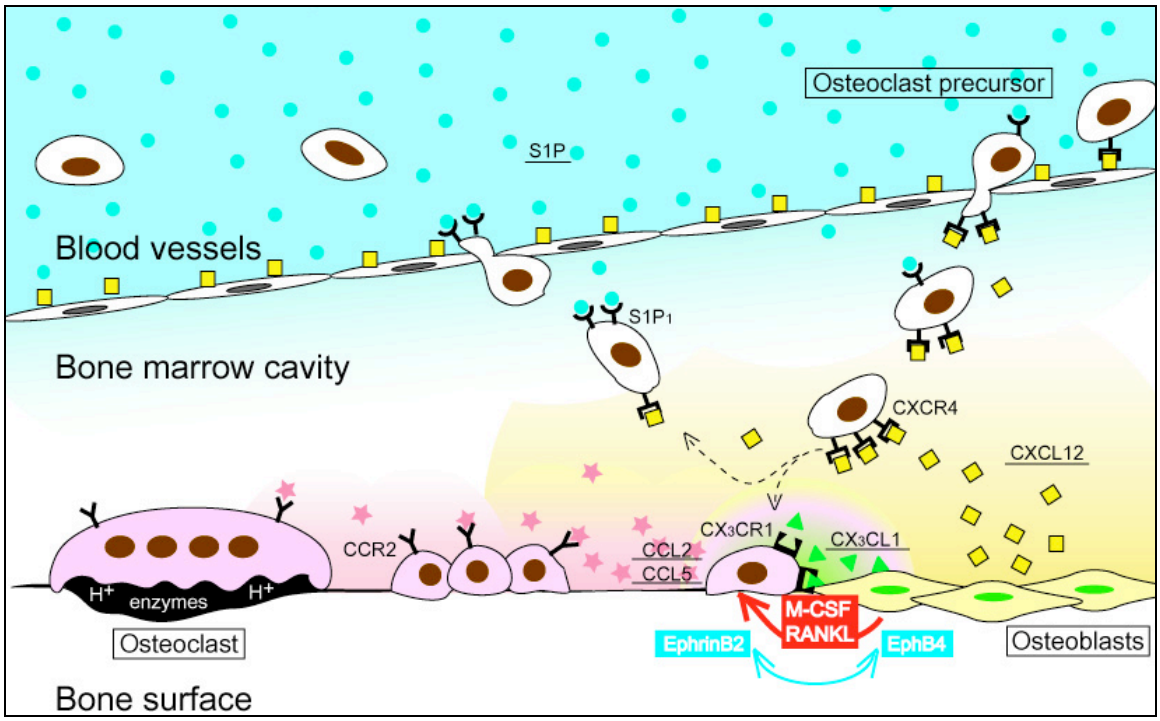

Fig. 1. Several chemoattractants control the behavior of monocyte/macrophage-lineage osteoclast precursors. Bone-attraction molecules such as CXCL12 attract osteoclast precursors into the bone marrow cavity from the bloodstream. Then, bone-attachment inducers such as CX3CL1 recruit and attach the precursors 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.

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