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Minireview

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

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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

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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 megakaryocyte 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

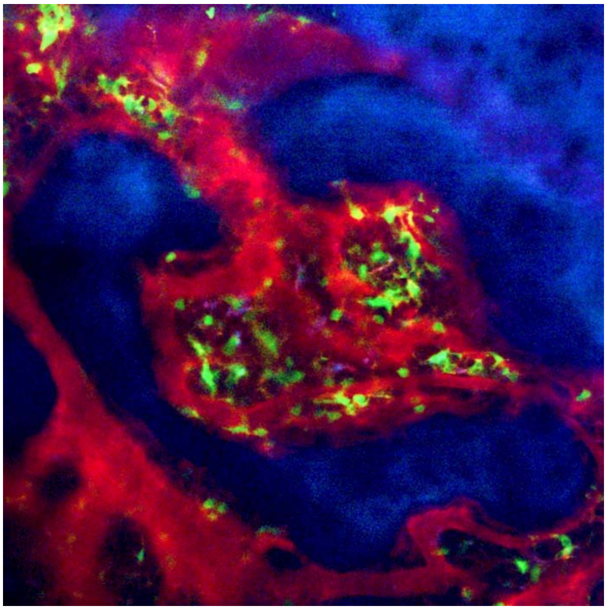

Fig. 1. Bone marrow structure visualized by intravital two-photon imaging. Murine skull bone tissues of heterozygous Cx_3CR1-EGFP knock-in mice. Collagen fibers in bone are detected by second-harmonic generation (in blue), and the microvasculature are visualized by intravenous injection of 70 kDa dextran-conjugated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

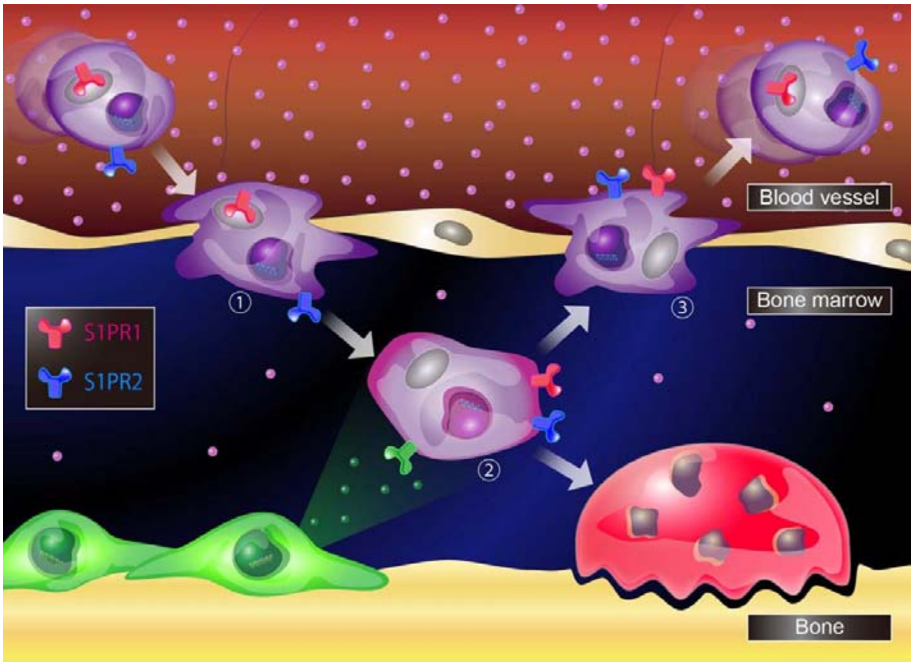

Fig. 2. A schematic model for S1P-mediated osteoclast precursors localization. The entry of osteoclast precursors from blood vessels where S1P is at high concentration, is initiated by chemorepulsion through S1PR2 (1). Once enter in bone marrow, osteoclast precursors migrate toward chemokines enriched 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.

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