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Systemic Circulation and Bone Recruitment of Osteoclast Precursors Tracked by Using Fluorescent Imaging Techniques

Manato Kotani,†*‡ Junichi Kikuta,†*‡ Frederick Klauschen, ‡ Takenao Chino,‡ Yasuhiro Kobayashi,† Hisataka Yasuda,‖ Katsuto Tamai,‡ Atsushi Miyawaki,‡ Osami Kanagawa,*,**, Michio Tomura,***† and Masaru Ishii*†

Osteoclasts are bone-resorbing polykaryons differentiated from monocyte/macrophage-lineage hematopoietic precursors. It remains unclear whether osteoclasts originate from circulating blood monocytes or from bone tissue–resident precursors. To address this question, we combined two different experimental procedures: 1) shared blood circulation “parabiosis” with fluorescently labeled osteoclast precursors, and 2) photoconversion-based cell tracking with a Kikume Green-Red protein (KikGR). In parabiosis, CX3CR1-EGFP knock-in mice in which osteoclast precursors were labeled with EGFP were surgically connected with wild-type mice to establish a shared circulation. Mature EGFP+ osteoclasts were found in the bones of the wild-type mice, indicating the mobilization of EGFP+ osteoclast precursors into bones from systemic circulation. Receptor activator for NF-κB ligand stimulation increased the number of EGFP+ osteoclasts in wild-type mice, suggesting that this mobilization depends on the bone resorption state. Additionally, KikGR+ monocytes (including osteoclast precursors) in the spleen were exposed to violet light, and 2 d later we detected photoconverted “red” KikGR+ osteoclasts along the bone surfaces. These results indicate that circulating monocytes from the spleen entered the bone spaces and differentiated into mature osteoclasts during a certain period. The current study used fluorescence-based methods clearly to demonstrate that osteoclasts can be generated from circulating monocytes once they home to bone tissues. The Journal of Immunology, 2013, 190: 000–000.
In the current study, to investigate whether circulating osteoclast precursors have the potential to differentiate into osteoclasts, we used two different experimental methods: a shared blood circulation “parabiosis” system (16, 17) and photoconversion-based cell tracking with a Kikume Green-Red protein (KikGR). Parabiosis enables one to determine whether cells/molecules are circulating or not (16–18). KikGR is a photobleachable GFP cloned from the coral *Favia fava* whose emission changes from green to red upon exposure to violet light (19). We generated monocyte/osteoclast (RANK)-specific KikGR-expressing mice, and cells in the spleen were marked by photoconversion to determine whether they can migrate to bones and differentiate into mature osteoclasts. These novel approaches using fluorescence imaging techniques are useful for tracking cellular dynamics in vivo (20–22).

**Materials and Methods**

Mice

CX3CR1-EGFP knock-in mice (23) were obtained from The Jackson Laboratory (Bar Harbor, ME). RANK-Cre knock-in mice were generated as previously reported (24). The ROSA-CAG-lox-stop-lox-KikGR knock-in mice were generated at RIKEN and are described elsewhere (M. Tomura, A. Miyawaki, and O. Kanagawa, manuscript in preparation). These two strains were crossed to generate RANK promoter-driven KikGR-expressing mice (RANK-KikGR). All mice were bred and maintained under specific pathogen-free conditions at the animal facilities of Osaka University, and all animal experiments were performed according to Osaka University animal experiment guidelines using approved protocols.

Parabiosis

Parabiotic surgery was performed according to a previously published protocol (25). A CX3CR1-EGFP knock-in mouse was surgically joined to a wild-type mouse. The two mice were housed together in a single cage for 1 wk preoperatively. At age 8 wk, the mice were anesthetized with isoflurane (Escaf; Mylan, Canonsburg, PA). The dorsal and lateral aspects of each mouse were shaved and treated with hair-removal lotion (Epilat; Kracie Holdings, Tokyo, Japan). Matching skin incisions were made from the shoulder to the knee joint of each mouse. Approximate 1-cm incisions in the peritoneum were made in each mouse, and the mice were attached using 3-0 coated Vicryl (Ethicon, Somerville, NJ). Next, the dorsal and ventral skin was stitched through continuous suture, and the matching skin was sterilized using Isodine 10% solution (Meiji Seika Pharma, Tokyo, Japan). To ensure the animals’ well-being for 4 wk, individual parabiotic mouse pairs were placed in clean cages, and food pellets were provided on the floor to minimize the strain of reaching for food while adjusting to parabiotic existence. Established shared blood circulation was confirmed by injection of Evans blue dye (Tokyo Chemical Industry, Tokyo, Japan). A total of 200 μl 0.5% Evans blue dye in saline was injected intravenously into the sides of CX3CR1-EGFP knock-in mice.

Receptor activator for NF-κB ligand (RANKL) and FTY720 treatment

FTY720 (Cayman Chemical, Ann Arbor, MI) was dissolved in DMSO (Nacalai Tesque, Kyoto, Japan). FTY720 solution was diluted in saline containing 30% fatty acid-free BSA (Sigma-Aldrich, St. Louis, MO) at 5 mg/ml (FTY720 solution). Parabiotic pairs were injected i.p. with FTY720 solution for 3 d (3 mg/kg body weight) and GST-RANKL (Oriental Yeast, Tokyo, Japan) (20 mg/kg body weight) using spot UV curing equipment (SP500; Usio, Tokyo, Japan). For spleen photoconversion, the three sides of the spleen were exposed for 10 min. The wound was closed with suture, and the matching skin was sterilized using Isodine 10%.

Flow cytometry analysis

Cells were washed with staining buffer [2% FBS and 0.035% sodium azide (both from Sigma-Aldrich) in saline]. The fluorescent cells were analyzed using a FACSAria flow cytometer (BD Biosciences, Franklin Lakes, NJ). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Immunofluorescence analysis

Femurs were removed and fixed through incubation overnight with 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer. The samples were then washed in chilled hexane (Wako, Osaka, Japan) using dry ice. Then, sections (10 μm thick) of noncalcified femur were prepared using a Multi-Purpose Cryosection Preparation Kit (Section-Lab, Hiroshima, Japan, and Leica Microsystems, Wetzlar, Germany) (26). The sections were subjected to fluorescence-based staining for tartrate-resistant acid phosphatase (TRAP) using ELF97 substrate (Life Technologies, Carlsbad, CA). The samples were observed using a TCS-SPS confocal microscope (Leica Microsystems) and were illuminated with a laser (wavelength: 405 nm for ELF97, 488 nm for EGFP and Green KikGR, and 561 nm for Red KikGR). Collagen-enriched bone matrices were visualized by the second harmonic emission from collagen fibers excited with infrared lasers. The sections were reconstructed, and bone volume/tissue volume (BV/TV; %) ratio was calculated using TRI/3D-BON software (RATOC Systems, Tokyo, Japan). Bone morphometric analysis was performed as described previously (28).

Quantitative RT-PCR analysis

Total RNA and cDNA were prepared using a NucleoSpin RNA XS kit (TaKaRa, Shiga, Japan) and SuperScript III reverse transcriptase (Life Technologies) according to the manufacturer’s instructions. Real-time PCR analysis was performed with a Thermal Cycler Dice Real Time system (TaKaRa) using SYBR Premix EX Taq (Tli RNaseH Plus) (TaKaRa). Gene expression values were calculated by the ΔΔCt method using Gapdh as an internal control. The following primers were used: Rank, 5′-TCCCGACCCAGAGATATCAGAAG-3′ and 5′-CATGGGGTCTACGAGGA-3′; and Gapdh, 5′- ACCACAGTCCATGACAC-3′ and 5′-TCCACCACGTGCTCTGA-3′. The Rank primer sequences were obtained using the Perfect Real-Time support system (TaKaRa).

Cell culture

To obtain osteoclast-like cells in vitro, bone marrow cells were obtained from the femurs and tibiae of C57BL/6J mice, and 5 × 10^5 of these were cultured for 48 h in 96-well plates in α-MEM (MP Biomedicals, Solon, OH) containing 10% FBS and 10 ng/ml human M-CSF (R&D Systems, Minneapolis, MN). Then the cells were cultured for 3 d in fresh medium containing 10 ng/ml human M-CSF and 50 ng/ml human RANKL (PeproTech, Rocky Hill, NJ). After being cultured for 5 d, the cells were fixed with 4% paraformaldehyde in 0.1 M PB and stained for TRAP (4, 5).

Statistical analysis

The Mann–Whitney rank sum test was performed using GraphPad PRISM software (GraphPad Software, La Jolla, CA) to calculate p values for skewed distributions. For Gaussian-like distributions, two-tailed t tests were used.

Distribution of materials

ROSA-CAG-lox-stop-lox-KikGR knock-in mice will be distributed from the RIKEN Bio-Resource Center Experimental Animal Division (http://www.brc.riken.go.jp/lab/animal/en/).
Results
Establishment of cross-blood circulation “parabiosis”
We generated parabiosis mouse pairs with heterozygous CX3CR1-EGFP knock-in and wild-type mice. Because the chemokine receptor CX3CR1 is known to be expressed in monocytoid cells, including osteoclast precursors (29), we were able to track the movement of osteoclast precursors between paired mice via the blood circulation. We showed by flow cytometry that 3.93 ± 0.71% (mean ± SEM; n = 3) of all bone marrow cells in CX3CR1-EGFP knock-in mice were EGFP+ (Fig. 1A). Such cells have been reported to readily differentiate into mature osteoclasts in vitro (15). We also showed that most of the mature TRAP+ osteoclasts in these mice expressed EGFP (Fig. 1Bd). Further, we confirmed that CX3CR1-EGFP+ cells could efficiently differentiate into mature osteoclast-like cells and have potency for resorbing bone tissues (dentin slices) in vitro (Supplemental Fig. 1). These results confirmed that EGFP+ cells include osteoclast precursors. Four weeks after surgery, we checked the sharing of blood circulation between the paired mice by i.v. injection of 0.5% Evans blue dye in one side (data not shown). Flow cytometric analyses showed that EGFP+ cells were present in the bone marrow of the wild-type mice. These monocytes, which included osteoclast precursors, had migrated from CX3CR1-EGFP knock-in mice via the bloodstream (Fig. 1C). The percentage of EGFP+ cells in wild-type mice [0.32 ± 0.09% (mean ± SEM), n = 3] was smaller than that in the joined CX3CR1-EGFP knock-in mice (Fig. 1A). These results suggested that substantial numbers of CX3CR1+ osteoclast precursors from wild-type mice remained without repopulation in the wild-type bone marrow, and recruitment and homing of monocytes into bone marrow tissues limited the number of EGFP+ osteoclast precursors in wild-type mice. We also checked sections of bone from joined wild-type mice (Fig. 1D). We detected mature EGFP+ TRAP+ osteoclasts lining the bone trabeculae (Fig. 1Dd, arrowhead), as well as TRAP+ osteoclasts (Fig. 1Dd, asterisks). We confirmed that EGFP+ osteoclasts originating from circulating precursors were localized along the eroded bone surface and participated in active bone resorption (data not shown). These results clearly suggest that circulating CX3CR1-EGFP+ osteoclast precursors could home to bone surfaces and differentiate into mature osteoclasts in situ.

The dynamic nature of osteoclast precursor migration
Next, we examined whether the mobilization of circulating osteoclast precursors was affected by bone-resorptive conditions. Intraperitoneal injection of recombinant RANKL has been shown to enhance osteoclastic bone resorption and potently to induce osteopenia (30). After establishment of a shared blood circulation, RANKL (20 μg/pair) or saline (control) was administered in 24-h intervals for 2 d to the sides of wild-type mice. The jointed pairs were killed 24 h after the last injection. In wild-type mice, bone mineral density, defined as the BV/TV ratio (%), was significantly reduced by application of RANKL (Fig. 2A, 2B).

To evaluate the contribution of circulating EGFP+ osteoclast precursors to RANKL-induced osteoclastogenesis, we first checked the number of EGFP+ cells in the bone marrow of wild-type mice. The number of EGFP+ cells was increased by RANKL treatment, indicating the increased recruitment of precursor monocytes into the bone marrow cavity (Fig. 2C). Next, we performed immunofluorescence analysis of sections of bone from control and RANKL-treated wild-type mice from the mouse pairs (Fig. 2D). Fluorescent image data were automatically processed as described in the Materials and Methods section to quantify objectively the proportion of TRAP+ osteoclasts that were also positive for EGFP; the proportion was significantly increased by RANKL application (Fig. 2E), indicating that the contribution of circulating precursors to osteoclast formation on the endosteum was increased under these conditions (Fig. 2F). These results suggest that sources of osteoclast precursors may change in association with several pathological bone conditions, including bone-resorptive disorders.

Next, we checked the effect of the S1P receptor agonist FTY720 on the proportion of osteoclasts that were EGFP+. As shown previously, migration of osteoclast precursors was regulated by blood S1P, and application of FTY720 may facilitate the recirculation of osteoclast precursors from bone to blood and thereby...

FIGURE 1. Generation of shared blood circulation in parabiotic animals. (A and C) Flow cytometric analysis of bone marrow cells from CX3CR1-EGFP knock-in mice (A) and wild-type mice (C). Bone marrow cells were analyzed for EGFP expression. Three pairs of animals were independently analyzed and gave similar results. The results of one representative analysis are shown. (B and D) Femoral bone tissues from CX3CR1-EGFP knock-in mice (B) and wild-type mice (D). Images of EGFP fluorescence (a), TRAP staining (b), second harmonic generation of bone tissue (c), and overlay with a transmission image (d). Arrowhead, mature EGFP+ TRAP+ osteoclast; asterisks, mature TRAP+ osteoclasts. The EGFP+ TRAP+ osteoclast/total TRAP+ osteoclast ratios are indicated by the white numbers in the bottom right corners. Scale bars, 30 μm.
prevent loss of bone density during ovariectomy-induced osteoporosis (14). We induced osteoclastic bone resorption in parabiotic mouse pairs through i.p. injection of RANKL (20 μg/pair) and further administered FTY720 (3 mg/kg) or vehicle in 24-h intervals to test their therapeutic effects (Fig. 3A). The jointed pairs were killed 48 h after the last injection, and we confirmed that the bone mineral density, defined as the BV/TV ratio (%), was significantly increased in FTY720-treated wild-type mice (Fig. 3B), as observed in the ovariectomy model (14). We also demonstrated that the total number of EGFP+ cells in bone marrow was significantly decreased by FTY720 treatment (Fig. 3C), which is also consistent with the literature (14).

We performed immunofluorescence analysis of bone sections from mice treated with RANKL with or without FTY720 (Fig. 3D). Although the total number of bone-lining osteoclasts was significantly decreased in mice treated with FTY720 (14), the proportion of osteoclasts that were EGFP+ did not differ significantly between the two groups (Fig. 3E). These results suggest that EGFP+ osteoclast precursors generated in wild-type bone marrow and EGFP+ precursors originally derived from CX3CR1-EGFP knock-in mouse bones and homed into wild-type bone marrow have comparable abilities to recirculate into the bloodstream after application of FTY720. However, we could not completely exclude the possibility that FTY720 may influence the differentiation of osteoclasts (Fig. 3F).

Tracking the movement of osteoclast precursors in photoconvertible fluorescence protein “KikGR” knock-in mice

To clarify whether osteoclast precursors were really circulating, we also used a different technique involving the photoconvertible fluorescent protein KikGR. We applied this method to study dynamic cell behavior using mouse strains in which KikGR is

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**FIGURE 2.** Mobilization of circulating osteoclast precursors under bone-resorptive conditions. (A) Characterization of the RANKL-induced bone loss model. Femurs were collected from wild-type mice after RANKL was i.p. injected (20 μg/pair) for 2 d (b). Control parabiotic pairs were injected with saline only (a). Scale bars, 1 mm. (B) Bone samples were measured by cone-beam μCT. BV/TV (%) was calculated from μCT images. *p < 0.05 (two-tailed unpaired t test). (C) Flow cytometric analyses of bone marrow cells collected from wild-type mice connected with CX3CR1-EGFP knock-in mice after i.p. injection of saline (a) or RANKL (b) (n = 4 and n = 3, respectively). *p < 0.05 (two-tailed unpaired t test). (D) Histological examination combined with computational analysis to measure the length of the white line. Upper panels, original images; lower panels, segmented images. Blue area, bone trabeculae (second harmonic fluorescent signal); orange and yellow area, EGFP+ TRAP+ osteoclasts attached to or detached from bone trabeculae, respectively; red and green area, TRAP+ osteoclasts attached to or detached from bone trabeculae; white line, osteoclast and bone attachment interface. The EGFP+ TRAP+ osteoclast/total TRAP+ osteoclast ratios are indicated by white numbers. Scale bars, 30 μm. (E) Proportion of TRAP+ osteoclasts that were EGFP+. Data represent the mean ± SEM (n = 52). **p < 0.01 (two-tailed unpaired t test). (F) Schematic illustration of osteoclast precursor mobilization. Osteoclast precursors can be generated from circulating monocytes (green + gray), as well as bone-resident precursors (gray). Under bone-resorptive conditions, recruitment of circulating monocytes into bones is increased, leading to an increase in proportion of osteoclasts on the bone surface that are positive for EGFP (green).
expressed in osteoclast precursors. We generated RANK-KikGR mice by crossing ROSA-CAG-lox-stop-lox-KikGR knock-in mice with RANK-Cre knock-in mice. RANK is a cognate receptor for RANKL, which is required for differentiation into mature osteoclasts (31). Bone marrow cells from RANK-KikGR mice were analyzed by flow cytometry (Fig. 4A). We confirmed that 0.659 ± 0.16% (mean ± SEM; n = 3) of all bone marrow cells expressed KikGR, and that KikGR+ cells could differentiate into TRAP+ osteoclast-like cells in vitro (Fig. 4B). Furthermore, Rank mRNA expression was higher in KikGR+ cells than in KikGR− cells (Fig. 4C), indicating that some KikGR+ cells in RANK-KikGR mice are osteoclast precursors.

We evaluated the efficiency of photoconversion of KikGR expressed in RANK+ cells in single-cell suspensions. Splenocytes from RANK-KikGR mice exposed to violet light (436 nm) for 10 min exhibited red-shifted emission signals compared with non-exposed cells (Fig. 4D), indicating successful photoconversion under this condition. Next, we assessed the photoconversion ef-
ficiency in the spleen after exposure to violet light in situ. Spleens were accessed by making small incisions in the skin and were exposed to violet light (436 nm). A total of \(11.28\pm1.90\%\) (mean \(\pm\) SEM; \(n=3\)) of KikGR in these cells was photoconverted and showed shifts in emission spectra (Fig. 4E). Histological examination of spleens showed that only KikGR-expressing cells near the surface of the spleen could be photoconverted (Fig. 4F), which explains the low efficiency compared with single-cell suspension.

Under bone-resorptive conditions induced by the application of RANKL, we detected a small number of mature TRAP⁺ osteoclasts.
that expressed red (photoconverted) KikGR (Fig. 4G). This result clearly demonstrates that osteoclast precursors from the spleen migrated to the bone marrow and differentiated into mature osteoclasts during these 2 d.

Discussion

Using two different experimental methods, we clearly demonstrated that circulating monocyte precursors are capable of differentiating into osteoclasts, although we think that osteoclasts can also be derived from precursors within the same bone marrow cavity. We showed that some osteoclast precursors exit the bone marrow after they have been produced by hematopoiesis, circulate via the bloodstream, and enter the bone spaces that need to be resorbed. What is the relevance of this detox for osteoclast formation? Bone resorption does not occur homogeneously in all bone tissues (32). This implies that the site of bone resorption is not necessarily the same as that of osteoclast precursor generation. The most extreme example is bone destruction in arthritic joints, such as in fingers, where hematopoiesis is not so dominant. The versatile circulation system of monocyte precursors would be reasonable and beneficial for delivering osteoclast precursors from their site of origin to the site that actually needs bone destruction (33). More importantly, we showed that the recruitment of circulating precursor monocytes into the bone marrow cavity was enhanced by bone-resorptive states, such as that induced by RANKL treatment. These results suggest that unknown mechanisms control precursor recruitment in a RANKL-dependent manner. In this regard, we have to be aware that parabiotic mice were under conditions of slightly increased bone resorption because of their restricted motion, as observed in the tail suspension model (34).

Now that osteoclast precursor localization (6, 7) has been reported to be involved in intravital two-photon microscopy, Mol. Cells 31: 399–404.)

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figure 1. CX3CR1-EGFP\(^+\) cells could differentiate into mature osteoclast-like cells and resorb bone tissues (dentin slices) \textit{in vitro}.

EGFP\(^+\) cells were collected from the bone marrow of CX3CR1-EGFP -knock-in mice using a cell sorter (FACS\texttrademark Aria, Becton Dickinson) for \textit{in vitro} differentiation on dentin slices. These EGFP\(^+\) cells were efficiently differentiated into mature osteoclast-like large cells and had potency for resorbing bone.
Supplementary Figure 2. FTY720 potently increases bone density in parabiotic mouse pairs.

The femur bones were measured for bone density (BV/TV, %) under the following conditions; (from right to left; untreated (unconnected), untreated (parabiosis), FTY720 treatment (parabiosis), RANKL treatment (parabiosis), treatment with RANKL and FTY720 treated (parabiosis) (3 or 4 pairs each). FTY720 potently increased bone density not only in RANKL-treated mice, but also in untreated mice in the parabiotic pairs. *p <0.05, ***p <0.001 (Newman-Keuls multiple comparison test).