Role of CX3CL1/Fractalkine in Osteoclast Differentiation and Bone Resorption

Keiichi Koizumi, Yurika Saitoh, Takayuki Minami, Nobuhiko Takeno, Koichi Tsuneyama, Tatsuro Miyahara, Takashi Nakayama, Hiroaki Sakurai, Yasuo Takano, Miyuki Nishimura, Toshio Imai, Osamu Yoshie and Ikuo Saiki

*J Immunol* 2009; 183:7825-7831; Prepublished online 18 November 2009;
doi: 10.4049/jimmunol.0803627
http://www.jimmunol.org/content/183/12/7825
Role of CX3CL1/Fractalkine in Osteoclast Differentiation and Bone Resorption

Keiichi Koizumi,†‡ Yurika Saitoh,*, Takayuki Minami,*, Nobuhiro Takeno,*
Koichi Tsurenaya,†‡ Tatsuro Miyahara,§ Takashi Nakayama,‖ Hiroaki Sakurai,∗††
Yasu Takano,‡ Miyuki Nishimura,‖ Tosio Imai,‖ Osamu Yoshie,‖ and Ikuo Saiki*††

The recruitment of osteoclast precursors toward osteoblasts and subsequent cell-cell interactions are critical for osteoclast differentiation. Chemokines are known to regulate cell migration and adhesion. CX3CL1 (also called fractalkine) is a unique membrane-bound chemokine that has dual functions for cells expressing its receptor CX3CR1: a potent chemotactic factor in its soluble form and a type of efficient cell adhesion molecule in its membrane-bound form. In this paper, we demonstrate a novel role of CX3CL1 in osteoblast-induced osteoclast differentiation. We found that osteoclast precursors selectively expressed CX3CR1, whereas CX3CL1 is expressed by osteoblasts. We confirmed that soluble CX3CL1 induced migration of bone marrow cells containing osteoclast precursors, whereas immobilized CX3CL1 mediated firm adhesion of osteoclast precursors. Furthermore, a blocking mAb against CX3CL1 efficiently inhibited osteoclast differentiation in mouse bone marrow cells cocultured with osteoblasts. Anti-CX3CL1 also significantly suppressed bone resorption in neonatal mice by reducing the number of bone-resorbing mature osteoclasts. Collectively, CX3CL1 expressed by osteoblasts plays an important role in osteoclast differentiation, possibly through its dual functions as a chemotactic factor and adhesion molecule for osteoclast precursors expressing CX3CR1. The CX3CL1-CX3CR1 axis may be a novel target for the therapeutic intervention of bone resorbing diseases such as rheumatoid arthritis, osteoporosis, and cancer bone metastasis. The Journal of Immunology, 2009, 183: 7825–7831.

Bone is a highly dynamic structure undergoing constant remodeling through the balance between bone resorption by osteoclasts and bone formation by osteoblasts at the specialized sites called bone multicellular units (1, 2). Osteoclasts are tartrate-resistant acid phosphatase (TRAP)3-positive multinucleated cells that are generated from osteoclast precursor cells of the monocyte/macrophage lineage through close cell-cell interactions with osteoblasts (1, 2). It is now known that osteoclast precursors express cell surface receptors such as c-Fms (the tyrosine kinase receptor for M-CSF), receptor activator of NF-κB (RANK), and osteoclast-associate receptor, while osteoclasts express their respective (1, 2). In particular, RANK ligand (RANKL), a transmembrane glycoprotein of the TNF-α superfamly expressed by osteoblasts, plays the major role in osteoclast differentiation via RANK by activating the osteoclastogenic cascade of transcription factors NF-κB, AP-1 (c-Fos), and NF-ATc1 (1, 2). Osteoblasts also produce M-CSF that promote the survival and proliferation of osteoclast precursors via c-Fms (1, 2). Nowadays, it is possible to generate osteoclast-like cells in vitro from purified monocytes by a mixture of soluble RANKL and M-CSF, thus greatly simplifying the analysis of osteoclast differentiation.

Chemokines are a group of structurally related cytokines that regulate the migration and activation of leukocytes and other types of cells expressing a group of seven transmembrane G protein-coupled receptors (3). According to the arrangement of the amino-terminal conserved cysteine residues, the chemokines are classified into four subfamilies: CXC, CC, C, and CX3C (3). Several chemokines have been shown to play an important role in osteoclastogenesis. For example, parathyroid hormone strongly stimulates osteoblasts to express CCL2, which in turn can potently recruit osteoclast precursors (4). CCL2 and CCL5 are also potently induced by RANKL during osteoclast differentiation and strongly promote the formation of TRAP+ multinuclear cells by an autocrine/paracrine manner (5). CCL3 is also induced by RANKL during osteoclast differentiation (6) and a known osteoclastogenic factor in multiple myeloma (7).

CX3CL1 (also called fractalkine) is a membrane-bound chemokine and the only known member of the CX3C subfamily (3). CX3CL1 can be cleaved from its membrane proximal site by the family of a disintegrin and metalloprotease ADAM10 and ADAM17 (8, 9). Importantly, CX3CL1 is a dual function molecule: it potently attracts CX3CR1-expressing cells by its soluble form and efficiently mediates firm adhesion CX3CR1-expressing...
cells by its membrane-bound form even without activation of integrins (10, 11). Therefore, the CX3CL1-CX3CR1 axis may be particularly useful in a biological situation where both attraction and subsequent cell-cell interactions are required. In this paper, we demonstrate for the first time that CX3CL1 expressed by osteoblasts plays an important role in osteoclastogenesis via CX3CR1 that are expressed by osteoclast precursors.

Materials and Methods

Mice

Female ddy strain mice, pregnant if required, were purchased from SLC. Eight-week-old mice were used for the isolation of bone marrow cells and osteoclast precursors. Neonatal mice were used for the isolation of primary osteoblasts and the treatment with anti-CX3CL1 Ab. All mice were kept under specific pathogen-free and laminar air flow conditions in the Laboratory for Animal Experiments, Institute of Natural Medicine, University of Toyama (Toyama, Japan). This study was conducted in accordance with the guideline for the Care and Use of Laboratory Animals of University of Toyama.

Cells

Primary osteoclast precursors were isolated as described previously (12). In brief, femora and tibiae from 8-wk-old mice were flushed with α-MEM (ICN Biomedicals) to release bone marrow cells. Cells were incubated in α-MEM supplemented with 10% FBS for 3 h and nonadherent cells enriched with osteoclast precursors were collected. Primary mouse osteoblasts were isolated as described previously (13). In brief, calvariae from 2-day-old mice were treated with 0.1% collagenase (Wako Pure Chemical) and 0.2% dispase (Godo Shuzo). PCR products were electrophoresed on 1.7% agarose gels and stained with ethidium bromide. This was performed as described previously (14). Briefly, total RNAs were isolated from mouse spleen and RAW 264.7 cell cultures (see above) were fixed with 4% paraformaldehyde, sequentially stained with mouse anti-cathepsin K (1/50; Santa Cruz), or control rabbit IgG and with peroxidase (PO)-conjugated goat anti-rabbit IgG polymer (Histofine for goat primary Abs; Nichirei). Tissue sections were treated with diaminobenzidine and counterstained with hematoxylin. To clarify the nature of CX3CL1- and CX3CR1-positive cells, a double-immunostaining method was performed in human bone samples. A rabbit polyclonal Ab against CXCL5 (1/2000) and Envision-PO, which, visualized by diaminobenzidine (brown color), was used for primary staining. CX3CR1 (1/300) and Envision-AP (Dako), which was visualized by fast blue (blue color) (Vector Laboratories), were used for secondary staining. To avoid interference due to double recognition of the primary Ab applied in the step of secondary Ab application, the specimens were soaked in boiling water for 10 min. We preliminarily confirmed the negative staining after soaking in boiling water by applying PO-conjugated secondary Abs against rabbit IgG. To evaluate the proportion of double-positive cells, the numbers of brown, blue, and dual-colored cells were counted within visual fields at ×400 magnification for each specimen. This study was approved by the institutional ethics committee and written informed consent was obtained from each patient.

Chemoattract assay

For the chemotaxis assay of mouse primary bone marrow cells, we used EZ-TAXIscan MIC-1000 (Effector Cell Institute) (16). In brief, cells were suspended in RPMI 1640 (Sigma-Aldrich) containing 10% FBS and cultured in a 24-well plate at 1 × 10^5 cells per well. After 48 h, the culture medium was replaced with fresh medium containing 10% FBS. Mouse osteoprogenitor cells and mouse osteoclast precursor cell line RAW 264.7 were cultured in α-MEM supplemented with 10% FBS. Mouse splenocytes and a mouse osteoclast precursor cell line RAW 264.7 were cultured in α-MEM supplemented with 10% FBS.

RANKL-induced differentiation of osteoclasts in vitro

RANKL-induced differentiation of osteoclasts in vitro was performed as described previously (12). In brief, primary osteoclast precursors (nonadherent mouse bone marrow cells, spleenocytes, and RAW 264.7) were suspended in α-MEM supplemented with 10% FBS and cultured in a 24-well culture plate at 1 × 10^5 cells per well. After 48 h, the culture medium was replaced with fresh culture medium with or without 100 ng/ml mouse recombinant RANKL (Wako Pure Chemical). After 4 days, cells were detached with ethylenediaminetetraacetic acid (1:1) for 1 min, rinsed and stained at room temperature with TRAP staining solution. TRAP-positive cells appeared dark red. We counted TRAP-positive multinucleated cells containing three or more nuclei as osteoclasts.

Effects of CX3CL1 on differentiation and CX3CR1 expression of osteoclast precursors

Osteoclast precursors (nonadherent mouse bone marrow cells) were cultured in α-MEM supplemented with 10% FBS with or without 10 ng/ml murine recombinant chemokine domain CX3CL1 (10 nM; R&D Systems). After 2 days, cells were stained with TRAP and RT-PCR analysis for CX3CR1 of the cells was performed.

RT-PCR

This was performed as described previously (14). Briefly, total RNAs were extracted from cultured cells using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from RNA template (1 μg) using oligo(dT)₅ primer and SuperScript II reverse transcriptase (Invitrogen). Reverse transcription was performed at 42°C for 50 min and then at 70°C for 15 min. PCR amplification was performed by denaturation at 94°C for 30 s, annealing at the indicated temperatures for 1 min, and extension at 72°C for 10 s using cDNA and TaKara Ex Taq HS PCR kit (Takara Shuzo). PCR products were electrophoresed on 1.7% agarose gels and stained with ethidium bromide. The sequences of the PCR primers are as follows: GAPDH sense, 5'-GGTGAAATGTCGGTAAAGCGATTT-3' and antisense, 5'-AATGCGCAAAGTTGTATGAGACC-3' (478 bp); CX3CR1 sense, 5'-TCTATACCGTCTACGATG-3' and antisense, 5'-TTAGCCGAAAGTCTGCGA-3' (511 bp); TRAP sense, 5'-TCCGTGATGTTCTGCTG-3' and antisense, 5'-GGATTTCCGCCT-3'.

Flow cytometric analysis

Mouse primary osteoblasts isolated from calvariae of neonatal mice (see above) were fixed with 4% paraformaldehyde, sequentially stained with hamster anti-mouse CX3CL1 mAb (clone 5H8-4) (15) and PE-conjugated mouse anti-hamster IgG (BD Pharmingen), and analyzed on a FACSCalibur (BD Biosciences).

Immunohistochemistry

Human bone biopsy specimens fixed with 20% buffered formalin and embedded into paraffin after decalcification were obtained from the surgical files of the Department of Pathology, Faculty of Medicine, University of Toyama (Toyama, Japan). After paraffinization, tissue sections (5-μm thick) were heated in a target retrieval solution (Dako) for 15 min using a microwave oven. Tissue sections were treated with 3% H₂O₂ in TBS for 10 min to inhibit endogenous peroxidase and with 5% BSA for 5 min to block nonspecific sites. Tissue sections were sequentially stained with rabbit anti-human CX3CL1 (1/200; Torrey Pines Biolabs), rabbit anti-human CX3CR1 (1/300; Torrey Pines Biolabs), goat anti-cathepsin K (1/50; Santa Cruz), or control rabbit IgG and with peroxidase (PO)-conjugated goat anti-rabbit IgG polymer (EnVision-PO for rabbit primary Abs; Dako) or PO-conjugated rabbit anti-goat IgG polymer (Histofine for goat primary Abs; Nichirei). Finally, tissue sections were treated with diaminobenzidine and counterstained with hematoxylin. To clarify the nature of CX3CL1- and CX3CR1-positive cells, a double-immunostaining method was performed in human bone samples. A rabbit polyclonal Ab against CX3CL1 (1/2000) and Envision-PO, which, visualized by diaminobenzidine (brown color), was used for primary staining. CX3CR1 (1/300) and Envision-AP (Dako), which was visualized by fast blue (blue color) (Vector Laboratories), were used for secondary staining. To avoid interference due to double recognition of the primary Ab applied in the step of secondary Ab application, the specimens were soaked in boiling water for 10 min. We preliminarily confirmed the negative staining after soaking in boiling water by applying PO-conjugated secondary Abs against rabbit IgG. To evaluate the proportion of double-positive cells, the numbers of brown, blue, and dual-colored cells were counted within visual fields at ×400 magnification for each specimen. This study was approved by the institutional ethics committee and written informed consent was obtained from each patient.

Cell adhesion assay

Cell adhesion to immobilized CX3CL1 was measured as described previously (10). Briefly, each well of the 96-well plate was precoated with anti-secretory alkaline phosphate Ab (10 μg/ml) in PBS at 4°C overnight. After washing with PBS, nonspecific binding sites were blocked with adhesion buffer (RPMI 1640 containing 1% BSA and 20 mM HEPES, pH 8.0, and applied to the holder assembly. Murine recombinant chemokine domain CX3CL1 (0–1000 nM; R&D Systems) was injected into the other side of the chamber. Cell migration was recorded for 1 h at 37°C using a charge-coupled device camera and a coaxial episcopic illumination system. For the chemotaxis assay of RAW 264.7 cells, we used Transwell plates (8-μm pore size; Nuclepore) essentially as described previously (17). Briefly, the filters of the upper wells were coated with collagen (Iwaki Glass) on their lower surface. R&D Systems was applied at 1 × 10⁵ cells/ml in RPMI 1640 (Sigma-Aldrich) containing 0.1% BSA and 20 mM HEPES, pH 8.0, and applied to the upper wells, while the lower wells contained medium with or without murine recombinant CX3CL1 (R&D Systems). After 4 h at 37°C, cells that had migrated to the lower surface were counted.

Osteoclast differentiation by coculture

Osteoclast differentiation by coculture was performed as described previously (18). Briefly, primary osteoblasts (see above) were suspended in α-MEM supplemented with 10% FBS and cultured in 96-well plates (1 × 10⁵ cells/well) overnight. Bone marrow cells were added to osteoblasts at 1 × 10⁶ cells/well, and the cells were cocultured for 6 days in α-MEM supplemented with 10% FBS and vitamin D₃ (10 nM). In some experiments, rat anti-mouse CX3CL1 mAb (20 μg/ml, clone 126315; R&D Systems) or control rat IgG (Jackson Laboratory) was added to the medium.
TRAP-positive cells containing more than three nuclei were counted as osteoclasts (18).

Treatment of neonatal mice with anti-CX3CL1 mAb

Neonatal mice (2 days old, n = 3) were injected i.p. with 500 μg hamster anti-mouse CX3CL1 mAb (clone 5H8-4) (15) or control hamster IgG (Jackson Laboratory) daily for 5 days. On day 8, mice were killed and the left femurs were fixed in 70% ethanol, decalcified, and embedded in glycol methacrylate. Serial 3-μm thick sections were made longitudinally in the distal region of femur and stained with toluidine blue and TRAP staining solution. Histomorphometric measurements were made at ×400 in a minimum of four optical fields in the secondary spongiosa area from the growth plate-metaphyseal junction using a semiautomatic image analyzing system (Osteoplan II; Carl Zeiss) linked to a light microscope. Eroded surface/bone surface (ES/BS), osteoclast number/bone perimeter (N.Oc/B.Pm), and osteoclast surface/bone surface (Oc.S/BS) were obtained. The nomenclature and symbols are those recommended by the Nomenclature Committee of the American Society for Bone and Mineral Research (19).

Statistical analysis

The Student’s two-tailed t test was applied to determine the significance of differences between groups, and a value of p < 0.05 was considered significant.

Results

CX3CR1 were highly expressed in osteoclast precursors but not mature osteoclasts

The nonadherent fraction of bone marrow cells and splenocytes is commonly used as the source of primary osteoclast precursors (20). When nonadherent primary bone marrow cells were cultured for 4 days with or without recombinant RANKL, we observed numerous TRAP-positive multinucleated cells (mature osteoclasts) in the cultures treated with RANKL (Fig. 1A). By RT-PCR, we found that CX3CR1 was strongly expressed in untreated cells (osteoclast precursors) but not in those treated with RANKL (mature osteoclasts). We next investigated whether CX3CR1 expression of osteoclast precursors but not mature osteoclasts is unique to the nonadherent fraction of bone marrow cells. As shown in Fig. 1B, similar expression patterns were also observed in osteoclast precursors and mature osteoclasts derived from mouse splenocytes and mouse osteoclast precursor cell line RAW 264.7.

As another chemokine receptor (Fig. 1A), CCR2 was also selectively expressed in untreated cells, whereas CCR1 was strongly up-regulated in mature osteoclasts as described previously (21). Because suitable Abs against mouse CX3CR1 were not available, we performed immunohistochemical staining of CX3CR1 in human bone sections. As shown in Fig. 1C, we observed CX3CR1-positive cells in close colocalization with osteoblasts. These CX3CR1-positive cells could be regarded as immature osteoclasts and/or the aggregation state of osteoclast precursors from the morphologic features such as two or three nuclei, attachment to the bone, and a clear outline of the cell surface (Fig. 1Ca). Cathepsin K is a representative marker of mature osteoclasts and its expression gradually increases from immature to mature osteoclasts. These CX3CR1-positive cells were almost negative and/or slightly positive for cathepsin K (Fig. 1Cb). In contrast, cells existing in the bone matrix and showing morphologic features of mature osteoclasts, such as multinucleated giant cells with an unclear undulating cell surface, were found to be negative for CX3CR1 expression but not strong positive for cathepsin K (Fig. 1Cc and 1Cd). These results suggested that CX3CR1 was highly expressed in osteoclast precursors.

CX3CL1 directly mediates the migration and adhesion but not differentiation of osteoclast precursors

The possible expression of CX3CR1 by osteoclast precursors prompted us to examine their biological responses to CX3CL1. We first examined chemotactic responses to soluble CX3CL1 using TAXIScan, a real-time chemotaxis system (16). As shown in Fig. 2A, soluble CX3CL1 vigorously induced migration of cells in the nonadherent fraction of mouse bone marrow cells containing osteoclast precursors with a typical bell-shaped dose-response curve. Separately, we confirmed vigorous migration of cells of RAW 264.7, a mouse osteoclast precursor cell line, to soluble CX3CL1, using a conventional chemotaxis chamber assay (data not shown).
We next examined cell adhesion to immobilized CX3CL1. Osteoclast precursors (Fig. 2B) as well as RAW 264.7 cells (data not shown) efficiently adhered to immobilized CX3CL1. Furthermore, a rat anti-mouse CX3CL1 mAb, but not control rat IgG, effectively suppressed the adhesion of cells to immobilized CX3CL1. These results supported that CX3CR1-expressing osteoclast precursors were highly responsive to CX3CL1. In contrast, soluble CX3CL1 did not directly induce differentiation of osteoclast precursors (Fig. 3A), and also did not down-regulate its CX3CR1 expression in contrast with RANKL by RT-PCR (Figs. 1Ac and 3B).

Close contact between osteoblasts and osteoclast precursors via CX3CL1-CX3CR1 interaction on bone surface
The possible selective expression of CX3CR1 by osteoclast precursors prompted us to examine whether osteoblasts expressed its ligand CX3CL1. We isolated osteoblasts from dissected neonatal mouse calvariae and stained for CX3CL1. As shown in Fig. 4A, osteoblasts were indeed strongly positive for CX3CL1. Immunohistochemical analysis of human bone sections also confirmed CX3CL1 expression by the typical consecutive osteoblast regions on the bone surface (Fig. 4B). Furthermore, the juxtaposition of CX3CL1-positive osteoblasts and CX3CR1-positive monocytes/macrophage-like cells, which are thought to be osteoclast precursors, was observed more often by double staining. CX3CL1-positive osteoblasts were elongated, and their CX3CL1-positive osteoclast precursors closely contacted with the osteoblasts on the bone surface (Fig. 4C).

FIGURE 2. CX3CL1 induces chemotaxis and cell adhesion of osteoclast precursors. Because of the lack of suitable surface markers, we used the non-adherent fraction of fresh mouse bone marrow cells as the source of osteoclast precursors. A. Chemotaxis assay. Using a real-time chemotaxis assay apparatus TAXIscan, chemotactic responses to the gradients generated by mouse recombinant chemokine domain CX3CL1 (0~1000 nM) were recorded every 1 min. After 1 h, cells that had migrated toward the opposite side were counted and the chemotaxis index was calculated. B. Cell adhesion assay. Cells were added to wells precoated with CX3CL1. After 1 h, bound cells were counted. Rat anti-CX3CL1 mAb (clone 126315) and control rat IgG (Cont. IgG) were used at 10 μg/ml to ascertain the binding specificity. Data represents the mean ± SD, *p < 0.05. Representative results from experiments repeated at least three times are shown.

FIGURE 3. No direct effect of CX3CL1 stimulation on differentiation and CX3CR1 expression of osteoclast precursors in vitro. A. Osteoclast precursors derived from mouse bone marrow cells were cultured in the presence of recombinant RANKL (100 ng/ml; a) or recombinant CX3CL1 (10 nM; b) for 2 days and stained for TRAP. Asterisks indicate TRAP-positive osteoclasts. B. RT-PCR of osteoclast precursors cultured in the presence of recombinant CX3CL1 (10 nM) for 2 days was performed for CX3CR1 gene. GAPDH served as the loading control.

FIGURE 4. Close contacts with CX3CL1-positive osteoblasts and CX3CR1-positive osteoclast precursors in vivo. A. FACS analysis. Primary osteoblasts isolated from neonatal mouse calvariae were stained for surface CX3CL1. FACS profiles by control hamster IgG (shaded area) and hamster anti-CX3CL1 mAb (clone 5H8–4; blue line) are shown. B. Immunohistochemical analysis of human bone tissue. Osteoblasts colocalized with the bone are clearly positive for CX3CL1. Arrowheads indicate osteoblasts and asterisks indicate bone. Original magnification, ×200. Representative results from experiments repeated at least three times are shown. C. Close contact of CX3CL1-positive osteoblasts (white arrowheads) and CX3CR1-positive osteoclast precursors (black arrows) by double staining for CX3CL1 (brown) and CX3CR1 (blue). Asterisks indicate bone. Original magnification, ×1000.
Inhibition of osteoclast differentiation in vitro by anti-CX3CL1 mAb

The possible expression of CX3CR1 by osteoclast precursors and the corresponding expression of its ligand CX3CL1 by osteoblasts prompted us to examine the role of CX3CL1 in osteoclast differentiation. Mouse bone marrow nonadherent cells and primary osteoblasts were cocultured in the presence of vitamin D₃ for 6 days. Rat anti-CX3CL1 mAb (clone 126315) or control rat IgG was added to the culture medium at 20 μg/ml. Asterisks indicate TRAP-positive osteoclasts. B, Effect of anti-CX3CL1 mAb on the number of TRAP-positive osteoclasts. TRAP-positive multinuclear cells containing three or more nuclei were counted as mature osteoclasts. C, Effect of anti-CX3CL1 mAb on the surface area of TRAP-positive osteoclasts. Surface areas of TRAP-positive osteoclasts were analyzed by image analysis software (NIH Image). Data represents the mean ± SD. *, p < 0.05. Representative results from experiments repeated at least three times are shown.

Inhibition of bone resorption in vivo by anti-CX3CL1 mAb

To examine the role of CX3CL1 in osteoclast differentiation in vivo, we injected neonatal mice with the hamster anti-CX3CL1 mAb or control hamster IgG daily for 5 days and examined TRAP-positive osteoclasts in the osteogenic zone of left femur. We observed a slight decrease in the number of TRAP-positive cells (N.Oc/B.Pm) in mice treated with anti-CX3CL1 mAb compared with those treated with control IgG but the differences were not statistically significant (Fig. 6, A and C, and Table I). This could be due to numerous TRAP-positive cells in vivo such as immature osteoclasts and some monocytes. We therefore focused on TRAP-positive invading and penetrating cells in the bone pit that could be invaded by anti-CX3CL1 (Fig. 5C). These results are consistent with the notion that CX3CL1 is involved in the osteoblast-induced differentiation of osteoclasts.

Table I. Inhibitory effects of anti-CX3CL1 mAb on bone resorption in mice

<table>
<thead>
<tr>
<th></th>
<th>N.Oc/B.Pm (/100 mm)</th>
<th>ES/BS (%)</th>
<th>Oc.S/BS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control IgG</td>
<td>505.6 ± 66.0</td>
<td>10.5 ± 1.2</td>
<td>10.3 ± 0.4</td>
</tr>
<tr>
<td>CX3CL1 Ab</td>
<td>440.5 ± 36.2</td>
<td>6.4 ± 1.1*</td>
<td>5.4 ± 1.5*</td>
</tr>
</tbody>
</table>

Five hundred micrograms of anti-mouse CX3CL1 mAb or control IgG was injected into the peritoneal cavity of three mice for 5 days. The bone resorption index was evaluated according to the Nomenclature Committee of the American Society for Bone and Mineral Research’s rules. Data represent the mean ± SD. *, p < 0.05 vs control IgG. Data are representative of three independent experiments.
regarded as activated mature osteoclasts in the process of bone resorption. We observed dramatic decreases in the percentage of bone surface covered by osteoclasts (Oc.S/BS) as well as that of eroded surface (ES/BS) in mice treated with anti-CX3CL1 mAb (Fig. 6, B and D, and Table I). These results clearly demonstrate that anti-CX3CL1 is capable of inhibiting the formation of mature osteoclasts in vivo.

Discussion

In the present study, we have presented strong evidence that osteoclast precursors but not mature osteoclasts express CX3CR1 (Fig. 1), whereas osteoblasts express its ligand CX3CL1 in both mice and humans. In addition, close contact between these cells on the bone surface was revealed (Fig. 4). The results suggest that CX3CL1, which is a dual functional chemokine, on osteoblasts works as an adhesion molecule toward osteoclast precursors. Furthermore, blocking of the CX3CL1-CX3CR1 axis by anti-CX3CL1 mAb strongly inhibited osteoblast-induced differentiation of osteoclasts in vitro (Fig. 5) and the numbers of mature osteoclasts actively resorbing the bone in vivo (Fig. 6). In particular, the typical bone resorption parameters, Oc.S/BS (active osteoclasts which dissolve bone) and ES/BS (pits on bone surface formed by active osteoclasts), were markedly decreased by the treatment with anti-CX3CL1 (Table I). These results clearly demonstrate for the first time that the CX3CL1-CX3CR1 axis plays an important role in the osteoclast differentiation and bone resorption. Unfortunately, we were unable to test the effect of anti-CX3CR1 to directly prove the involvement of CX3CR1 in the osteoclast differentiation because of the lack of suitable neutralizing anti-mouse CX3CR1 Abs. Moreover, it would be interesting to study the bone tissues of CX3CR1- or CX3CL1-knockout mice, even though no gross bone abnormalities have been reported in these mice to date.

CX3CR1 is known to be expressed by NK cells, a subset of monocytes/macrophages, and mature mucosal dendritic cells. Therefore, it may be not so surprising that osteoclast precursors, which represent a cell lineage closely related to monocytes/macrophages (22), do express CX3CR1. However, it is rather striking that mature osteoclasts cease to express this receptor (Fig. 1). Therefore, the expression of CX3CR1 in the osteoclast lineage is highly regulated, suggesting its critical role at the stage of osteoclast precursors and immature osteoclasts. In contrast, CX3CL1 is known to be expressed by activated endothelial cells (23), mature dendritic cells (24), and intestinal epithelial cells (25). As shown in Fig. 4, CX3CR1-expressing monocyes/macrophage-like cells closely colocalize with CX3CL1-positive osteoclasts on the bone surface. Collectively, given that CX3CL1 is a dual function molecule, serving as a potent chemoattractant by its soluble form and an efficient adhesion molecule by its membrane-bound form (Fig. 2), the CX3CL1-CX3CR1 axis may be particularly suited for the initial attraction and the subsequent adhesion of osteoclast precursors by osteoblasts.

However, the possibility that CX3CL1-positive osteoblasts directly induce osteoclast maturation and CX3CR1 down-regulation in vivo is unclear in the present study, although CX3CL1 stimulation alone did not affect the differentiation and CX3CR1 mRNA expression of osteoclast precursors in vitro (Fig. 3). Therefore, further studies are needed to clarify the CX3CR1 expression and the osteoclast differentiation grade in the proximity of CX3CL1-producing cells using bone sections by multiple immunohistochemical staining of both CX3CR1 and cell differentiation molecules in osteoclast precursors and CX3CL1 in osteoblasts, especially with the difficulty of multiple staining against of the bone section.

Previously, several chemokines have been shown to promote osteoclast differentiation (5, 7, 26, 27). In particular, CCL2 is produced by osteoblasts upon stimulation with parathyroid hormone and attract osteoclast precursors via CCR2 (4). The expression of CCR2 by osteoclast precursors is further up-regulated by stimulation with RANKL (6). Furthermore, CCL2 and CCL5 can induce the formation of TRAP-positive multinuclear cells even in the absence of RANKL, although these cells are negative for bone resorption (5). Yang et al. reported that paracrine interaction of CCL9 and CCR1 in osteoclasts had a genuine physiologic role in osteoclast differentiation and that anti-CCL9 Ab inhibited osteoclast differentiation in rats (27). However, attention to the significant species differences in osteoclastogenic chemokines is needed to develop a new therapeutic approach by inhibition chemokines and their receptor interaction in bone diseases because of a lack of CCL9 in human.

From the findings of the present study, osteoblasts of both mice and humans, which constitutively express CX3CL1 at high levels (Fig. 4), may also use the soluble form of CX3CL1 to attract osteoclast precursors and the membrane-bound CX3CL1 to adhere with osteoclast precursors (Fig. 2). However, we observed no clear-cut reduction in the gross numbers of TRAP-positive cells in the bone of neonatal mice treated with anti-CX3CL1 (Fig. 6 and Table I). In contrast, mature bone-resorbing osteoclasts were clearly decreased by anti-CX3CL1. Even though the role of CX3CL1 as a chemoattractant for osteoclast precursors is not formally excluded, these results suggest that the initial attraction of osteoclast precursors by osteoblasts is less dependent on CX3CL1, and the CX3CL1-CX3CR1 axis is more involved in the later stages of osteoclast differentiation, i.e., after the initial interactions between osteoblasts with osteoclast precursors. Given that the membrane-bound form of CX3CL1 can induce firm adhesion of cells expressing CX3CR1 even without intracellular signaling, the CX3CL1-CX3CR1 axis may be primarily involved in the stabilization of adhesion between osteoblasts and osteoclast precursors that allows the close cell-cell interactions necessary for osteoclast differentiation. Another possibility may be that CX3CL1 is required for the survival of osteoclast precursors (28). Obviously, further studies are necessary to elucidate the exact role of the CX3CL1-CX3CR1 axis in the osteoclast differentiation and bone resorption.

Pathologic bone resorption is a serious problem in diseases such as rheumatoid arthritis (29), osteoporosis (30), and cancer bone metastases (31). Previously, one of the present authors demonstrated that anti-CX3CL1 mAb significantly lowers the clinical arthritis score and reduces synovial inflammatory cell infiltration in the mouse model of collagen-induced arthritis (15). Our present study has further shown that the treatment with anti-CX3CL1 Ab can reduce the bone resorption by mature, activated osteoclasts. Therefore, the blocking of the CX3CL1-CX3CR1 axis may be useful for the therapy of rheumatoid arthritis in two ways: suppression of cellular infiltrates in inflamed synovium (32) and reduction of bone resorption by mature osteoclasts in affected joints (33). Similarly, the blocking of the CX3CL1-CX3CR1 axis may be an attractive therapeutic strategy for other bone resorbing diseases such as osteoporosis (30) and cancer bone metastases (34), where reduction of mature osteoclasts would be beneficial. Further studies are necessary to test these possibilities.

Acknowledgments

We thank Kureha Special Laboratory (Tokyo, Japan) and Effector Cell Institute (Tokyo, Japan) for helpful advice and technical support.
Disclosures
The authors have no financial conflict of interest.

References