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ORIGINAL PAPER

RANKL-induced CCL22/macrophage-derived chemokine produced from osteoclasts potentially promotes the bone metastasis of lung cancer expressing its receptor CCR4

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Abstract Chemokines are now known to play an important role in cancer growth and metastasis. Here we report that differentiating osteoclasts constitutively produce CCL22 (also called macrophage-derived chemokine) and potentially promote bone metastasis of lung cancer expressing its receptor CCR4. We first examined expression of chemokines by differentiating osteoclasts. CCL22 was selectively upregulated in osteoclast-like cells derived from RAW264.7 cells and mouse bone marrow cells upon stimulation with RANKL (receptor activator of nuclear factor-κB ligand). In addition, a human lung cancer cell line SBC-5 that efficiently metastasized to bone when intravenously injected into NK cell-depleted SCID mice was found to express CCR4. Stimulation of SBC-5 cells with CCL22 induced cell migration and also enhanced phosphorylation of protein kinase B/Akt and extracellular signal-regulated kinase (ERK). Furthermore, immunohistochemical analysis of bone metastasis lesions demonstrated close co-localization of tartrate-resistant alkaline phosphatase (TRAP)-positive osteoclasts expressing CCL22 and SBC-5 cells expressing CCR4. Collectively, these results suggest that osteoclasts may promote bone metastasis of cancer cells expressing CCR4 in the bone marrow by producing its ligand CCL22.

Keywords Osteoclast differentiation · CCL22/MDC · CCR4 · Bone metastasis · Human lung cancer

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Introduction

Metastasis is a non-random process where various molecules are involved in leading cancer cells to target organs [1–3]. In particular, bone metastases are a frequent complication of cancers such as carcinoma of lung, breast and prostate. Bone metastasis lesions of cancers such as lung and breast cancers are predominantly osteolytic, while those of prostate cancer are predominantly osteoblastic [4–6]. Vicious cycles involving various soluble factors have been implicated in the formation of such bone lesions. For example, parathyroid hormone-related peptide (PTHrP) is a critical factor produced by various solid tumors to stimulates the formation of osteolytic lesions. On the other hand, various factors such as insulin-like growth factor and transforming growth factor β are factors involved in the formation of osteoblastic lesions.

It is now considered that chemokines play a significant role in organ-selective cancer metastasis [7]. Chemokines are a family of small cytokines that primarily induce directed migration of hematopoietic cells through interactions with a

group of seven transmembrane, G protein-coupled receptors [8]. Previously, CXCL12 (also called stromal cell-derived factor 1), which is selectively produced by osteoblasts and marrow endothelial cells [9], has been implicated in the bone metastasis of prostate cancer [10]. Given that bone lesions of lung cancer are mostly in a close association with osteoclasts [4–6], we hypothesized that osteoclasts produce some chemokines that may promote bone metastasis of lung cancer cells expressing the cognate chemokine receptors.

In the present study, we have shown for the first time that differentiating osteoclasts selectively upregulate CCL22 (also called macrophage-derived chemokine). Furthermore, a bone-metastasizing human lung cancer cell line SBC-5 expresses its receptor CCR4 and responds to CCL22 in cell migration and phosphorylation of protein kinase B/Akt and extracellular stress-regulated kinase (ERK). Consistently, we observed a close co-localization of tartrate-resistant alkaline phosphatase (TRAP)-positive osteoclasts expressing CCL22 and SBC-5 cells expressing CCR4 in bone metastasis lesions. Our findings thus imply a novel role of CCL22 in the physiological function of osteoclasts and also in bone metastasis of cancer cells expressing its receptor CCR4.

Material and methods

Reagents and antibodies

Rabbit anti-asialo GM1, recombinant human soluble RANK ligand (rhsRANKL) and recombinant mouse soluble RANK ligand (rmsRANKL) were purchased from Wako Pure Chem. Ind., Osaka, Japan. Human and mouse recombinant chemokines were purchased from R&D Systems, Inc., Minneapolis, USA (human CCL22/MDC) and Peprotech EC Ltd., London, UK (human and murine CCL22/MDC). Recombinant murine macrophage colony-stimulating factor (M-CSF) was purchased from Peprotech. Rat monoclonal anti-mouse CCL22/MDC antibody (clone 158113) and rat IgG2a isotype control (clone 54447) were purchased from R&D Systems. Antibodies used in immunoprecipitation assay and Western blotting were as follows. Rabbit anti-human CCR4 (CKR-4, H-48, sc-7936) was from Santa Cruz Biotechnology Inc., while rabbit immunoglobulin (code no. X0903), horseradish peroxidase (HRP)-conjugated rabbit immunoglobulin (code no. P0448) and HRP-conjugated mouse immunoglobulin (code no. P0260) were from DakoCytomation Denmark A/S, Glostrup, Denmark. Akt, phospho-Akt (Ser473), ERK1 and phospho-ERK antibodies were from Cell Signaling Technology Inc.

Mice

SPV/VAF mice (6–7-week old female), strain C.B-17/Icr Crj-scid, were purchased from Charles River Co., Yokohama, Japan. Severe combined immunodeficient (SCID) mice were fed freely with autoclaved water and food. All mice were kept in SPF and laminar air flow conditions at the Laboratory for Animal Experiments, Institute of Natural Medicine, University of Toyama. This study was conducted in accordance with the guidelines for the Care and Use of Laboratory Animals of University of Toyama.

Cell culture

A human lung cancer line SBC-5 was maintained in monolayer culture in MEM (Invitrogen Corp., NY, USA) supplemented with 10% fetal bovine serum (FBS) (ICN Biomedicals Inc., Ohio, USA). A murine macrophage-like cell line RAW264.7 was cultured in alpha-MEM (ICN Biomedicals) supplemented with 10% FBS, 0.01% Penicillin G, 0.006% Streptomycin and 0.225% Sodium bicarbonate. Primary culture of bone marrow cells was performed as described previously [11]. In brief, mice were sacrificed by cervical dislocation. Femora and tibiae were taken out and kept in a 6-cm culture dish containing alpha-MEM supplemented with 10% FBS, while the remaining bones were prepared. Under sterile conditions, bone marrow cells were flushed out with FBS-free medium. Cells were collected in a 50-ml Falcon tube and filled up to 50 ml with FBS-free medium. Cell suspension was vigorously pipeted (approx. 30 times) and centrifuged at 2000 rpm/10 min. Cells were resuspended in alpha-MEM supplemented with 10% FBS. To determine total cell number, cells were diluted in 2% acetic acid and counted in a hemacytometer chamber.

RANKL-induced formation of osteoclast-like cells

RAW264.7 cells (1×10^4/ml) or primary bone marrow cells (1×10^6/ml) in alpha-MEM supplemented with 10% FBS were cultured in a 24-well culture plate (Corning Inc., NY, USA) (500 µl/well). At 24 h for RAW264.7 cells and at 48 h for primary bone marrow cells, the culture medium was replaced with fresh culture medium with or without rhsRANKL (100 ng/ml). For RAW264.7 cells, fresh culture medium with or without rhsRANKL was re-supplied on day 3 or 4. On day 6–8, conditioned media were collected for analysis with ELISA and adherent cells were stained for TRAP.

TRAP-staining assay

Adherent cells were fixed with 10% formalin in a buffer solution (pH 7.2) containing 37 mM sodium dihydrogenphosphate and 180 mM disodium hydrogenphosphate for 10 min. Cells were dehydrated with ethanol–acetone (1:1) for 1 min, dried and stained at room temperature with tartrate-resistant acid phosphatase (TRAP) staining solution filtered through a paper filter. TRAP staining solution was prepared according to a previous report [12] with slight modifications. In brief, acetate buffer (0.1 M sodium acetate, adjusted to pH 5.0 with 0.1 M acetic acid) was added with 10 mM sodium tartrate (Nacalai Tesque Inc., Kyoto, Japan), 0.008% (w/v) of naphthol AS-MX phosphate (ICN Biomedicals), 0.4% (v/v) of *N*,*N*-dimethylformamide (Wako Pure Chem. Ind., Osaka, Japan) and 0.07% (w/v) of Fast Red Violet LB salt (Sigma-Aldrich Co., MO, USA). TRAP-positive cells stained as dark red multinucleated cells were then counted. In each well, 4 randomly chosen fields were photographed with a digital camera and printed out. The sum of cells in the 4 fields was obtained and average was calculated from 3 to 4 wells.

ELISA

Conditioned media of cells stimulated with or without RANKL were collected in 1.5 ml-Eppendorf tubes, centrifuged at 2000 rpm/5 min to remove cell debris and kept at −30°C until assay. CCL22 was measured with mouse MDC/CCL22 DuoSet ELISA Development kit (R&D Systems). Assays were done in triplicate and optical density at 450 nm was read on Wako/Tecan immunoplate reader (Osaka, Japan).

Bone metastasis model

A bone metastasis model using SBC-5 was done essentially as described previously [13]. In brief, SCID mice were intraperitoneally injected with anti-asialo GM1 (200 μg/200 μl PBS/mouse) to deplete NK cells. On day 3, SBC-5 cells were intravenously injected into mice (1×10^6 cells in 200 μl PBS/mouse). On day 10, mice were injected with anti-asialo GM1 again. The animals were daily monitored and, from the 4th to 9th week after inoculation of tumor cells.

Cell migration assay

This was done using Transwell plates (8.0-μm pore size, Nucleopore, Pleasanton, CA, USA) essentially as described previously. Briefly, filters were precoated with 2 μg/50 μl/filter of fibronectin (Iwaki Glass, Tokyo, Japan) on their lower surface, dried at room temperature, washed in phosphate-buffered saline (PBS) and dried until use. Exponentially growing SBC-5 cells were harvested using 1 mM EDTA in PBS, washed twice with FCS-free medium and resuspended in 0.1% BSA medium at a density of 1×10^6 cells/ml. One-hundred-microliter aliquots of cell suspension were added to the upper compartment, while the lower compartments contained 600 μl medium with or without test samples. After incubation at 37°C in a 5% CO_2/95% air atmosphere for 24 h, cells migrated to the lower surface were stained with hematoxylin for 4 min, washed in water and stained again with eosin for 1 min. For each filter, cells in 5 randomly chosen fields were counted and averages were calculated. The final averages were calculated from the averages of 3 filters.

Reverse transcription-polymerase chain reaction (RT-PCR)

This was performed as described previously [14]. Briefly, total RNA was extracted from cultured cells using Trizol reagent (Invitrogen Corp., CA, USA). First strand complementary DNA (cDNA) was prepared from RNA template (1 μg) using oligo$(dT)_{18}$ 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 indicated temperatures (Table 1) for 1 min, and extension at 72°C for 105 s, using template cDNA and TaKara Ex TaqTM HS PCR kit (TaKaRa Shuzo Co., Ltd., Otsu, Japan). The primer sequences are listed in Table 1. All primers were verified to yield the expected products under the indicated conditions. PCR products were electrophoresed on 1.7% agarose gels and stained with ethidium bromide.

Protein immunoprecipitation

The procedure for immunoprecipitation of CCR4 was modified from that of a previous report [15]. PBMCs or SBC-5 cells were washed twice in cold Tris-buffered saline. Cells were lysed by lysis buffer containing 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM NaF, 0.1% SDS, 0.1% Nonidet P-40, 0.05% sodium deoxycholate, 10% glycerol, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of aprotinin and 10 μg/ml of leupeptin). After centrifugation, cell lysates were incubated on ice for 4 h with 10 μg/ml of anti-human CCR4, and then concentrated overnight at 4°C by addition of 60 μl of Protein G-Sepharose (Amersham Biosciences, Uppsala, Sweden). The beads were washed three times with wash buffer containing 10 mM Tris–HCl (pH 7.5), 0.1% Nonidet P-40, 0.05% sodium deoxycholate, 10 μg/ml of aprotinin and

Table 1 Primer sequences and conditions used in RT-PCR analysis of mouse chemokines and human chemokine receptor, CCR4

Targets		Sequences of primers	Annealing temperature (°C)	Product length (bp)
Mouse chemokines				
CCL1/I-309	Sense	5′-CCGTGTGGATACAGGATGTTG-3′	62	133
	Antisense	5′-TCAGGACAGGAGGAGCCC-3′		
CCL3/MIP-1 alpha	Sense	5′-ACCACTGCCCTTGCTGTTC-3′	62	202
	Antisense	5′-TCTGCCGGTTTCTCTTAGTCAG-3′		
CCL4/MIP-1 beta	Sense	5′-CTCTCCTCTTGCTCGTGGC-3′	62	229
	Antisense	5′-GTACTCAGTGACCCAGGGCTC-3′		
CCL5/RANTES	Sense	5′-GCTGCCCTCACCATCATCC-3′	60	237
	Antisense	5′-GTATTCTTGAACCCACTTCTTCTCTG-3′		
CCL7/MCP-3	Sense	5′-GCTCATAGCCGCTGCTTTC-3′	60	257
	Antisense	5′-GCTTTGGAGTTGGGGTTTTC-3′		
CCL8/MCP-2	Sense	5′-CCAGATAAGGCTCCAGTCACC-3′	60	145
	Antisense	5′-AGAGAGACATACCCTGCTTGGTC-3′		
CCL9/MIP-1 gamma	Sense	5′-CAACAGAGACAAAAGAAGTCCAGAG-3′	62	195
	Antisense	5′-CTTGCTGATAAAGATGATGCCC-3′		
CCL17/TARC	Sense	5′-CTGCTCTGCTTCTGGGGAC-3′	62	211
	Antisense	5′-TGTTTGTCTTTGGGGTCTGC-3′		
CCL20/LARC	Sense	5′-TGCTCTTCCTTGCTTTGGCATGGGTA-3′	62	365
	Antisense	5′-TCTGTGCAGTGATGTGCAGGTGAAGC-3′		
CCL22/MDC	Sense	5′-GGTCCCTATGGTGCCAATG-3′	62	133
	Antisense	5′-TTATCA AAACAACGCCAGGC-3′		
CXCL12/SDF-1	Sense	5′-TAAACCAGTCAGCCTGAGC-3′	62	204
	Antisense	5′-TTGTTTAAAGCTTTCTCCAGG-3′		
Trap	Sense	5′-TCCCCTGGTATGTGCTGG-3′	62	220
	Antisense	5′-GCATTTTGGGCTGCTGA-3′		
GAPDH	Sense	5′-GGTGAAGGTCGGTGTGAACGGATTT-3′	60	478
	Antisense	5′-AATGCCAAAGTTGTCATGGATGACC-3′		
Human				
CCR4	Sense	5′-AAGAAGAACAAGGCGGTGAAGATG-3′	62	269
	Antisense	5′-AGGCCCCTGCAGGTTTTGAAG-3′		
GAPDH	Sense	5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′	60	982
	Antisense	5′-CATGTGGGCCATGAGGTCCACCAC-3′		

10 μg/ml of leupeptin. The immunoprecipitates were eluted by addition of 50 μl of SDS-PAGE sample buffer.

Western blotting

Aliquots of PBMCs or SBC-5 cell lysates, obtained as described above, were resolved by SDS-PAGE and transferred to Immobilon-P nylon membrane (Millipore, Bedford, MA, USA), The membrane was treated with BlockAce (Dainippon Pharmaceutical Co. Ltd., Suita, Japan) for 1 h and probed with primary antibodies (anti-human CCR4, 1:400 dilution; anti-PCNA, 1:1000 dilution) for 2 h. The antibodies were detected using HRP-conjugated anti-rabbit and anti-mouse IgG, respectively (1:1000 dilution) and visualized with the ECL system (Amersham Biosciences). For detection of Akt, ERK and their phosphorylated forms, SBC-5 cells were seeded at 3×10^5 on 35-mm dishes. After 24-h incubation cells were rinsed twice with FCS-free medium and then stimulated with recombinant human CCL22 at the indicated concentrations for 30 min. Cell lysates were obtained as described above. Membranes were probed with primary antibodies (anti-Akt, anti-phospho Akt, anti-ERK1, anti-phospho ERK; 1:500 dilutions) for 2 h. The antibodies were detected using HRP-conjugated anti-rabbit IgG (1:1000 dilution) and visualized with the ECL system (Amersham Biosciences).

Immunohistochemistry

Tissue sections of mouse normal bone or metastatic bone lesions were made from formalin-fixed and glycolmethacrylate (GMA)-embedded tissue blocks, and were stained with tartrate-resistant acid phosphatase (TRAP)-staining solution, as described above, for 30 min at 37°C. TRAP-positive cells (osteoclasts) stained as dark red cells. After TRAP staining, endogenous peroxidase was quenched by pretreating the tissue sections in a water bath in target retrieval solution (DakoCytomation) for 40 min at 95°C and peroxidase blocking reagent (DakoCytomation) for 15 min at room temperature. To prevent non-specific binding, sections were treated with 50% BlockAce containing 0.5% anti-mouse CD16/CD32 (Fcγ III/II receptor, clone 2.4G2) (BD Biosciences Pharmingen) and 5% normal rabbit serum or normal goat serum (DakoCytomation) for 15 min at room temperature. Tissue sections were then incubated at 4°C overnight with goat anti-mouse CCL22 antibody

(MDC, M-15) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Isotype-matched immunoglobulins obtained from DakoCytomation were used as negative controls.

Statistical analysis

The significance of differences between groups was determined by applying Student's two-tailed *t*-test. A *P*-value lower than 0.05 was considered to be significant.

Results

RANKLl-induced osteoclast differentiation selectively upregulate CCL22MDC expression

We first examined expression of chemokines by osteoclast-like cells that were generated in vitro from a murine macrophage-like cell line (RAW264.7) and primary cultures of murine bone marrow cells upon stimulation with RANKL. As shown in Fig. 1A, RANKL-stimulated RAW264.7 cells progressively differentiated into TRAP-positive osteoclast-like multinucleated giant cells. Therefore, we examined expression of chemokines in RAW264.7 cells stimulated with or without RANKL by using RT-PCR. The results are shown in Fig. 1B. First, RAW264.7 cells or those treated with RANKL hardly expressed CCL8 (also called monocyte chemotactic protein-2), CCL17 (also called thymus and activation-regulated chemokine), CCL20 (also called liver and activation-regulated chemokine) or CXCL12. Second, RAW264.7 cells constitutively expressed CCL1 (also called I-309), CCL3 (also called macrophage inflammatory protein-1α), CCL4 (also called macrophage inflammatory protein-1β), CCL5 (also called regulated upon stimulation, normal T cell expressed and secreted), CCL7 (also called monocyte chemotactic

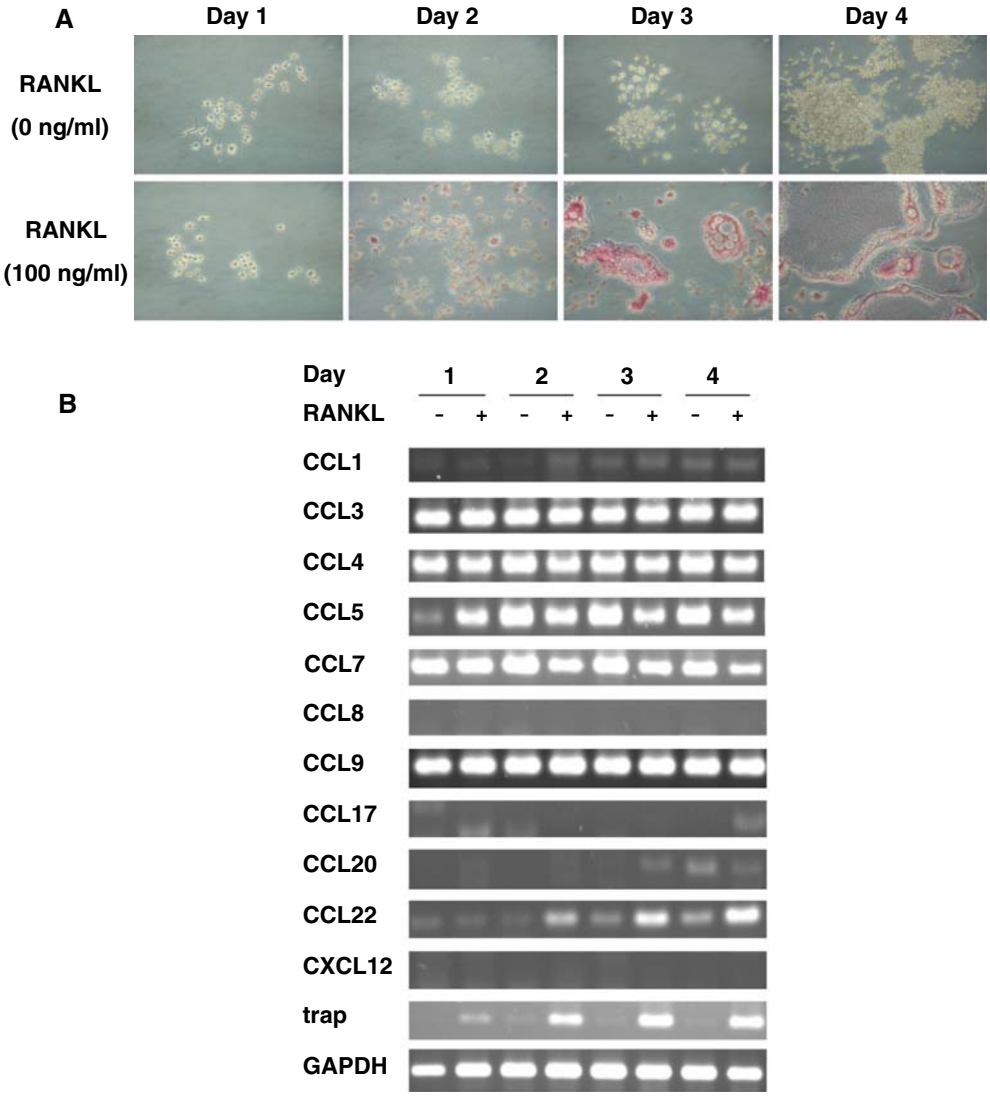

Fig. 1 RANKL-induced differentiation of RAW264.7 cells into osteoclast-like cells and enhanced expression of CCL22. RAW264.7 cells were stimulated with or without recombinant soluble RANKL (100 ng/ml) for 1–4 days. Cell differentiation into osteoclast-like cells was analyzed by a TRAP staining assay (**A**). Cells were collected on each day during stimulation. Their total RNA was extracted and converted to cDNA, which was used in RT-PCR analysis of various chemokine ligands (35 amplification cycles), TRAP and GAPDH (24 amplification cycles) (**B**)

protein-3) and CCL9 (also called macrophage inflammatory protein-1γ) with little further changes upon stimulation with RANKL. Third, RAW264.7 cells hardly expressed CCL22 but progressively upregulated its expression upon stimulation with RANKL. Accordingly, RANKL-stimulated but not control RAW264.7 released CCL22 into culture medium (Table 2). We also confirmed that RANKL-stimulated primary bone marrow cells derived from SCID mice progressively generated TRAP-positive osteoclast-like cells and produced an elevated amount of CCL22 (Table 2).

A human lung cancer cell line SBC-5 metastasizes to bone in NK-cell depleted SCID mice

We next examined bone metastasis of human lung cancer cell SBC-5 in NK-cell-depleted SCID mice. The hind legs were most frequently involved. The bone lesions were also typically osteolytic with tumor cells in close association with osteoclasts (Fig. 5).

SBC-5 cells express CCR4 and respond to CCL22

Given that expression of CCL22 was selectively upregulated upon osteoclast differentiation, we examined whether the expression of CCR4 in SBC-5 cells. RT-PCR analysis indeed detected CCR4 expression in SBC-5 cells (data not shown), and immunoblotting experiments using anti-CCR4 revealed a band of about 37-kDa in the SBC-5 cell lysate (Fig. 2A). Anti-CCR4 but not control IgG precipitated a band of the same size from the SBC-5 cell lysate (Fig. 2B). We subsequently examined in vitro migratory responses of SBC-5 cells to CCL22. The migratory responses of SBC-5 cells were only seen when lower surface of filters were coated with fibronectin. However, also highly enhanced their spontaneous migration which is referred as haptotactic migration. The representative results are shown in Fig. 3A. CCL22 significantly enhanced migration of SBC-5 cells, in spite of high backgrounds. Moreover, the conditioned media of RANKL-stimulated bone marrow cells from SCID mice induced migration of SBC-5 cells more efficiently than those of control bone marrow cells, and anti-CCL22 but not control IgG effectively abrogated the migration-stimulatory activity of the conditioned media from RANKL-stimulated bone marrow cells (Fig. 3B).

We also examined activation of signaling pathways of survival and/or proliferation in SBC-5 cells by CCL22. As shown in Fig. 4, phosphorylation of Akt as well as ERK was enhanced by CCL22 in a concentration-dependent manner.

Table 2 Differentiation of osteoclast-like cells induced by RANKL and release of CCL22

Precursor cell	Number of TRAP (+) cells		Release of CCL22 (pg/ml)	
	RANKL (ng/ml)		RANKL (ng/ml)	
	0	100	0	100
Murine macrophage RAW 264.7 cells	0	70.3 ±15.0	5.8 ± 1.9	132.2 ± 40.4[a]
Bone marrow cells	0	62.2 ±20.1	45.2 ±0.4	151.3 ±2.1[a]

[a] $P < 0.01$ in relation to control

Juxtaposition of osteoclasts producing CCL22 and SBC-5 cells in bone metastasis

After intravenous injection of SBC-5 cells into NK-cell depleted SCID mice, metastasis bone lesions with intense osteolysis were observed (Fig. 5A). In tumor-metastasized bone sections, TRAP-positive osteoclasts were elongated and stood in a layer on the boundary between the bone and tumor masses. In addition, CCL22 was produced by TRAP-positive osteoclasts located around tumor masses and resorbed bone (Fig. 5B, C). Using RT-PCR, SBC-5 cells which metastasized to the bone expressed CCR4 mRNA just as cultured SBC-5 cells did. On the other hand, SBC-5 cells mass of bone did not expressed CCL22 mRNA (data not shown).

Discussion

In this study, we first demonstrated that differentiating osteoclasts selectively upregulate production of a chemokine CCL22. This was shown by RANKL-induced osteoclast-like cells differentiated from RAW264.7 cells and from mouse primary bone marrow cells.

Previously, CCL9/MIP-1γ was shown to be expressed by osteoclasts [16] and was suggested to have a role in the formation and survival of osteoclasts via an autocrine pathway [17]. We examined CCL9 expression in RAW264.7 cells with or without RANKL-stimulation (Fig. 1B), but found no difference in its expression level between undifferentiated and differentiated cells. RT-PCR as well as ELISA showed that there was no difference in CCL9 expression between non-stimulated and RANKL-stimulated bone marrow cells (data not shown). Thus, CCL22 appears to be a unique chemokine that is selectively upregulated upon osteoclast differentiation, possibly playing an important role in osteoclast function. It does not

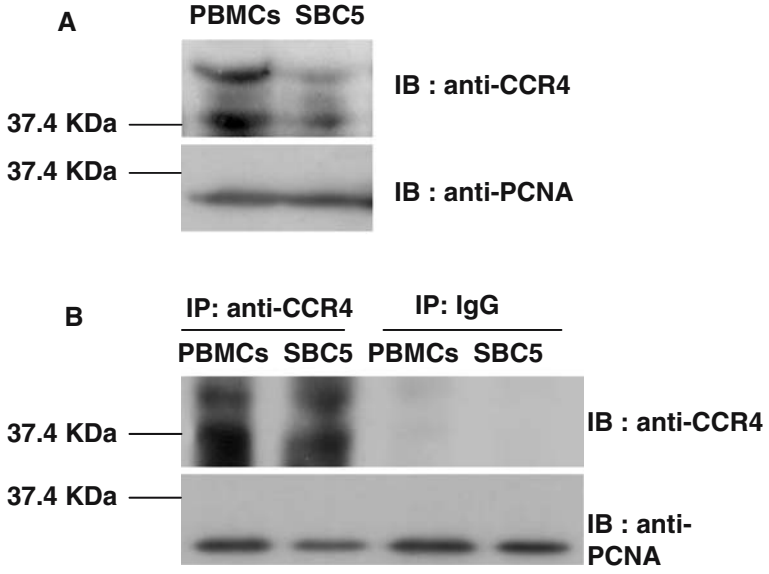

Fig. 2 Expression of CCR4 in SBC-5 cells. (**A**) Cell lysates of human peripheral blood mononuclear cells (PBMCs) and SBC-5 cells were resolved by 12.5% SDS-PAGE, electrophoretically transferred to filters, and immunoblotted with anti-human CCR4 or anti-PCNA. (**B**) Aliquots of the cell lysates shown in panel A were immunoprecipitated with anti-human CCR4 or control IgG, resolved by 12.5% SDS-PAGE, electrophoretically transferred to filters, and immunoblotted with anti-human CCR4 or anti-PCNA. IB, immunoblotting; IP, immunoprecipitation

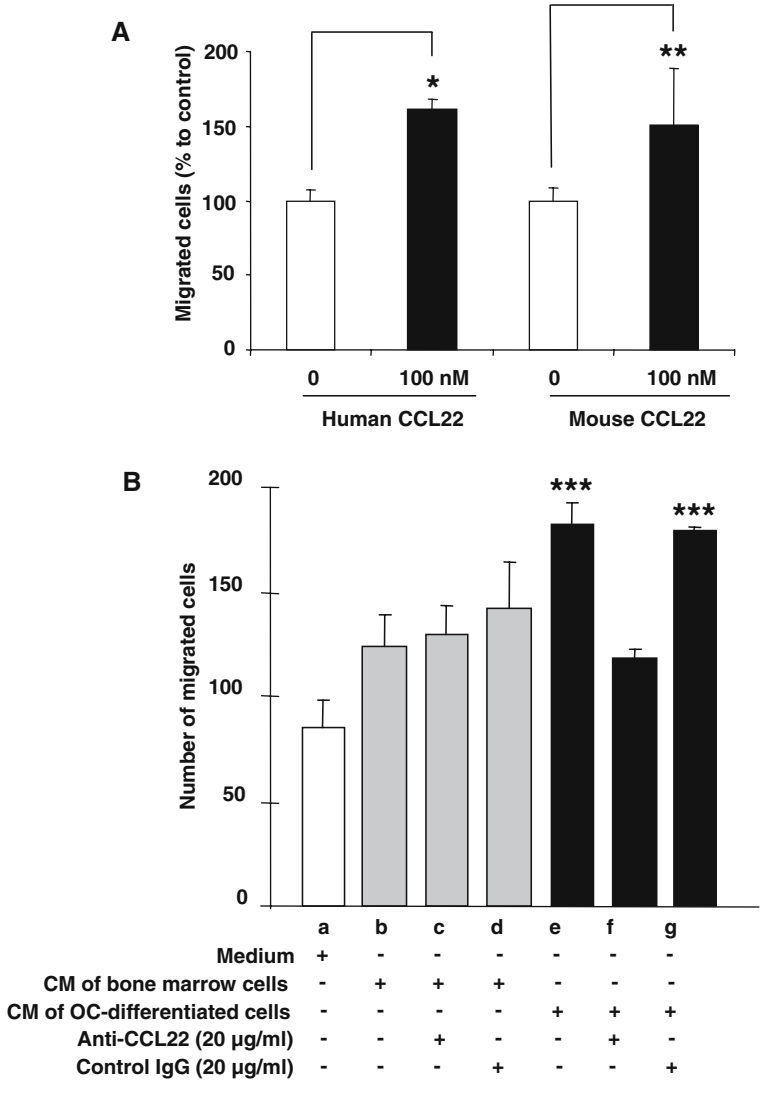

Fig. 3 Enhanced migration of SBC-5 cells by recombinant CCL22 and CCL22 released by bone marrow cells that differentiated into osteoclast-like cells upon RANKL stimulation. (**A**) Recombinant CCL22 promoted migration of SBC-5 cells, as determined by migration through Transwell chambers. (**B**) Migration of SBC-5 cells was examined in the presence of conditioned medium of undifferentiated bone marrow cells and OC-differentiated cells. $*P < 0.005$; $**P < 0.05$; $***P < 0.05$ in relation to bars a, b, c and f, respectively. CM, conditioned medium

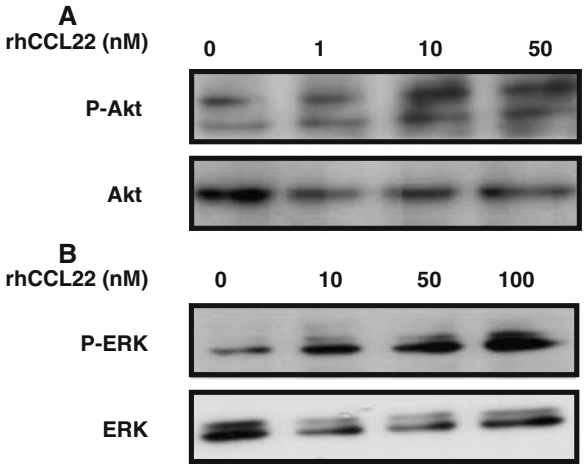

Fig. 4 Phosphorylation of Akt and ERK in SBC-5 cells stimulated with CCL22. (**A**) SBC-5 cells were stimulated with CCL22 (0, 1, 10, and 50 nM) and, at 30 min, cell lysates were prepared. Immunoblotting analysis was performed using anti-phospho Akt and anti-Akt antibody. (**B**) SBC-5 cells were stimulated with CCL22 (0, 10, 50, and 100 nM) and, at 30 min, cell lysates were prepared. Immunoblotting analysis was performed using anti-phospho ERK and anti-ERK antibody

exclude the evident importance of other chemokines such as CCL9 in the same function.

CCL22 is known to be mainly produced by macrophages and mature dendritic cells [18]. Thus, it is likely that osteoclasts, which also represent a cell lineage closely related to these types of cells [4–6], produce CCL22.

Given that osteoclasts are postivley related to the osteolytic bone metastasis, we next asked whether CCL22 might play a role in bone metastasis of cancer. The bone lesions were predominantly osteolytic. Importantly, SBC-5 cells produced PTHrP, a factor closely associated with osteolytic bone metastasis [6], at high levels (data not shown), and also expressed CCR4 (Fig. 2). SBC-5 cells also responded to CCL22 in cell migration (Fig. 3) and phosphorylation of Akt and ERK (Fig. 4). It is known that activation of Akt and ERK play important roles in survival and proliferation of various cells. Activation of Akt led to the promotion of survival and prevention of apoptosis of glioma cells [19, 20]. Phosphorylation of Akt suppressed various types of apoptotic molecules, including caspase-9, -3 [21, 22]. CXCL12-dependent proliferation has been shown to correlate to the phosphorylation and activation of ERK of ovarian cancer cell [23]. On the other hand, SBC-5 cells did not respond to CCL1, CCL3 as well as CXCL12 in migration assays (data not shown). As shown in Fig. 5, CCR4-expressing SBC-5 cells closely co-localized with TRAP-positive osteoclasts producing CCL22 in the bone metastasis lesions. Collectively, these results suggest that CCL22 has a potential role in bone metastasis of cancer cells expressing CCR4.

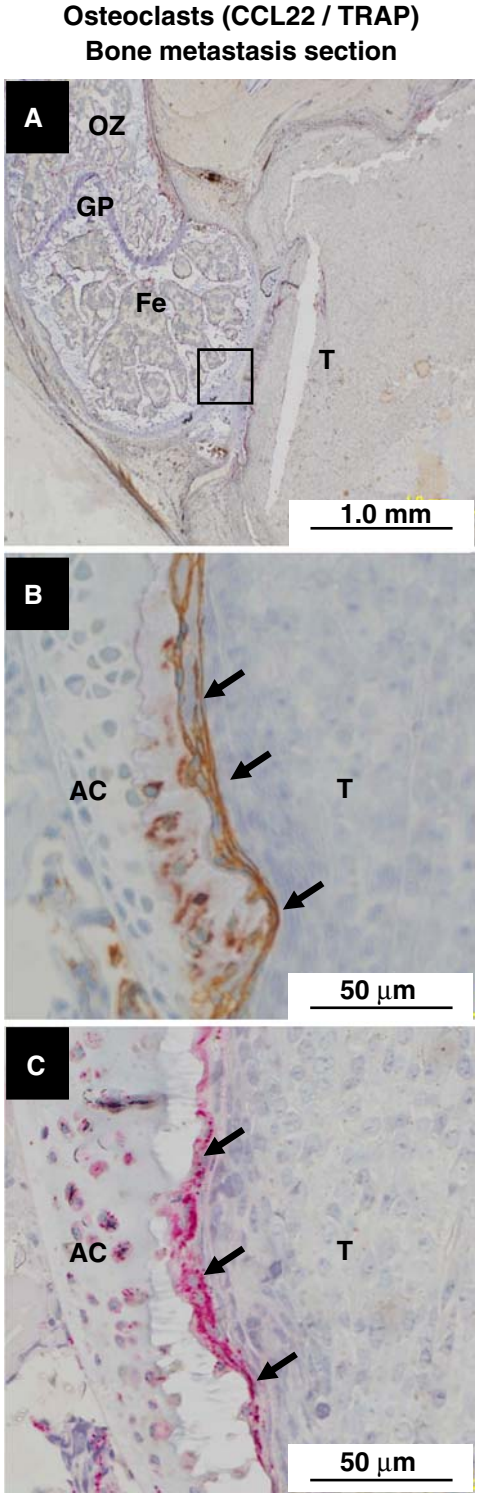

Fig. 5 Immunohistochemical analysis of bone sections of SCID mice. Bone sections of mice that developed bone metastases (**A**, **B**) were double stained for TRAP (red) and CCL22 (brown), and then counterstained with hematoxylin. This staining procedure was also done in panels C, but with omission of anti-mouse CCL22 antibody. Panels B, C, show the areas in square in panels A magnified. Magnifications, ×20 (**A**), ×200 (**B**, **C**) Arrows indicate osteoclasts. Fe, Femur; T, tumor; OZ, osteogenic zone; GP, growth plate; AC, articular cartilage

CXCL12 has been shown to participate in cancer metastases including bone metastasis [7, 9, 10, 24–28]. In the present study, however, CXCL12 did not appear to play an important role in cancer cell migration. RAW264.7 cells or primary bone marrow cells did not express CXCL12 nor were induced to express it upon RANKL-induced differentiation to osteoclasts (Fig. 1B). In addition, recombinant CXCL12 did not induce migration of SBC-5 cells.

How could CCL22 produced by osteoclasts play a role in the bone metastasis of cancer? The fact that SBC-5 produced PTHrP at high levels (data not shown) and indicates it is critical for the cell ability to metastasize into the bone. First, circulating CCR4-positive tumor cells are arrested in the bone (possibly by CCL22 produced by osteoclasts). Second, PTHrP produced by cancer cells activates osteoblasts and induce their expression of RANKL. Third, RANKL promotes osteoclast differentiation and production of CCL22. Fourth, CCL22 further promotes localization and survival of CCR4-expressing tumor cells in the bone marrow possibly through the activation of Akt and ERK (Fig. 4). Additionally, the elevation of tumor cell motility by osteoclast derived-CCL22 would be advantageous for the formation of bone metastasis because tumor cells could quickly move into the spaces of bone resorption generated by abnormally activated osteoclasts. Future study employing the techniques such as introducing siRNA for CCR4 in SBC-5 might be useful to prove our hypothesis, including CCR4 between primary and bone metastasis lesions of human cancers.

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