Role of chemokine CX3CL1 in progression of multiple myeloma via CX3CR1 in bone microenvironments

AKINORI WADA1,2*, AYA ITO2*, HIROFUMI IITSUKA2, KOICHI TSUNEYAMA3, TAKAYOSHI MIYAZONO1, JUN MURAKAMI1, NAOTOSHI SHIBAHARA2, HIROAKI SAKURAI3, IKUO SAIKI4, TAKASHI NAKAYAMA5, OSAMU YOSHIE6, KEIICHI KOIZUMI2 and TOSHIRO SUGIYAMA1

1Department of Gastroenterology and Hematology, Graduate School of Medicine and Pharmaceutical Science; 2Division of Kampo Diagnostics, Institute of Natural Medicine; 3Department of Cancer Cell Biology, Graduate School of Medicine and Pharmaceutical Sciences; 4Division of Pathogenic Biochemistry, Institute of Natural Medicine, University of Toyama, Toyama; 5Division of Chemotherapy, Kinki University School of Pharmaceutical Sciences; 6Department of Microbiology, Kinki University Faculty of Medicine, Osaka, Japan

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Correspondence to: Dr Keiichi Koizumi, Division of Kampo Diagnostics, Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan
E-mail: kkoizumi@inm.u-toyama.ac.jp

*Contributed equally

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Abstract. Several chemokines/chemokine receptors such as CXCL12, CCL3, CXCR4 and CCR1 attract multiple myelomas to specific microenvironments. In the present study, we investigated whether the CX3CL1/CX3CR1 axis is involved in the interaction of the multiple myeloma cells with their microenvironment. The expression of CX3CR1 (also known as fractalkine) was detected in three of the seven human myeloma cell lines. CX3CL1-induced phosphorylation of Akt and ERK1/2 was detected in the CX3CR1-positive cell lines, but not in the CX3CR1-negative cell lines. In addition, CX3CL1-induced cell adhesion to fibronectin and vascular cell adhesion molecule-1 (VCAM-1) in the human myeloma RPMI-8226 cell line. We also investigated whether a relationship existed between myeloma cells and osteoclasts that may function via the CX3CL1/CX3CR1 axis. Conditioned medium from CX3CL1-stimulated RPMI-8226 cells drastically increased the osteoclast differentiation. Collectively, the results from the present study support the concept of the CX3CL1-mediated activation of the progression of the multiple myeloma via CX3CR1. Thus, CX3CR1 may represent a potential therapeutic target for the treatment of multiple myeloma in a bone microenvironment.

Introduction

The multiple myeloma cell is a neoplastic plasma-cell disorder that is characterized by clonal proliferation of malignant plasma cells in the bone marrow microenvironment. This disorder causes monoclonal protein proliferation in the blood or urine and it is associated with organ dysfunction (1). It accounts for ~1% of neoplastic diseases and ~10% of hematologic cancers. The median age of diagnosis is ~70 years (2). The recent introduction of autologous stem-cell transplantation and the availability of agents such as thalidomide, lenalidomide and bortezomib have changed the management of myeloma (3,4). Although overall survival has increased, the disease is not curable.

Chemokines are low molecular weight cytokines that are specialized for recruiting leukocyte to inflammatory sites and for correctly positioning lymphocytes in secondary lymphoid organs (5). Chemokines are also involved in different pathological processes including the growth and dissemination of solid tumors and hematological malignancies (6,7). For example, several chemokines/chemokine receptors are associated with multiple myeloma activity. The migration of myeloma cells to the bone marrows is mediated by CXCR4, which is highly expressed in myeloma cells, and by its ligand CXCL12 which is produced by stromal cells (8). CCR1, the receptor for CCL3/MIP-1α, is involved in osteolytic bone diseases and is highly expressed in myeloma patients (9).

CX3CL1 (also known as fractalkine) is a chemokine constitutively expressed in many hematopoietic and non-hematopoietic tissues. It is synthesized as a membrane-bound protein, but can also be released by proteolytic cleavage (10,11). Membrane-bound CX3CL1 functions as an adhesion molecule, whereas the secreted form triggers chemotaxis of lymphocytes and monocytes to inflammatory sites (10,12). The receptor for CX3CL1, CX3C chemokine receptor 1 (CX3CR1), is expressed on human NK cells, monocytes, T lymphocytes and mast cells (10,13).
Previous studies have shown that CX3CR1 expression is upregulated in solid tumors such as in breasts or prostate (14,15), while pancreatic adenocarcinoma models (16) have shown that CX3CR1 is involved in the metastatic spread of tumor cells to specific tissues expressing CX3CL1. Several studies of CX3CR1 have been performed with different types of B cell lymphoma (17) and chronic lymphocytic leukemia (CLL) (18) in hematological malignancies. However, the expression of CX3CR1 has not been investigated in multiple myeloma, since CX3CR1 expression has not been confirmed in B cell lineages from pro B cells to plasma cells (19). Therefore, the functional role of CX3CR1 in multiple myeloma remains unclear. The present study, which was an investigation of the role of the CX3CL1/CX3CR1 axis in multiple myeloma, indicates that this axis is involved in the interaction between the tumor cells and their bone microenvironment.

Materials and methods

Reagents and antibodies. Recombinant human CX3CL1 and anti-human CX3CL1 were purchased from R&D Systems (Minneapolis, MN, USA). The following mAbs were used: anti-AKT1 (C-20), anti-ERK1/2 (C-16), anti-PCNA (PC-10) (Minneapolis, MN, USA). The following mAbs were used: anti-human CX3CL1 were purchased from R&D Systems Ltd., Tokyo, Japan) and converted to cdNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies Corporation, Carlsbad, CA, USA). The expres-

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Cells. Human multiple myeloma cell lines included RPMI-8226, KMS-12BM, KMS-12PE, L-363, OPM-2, KARPAS-620 and AMO-1 cells. All the cell lines were maintained in RPMI-1640 medium (Wako Pure Chemical Industries, Inc., Osaka, Japan) supplemented with 20% fetal bovine serum, 50 µM 2-mercaptoethanol (both from Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (both from Meiji Seika Pharma, Tokyo, Japan). The cells were cultured at 37°C in an incubator with a humidified 5% CO₂ atmosphere.

Reverse transcription-polymerase chain reaction (RT-PCR). RNA was extracted using an RNasy Plus Mini kit (Qiagen, Hilden, Germany) and converted to cdNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies Corporation, Carlsbad, CA, USA). The expres-

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RT-PCR. The western blot analysis was performed following incubation with or without 10 nM rCX3CL1. Antibodies to phosphorylated (p) or unphosphorylated JAK, Stat, Akt and Erk1/2 were used. Images of immunoblots were scanned and quantified with an ImageQuant LAS 4000 Lumino Image Analyzer from Fuji Film Corporation (Tokyo, Japan).

Adhesion assay. Triplicate wells of 96-well plates were coated with 1 µg fibronectin (Asahi Techno Glass, Co., Ltd., Tokyo, Japan) and vascular cell adhesion molecule-1 (VCAM-1) (R&D Systems). RPMI-8226 cells were stimulated by recombinant human CX3CL1 (10 nM) for 5 min. After stimulation, RPMI-8226 cells (1x10⁵ cells/100 µl in EMEM with 0.1% BSA) were seeded and incubated for 20 min at 37°C. Cells that adhered to the well were evaluated as previously described (20).

Osteoclast differentiation. RPMI-8226 cells were cultured in the absence or presence of recombinant human CX3CL1 (10 nM) for 48 h, and then the conditioned cell culture medium was collected. Osteoclast precursors (RAW 264.7) were suspended in a-MEM supplemented with 10% FBS and cultured in a 24-well culture plates at 1x10⁵/well. After 48 h, the culture medium was replaced with 50% conditioned medium with or without 100 ng/ml of mouse recombinant soluble RANKL (Wako Pure Chemical Industries, Inc.). After 4 days, the 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. TRAP-positive cells appeared dark red. We counted TRAP-positive multinucleated cells containing three or more nuclei as osteoclasts.

Statistical analysis. Data were analyzed for statistical significance using the Student's t-test. P<0.05 was considered to indicate a statistically significant result. The mean and SD were calculated for all variables.

Results

CX3CR1 expression in multiple myeloma. We first compared the expression of CX3CR1 in seven human multiple myeloma cell lines and plasma cells (CD138-positive cell fraction) by RT-PCR (Fig. 1). CX3CR1 expression was detected in the plasma cells that derived from healthy donors. CX3CR1 expression was apparently induced during the process of malignant transformation of normal plasma cells to multiple myeloma.

Activation of Akt and Erk signaling by CX3CL1 in multiple myeloma. The observation of the CX3CR1 expression in human multiple myeloma cells prompted us to examine the biological responses of these cells to CX3CL1. The mechanisms underlying the differences in CX3CL1-induced progression and cell survival were investigated by analyzing signal transduction. Seven multiple myeloma cell lines were incubated with or without recombinant CX3CL1 and were subjected to western blotting using antibodies against unphosphorylated and
phosphorylated Akt, Erk1, Erk2 and STAT3. Akt, Erk1 and Erk2, but not STAT3, were constitutively phosphorylated in the two cell lines (Fig. 2A). Incubation of RPMI-8226 cells with CX3CL1-induced p-Erk1/2 expression after 1 min; this expression peaked from 2 to 5 min, while p-Akt expression peaked after 2 min (Fig. 2B). Next, the RPMI-8226 cells were stimulated with the antibody for 2 min. We also observed that CX3CL1-induced activation of Akt and ERK in RPMI-8226 cells was inhibited selectively by an anti-CX3CL1 antibody (Fig. 3). These results indicate a rapid CX3CL1-driven signaling for progression and cell survival after stimulation of CX3CL1 in multiple myeloma.

Increased adhesion to the extracellular matrix (ECM) by CX3CL1 in RPMI-8226. We next examined whether CX3CL1 regulates cell adhesion of human multiple myeloma. The number of RPMI-8226 cells adhering to fibronectin and VCAM-1 increased by ~17- and 3-fold respectively, in response to pretreatment with recombinant CX3CL1 (Fig. 4). The adhesion of multiple myeloma cells to fibronectin and VCAM-1, which are mainly expressed in bone marrow stromal cells, activates many pathways and results in the upregulation of the cell cycle regulating proteins and anti-apoptotic proteins (9,21). These results suggest that CX3CL1-induced progression of multiple myeloma in bone microenvironments.
Induction of osteoclast differentiation by multiple myeloma via CX3CL1. We previously reported that CX3CL1 expressed by osteoblasts plays an important role in osteoclast differentiation, possibly acting through its dual functions as a chemotactic factor and adhesion molecule for osteoclast precursors expressing CX3CR1 (22,23).

Given the apparent expression of CX3CR1 by multiple myeloma cells, along with the inducible effect of its ligand CX3CL1 on multiple myeloma cell adhesion to bone microenvironment ECM. We also investigated the possible synergy between multiple myeloma and osteoclast precursors in osteoclast differentiation via CX3CL1. Osteoclast precursors, RAW 264.7 were differentiated by RANKL in conditioned medium collected from multiple myeloma cells stimulated by CX3CL1. After 4 days, TRAP-positive multinuclear osteoclasts were counted (Fig. 5). The conditioned medium increased the number of TRAP-positive multinuclear osteoclasts, while treatment with rat anti-CX3CL1 mAb suppressed the induction of TRAP-positive multinuclear osteoclasts. These results suggest that CX3CL1 indirectly induces osteoclast differentiation by promoting the secretion of a factor from multiple myeloma in bone microenvironments.

Discussion

In the development of multiple myeloma, several cytokines, such as IL-6, IGF-1, VEGF and TNF-α directly promote cell survival and angiogenesis. JAK-STAT and IL-6 in particular, are believed to play a central role in cell survival and disease progression in multiple myeloma (24). Each chemokine and its receptor forms an axis that promotes cancer progression via effects on cell survival and angiogenesis (25). The role of the CX3CL1/CX3CR1 axis in the interaction between tumor cells and their microenvironment has been examined in non-Hodgkin lymphoma (17) and CLL (18), but not in multiple myeloma. In the present study, we confirmed expression of the chemokine receptor CX3CR1 in the multiple myeloma cell lines. No CX3CR1 was expressed in the plasma cells that derived from healthy donors (19).

We therefore investigated whether the chemokine CX3CL1 and its ligand CX3CR1 may be associated with cell survival and disease progression. As shown in Figs. 1-3, rapid phosphorylation of Akt and ERK1/2, which is related to signaling for survival and progression, as well as JAK-STAT, was observed following chemokine CX3CL1 treatment. These results may indicate that CX3CR1-positive myeloma cells have advantages regarding survival and progression.

However, the reason why multiple myeloma upregulates CX3CR1, but not in plasma cells from healthy donors, remains unclear. Therefore, further studies are needed to clarify the regulation of CX3CR1 expression by analysis of transcriptional factors (26) and chromosomal translocation related to multiple myeloma progression (27). The CX3CL1/CX3CR1 axis has a known association with several diseases caused by abnormal inflammation, such as rheumatoid arthritis (28). In fact, an animal model of CIA (collagen induced arthritis)
showed a dramatic improvement following the administration of the anti-CX3CL1 antibody. In this case, inflammatory cells permeated into the synovium and bone destruction were controlled (29).

The progression of multiple myeloma requires that the cells adhere to the extracellular matrix components such as fibronectin and VCAM-1 in the bone marrow. Many signaling pathways are activated when multiple myeloma adheres to ECM, resulting in upregulation of cell cycle regulating and anti-apoptotic proteins (9,21). In the present study, we showed that the CX3CL1/CX3CR1 axis aids in the co-operation between multiple myeloma and osteoclasts. Treatment with CX3CL1-induced adhesion of multiple myeloma cells to bone ECM and also induced osteoclast differentiation by a secretion factor produced by multiple myeloma cells (Figs. 4 and 5). We previously reported that the CX3CL1/CX3CR1 axis also plays an important role in osteoclast differentiation. Osteoclast precursors selectively expressed CX3CR1, whereas CX3CL1 was expressed by osteoblasts (22,23). The demonstration of CX3CR1 expression in multiple myeloma and CX3CL1 in osteoclast precursors observed in the previous, and the present study strongly indicates that the CX3CL1/CX3CR1 axis may be an attractive therapeutic target for prevention of the progression of multiple myeloma in bone microenvironments. Future studies should be aimed at investigating the blocking of this axis as a means of inhibiting myeloma progression, as well as suppressing the adverse skeletal-related events common in multiple myeloma.

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References

15. Andre F, Cab有意の情報が含まれていない文章です。