Targeting FSTL1 Prevents Tumor Bone Metastasis and Consequent Immune Dysfunction

Chie Kudo-Saito, Takafumi Fuwa, Kouichi Murakami, and Yutaka Kawakami

Abstract

Bone metastasis greatly deteriorates the quality of life in patients with cancer. Although mechanisms have been widely investigated, the relationship between cancer bone metastasis and antitumor immunity in the host has been much less studied. Here, we report a novel mechanism of bone metastasis mediated by FSTL1, a follistatin-like glycoprotein secreted by Snail+ tumor cells, which metastasize frequently to bone. We found that FSTL1 plays a dual role in bone metastasis—in one way by mediating tumor cell invasion and bone tropism but also in a second way by expanding a population of pluripotent mesenchymal stem-like CD45+ALCAM+ cells derived from bone marrow. CD45+ALCAM+ cells induced bone metastasis de novo, but they also generated CD8low T cells with weak CTL activity in the periphery, which also promoted bone metastasis in an indirect manner. RNA interference-mediated attenuation of FSTL1 in tumor cells prevented bone metastasis along with the parallel increase in ALCAM+ cells and CD8low T cells. These effects were accompanied by heightened antitumor immune responses in vitro and in vivo. In clinical specimens of advanced breast cancer, ALCAM+ cells increased with FSTL1 positivity in tumor tissues, but not in adjacent normal tissues, consistent with a causal connection between these molecules. Our findings define FSTL1 as an attractive candidate therapeutic target to prevent or treat bone metastasis, which remains a major challenge in patients with cancer. Cancer Res; 73(20); 1–9. ©2013 AACR.

Introduction

Bone metastasis of tumor cells is frequently seen in patients with cancer, particularly with breast cancers and prostate cancers, and greatly deteriorates the quality of life in patients, leading to poor prognosis (1). Chemokines and its receptors are one of the representative molecules regulating bone tropism of tumor cells. For example, CXCR4+ tumor cells are attracted by CXCL12 secreted from stromal cells in bone marrow (2), and CXCL12 maintains proliferation and survival of the tumor cells (3). Bone-derived TGFβ and PDGF also promote tumor progression in bone marrow (4). CCL2 is known as another chemokine critical for bone metastatic mechanism in various cancers (5, 6). The excess of tumor growth in bone marrow results in disruption of skeletal integrity, and causes abnormal osteogenesis or osteolysis in patients with cancer (1). One of the most influential molecules is RANKL, which is highly expressed in normal mesenchymal cells including osteoblasts and stromal cells, and metastatic tumor cells undergoing epithelial-to-mesenchymal transition (EMT; ref. 7).

Materials and Methods

Cell lines and mice

Murine melanoma B16-B10 cells were kindly provided by Cell Resource Center for Biomedical Research at Tohoku University in Japan. Human tumor cells including melanoma HS294T and pancreatic cancer Panc1 were purchased from American Type Culture Collection, and were authenticated by
short tandem repeat profiling. Some clones were transfected with plasmid vector pcDNA3.1 encoding murine or human snail gene as described before (9), and/or with lentiviral vector encoding GFP gene (Biogenova) for in vivo study. Tumor cells were cultured routinely in 10% FBS/Dulbecco’s Modified Eagle Medium (Invitrogen), and sometimes in 2% FBS/Opti-MEM (Invitrogen) for preparation of supernatant used for assays. Female C57BL/6 and C57BL/6-CAG-EGFP mice (designated EGFP+ mice) were purchased from SLC, and were maintained under pathogen-free conditions until use according to the protocols approved by the Animal Care and Use Committee at Keio University School of Medicine (Tokyo, Japan).

**Stimulation and characterization of bone marrow cells**

Bone marrow cells (BMC) were stimulated with 25% tumor supernatant (1 × 10^6 cells/10 mL/3 days, 0.22 μm filtration) or FSTL1 (5 ng/mL; Thermo Scientific) in 2% FBS/Opti-MEM for 7 to 10 days. CD45− BMCs were sorted from the culture using a MACS system with microbeads-conjugated monoclonal antibody (mAb; Miltenyi Biotec), and were tested for MSC activities: differentiation activity into mesenchymal lineages using Mesenchymal Stem Cell Functional Identification Kit (R&D Systems), self-renewal activity using 2% agarose medium, and immunoregulatory activity using coculture with splenic T cells in vitro, and coinjection with tumors in vivo. The details were described in Supplementary Data. For tracking CD45− ALCAM+ cell division, the sorted CD45− ALCAM+ cells were stained with red fluorescent dye PKH26 (Sigma) before assay.

**Functional analysis of tumor cells**

Tumor cells (5 × 10^6) were assessed for Matrigel invasion (6 hours) and chemotaxis (4 hours) to CCL2 (R&D Systems) or CXCL12 (R&D Systems) using a Transwell chamber with a membrane (pore size, 8 μm; BD Biosciences). FSTL1 in the supernatant fluids was measured using the ELISA kit (R&D Systems) according to the manufacturer’s instructions. For knockdown of fstl1 or snail expression, the specific siRNAs or the scrambled oligonucleotides as a control (3 μg; Invitrogen) were complexed with jetPEI (PolyPlus), and were then transfected into tumor cells. The transfection efficacy was validated by reverse transcriptase (RT)-PCR 1 to 2 days after transfection. Several kinds of siRNAs targeting on different sequence position of the genes were initially used, and a siRNA having the highest knockdown efficiency was mainly used. In a setting, tumor cells were stimulated with FSTL1 (5 ng/mL) in combination with/without CCL2 (5 ng/mL) for 3 days before assay.

**Flow-cytometric analysis**

After Fc blocking, cells were stained with immunofluorescence (Fluorescein isothiocyanate, phycoerythrin, or CyChrome)-conjugated mAbs specific for mouse and/or human ALCAM (eBioscience), CCR2 (R&D Systems), CCR5, CD146 (Abcam), CD271, CD45, CD8, CXC4, DIP2A (Santa-Cruz), Foxp3, PDGFαR (R&D Systems), RANKL (R&D Systems), RANKL, tetramer for gp70 (a tumor antigen expressed in B16-F10; MBL), or the appropriate isotype control antibodies. The immunofluorescence-conjugated secondary antibodies were used if necessary. Antibodies except designated ones were purchased from BD Pharmin- gen. For intracellular staining, cells were treated with Cytofix/Cytoperm solution (BD). The immunofluorescence was analyzed and compared with the isotype controls by CellQuest software using a FACScanibur cytometer (BD).

**Analysis of clinical tissues**

Paraffin-embedded tumor tissue sections obtained from patients with stage III breast cancer (n = 26) and normal mammary tissue sections (n = 6) were purchased (Human Cancer Tissue Array; MBL). To analyze mRNA expression, semi-quantitative RT-PCR was conducted using Ampdirect Plus (Shimadzu) and the specific paired primers for human fstl1, alcam, and gapdh (Supplementary Data) as described before (9). The digital images of the bands were quantified using NIH ImageJ software, and the signal intensity was normalized to gapdh expression as a control (normal tissues, < 0.1). To analyze protein expression, immunohistochemical analysis was conducted with anti-FSTL1 mAb (Abcam) and anti-ALCAM mAb (LSBio) according to a general protocol (Supplementary Data). As immunofluorescence intensity, pixel counts were automatically measured at two fields under the same 4’, 6-diamidino-2-phenylindole level using a LSM700 Laser Scanning Microscope (Carl Zeiss), and the average data were plotted in graphs (FSTL1, < 66, ALCAM, < 233 in normal tissues).

**FSTL1 blocking therapy in vivo**

To evaluate siRNA efficacy on tumor dissemination from the primary tumor site, and on tumor metastatic nodule formation, tumor cells were implanted into mice both subcutaneously (5 × 10^6) to prepare easy accessible sites for siRNA injection, and intravenously (1 × 10^5) to make visible bone metastasis for a short term while F10-snail+ tumor-implanted mice were living. Seven days later, poly-ethyleneamine-complexed siRNAs (5 μg) were injected into the subcutaneous tumors (n = 3–5/group), and the transfection efficacy was validated by RT-PCR or Western blotting 1 to 2 days after injection. Tumor volume (0.5 × length × width^2, mm^3) was measured 1 to 2 times a week. Ten days after siRNA injection, the subcutaneous tumors, bone marrow, and spleens were analyzed by flow cytometry, and splenic CD8+ T cells were tested for IFNγ production (24 hours) and tumor killing (4 hours) as described before (9).

**Statistical analysis**

Significant differences (P < 0.05) were evaluated using the unpaired two-tailed Student t test. The significance was confirmed by the nonparametric Mann–Whitney U test using the cumulative data of the repeated experiments, when the number of n was small in one experiment. Mouse survival was analyzed by Kaplan–Meier method and ranked according to the Mantel–Cox log-rank test. Correlation between tumor bone metastasis and ALCAM+ cells in mice, or between FSTL1 and ALCAM expressions in clinical tissues was evaluated by the nonparametric Spearman rank test.
Results

Snail\(^{+}\) tumor cells generate CD45\(^{-}/C0\) ALCAM\(^{+}\) mesenchymal stem cells in bone marrow

We previously established murine snail-transduced B16-F10 melanoma (designated F10-snail\(^{+}\)) having typical EMT features (9). When the F10-snail\(^{+}\) tumor cells were implanted both subcutaneously and intravenously in C57BL/6 mice, tumor metastatic dissemination was more severely observed in various tissues such as lymph node, lung, and bone marrow, as compared with that of the mice implanted with B16-F10 tumors transduced with empty vectors (designated F10-mock; Supplementary Fig. S1A), although the subcutaneous tumor growth was slower than F10-mock growth as shown before (12). Particularly, there are many mice having black bones due to invading melanoma tumor cells.
to melanoma metastasis in the subcutaneous/intravenous tumor model (41/48 = 85.4%; F10-mock, 1/12 = 8.3%), and the F10-snail+ tumor cells spontaneously disseminated to bone rather than other tissues even after only subcutaneous implantation (Supplemental Fig. S1B). These results suggest that Snail regulates bone tropism of B16-F10 tumor cells.

To examine how Snail+ tumor cells would affect bone marrow microenvironment, we stimulated BMCs with F10-snail+ supernatant for 7 to 10 days. Many colony-forming cells were observed in the culture, and CD45+ cells significantly increased as compared with the culture with F10-mock supernatant (P = 0.0002; Fig. 1A). Indeed, in addition to tolerogenic dendritic and regulatory T cells (Treg; but not MDSCs) reported before (9), CD45+ cells also increased in the F10-snail+ implanted mice (Supplementary Fig. S1C). CD45+ BMCs sorted from the culture with F10-snail+ supernatant more frequently differentiated into mesenchymal lineages such as adipocytes and osteoblasts, and formed large sphere colonies as compared with the CD45- BMCs treated with none or F10-mock supernatant (Fig. 1B). This indicates that F10-snail+ induced CD45+ BMCs are MSCs having pluripotency and self-renewal activity. The Snail+ tumor-induced MSCs (designated sMSCs) significantly suppressed splenic T-cell proliferation and cytokine production, indicating immunosuppressive activity (P < 0.002 vs. F10-mock–treated BMCs; Fig. 1C). These results suggest that Snail+ tumor cells expand MSCs in bone marrow microenvironment.

The sMSCs highly expressed some MSC markers reported in human MSCs (13), particularly ALCAM and CD146, as compared with the F10-mock–treated BMCs (Fig. 1D). However, the CD45+ CD146+ subpopulation hardly differentiated into osteoblasts (Supplemental Fig. S2). In contrast, the CD45+ ALCAM+ subpopulation showed high differentiation activity (Supplemental Fig. S2), proliferated in response to F10-snail+ supernatant (Fig. 1E). ALCAM blocking with the specific mAb significantly suppressed the sMSC increase (P = 0.001; Fig. 1E).
Data in each panel are representative of three independent experiments.

and significantly improved T-cell responses ($P < 0.002$; Fig. 1C). These data suggest that ALCAM is partly but critically responsible for the sMSC properties. ALCAM is a member of the immunoglobulin (IgG) superfamily, and binds to CD6 expressed T cells with high affinity, and to ALCAM-expressed various cells with low affinity (14). The ALCAM–ALCAM homophilic binding is strengthened by ALCAM clustering on the cell surface, and has been recently reported as a critical molecular interaction for proliferation and colonization of cancer stem cells (14). However, the functional role of ALCAM in MSCs remains unclear. Probably, sMSC expansion may be mediated by this homophilic interaction via ALCAM clustering, and T-cell suppression may be mediated by the homophilic interaction.

The sMSCs induce both tumor bone metastasis and immune dysfunction via generation of impaired CD8$^{low}$ T cells

We next conducted in vivo study using GFP$^+$ tumor cells or EGFP$^+$ BMCs for cell tracking after injection in mice. When EGFP$^+$ sMSCs were intravenously injected in mice on day 7 after F10-mock subcutaneous implantation, the sMSCs mainly infiltrated in the tumors, and tumor growth was significantly promoted ($P = 0.002$ vs. F10-mock–treated BMCs; Fig. 2A). To see how the infiltrating sMSCs would affect tumor microenvironment more clearly, the sMSCs were mixed with F10-mock tumor cells, and injected subcutaneously in mice. The tumors grew more aggressively ($P = 0.013$ vs. F10-mock–treated BMCs), and CD8$^{low}$ T cells predominantly increased in the tumors (Supplementary Fig. S3A). CD8$^{low}$ T cells also increased in spleen that was hypertrophic with abundant adipose tissues having increased CD45$^+$ cells including the injected sMSCs (Fig. 2B and Supplementary Fig. S3B). The CD8$^{low}$ T cells sorted from spleen hardly produced IFN$\gamma$ in response to a tumor antigen gp70 peptide, although weakly reacted to gp70 tetramer (Fig. 2B). It is inferred that such CTL dysfunction would be induced by the sMSCs infiltrating in spleen. In the sMSC-injected mice, tumor cells increased in bone marrow (Fig. 2B and Supplementary Fig. S3C), and the significant correlation was seen between the number of tumor cells and ALCAM$^+$ cells in bone marrow and the primary tumor site ($P < 0.005$; Fig. 2C). This suggests that sMSCs could induce bone metastasis de novo followed by further increase of ALCAM$^+$ sMSCs. Indeed, stimulation with the sMSC supernatant significantly enhanced tumor invasion in vitro ($P = 0.001$), and bone metastasis in vivo ($P = 0.006$; Fig. 2D). We found that the sMSCs significantly more produced CCL2 among soluble factors tested as EMT-inducible molecules ($P = 0.004$ vs. F10-mock–treated BMCs; Fig. 2D). CCL2 blocking with the specific mAb significantly inhibited the sMSC-induced tumor invasion in vitro ($P = 0.002$ vs. control IgG) and bone metastasis in vivo ($P = 0.006$; Fig. 2D). These results suggest that CCL2 is, at least in part, involved in the sMSC-induced bone metastasis mechanism. There may be a molecule that directly regulates this mechanism more significantly. The further study is needed. Tumor metastasis-promoting activity of MSCs has been already shown using xenograft model implanted with human breast cancer cells and human bone marrow-derived MSCs, and CCL5 has been shown as a molecule essential for the activity (15). However, the sMSCs produced no CCL5, and no lung metastatic nodules were observed in the coinjected mice in our study (data not shown).
FSTL1 is a key molecule governing Snail-induced bone metastasis

To identify the specific molecule regulating the Snail-induced bone metastasis, we reviewed literature focusing on molecules that associate with "bone"-related diseases such as arthritis, leukemia, and osteosarcoma, and about 20 candidates were chosen for analysis of mRNA and protein expressions. FSTL1 was the molecule that significantly and widely upregulated in murine and human Snail tumor cells (Fig. 3A and Supplementary Fig. S4). FSTL1 is a follistatin-like glycoprotein that binds to DIP2A receptor (16, 17). FSTL1 is originally known to regulate organ tissue formation in embryos (18), and its increase has been reported in some diseases such as rheumatoid arthritis (19, 20) and osteosarcoma (21). However, the functional role in cancer and metastasis has never been shown. Transfection with fstl1-specific siRNAs into Snail tumor cells significantly inhibited tumor invasion (P < 0.004), bone metastasis-associated molecule expressions such as CCR2 and CXCR4, and migration to the ligand such as CCL2 and CXCL12 (Fig. 3B and C and Supplementary Fig. S5), in addition to the typical reversal changes of EMT (e.g., increased adhesion, and decreased CD44 expression; data not shown). When Snail tumor cells were stimulated with FSTL1, these molecular expressions and tumor invasion were significantly enhanced (P < 0.002; Fig. 3D). This suggests that FSTL1 is a critical effector molecule for the high metastatic activity of Snail tumor cells. CCL2 combination synergistically elevated CXC4 and RANK expressions (Fig. 3D). It is inferred that CCL2 produced from the sMSCs and Snail tumor cells (12) may collaborate for promotion of bone metastasis. More interestingly, CD8low T-cell–inducible sMSC-like cells increased when BMCs were stimulated with FSTL1 (Fig. 4). The sMSCs highly expressed DIP2A (Fig. 1D). These results suggest that FSTL1 is responsible for both tumor metastasis and immune dysfunction in cancer bone metastasis mechanism.

We validated the FSTL1 effect in human system. When human peripheral blood mononuclear cell (PBMC) were stimulated with FSTL1 for 7 days, CD45–/lowALCAM+ cells increased in the culture (Supplementary Fig. S5D). When the sorted CD45–/lowALCAM+ cells were cocultured with FSTL1 (Fig. 4). The sMSCs highly expressed DIP2A (Fig. 1D). These results suggest that FSTL1 is responsible for both tumor metastasis and immune dysfunction in cancer bone metastasis mechanism.

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frequently occurs in patients with breast cancer (1). Colonizing ALCAM+ cells significantly increased in FSTL1+ tumor tissues, but not normal tissues, and the significant correlation between FSTL1 and ALCAM increase was seen (P < 0.01; Fig. 5). This suggests a causal connection between these molecules in tumor microenvironment. To clarify the usefulness of these molecules for diagnosis and prognosis of patients with cancer, the further studies are needed using other cancers and bone marrow samples of patients at varying stages.

**FSTL1 blockade inhibits bone metastasis and immune dysfunction in vivo**

To evaluate the antitumor efficacy of FSTL1 blocking, F10-snail+ cells were implanted both subcutaneously and intravenously in mice, and polyethyleneimine-complexed siRNA-fstl1 was injected into the subcutaneous tumors on day 7. Tumor growth (P = 0.012; Fig. 6A) and bone metastasis (P = 0.009; Fig. 6B) were suppressed, and the mouse survival was prolonged by (P < 0.01). This suggests a causal connection between FSTL1 blocking and antitumor immune dysfunction in vivo. F10-mock tumors were implanted both subcutaneously (5 × 10^5) and intravenously (1 × 10^5) into mice, and 7 days later, siRNA was injected into the subcutaneous tumors (n = 5–10 per experiment). Knockdown efficacy on day 2 was confirmed by Western blotting (S, Snail; F, FSTL1; A, Actin; A). In the mice having F10-snail+ tumors injected with siRNA-fstl1 (closed triangles, siF) or siRNA-alcam (closed squares, siA), the subcutaneous tumor growth (n = 10, mean ± SD; A) and bone metastasis (n = 9, mean ± SD; B) were suppressed, and the mouse survival was prolonged by (n = 10; C). Open circles (M), F10-mock tumors. Closed circles (C), F10-snail+ tumors injected with control siRNA. Ten days after siRNA injection, the subcutaneous tumors, bone marrow, and spleens of the mice were analyzed for CD8+ cells as MSCs (n = 6, mean ± SD; D). SPCs were further analyzed for CD8+gp70-tetramer+ T cells and CD8+Foxp3+ T cells by flow cytometry (E), and the sorted CD8+ T cells were tested for F10-mock tumor killing (ET ratio = 50:1; n = 3, mean ± SD; F) and IFNγ production (n = 3, mean ± SD; F), (P < 0.02 versus control siRNA. Data in each panel are representative of three independent experiments.
mechanism (5, 6). Surely, CCL2 is a useful marker for "mesenchymal" cells regardless of normal or cancerous cells (Fig. 6E) was not seen, and tumor-specific CD8⁺ T-cell responses were significantly elevated (P < 0.002; Fig. 6F). The siRNA-fstl1 efficacy was higher than that of siRNA-snail, particularly on induction of ALCAM⁺ sMSCs and CD8low T cells. These results suggest that FSTL1 knockdown prevents not only tumor metastasis, but also immune dysfunction through ALCAM⁺ sMSC decrease, and therefore antitumor CTLs are induced right for eliminating tumor cells from the mice. FSTL1 blockade may be a promising strategy for treating cancer bone metastasis of patients.

Discussion

The relationship between cancer bone metastasis and antitumor immunity in the host has been rarely investigated. This study revealed a novel mechanism, which is governed by FSTL1 produced from Snail⁺ tumor cells undergoing EMT. FSTL1 plays a dual role in cancer bone metastasis, in one way by mediating tumor invasion and bone tropism, and in a second way by expanding pluripotent and multifunctional CD45⁻ ALCAM⁺ MSC-like cells, named as sMSCs. The sMSCs are possibly derived from BMCs, and disseminate all over the body. Tumor-infiltrating sMSCs induce bone metastasis de novo followed by further increase of sMSCs in bone marrow. The sMSCs are able to generate CD8low T cells with almost no CTL activities, leading to dysfunction of antitumor immune responses. The number of ALCAM⁺ cells is correlated with the amount of tumor bone metastasis in a murine model, and ALCAM⁺ cells correlatively increase in FSTL1⁺ tumor tissues of patients with advanced breast cancer, who may have bone metastasis and deteriorated immune status frequently. FSTL1 blocking in vivo significantly prevents both tumor bone metastasis and increase of ALCAM⁺ MSCs (13). However, we found that murine MSCs, which were phenotypically and functionally similar to human MSCs (13), increased in mice having Snail⁺ FSTL1⁺ tumors in vivo, and are expanded by tumor-derived FSTL1 for a short term in vitro. FSTL1 was selectively expressed in tumor cells of patients, and stimulation with FSTL1 induced sMSC-like CD45⁻ CD8low ALCAM⁺ cells in human PBMCs. A specific circumstance under cancer would lead to the finding of the sMSCs.

Some reports have shown induction of immunosuppressive T cells by MSCs. However, CD8 reduction was not shown in the report of Foxp3⁺ Treg induction (23), and Foxp3 expression was not shown in the report of CD4low or CD8low T-cell induction (24). Our study additionally showed that the sMSCs generate CD8low T cells partly including CD8lowFoxp3⁺ cells. CD8 reduction in T cells may be mediated by zince-finger protein MAZ (25) and/or cKrox (26), which are negative regulators of CD8 expression in thymocyte lineage differentiation. The signaling pathway of ALCAM, which is possibly required for CD8low T-cell induction, may be of interest for elucidation of this mechanism.

Taken together, FSTL1 is a critical determinant governing cancer bone metastasis accompanied by immune dysfunction, and may be an attractive target for treating cancer via reprogramming of antitumor immune responses in patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C. Kudo-Saito
Development of methodology: C. Kudo-Saito, T. Fuwa
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Kudo-Saito, K. Murakami
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Kudo-Saito
Writing, review, and/or revision of the manuscript: C. Kudo-Saito
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Kudo-Saito, T. Fuwa
Study supervision: C. Kudo-Saito, Y. Kawakami

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Role of FSTL1 in Cancer Bone Metastasis

References

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