Role of CXCR4 and SDF-1 in Mammary Tumor Metastasis in the Cat

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ABSTRACT. It has recently been suggested that the chemokine receptor CXCR4 and its ligand SDF-1 (CXCL12) promote metastasis of various cancers in humans. Since feline mammary tumors also metastasize to distant organs frequently, we used real-time quantitative PCR to examine the expression of feline CXCR4 (fCXCR4) in ten feline mammary tumor cell lines and seven feline mammary tumor tissues, and also the expression of feline SDF-1 (fSDF-1) in various organs. Cell lines derived from metastatic regions expressed more fCXCR4 than those derived from primary tumors. Mammary tumor tissues overexpressed more fCXCR4 than normal mammary tissues. Organs with high levels of fSDF-1 expression represent common sites of metastasis. Migration assays using the feline mammary tumor cell line NAC were also performed to test the activity of TN14003 and TC14012, antagonists of human CXCR4, to antagonize fCXCR4 expressed on NAC cells. TN14003 and TC14012 inhibited migration of NAC cells. We conclude that fCXCR4 may be a therapeutic target for feline mammary tumors.

KEY WORDS: CXCR4, feline, mammary tumor, metastasis, SDF-1.

In cats, mammary tumors are the third most frequently occurring neoplasm, following hematopoietic and skin tumors. Almost all feline mammary tumors are aggressively malignant: they metastasize easily, relapse frequently and progress rapidly, resulting in a generally poor prognosis. This degree of malignancy, in particular the frequent metastasis, poses a serious obstacle to effective cancer therapy, and it is consequently important to prevent the cancer cells from metastasizing to distant organs [5, 9].

Chemokines are small, secreted peptides that control the migration of leukocytes, especially during immune and inflammatory reactions [13]. They are divided into two major subfamilies, CC and CXC, based on the position of their amino-terminal cysteine residues, and bind to G-protein-coupled receptors, whose two corresponding major subfamilies are designated CCR and CXCR. Recently it has been suggested that human breast cancer cells make use of the chemokine receptor CXCR4 and chemokine SDF-1 axis to form metastatic foci [11]. Although feline CXCR4 (fCXCR4) on CD4+ T cells is well known as a feline immunodeficiency virus (FIV) co-receptor, there is as yet no information available concerning a potential association between ICXCR4 and tumor metastasis.

In this study, we examined the possible role in metastasis of fCXCR4 expressed on mammary tumor cells. We also examined the antimetastatic potential of two human CXCR4 antagonists, TN14003 and TC14012.

MATERIALS AND METHODS

Cell lines: Feline cell lines NAC and FRM have been described previously [10]. All other cell lines used in this study were established at the Laboratory of Veterinary Surgery, University of Tokyo, from five primary tumors (FON-p, FMC-p1, FMC-p2, FYM, and PKN) or five metastatic regions (FON-m, FMC-m, FRM, NAC, and FMY2) of feline mammary tumors. Detailed information on these cell lines will be published elsewhere. All cell lines were grown in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (EQUITECH-BIO, Kerrville, TX, U.S.A.), 1.5 µg/ml fungisone, and 50 µg/ml gentamicin. Cells were maintained in 95% air/5% CO2 at 37°C.

RT-PCR: Total RNA was extracted from cells or homogenized tissue samples using TRIZol reagent (Invitrogen, Leek, Netherland), and reverse transcribed with M-MLV reverse transcriptase (Invitrogen) and random primers (TAKARA, Tokyo, Japan) for 50 min at 37°C, according to the manufacturers’ instructions. The reaction was stopped by heat-inactivation for 15 min at 75°C. Complementary DNA was quantitatively analyzed for the expression of fCXCR4 and fSDF-1 by the fluorogenic 5′-nuclease PCR assay as reported [6]. Specific primers and probes were obtained from Hokkaido System Science (Sapporo, Japan). For fCXCR4, the forward primer (5′-CGGCTGGAGAGCTAGGTAAGGT-3′), reverse primer (5′-TAGTGCTCGCTGAGCCCCAAGTC-3′), and probe (5′-CCATGGACGGGCTTCGTTATACCCCTCA-3′) were used. For fSDF-1 the forward primer (5′-GAGCCAAGCTCAAGCATCTCA-3′) and reverse primer (5′-GAGCCCAAGTC-3′) and probe (5′-CCATGGACGGGCTTCGTTATACCCCTCA-3′) were used.
3'), reverse primer (5'-CGGGTCAATGCACACTTGTCTA-3'), and probe (5'-TGTTGTTCTTCAGCCACGA-3') were used. Gene-specific PCR products were measured continuously for 40 cycles by means of an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, U.S.A.). The PCR products were also electrophoresed in 1.5% agarose gel and identified as single bands (data not shown).

Standard curves for fCXCR4 and fSDF-1 mRNA were generated using serially diluted solutions (10⁵ to 10¹⁰) of plasmid clones bearing full-length copies of either fCXCR4 or fSDF-1 as template. The fCXCR4 plasmid clone was a gift from Dr. T. Miyazawa, Osaka University. The fSDF-1 plasmid was made by amplification of full-length clones by PCR using forward and reverse primers, followed by subcloning the PCR products into the pGEM-T Easy Vector (Promega, Madison, WI, U.S.A.). The amount of target gene expression in each cell or tissue sample was calculated from the standard curves and normalized using expression of the 18S ribosomal RNA gene as an internal control. Accordingly, fCXCR4 and fSDF-1 mRNA levels are reported as fCXCR4/18S rRNA or fSDF-1/18S rRNA ratios, respectively. Real-time PCR assays were conducted in duplicate for each sample and the mean value was used for the calculation of mRNA expression levels.

Migration assay: The migration of NAC cells was assessed using a microchemotaxis chamber technique, as previously described [14]. Briefly, chambers 6.5 mm in diameter with 8 μm pore filters were used (KURABO, Osaka, Japan). The lower surfaces of the filters were coated with 50 μl of fibronectin (100 μg/ml) and then dried with HEPA-filtered air. Subconfluent (50–70%) NAC cells were detached and resuspended at 2 × 10⁵ cells/ml in serum-free media (RPMI1640 containing 1% BSA), and added in 100 μl aliquots to the upper chambers. The lower wells were filled with 600 μl of serum-free media containing 0 to 500 ng/ml SDF-1. Previously synthesized human SDF-1 was used because the sequence of human SDF-1 is identical to that of fSDF-1, and we regard them as indistinguishable [22]. To evaluate the inhibitory activity of the T140-type CXCR4 antagonists, cells were preincubated for 30 min at 37°C in the presence of 1 μM of one of two T140 derivatives, TN14003 and TC14012, which were synthesized as described previously [21]. The microchemotaxis chambers were then incubated for 12 hr at 37°C in 95% air/5% CO₂. After incubation, the filters were fixed in 70% ethanol and stained with 0.4% trypan blue. The upper surfaces of the filters were scraped twice with cotton swabs to remove nonmigrating cells. The number of migrating cells was then counted in five high-power (× 200 magnification) microscope fields per filter.

Statistical analysis: Statistical differences between the means for the different groups were evaluated by Student’s t test. All experiments were repeated at least twice, with triplicate samples, and similar results were obtained.

RESULTS
fCXCR4 mRNA expression: We examined fCXCR4 mRNA expression in ten feline mammary tumor cell lines; five were derived from primary tumors and five from metastatic regions. The frequency and amount of fCXCR4 expression were higher in cell lines derived from metastatic regions than in those from primary tumors (Fig. 1A). Only one primary tumor cell line expressed a detectable level of fCXCR4, while three cell lines from metastatic regions expressed it. In addition, the expression levels of fCXCR4 in NAC and FYM2 cells were ten times higher than the expression level of FON-p cells, the only cell line that expressed fCXCR4 among the five primary tumor cell lines. The expression of fCXCR4 was upregulated in feline mammary tumor tissues (1.13 ± 0.33; mean ± SD) as compared with normal mammary tissues (0.21 ± 0.10) (Fig. 1B). Though some normal tissues expressed considerable amounts of fCXCR4, the highest level noted in normal tis-
sues (0.66) was still lower than the mean value found in tumor tissues.

**fSDF-1 mRNA expression**: Quantitative analysis of fSDF-1 expression in various normal feline tissues revealed that fSDF-1 mRNA is expressed preferentially in lymph nodes, liver, and lung while showing markedly lower expression in kidney, ovary, heart, stomach, pancreas, and skin (Fig. 2). Organs exhibiting the highest fSDF-1 expression represent the most common sites of metastasis of mammary tumors.

**Migration and migration inhibition of NAC cells**: fSDF-1 induced directional migration of feline mammary tumor cell line NAC, which expressed high levels of fCXCR4, in a dose-dependent manner (Fig. 3). TN14003 and TC14012, antagonists of CXCR4, inhibited fSDF-1-induced directional migration by 80% and 70%, respectively (Fig. 4).

**DISCUSSION**

Recently, the involvement of the CXCR4-SDF-1 axis in human metastatic breast cancer has been postulated [11]. However, the role of this axis in breast cancer has not yet been reported in other species. In this study, we examined the possible role of fCXCR4 expressed by mammary tumor cells during metastasis in the cat. We observed that in the cat (1) fCXCR4 expression was upregulated in tumor tissues and in some cell lines, (2) organs with high fSDF-1 expression represent common sites of metastasis, and (3) directional migration of a tumor cell line was induced by fSDF-1 and inhibited by the CXCR4 antagonist. These results reveal a role of the CXCR4-SDF-1 axis in mammary cancer metastasis in the cat. Therefore, this suggests that involvement of the CXCR4-SDF-1 axis in metastasis is present not only in humans, but also in other mammals.

We observed that feline mammary tumor cell lines derived from metastatic regions expressed more fCXCR4 than did cell lines derived from primary tumors, which supports the idea that upregulation of fCXCR4 is related to metastasis of tumor cells. Although fCXCR4 expression in some cell lines was below the threshold for detection, a similar lack of expression in some cell lines has been reported in a human study [11]. It is possible that upregulation of fCXCR4 is a cell-specific event, or that expression is diminished during repeated passages of cell lines. Consistent with the observations in cell lines, mammary tumor tissues...
expressed high levels of fCXCR4 as compared with levels in normal tissues. However, two of six normal mammary tissue samples expressed more fCXCR4 than some tumor tissue samples. The reason for relatively high expression levels in normal tissues is not clear at present. The estrus cycle, age, ovariectomy, and other factors may influence fCXCR4 expression in the mammary gland. Indeed, high expression levels in some normal tissues were also reported in humans [11].

In this study, we could not obtain the set of specimens, primary tumor and metastatic region. However, it was reported that there was significant expression of human CXCR4 mRNA in both primary tumors and metastases-infiltrated lungs of SCID mice that had been injected with tumor cells [11]. This result supports the involvement of human CXCR4 in breast cancer metastasis. So, we speculate that fCXCR4 expressed in tumor tissues may also facilitate metastasis of mammary tumor in the cat.

Because specific antibodies against fCXCR4 are not available at present, we could not explore the presence of fCXCR4 protein. However, fCXCR4 expressed in NAC cells was shown to be functional. The fSDF-1 concentration range that induced directed migration of NAC cells was 50 to 500 ng/ml. This range is similar to that reported for induction of migration in human cancer cell lines [4, 11, 16]. In addition, FMC-p1 cells, expressing under the detectable level of fCXCR4, were not induced directed migration by fSDF-1 although FYM2 cells, another cell line expressing high levels of fCXCR4, were (data not shown). The tissues exhibiting the highest fSDF-1 expression levels represented the most common sites of metastasis for mammary cancer. Taken together, these findings show that the fCXCR4-fSDF-1 axis may facilitate metastasis and determine the specific target organ for metastatic foci.

In 1996, CXCR4 was identified as a co-receptor of HIV [3]. Since then, intense efforts have been underway to identify small-molecule antagonists for CXCR4 [1, 2, 21]. In this study, we used TN14003 and TC14012, which are the results of refinement of T140, as CXCR4 antagonists. TN14003, TC14012, and T140 all antagonize CXCR4 to the same degree, however TN14003 and TC14012 were more stable in feline serum than T140 [21]. Unlike other CXCR4 antagonists having partial agonist activity, T140 has inverse agonist activity. In this respect, TN14003 and TC14012, as well as T140, are superior to other antagonists [24]. In the present study TN14003 and TC14012 inhibited the migration of NAC cells (Fig. 4) and FYM2 cells (data not shown) in vitro, indicating that these antagonists may be effective in the prevention of feline mammary tumor metastasis. However, the effectiveness of these molecules in vivo is still unclear, and it will be necessary to analyze the inhibition of metastasis by these inhibitors in animal models in the future. The CXCR4-fSDF-1 axis has many functions that can promote malignancies, such as mediating tumor cell survival [25], inhibition of antitumor immunity [26], acceleration of tumor cell proliferation [18, 19], and induction of angiogenesis [15, 20]. By inhibiting these functions, CXCR4 antagonists may have antitumor effects in addition to antimetastatic activity.

It has been reported that CXCR4 is upregulated in many types of cell lines, in addition to breast cancer, including ovarian cancer [16, 17, 26], glioma [25], pancreatic cancer [7], melanoma [12], neuroblastoma [4], and rhabdomyosarcoma [8]; CXCR4 also promotes migration or invasion of these cells in vitro. Other chemokine receptors, such as CCR7, are also involved in tumor metastasis [11, 12, 23]. By analyzing chemokine receptors comprehensively and by comparing animal receptors with their human homologues, we should be able to unravel specific mechanisms of tumor metastasis, as well as mechanisms common to mammals as a group.

On the basis of our observations we conclude that the fCXCR4-fSDF-1 axis very likely plays an important role in tumor dissemination and metastasis. Hence, molecular strategies aimed at inhibiting this axis, e.g., the use of small-molecule inhibitors, may lead to therapies that complement conventional radiotherapy or chemotherapy in preventing dissemination of feline mammary tumors.

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