Involvement of Chemokine Receptor 4/Stromal Cell-derived Factor 1 System in Human Salivary Gland Carcinoma Motility

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Abstract: Salivary gland carcinoma such as adenoid cystic carcinoma (AdCC) is characterized by slow growth, diffuse invasion and lung metastasis, which determine the patient’s prognosis. It is important to clarify an attractant molecule leading tumor cells to migrate. We examined the effects of stromal cell-derived factor (SDF)-1, a chemokine, on salivary gland carcinoma cell clone HSG and its subclone HSG-AZA3. SDF-1 promoted the invasion and migration of HSG and HSG-AZA3 cells dose-dependently. Immunocyto-staining and RT-PCR indicated that HSG and HSG-AZA3 cells expressed SDF-1 receptor, CXCR4, both in protein and mRNA level, respectively. CXCR4 was present on the cell surface of HSG cells, and was downregulated by SDF-1 addition. Finally, we confirmed that CXCR4 was expressed in the tissue of AdCC. Our study suggests that SDF-1 and CXCR4 play a role in the migration of carcinoma of salivary gland origin.

Key words: salivary gland carcinoma, chemokine, SDF-1, CXCR4, metastasis

Introduction

Salivary gland carcinoma such as adenoid cystic carcinoma (AdCC), which is the second most common oral cancer following squamous cell carcinoma (SCC), is characterized by diffuse invasion and metastasis to the lungs and regional lymph nodes. According to in vitro data published so far, AdCC cells seem to require an abundant production of extracellular matrix, adhesion to matrix by forming the focal adhesion assembly through integrin-urokinase type plasminogen activator (uPA) receptor, and degradation of matrix by uPA in order to migrate and invade locally.

On the other hand, AdCC of the salivary gland prefers to metastasize to the lungs via the blood stream. The sites of distant metastasis are determined not only by the characteristics of carcinoma cells, but also by the microenvironment of the specific organs. Organ-specific attractant molecules may induce homing of carcinoma cells to particular sites. However, it is uncertain which molecules contribute to metastasis of salivary gland carcinoma.

Stromal cell-derived factor (SDF)-1 (CXC chemokine ligand 12) is a member of the CXC chemokine family. SDF-1 exerts effects through its cognate receptor CXC chemokine receptor (CXCR4). Recently, the SDF-1/CXCR4 system was reported to be involved in tumor metastasis including breast cancer, prostate cancer, colon cancer, osteosarcoma, melanoma, and oral, head and neck SCC, although it was initially characterized in murine bone marrow as a pre-B cell growth stimulating factor. In breast cancer and oral SCC, high expression of CXCR4 in tumor tissues is reported to correlate with metastasis and poor prognosis of the
patients\textsuperscript{9,15}, and the cultured carcinoma cells expressing CXCR4 were mobilized by SDF-1\textsuperscript{8,14}. Moreover, it has been shown that CXCR4 is involved in homing of carcinoma cells to specific organs in breast cancer\textsuperscript{9}, prostate cancer\textsuperscript{8} and osteosarcoma\textsuperscript{11}. However, there have been few studies on the role of the SDF-1/CXCR4 system in salivary gland carcinoma.

In the present study, we investigated the involvement of the SDF-1/CXCR4 system in cell motility of salivary gland carcinoma. We found that SDF-1 stimulates the migration of salivary gland carcinoma cells dose-dependently through CXCR4.

Materials and Methods

1) Reagents

Mouse monoclonal anti-human CXCR4 antibody was purchased from Zymed Laboratories Inc., South San Francisco, USA. Mouse monoclonal anti-human SDF-1 antibody and human recombinant SDF-1 were purchased from R & D Systems Inc., Minneapolis, USA.

2) Cell culture

HSG is a human salivary gland adenocarcinoma cell line which was derived from an irradiated submandibular gland and expresses a phenotype of intercalated duct cell\textsuperscript{17}. HSG-AZA3 is a subclone of HSG treated with 5-azacytidine, which expresses a phenotype of acinar cell\textsuperscript{18}. B88\textsuperscript{14} is an oral SCC cell line, and B32F\textsuperscript{19} is a human fibroblast line. The cell lines HSG and HSG-AZA3 were kindly provided by Professor Mitsunobu Sato, Second Department of Oral and Maxillofacial Surgery, Tokushima University, Japan. These cell lines were cultured in a Dulbecco’s Modified Eagle’s Medium (DMEM) (GIBCO Life Technologies Inc., NY, USA) containing 10% fetal bovine serum (SIGMA). Approximately 80% confluent cells were used in the following experiments.

3) Invasion assay

The double chamber method was used for the cell invasion assay. BD BioCoat\textsuperscript{TM} Matrigel\textsuperscript{TM} Invasion Chamber insert (BD Biosciences, Bedford, MA, USA) (upper chamber) whose bottom was a Matrigel\textsuperscript{TM}-coated PET membrane with an 8 \(\mu\)m pore size, was put in one well of a 24-well plate (lower chamber). The cells \((5 \times 10^5)\) were seeded onto the upper chamber membrane. Thereafter, various concentrations of SDF-1 (0, 10 and 100 ng/ml) were loaded into the lower chamber. After 24 h culture, the cells attached to the upper surface of the membrane were scraped off, and the cells migrating onto the lower surface through Matrigel\textsuperscript{TM} were fixed and stained. The invasion activity was determined by averaging the number of cells present on the lower surface in five high-power fields under a microscope.

4) Wound healing assay

The cells were cultured in a 60 mm-diameter dish until becoming confluent. Then, a part of the cell monolayer was scratched off with a cell scraper (Iwaki, Kyoto, Japan), forming a wounded area (approximately 12 mm x 12 mm square), and was cultured with or without SDF-1 (100 ng/ml). Six or twelve hours later, the number of cells migrating into the wounded area was counted.

5) Immunocytostaining

The cells on a glass slide were fixed with 4% paraformaldehyde for 6 h at 4\textdegree C. Then, they were incubated with primary monoclonal antibody against human CXCR4 or human SDF-1 overnight at 4\textdegree C. After washing with PBS, they were stained with the avidin-biotin peroxidase complex method using a Dako Envision kit (Dako Cytomation, Kyoto, Japan).

6) Confocal laser microscopy

The cells were cultured on a glass slide, air-dried for 30 min, fixed with 30% acetone PBS for 10 min at 4\textdegree C, and then incubated with mouse monoclonal anti-CXCR4 antibody or anti-SDF-1 antibody overnight at 4\textdegree C, followed by incubation with FITC-conjugated goat anti-mouse IgG antibody. The presence of CXCR4 and SDF-1 was visualized with Alexa Flor VECTA SHIELD PI (Invitrogen Co., CA, USA) and observed with a confocal laser microscope (LMS, Carl Zeiss, Germany).

7) Flow cytometric analysis

Cultured cells were detached from the culture dishes after being treated with 0.05% trypsin including 0.53 mM EDTA. Single cell suspensions were incubated with anti-CXCR4 monoclonal antibody for 30 min at room temperature. FITC-conjugated goat anti-mouse IgG antibody was used as a second antibody. Ten thousand gated cells were analyzed by a FACSort and the
software CellQuest (Becton-Dickinson Biosciences, Texes, USA).

8) **Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis**

Total RNA was isolated from $5 \times 10^6$ cells using a RNase Mini Kit (Qiagen Inc., MD, USA) according to the manufacturer’s instruction. cDNA was synthesized from 5 μg of RNA with the Super Script 3 First-Strand Synthesis System (Invitrogen Co., CA, USA). PCR was carried out in a Takara thermal cycler MP (Takara Biomedicals, Kusatsu, Japan) with specific primers for CXCR4 and SDF-1. Amplification conditions were as follows: 95°C for 9 min, followed by 45 cycles of 94°C for 45 sec and 57°C for 45 sec. This was followed by a final extension for 45 sec at 72°C. The products were visualized on 2% agarose gel with SYBER GREEN II. The densitometric analyses for PCR bands were performed with NIH images.

9) **Immunohistochemical staining**

Four micrometer-thick histological sections of 4% parafomaldehyde fixed, paraffin embedded tissues of AdCC were formed, mounted, and air-dried. The sections were deparaffinized in xylene and rehydrated in ethanol, and endogenous peroxidase was blocked by immersion in methanol containing 0.3% hydrogen peroxide for 30 min. The sections were incubated overnight at 4°C with primary anti-CXCR4 antibody, then for 30 min at room temperature with biotinylated anti-mouse IgG antibody, followed by staining with peroxidase-conjugated streptavidin (Histofine SAB-PO kit, Nichirei Co., Tokyo, Japan).

**Results**

1) **SDF-1 promotes migration of salivary gland carcinoma cells**

In order to study the effect of SDF-1 on salivary gland carcinoma cells, we performed invasion assay using the double chamber method. We seeded five kinds of cell lines (HSG, HSG-ZA3, B88, HeLa and B32F) on the Matrigel™-coated membrane of an upper chamber, and added various doses of SDF-1 into a lower chamber. The number of cells migrating from the upper surface of the membrane to the lower surface through Matrigel™ was counted. Figure 1 (A) shows that SDF-1 promoted the invasion of HSG, HSG-ZA3 and B88 cells dose-dependently. HSG-ZA3 was the best responder to SDF-1 among the three cell lines. However, HeLa and B32F cells were not stimulated by SDF-1 (Fig. 1 (A)).

We then confirmed SDF-1-mediated motility of salivary gland carcinoma cells by wound healing assay. An approximately 12 mm × 12 mm square of confluent cell sheet was scraped off to form a wounded area. Then, the number of cells migrating into the wounded area was counted. As a result, SDF-1 stimulated the migration of HSG, HSG-ZA3 and B88 cells (Fig. 1 (B)). These data demonstrate that SDF-1 acts not only on SCC cells, but also on salivary gland carcinoma cells.

2) **Expression of CXCR4 protein in salivary gland carcinoma cells**

Because SDF-1 caused salivary gland carcinoma cells to migrate through Matrigel™, we studied the expression of CXCR4, a SDF-1 receptor, in HSG and HSG-ZA3 cells. Immunocytochemical staining of the cultured cells showed that HSG, HSG-ZA3 and B88 cells expressed CXCR4 protein, but not SDF-1 (Fig. 2). However, it was uncertain whether CXCR4 was expressed cell-surface CXCR4. Among the three cell lines, HSG-ZA3 seemed to express the highest level of CXCR4 (Fig. 3 (A)). Furthermore, cell surface CXCR4...
was downregulated 1 h after SDF-1 addition, while the expression of CXCR4 recovered 12 h later (Fig. 3 (B)), showing that SDF-1 actually binds to CXCR4 on salivary gland carcinoma cells. This data may also imply the specificity of anti-CXCR4 antibody.

4) Expression of CXCR4 mRNA in salivary gland carcinoma cells

CXCR4 mRNA, but not SDF-1 mRNA, was identified in HSG, HSG-AZA3 and B88 cells (Fig. 4 (A)). When we measured the density of the PCR bands semi-quantitatively by densitometer, HSG-AZA3 cells expressed a
Fig. 3 Flow cytometric analysis for CXCR4. (A) Cell surface expression of CXCR4. Single cell suspension of each cell line was incubated with monoclonal anti-CXCR4 antibody, then followed by FITC-conjugated goat anti-mouse IgG antibody, and subjected to flow cytometry. Gray shaded area, isotype control antibody; black line, anti-CXCR4 antibody. (B) Down regulation of cell surface CXCR4. Each cell line was pretreated with 100 ng/ml of SDF-1 for 1 h, 3 h or 12 h. Thereafter, cell surface expression of CXCR4 was measured by flow cytometry. Gray shaded area, isotype control antibody; black line, anti-CXCR4 antibody without SDF-1; dotted line, anti-CXCR4 antibody with SDF-1.

Fig. 4 Expression of CXCR4 mRNA. cDNA was synthesized from 5 µg of RNA from each cell line. PCR was performed as described in Materials and Methods. The products were visualized on 2% agarose gel (A). The density of PCR bands of CXCR4 was measured with a densitometer using NIH images and evaluated in comparison with those of GAPDH (B).
higher amount of CXCR4 mRNA than HSG and B88 cells did (Fig. 4 (B)), which appeared to correspond to the results of SDF-dependent motility as shown in Figure 1.

5) Expression of CXCR4 in adenoid cystic carcinoma tissue of a patient

Finally, we confirmed the expression of CXCR4 protein in the tissue of a patient with AdCC arising from palatal salivary gland (Fig. 5). Immunohistochemical staining for CXCR4 was remarkably positive in tumor cells.

Discussion

The present study indicates that the SDF-1/CXCR4 system may contribute to the spread of salivary gland carcinoma. Although SDF-1 stimulates the migration and invasion of HSG, HSG-AZA3 and B88 cells, HSG-AZA3 and HSG cells were more strongly stimulated by SDF-1 dose-dependently. In particular, the invasion of HSG-AZA3 cells was more dependent on SDF-1 concentrations whereas B88 cells appeared to invade rather spontaneously, independently of SDF-1. It remains unknown why HSG-AZA3 cells are a better responder to SDF-1 than HSG cells. Sato et al. established HSG-AZA3 cells by treating HSG cells with 5-azacytidine. The gene mutation occurring with 5-azacytidine might change the phenotype of HSG as well as the reactivity to SDF-1. We speculate that this dependency of HSG-AZA3 cell invasion on SDF-1 may be related to metastasis of salivary gland carcinoma.

The SDF-1/CXCR4 system is reported to play an important role in organ-specific metastasis of cancer. This system has been more extensively studied in oral SCC, in which it has been shown that the metastasis of oral SCC to regional lymph nodes is associated with SDF-1/CXCR4. If SDF-1 causes salivary gland carcinoma cells to metastasize to lymph nodes and lungs, the cells will require several steps for metastasis including loss of cell-cell contact, degradation of extracellular matrix, cell motility, homing to specific organ, and adhesion to vascular endothelial cells. Because our invasion assay suggested that salivary gland carcinoma cells degraded Matrigel matrix, SDF-1 may stimulate them to produce matrix-degrading protease such as matrix metalloproteinase (MMP). In osteosarcoma and head and neck SCC, SDF-1 is reported to promote the expression of MMP-9 which can degrade type IV collagen.

Seki et al. demonstrated that a transcription factor NF-κB should be involved in the adhesion of AdCC cells to vascular endothelial cells during lung metastasis. Because activation of NF-κB promotes the expression of CXCR4 in breast cancer cells, the high expression of CXCR4 seen in HSG-AZA3 cells may also be induced by NF-κB. HSG cells are reported to express NF-κB which is activated by tumor necrosis factor. However, it is unknown whether the expression level of NF-κB is higher in HSG-AZA3 cells than HSG cells and SCC cells.

We used only HSG as a salivary gland carcinoma cell line because it is the first cell line established from salivary gland origin and is used commonly in the world. HSG cells originated from irradiated submandibular gland, not from AdCC. In order to confirm our hypothesis that the SDF-1/CXCR4 system is involved in invasion and metastasis of salivary gland carcinoma, we should investigate various types of salivary gland carcinoma cell lines including the AdCC cell line. Such experiments remain to be done in future.

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