Involvement of an Autocrine Stromal Cell–Derived Factor-1/CXCR4 System on the Distant Metastasis of Human Oral Squamous Cell Carcinoma

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Abstract

We have previously shown that a stromal cell–derived factor-1 (SDF-1; CXCL12)/CXCR4 system is involved in the establishment of lymph node metastasis, but not in that of distant metastasis, in oral squamous cell carcinoma (SCC). In this study, we investigated the role of the autocrine SDF-1/CXCR4 system, with a focus on distant metastasis in oral SCC cells. The immunohistochemical staining of SDF-1 and CXCR4 using primary oral SCCs and metastatic lymph nodes showed a significantly higher number of SDF-1–positive cases among the metastatic lymph nodes than among the primary SCCs, which was associated with a poor survival rate among those of the former group. The forced expression of SDF-1 in B88 cells, which exhibit functional CXCR4 and lymph node metastatic potential (i.e., the autocrine SDF-1/CXCR4 system), conferred enhanced cell motility and anchorage-independent growth potential onto the cells. Orthotopic inoculation of the transfectant into nude mice was associated with an increase in the number of metastatic lymph nodes and more aggressive metastatic foci in the lymph nodes. Furthermore, the SDF-1 transfectant (i.e., the autocrine SDF-1/CXCR4 system) exhibited dramatic metastasis to the lung after i.v. inoculation, whereas the mock transfectant (i.e., the paracrine SDF-1/CXCR4 system) did not. Under the present conditions, AMD3100, a CXCR4 antagonist, significantly inhibited the lung metastasis of the SDF-1 transfectant, ameliorated body weight loss, and improved the survival rate of tumor-bearing nude mice. These results suggested that, in cases of oral SCC, the paracrine SDF-1/CXCR4 system potentiates lymph node metastasis, but distant metastasis might require the autocrine SDF-1/CXCR4 system. (Mol Cancer Res 2007;5(7):685–94)

Introduction

Chemokines are a large family of small (7-15 kDa), structurally related heparin-binding proteins that have been identified as attractants of different types of blood leukocytes to sites of infection and inflammation (1, 2). They are produced locally in the tissues and act on leukocytes through selective membrane-bound G–protein–coupled receptors, the two major subfamilies of which have been designated as CCR and CXCR. Among these chemokines and their receptors, the stromal cell–derived factor-1 (SDF-1; also referred to as the CXCL12)/CXCR4 system has been shown to be involved primarily in the site-specific distant metastasis of several types of cancer (3–9). We have reported that SDF-1 produced in lymph nodes exerts an attractive force on lymph node metastasis in CXCR4-expressing oral squamous cell carcinoma (SCC; refs. 10–12), and we also showed frequent expression of CXCR4 in ~60% of oral SCCs at the primary site (13). Moreover, as regards cases of head and neck SCCs, including those in the oral, nasopharyngeal, and esophageal regions, several investigators have shown that this system is involved in lymph node metastasis (14–17). However, a recent report noted that a SDF-1/CXCR4 gradient regulated primarily hematogenous distant metastasis rather than lymph node metastasis (18). Clinically, oral SCCs are known to frequently metastasize to the lymph nodes, but rarely to the distant organs; the rate of the latter type of metastasis has been reported to be <20% of oral SCC (19–21). Based on these observations, it is likely that a SDF-1/CXCR4 gradient might regulate lymph node metastasis, but only in certain types of cancer such as head and neck SCCs; however, the reasons for which head and neck SCCs do not metastasize to the distant organs, despite the frequent expression of CXCR4, remains largely unknown.

One possible explanation for this phenomenon is that ectopic growth potential at distant sites in oral SCC is weaker than that in other types of cancer, even in cases when the cancer cells are able to reach the distant organs via the SDF-1/CXCR4 gradient. Previously, it has been suggested that at early stages of metastatic progression, paracrine growth mechanisms might dominate growth signals, whereas at later stages of metastatic progression, autocrine growth mechanisms might dominate the
growth responses of metastatic cells (22, 23). Recently, several growth factors and their receptor systems have been suggested to contribute via an autocrine mechanism to distant metastatic spread in several types of cancer (24-28). As described above, the SDF-1 produced by metastatic sites plays an attractive and paracrine role in the cancer cells with CXCR4 expression; however, the autocrine role played by this system in distant metastasis remains unknown. In this study, we examined whether the autocrine SDF-1/CXCR4 system contributes to the distant metastasis of oral SCC.

**Results**

**Expression of SDF-1 and CXCR4 in the Metastatic Lymph Nodes of Patients with Oral SCC**

In primary oral SCC tissue samples, SDF-1 expression was frequently detected in normal and cancer stromal cells, but was infrequently detected in cancer cells (13). For the establishment of distant metastasis, cancer cells must intravasate into (newly synthesized) micro–blood vessels or flow out from the metastatic lymph nodes. As regards cases of oral SCCs, the latter mechanism is thought to be critical because patients with distant metastases had high incidence (at the rate of 93%) of lymph node metastases (21). Thus, considering the hypothesis of the contribution of autocrine SDF-1/CXCR4 signaling on the distant metastatic spread of oral SCC, we first examined the expression of SDF-1 and CXCR4 proteins in primary oral SCC cells and also in metastatic cancer cells of lymph nodes (Fig. 1Aa-Ad). Thirty cases of oral SCC with metastatic lymph nodes were examined, and the expression of CXCR4 was found to be almost equivalent in the primary tumors and in the metastatic lymph nodes (Table 1). However, as regards SDF-1, the rate of positive expression in the metastatic lymph nodes (16 of 30) was significantly higher than that in the primary tissues (2 of 30; Table 1; \( P < 0.001 \), Wilcoxon signed rank test). Among these samples, some spindle cancer cells expressing both CXCR4 and SDF-1 were detected (Fig. 1Ac and Ad). The number of SDF-1– and CXCR4-positive metastatic lymph nodes was 14 out of a total of 30 samples. To confirm the specificity of these immunohistochemical results, we did immunohistochemistry, using metastatic lymph nodes formed in nude mice inoculated with mock or SDF-1 transfectants described in Fig. 2. SDF-1 staining could be dramatically detected only in the SDF-1 transfectant (data not shown). Thus, these results indicated that approximately half of the cases of oral SCC with metastatic lymph nodes had acquired autocrine SDF-1/CXCR4 signaling in those lymph nodes.

**Association between Expression of SDF-1/CXCR4 and Survival**

Next, we investigated the association between the expression of SDF-1/CXCR4 and the survival of patients with oral SCC. The 5-year survival rate was 25.0% for the SDF-1–positive group and 71.4% for the SDF-1–negative group; that is, a significantly poorer outcome was observed for the SDF-1–positive group (\( P = 0.023 \), Fig. 1B). Among the cases of mortality involving SDF-1–positive cancer cells in the lymph nodes, secondary lung metastases were identified in six patients by computed tomography, indicating that autocrine SDF-1/CXCR4 might be involved in the distant metastasis. In addition, we also found a significant association in terms of the 5-year survival rate between the CXCR4-negative group (71.4%) and the CXCR4-positive group (39.1%) in the primary tumor (\( P = 0.035 \), data not shown).

**Phenotypic Switch Induced by the Overexpression of SDF-1 in Oral SCC Cells**

Because autocrine SDF-1/CXCR4 might contribute to the distant metastasis and poor prognosis of oral SCC, we isolated a SDF-1 stable transfectant in the CXCR4 positive oral SCC cells, B88, which expressed high levels of CXCR4. By means of reverse transcription-PCR (RT-PCR) and ELISA, this transfectant (B88-SDF-1) was found to express high levels of SDF-1 mRNA and protein (Fig. 2A and B). Autonomous extracellular signal-regulated kinase 1/2 and Akt/PKB activation were observed in B88-SDF-1 cells, indicating that this transfectant
had acquired an autocrine SDF-1/CXCR4 system (Fig. 2C). We examined the expression of CXCR4 protein on the cell surface in the mock and B88-SDF-1 cells by use of fluorescence-activated cell sorting. However, we could not detect the significant change of CXCR4 expression in our assay system (data not shown). Although the overexpression of SDF-1 in B88 did not alter the anchorage-dependent growth of the cells (Fig. 5A), this transfectant significantly acquired chemokinetic response (Fig. 3A) and enhanced cell motility (Fig. 3B). Moreover, B88-SDF-1 cells also acquired the anchorage-independent growth potential compared with that of mock cells (Fig. 3C and D). We did the same experiment using an oral SCC cell line, HNt, in which the expression of CXCR4 is 7.5-fold lower than that in B88 cells (13). However, HNt-SDF-1 cells did exhibit slight, but not significant, phenotypic changes in vitro and in vivo, probably due to the reduced expression of CXCR4, compared with that of B88 cells (data not shown).

**Metastatic Potential in B88-SDF-1 Cells in vivo**

Mock and B88-SDF-1 cells were orthotopically inoculated into the masseter muscle of nude mice (29). The size of the primary tumor did not differ between the two groups (data not shown). Although both mock and B88-SDF-1 cells histopathologically metastasized to the cervical lymph nodes, the weight of lymph nodes containing B88-SDF-1 cells was significantly heavier than that of lymph nodes containing mock cells (P = 0.0033; Fig. 4A). Moreover, we observed an increase in the number of metastatic lymph nodes and more aggressive metastatic foci in the lymph nodes containing B88-SDF-1 cells (Fig. 4B; Table 2). However, in the orthotopic inoculation experiment, no distant metastasis of either type of cell was detected at 30 days. Because nude mice with orthotopically inoculated mock or B88-SDF-1 cells were unable to survive for a long period of time due to the obstruction of the airway and/or the blockage of food intake, we next did i.v. inoculation using this transfectant. Consequently, the lungs with B88-SDF-1 cells were significantly heavier than those with mock cells (P = 0.0008; Fig. 4C). Moreover, numerous metastatic nodules were detected in the lungs in all of the mice inoculated with B88-SDF-1 cells (Fig. 4D, right). In contrast, lung metastasis was not observed in any of the mice inoculated with the mock transfectant (Fig. 4D, left). These results indicate that autocrine, rather than paracrine, SDF-1/CXCR4 signaling is necessary for the distant metastasis of oral SCC cells.

**Effect of a CXCR4 Antagonist, AMD3100, on the Metastasis of B88-SDF-1 Cells to the Lung**

To assess the direct involvement of the autocrine SDF-1/CXCR4 system on the metastasis of B88-SDF-1 cells to the lungs, we first examined the effect of AMD3100, an antagonist for CXCR4, on the in vitro cell growth and motility. Although AMD3100 (1 μg/mL) did not influence the growth of the cells (Fig. 5A), AMD3100 significantly inhibited the enhanced motility of B88-SDF-1 cells both in wound assay (data not shown) and in transwell assay (Fig. 5A). Thus, we next treated the B88-SDF-1 tumor–bearing mice with AMD3100. Metastatic nodules in the lungs were markedly inhibited by treatment with AMD3100, according to both macroscopic (data not shown) and histopathologic (Fig. 5B, top) data during the 50 days of observation. We also confirmed the presence of the metastatic cancer cells at the molecular level by use of RT-PCR extracted from the lungs at day 28 (Fig. 5C, middle). During the course of 25 cycles amplified by RT-PCR, no expression of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected in three of four mice treated with AMD3100, but human GAPDH was detected in three of four mice treated by saline. Moreover, the treatment with AMD3100 significantly ameliorated the body weight loss of tumor-bearing nude mice (Fig. 5D). AMD3100 also significantly prolonged the survival rate of B88-SDF-1 tumor–bearing nude mice (Fig. 5E). It cannot be ruled out that the effects of AMD3100 were due to a combined inhibitory effect on both autocrine and paracrine SDF-1; however, these results indicated that the autocrine SDF-1/CXCR4 system was indispensable for the lung metastases induced by B88-SDF-1 and that AMD3100 may be an effective inhibitor of this autocrine SDF-1/CXCR4 system both in terms of distant metastasis and on the tumor-induced cachexia.

**Discussion**

In this study, we investigated the role of the autocrine SDF-1/CXCR4 system, focusing on the distant metastasis of oral SCC

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**Table 1. Expression of SDF-1 and CXCR4 in Primary Tumor and Metastatic Lymph Node**

<table>
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<tr>
<th>No.</th>
<th>Age/Gender</th>
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<th>CXCR4</th>
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<tr>
<td>30</td>
<td>59/M</td>
<td>–</td>
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NOTE: –, negative staining; +, positive staining. Abbreviations: ND, not determined; LN, lymph node.

*Significantly increased than primary tissues (P < 0.001, by Wilcoxon signed rank test).
The findings obtained from the present series of experiments were as follows. First, the number of SDF-1–positive metastatic lymph nodes was significantly higher than that of SDF-1–positive primary oral SCCs, which was associated with a poor survival rate among those of the former group. Second, the overexpression of SDF-1 in B88 cells conferred enhanced cell motility and anchorage-independent growth potential. Third, the transfectant acquired aggressive metastatic potential, including metastasis to the lymph nodes and lungs. Fourth, AMD3100, a CXCR4 antagonist, significantly inhibited the metastasis to the lungs of this transfectant as well as reducing tumor-induced cachexia. These results suggested that, in cases of oral SCC, the paracrine SDF-1/CXCR4 system potentiates lymph node metastasis, but the acquisition of an autocrine loop might be required for distant metastasis.

The clinical results showed a significantly higher number of SDF-1–positive cases among the metastatic lymph nodes than among the primary oral SCCs, which was associated with the poor survival rate among those of the former group. Unlike other CXC chemokines, SDF-1 is basally expressed in numerous tissues and in particular in mesenchymal cells (30, 31). The promoter of sdf1 contains several CpG islands, a transcription factor–binding motif commonly observed in housekeeping genes (30). Moreover, binding motifs for the transcription factors nuclear factor-κB and activator protein-1, which are commonly observed in other CXC chemokines, have not yet been found in the 19-kb sdf1 genomic clone (30). Therefore, it has generally been assumed that SDF-1 is a constitutively expressed chemokine; however, recent reports have suggested that SDF-1 expression is potently regulated by hypoxia-inducible factor-1 (32, 33). In addition, Staller et al. (34) showed that hypoxia-inducible factor positively regulates CXCR4 expression under hypoxic conditions, but the von Hippel-Lindau tumor suppressor protein pVHL negatively regulates CXCR4 expression, due to the capacity of the latter to target hypoxia-inducible factor for degradation under normoxic conditions. Because cancer cells that have escaped from a primary site are generally exposed to hypoxia due to a poor blood supply, not only SDF-1 but also CXCR4 expression in oral SCC might be regulated by certain tumor environmental factors such as hypoxia-inducible factor-1 at the metastatic lymph node, resulting in the acquisition of the autocrine loop of the SDF-1/CXCR4 system.

Oral SCCs are characterized by a high degree of local invasiveness and a high rate of metastases to the cervical lymph nodes; however, a low rate of metastases to distant organs is observed. Patients with distant metastases had high incidence of lymph node metastases (21). Moreover, we have previously reported that oral SCC cells easily intravasated into the circulation at early stage, despite the establishment of distant metastasis (35). These results indicating that distant metastasis in oral SCC is commonly arisen from the cancer cells which flow in from the metastatic lymph node, and that the failure of distant metastasis is due to a weak potential of extravasation or ectopic growth in oral SCC cells. In the present study, the autocrine SDF-1/CXCR4 system was found to enhance the cell migration and anchorage-independent (ectopic) growth potential in vitro.

Furthermore, Iikura et al. (36) reported that the activation of the SDF-1/CXCR4 system caused strong transendothelial migration (extravasation) in basophils. These observations suggest that the autocrine SDF-1/CXCR4 system might induce extravasation or the ectopic growth potential of oral SCC cells, which are considered indispensable for the establishment of distant metastasis. During these processes, oral SCC cells are also...
exposed to disconnected paracrine SDF-1 gradient; however, constitutive autocrine signaling might be critical for the establishment of distant metastasis. Collectively, it is assumed that the following steps are required for the distant metastasis of oral SCC cells via SDF-1/CXCR4 system. First, CXCR4-related oral SCC cells metastasize to cervical lymph nodes via paracrine SDF-1 gradient, and acquire SDF-1 expression in the lymph node. Second, the cancer cells flow out from the lymph node, and

![Figure 3](image)

**FIGURE 3.** Phenotypic switch by the autocrine SDF-1/CXCR4 system in oral SCC cells. **A.** Enhanced migration of B88-SDF-1 cells (black columns) compared with that of mock transfectant cells (white columns). Cells were seeded at $5 \times 10^4$ per well. After staining, the cells that plugged the pores and the cells attached to the lower surface of the membrane were counted in 10 fields under high-power magnification ($\times 400$). Bars, SD of triplicate samples. **B.** Enhanced wound healing associated with the B88-SDF-1 cells (middle) compared with that of mock (top) transfectants. After a generation of a linear wound on the confluent monolayers, cells were photographed at the same location on a grid 48 h later. The wound areas in each transfectant were calculated by NIH image (bottom). $^*P = 0.0043$. **C.** Acquisition of colony formation in soft agar of the B88-SDF-1 (right) transfectants. Top and bottom, cell colony formation at days 14 and 28, respectively. Magnification, $\times 400$. **D.** The number of mock-transfectant (white columns) and B88-SDF-1 (black columns) colonies that formed. Columns, mean of triplicate samples; bars, SD. Data are representative of two separate experiments with similar results. $^*P < 0.001$ (statistically significant by one-way ANOVA).
enter superior vena cava and right atrial of the heart, which are pushed out from right ventral into the lungs, and attach to the alveolar capillary wall. Third, only the cells exhibiting SDF-1/CXCR4 autocrine loop could dominantly extravasate and grow into the alveolar space of the lungs.

Muller et al. (3) showed that paracrine SDF-1 produced by target organs such as the lung, liver, and bone marrow attracts CXCR4-expressing breast cancer cells. However, in the present study, oral SCC cells with an autocrine SDF-1/CXCR4 loop acquired aggressive metastatic potential, including that to the lymph nodes and lungs; however, cells exhibiting a paracrine loop did not metastasize to any distant organs, not even when introduced by i.v. inoculation. These observations suggest that paracrine SDF-1/CXCR4 signaling is insufficient for achieving distant metastasis in the case of oral SCC. Recent reports have shown that SDF-1 transactivates HER2-neu (37), which was shown to be amplified in 30% of breast cancers (38). Moreover, Li et al. (39) showed that HER2 enhances the expression of CXCR4, which is required for HER2-mediated invasion in vitro and lung metastasis in vivo. However, in the case of oral SCC, HER2 overexpression has been reported to be very rare; that is, rates of 3.6% (40), and even 0%, have been reported (41). Thus, breast cancer cells might use both paracrine SDF-1/CXCR4 and SDF-1/HER2 costimulatory pathways for the establishment of distant metastasis. In contrast to breast cancer, oral SCC might require strong constitutive SDF-1/CXCR4 autocrine signaling for the establishment of distant metastasis. Indeed, the autocrine mechanism of the SDF-1/CXCR system has been reported in studies of prostate cancer cells (42), thyroid cancer cells (9), osteosarcoma cells (43), and Kaposi’s sarcoma cells (44), all of which are aggressive and highly distant metastatic tumors. These results indicate that

**FIGURE 4.** Enhancement of lymph node metastasis and acquisition of lung metastasis in B88-SDF-1 cells. A and B. Cells were inoculated into the masseter muscle of nude mice ($2 \times 10^6$), which were sacrificed at day 30. A. Mean weight of the bilateral cervical lymph nodes extracted from tumor-bearing nude mice. *, $P = 0.0033$ (one-way ANOVA). B. Metastatic cancer cells (brown zone) in the lymph nodes were immunostained by cytokeratin AE1/AE3 antibody. Data are representative of four lymph nodes, and the percentage staining area in all the lymph nodes is shown in Table 2. C and D. Cells ($1 \times 10^6$) were inoculated into the blood vessels of nude mice, which were sacrificed at day 30. C. Mean weight of the lung in mock transfectant (white column) and B88-SDF-1 (black column) cells. **, $P = 0.0008$ (one-way ANOVA). D. Macroscopic (top) or histopathologic (bottom) lung features extracted from mock transfectant–inoculated (left) or B88-SDF-1–inoculated (right) nude mice.
The metastatic area in the lymph nodes was counted by the NIH defined as immunohistochemistry positive those cases in which antibody (R&D Systems, Inc.) as described previously (13). We might be used as an effective chemotherapeutic agent for patients with CXCR4-related oral SCC, and may also be a useful palliative drug for patients with advanced oral SCC. With AMD3100 sequencer (Applied Biosystems). Fidelity of the cloned sequences were confirmed by use of ABI reading frame, Xho. This fragment, containing the human SDF-1 open reading frame, was ligated to the cloning site of pEB6-CAG-MCS. The integrity and replication (53). The human SDF-1 cDNA fragment was amplified from placenta cDNA using a pair of specific primer, SDF-1UP, CCGCTCGAGCGCGCCATGAACGCCAAGGTC-GTG, and SDF-1DN, CGGAATTCCATCTTGAACCTCTGGAGGCT, and mGAPDH-DN, TGCATTGCTGACAAATCTT-GAGTGA, respectively. These findings indicate that AMD3100 might also ameliorate tumor-induced cachexia in affected patients. In the future, AMD3100 might be used as an effective chemotherapeutic agent for patients with CXCR4-related oral SCC, and may also be a useful palliative drug for patients with advanced oral SCC.

Materials and Methods

Patients and Immunohistochemistry

Thirty patients with lymph node metastasis of advanced oral SCC classified stage IV (by International Union Against Cancer) were included in this study. Biopsy specimens and metastatic lymph nodes were obtained from 1990 to 1999 at the Second Department of Oral and Maxillofacial Surgery, Tokushima University Dental Hospital. All the patients selected in this study were primary treated by radiation, chemotherapy, and immunotherapy, as described previously (52). Immunohistochemistry was done with anti-CXCR4 monoclonal antibody (12G5; BioSource International) or anti–SDF-1α monoclonal antibody (R&D Systems, Inc.) as described previously (13). We defined as immunohistochemistry positive those cases in which over 25% of the cancer cells were stained for CXCR4 or SDF-1. The metastatic area in the lymph nodes was counted by the NIH image 1.63 after staining by the cytokeratin AE1/AE3 antibody (Dako Corporation).

Mice and In vivo Study

BALB/c nude mice were purchased from CLEA Japan. The mice were maintained under pathogen-free conditions and were handled in accordance with the Guidelines for Animal Experimentation of Tokushima University. The experiments were initiated when the mice were 8 weeks of age and were done as described previously (29). The presence or absence of lymph node and distant metastasis was confirmed by the H&E staining. In the experimental chemotherapy, every mouse was s.c. treated by an AMD3100 (2.5 mg/kg; Sigma) or same volume of saline at 48 h after the i.v. inoculation of the cells, as described previously (49). In some experiments, total RNA was prepared from lung using TRIzol (Invitrogen), and RT-PCR was done using a common upper primer, GAPDH-UP, CATCAC-CATCTTCCAAGGAGCA; and human and mouse specific lower primers, hGAPDH-DN, GCCATTGCTGATGACCTTG-GAGGCT, and mGAPDH-DN, TGCATTGCTGACAATCTT-GAGTGA, respectively.

Cells and Cell Culture

B88 cells and HNt cells were maintained in DMEM supplemented with 10% FCS, 100 µg/mL streptomycin, and 100 units/mL penicillin in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Construction of a Mammalian Expression Vector, pEB6-SDF-1

EBV-based vector, pEB6-CAG-MCS, is an extrachromosomal vector carrying a replicational origin, oriP and a replication initiation factor (EBNA-1), which are sufficient for autonomous replication (53). The human SDF-1 cDNA fragment was amplified from placenta cDNA using a pair of specific primer, SDF-1UP, CCGCTCGAGCGCGCCATGAACGCCAAGGTC-GTG, and SDF-1DN, CGGAATTCATCCATGACCCCTG-TTTAAGC. This fragment, containing the human SDF-1 open reading frame, XhoI site at 5’ end, and EcoRI site at 3’ end, was ligated to the cloning site of pEB6-CAG-MCS. The integrity and fidelity of the cloned sequences were confirmed by use of ABI 3100 sequencer (Applied Biosystems).

Stable Transfection

After seeding cells (5 × 10⁶ per dish), the cells were transfected with 5 µg of pEB6-SDF-1 or pEB6-CAG-MCS, using Superfect (Qiagen). Forty-eight hours later, the cells were switched to a selective medium containing geneticin (400 µg/mL G418; Invitrogen Corp.). All of the G418 resistant clones were collected after 14 days of cultivation in the selective medium.

Enzyme-Linked Immunosorbent Assay

ELISA for SDF-1α was done by Quantikine kit (R&D) as described previously (10).

Western Blotting

Western blotting was done as described previously (10). The nitrocellulose membrane (Amersham Pharmacia Biotech)
was incubated with primary antibodies against phosphorylated-or total-extracellular signal-regulated kinase 1/2 and Akt/PKB (Cell Signaling Technology), followed by horseradish peroxidase–conjugated secondary antibodies. Detection was then done using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

**Wound Assay**

At 24 h after seeding the cells, a linear wound was generated on the confluent monolayers by scraping with a pipette chip with our without 1 μg/mL of AMD3100. Unattached cells were washed off with agitation. Cells were photographed at the same point on a grid at 48 h later. Each line was plated and wounded in triplicate.
In vitro Cell Migration Assay

The in vitro migration of oral SCC cells was evaluated using the Transwell (Corning) as described previously (10). The plugged cells in the pore or the cells attached to the lower surface of the membrane were counted in 10 fields at high-power view (×400) by a third person without any knowledge of the treatments. In some experiments, 1 μg/mL of AMD3100 was co-incubated with the cells, which were seeded on the upper chamber.

Soft Agar Assay

Transfectants (B88-SDF1 or B88-EB6) were seeded at a density of 1 × 10⁴, 1 × 10⁵, or 1 × 10⁶ per well in six-well plates in 2 mL of 0.6% agar (Wako) supplemented with DMEM in the presence of 10% FCS. After 14 and 28 days, colonies containing >20 cells were counted.

Statistical Analysis

Statistical differences between the means for the different groups were evaluated with StatView 4.5 (Abacus Concepts) using one-way ANOVA, with the level of significance at P < 0.05. The cause-specific survival rates were calculated by the Kaplan-Meier method and compared using the log-rank test. The significance level was set at 5% for each analysis. All experiments were repeated twice to thrice, and similar results were obtained.

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References

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