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Blockade of CXCR4 in oral squamous cell carcinoma inhibits lymph node metastases

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ABSTRACT

We have previously demonstrated that a stromal cell-derived factor-1 (SDF-1; CXCL12)/CXCR4 system is involved in the establishment of lymph node metastasis in oral squamous cell carcinoma (OSCC). In this study, we investigated whether the blockade of CXCR4 inhibits lymph node metastasis in B88 OSCC cells. These cells harbour a functional CXCR4 and have the potential to metastasise to the lymph node *in vivo*. Following introduction of a vector that expresses short hairpin small interfering RNA (shRNA) against CXCR4, we isolated three clones (shCXCR4-16, -17 and -21) that showed decreased expression of CXCR4 mRNA. These clones also had reduced CXCR4 protein levels and showed impairments in calcium flux and cell migration in response to SDF-1. These cells were orthotopically inoculated into the masseter muscle of nude mice. Lymph node metastases, loss in body weight and tumour volumes were significantly inhibited in mice inoculated with shCXCR4-17 cells compared to mice inoculated with control cells. SDF-1-induced migration of B88 cells was significantly inhibited *in vitro* by the treatment with 1,1′-[1,4-phenylenebis(methylene)]bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride (AMD3100), a CXCR4 antagonist. Subcutaneous administration of AMD3100 significantly inhibited the lymph node metastases of B88 cells when they were orthotopically inoculated into the masseter muscle of nude mice. Moreover, the enhanced production of interleukin (IL)-6 and IL-8 in response to SDF-1 was inhibited by shRNA against CXCR4 or by treatment with AMD3100. These results suggest that blockade of CXCR4 may be a potent anti-metastatic therapy against lymph node metastases in cases of CXCR4-related OSCC.

1. Introduction

Oral cancer, excluding pharyngeal cancer, represents 2.1% of all human malignancies worldwide.[1] Most oral cancer is histopathologically diagnosed as squamous cell carcinoma (SCC). Oral SCCs (OSCCs) are characterised by a high degree of local invasion and a high rate of metastases to cervical lymph nodes. In particular, lymph node metastasis has been shown to directly affect the prognosis of patients with OSCC.[2,3] This effect is a result of the extracapsular spread of cancer cells or secondary distant metastasis. Thus, novel therapies that target lymph node metastases in OSCC are desired.

Chemokines are a large family of small (7–15 kDa), structurally related heparin-binding proteins that are attractants of different types of blood leucocytes to sites of infection and inflammation.[4] They are produced locally in the tissues

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and act on leucocytes through selective membrane-bound G protein-coupled receptors whose two major subfamilies are designated CCR and CXCR. Among these chemokines and their receptors, the stromal cell-derived factor-1 (SDF-1; also known as CXCL12)/CXCR4 system is involved in lymph node or distant metastasis in several types of cancer.[5–11] We have also reported that lymph node metastatic OSCC cells specifically express functional CXCR4, and the SDF-1/CXCR4 system is involved in the metastatic process of OSCC to the lymph node.[12–16]

In view of the importance of CXCR4 in a variety of cancers, including OSCC, blockade of CXCR4 has been investigated as a potential therapeutic target. For example, Müller and colleagues have shown that administration of a CXCR4-neutralising antibody into tumour-bearing mice inhibits lymph node and lung metastases in their breast cancer model.[5] However, administration of a neutralising antibody to patients would incur a huge expense due to the large volume that would be needed. Therefore, synthetic compound(s) that could be easily produced are desirable for cancer treatment. Recently, small interfering RNA (siRNA) against CXCR4 or synthetic CXCR4 antagonists such as bicyclam 1,1′-[1,4-phenylenebis(methylene)]bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride (AMD3100),[17] horseshoe crab-derived peptide T22[18] and the non-peptide TAK-779[19] have been developed. These agents are being clinically tested for their ability to inhibit HIV infection or to mobilise haematopoietic stem cells. However, it is unknown whether the inhibition of CXCR4 by siRNA or synthetic compounds has any effect on lymph node metastasis in OSCC. In this study, we investigated whether the blockade of CXCR4 by siRNA and the AMD3100 synthetic compound inhibits lymph node metastasis in B88[12] OSCC cells, which contain functional CXCR4 and have the potential to metastasise to lymph nodes.

2. Materials and methods

2.1. Mice and in vivo study

BALB/c nude mice were purchased from CLEA Japan (Osaka, 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 performed as described previously.[13] Briefly, B88 cells (2×10^6) containing shRNA against CXCR4 (shCXCR4-17), or containing constitutively expressed firefly luciferase (B88-luc), were orthotopically inoculated into the masseter muscle of nude mice. In the experimental chemotherapy regimen, every mouse was subcutaneously treated with AMD3100 (2.5 mg/kg; Sigma), or the same volume of saline, 24 h after cell inoculation, as described previously with a slight modification.[16,20] The tumour volume was estimated by measuring the tumour size and using the following formula: tumour volume = $1/2 \times L \times W^2$, where L and W represent the largest diameter and the smallest diameter, respectively. The presence or absence of lymph node metastases was confirmed by the haematoxylin–eosin staining in shCXCR4-17 cells or by luciferase assay in B88-luc cells.

2.2. Cells and cell culture

B88 cells were originally established from a patient with tongue cancer[12] and deemed free of mycoplasma and bacterial contaminants. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (V/V) faetal calf serum (FCS), 100 μg/ml streptomycin, and 100 U/ml penicillin in a humidified atmosphere of 95% air (V/V) and 5% CO_2 (V/V) at 37 °C.

2.3. Transfection

A CXCR4 shRNA expression vector and a control vector were generous gifts from Drs. Jianhua Wang and Russell S. Taichman (University of Michigan School of Dentistry, Ann Arbor, MI).[21] Cells (5×10^5 cells/dish) were seeded in 100 mm culture dishes (Falcon; Becton Dickinson Labware) in DMEM supplemented with 10% FCS. Twenty-four hours later, the cells were transfected with 5 μg of the CXCR4 shRNA expression vector or the control vector, using superfect transfection reagent (Qiagen). The cells were incubated for 24 h in DMEM containing 10% FCS (V/V) and were subsequently trypsinised and seeded (1:5 ratio) in 100 mm culture dishes in DMEM medium containing 10% FCS (V/V). Forty-eight hours later, the cells were placed in a selective medium containing Geneticin (700 μg/ml G418; Invitrogen Corp.). After 14 days of culture in the selective medium, 24 representative mock or shCXCR4 G418-resistant clones were isolated and expanded in a 24-well cluster dish (Falcon).

2.4. Quantitative RT-PCR

After the isolation of stable transfectants, the preparation of total RNA and reverse transcription was performed as described previously.[14] In quantitative PCR, CXCR4 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were detected using QuantiTect™ Gene Expression Assays (Hs-CXCR4; Qiagen) or the Taqman™ Gene Expression Assay (Applied Biosystems), respectively. Gene-specific products were measured continuously by an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) during 40 cycles. Experiments were repeated at least three times.

2.5. Flow cytometric analysis

Logarithmically growing cells were trypsinised and fixed in 4% paraformaldehyde (V/V) on ice for 10 min. The cells were washed and incubated with anti-CXCR4 mAb (dilution 1:100; 12G5; BioSource International, Inc.) for 30 min at room temperature. After being washed twice with D-PBS (–), the cells were incubated with PE-labelled goat anti-mouse IgG (Serotec) for 30 min at room temperature and analysed with an EPICS flow cytometer (Coulter).

2.6. Calcium flux assay

Calcium flux was performed using an EPICS flow cytometer as described previously.[12]

2.7. MTT assay

Cells were seeded on a 96-well plate (Falcon; Becton Dickinson Labware) at 5×10^3 cells per well in DMEM containing 10% FCS. Twenty-four hours later, the cells were treated with or without 1 μg/ml of AMD3100 for 48 h. The number of cells was quantified by an assay using MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma].

2.8. In vitro cell migration assay

The *in vitro* migration of B88 cells was evaluated using the Transwell (Corning) as described previously.[12] 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 blinded to treatment conditions. In some experiments, 1 μg/ml of AMD3100 was co-incubated with the cells seeded on the upper chamber.

2.9. Enzyme-linked immunosorbent assay (ELISA)

ELISA for interleukin (IL)-6, IL-8, vascular endothelial growth factor (VEGF), tumour necrosis factor (TNF)-α or leukaemia inhibitory factor (LIF) was performed by an ELISA kit (Biosource) according to the manufacturer's instructions. Conditioned media from the cells were collected and prepared as described previously.[16]

2.10. Statistical analysis

Statistical differences between the means for the different groups were evaluated with StatView 4.5 (Abacus Concepts) using one-way ANOVA significance set at $p < 0.05$.

3. Results

3.1. Expression of CXCR4 in the transfectants containing shRNA against CXCR4

In order to investigate whether the blockade of CXCR4 inhibits lymph node metastases, an shRNA expression vector against CXCR4 was transfected into B88 cells. Twenty-four transfectants were isolated and the expression of CXCR4 mRNA was examined by a quantitative RT-PCR. We isolated three clones (shCXCR4-16, -17 and -21) that showed effective downregulation of CXCR4 mRNA (Fig. 1A). Flow cytometric analysis was used to examine the CXCR4 protein expression. Similar to the quantitative RT-PCR results, the levels of CXCR4 protein were downregulated in shCXCR4-16 (39.4% positive) and shCXCR4-17 (32.6% positive) compared to mock cells (77.6% positive; Fig. 1B).

3.2. Functional downregulation of CXCR4 in the transfectants with shRNA against CXCR4

To confirm that CXCR4 expression in these transfectants was functionally suppressed, mock and shCXCR4-17 cells were treated with the CXCR4 ligand, SDF-1α, and calcium fluxes were monitored by flow cytometry. As shown in Fig. 2A, recombinant human SDF-1α treatment rapidly induced characteristic calcium fluxes in mock cells, an effect that was only faintly observed in shCXCR4-17 cells. Next, migration assays were performed to examine the effects of SDF-1α on the motility of the transfectants. Mock cells migrated significantly towards recombinant SDF-1α. In contrast, shCXCR4-16 and -17 cells did not migrate towards SDF-1α (Fig. 2B). We also evaluated the effect of shRNA against CXCR4 on the growth of B88 cells. The shRNA against CXCR4 did not inhibit the growth of these transfectants regardless of the absence or presence of SDF-1α (data not shown).

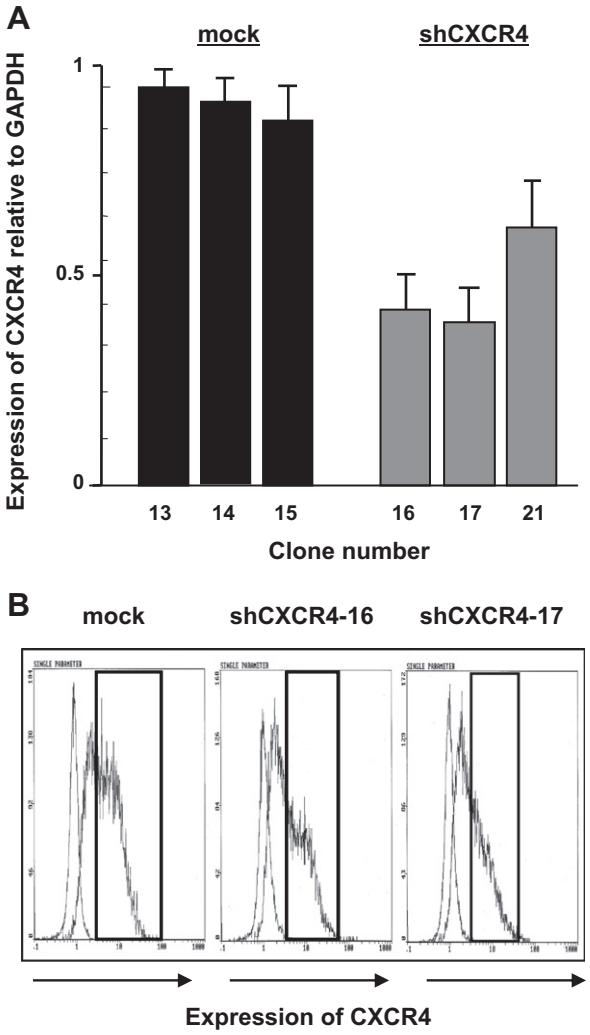

Fig. 1 – Downregulation of CXCR4 expression by the introduction of shRNA against CXCR4. B88 cells were transfected with a CXCR4 shRNA expression vector or a control vector. After selection with G418, stable transfectants were isolated. The results of quantitative RT-PCR (A) and flow cytometric analysis (B) in the independent clones are shown.

3.3. Effect of decreased-CXCR4 expression on lymph node metastasis in B88 cells

Mock and shCXCR4-17 cells were orthotopically inoculated into the masseter muscle of nude mice. The number and

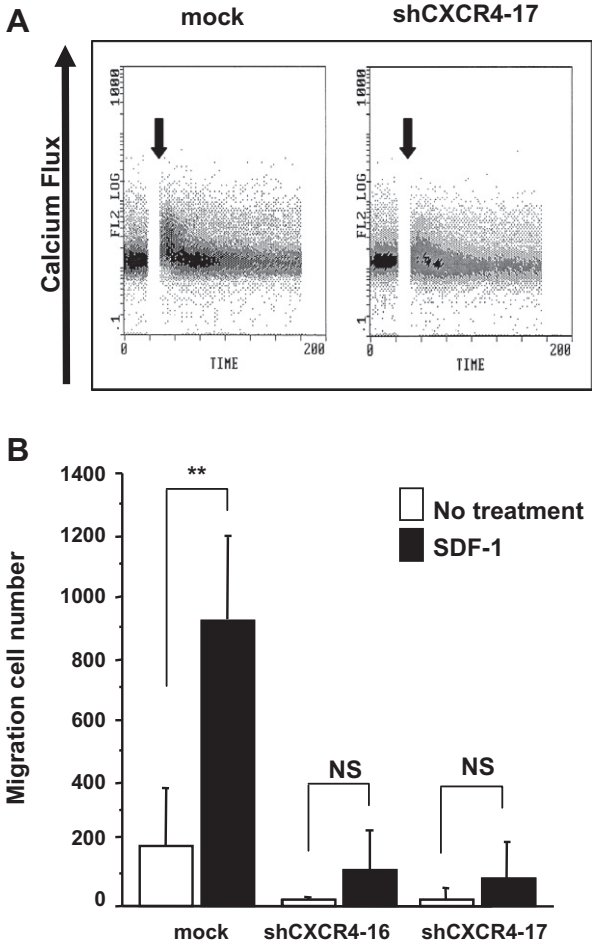

Fig. 2 – Functional downregulation of CXCR4 in the shRNA transfectants against CXCR4. (A) Ca^{2+} flux on logarithmically growing mock (*left panel*) and shCXCR4-17 (*right panel*) cells in response to SDF-1α (100 ng/ml) were determined with flow cytometry at 525 nm. *Arrow* indicates the addition of SDF-1α. (B) Mock (*white box*) and shCXCR4 (*black box*) cells were seeded on the upper membrane in the presence or absence of the indicated concentrations of SDF-1α in the lower compartment. After 24 h, cells attached under the surface of the membrane were stained by haematoxylin–eosin and counted. The bars show the SD of triplicate samples. Data are representative of three separate experiments with similar results. $^{**}p < 0.01$ when compared to the control by one-way ANOVA and NS; not significant.

the weight of metastatic lymph nodes significantly increased in the mice inoculated with mock cells compared to those harbouring shCXCR4-17 cells (Table 1, Fig. 3). Although both mock cells and shCXCR4-17 cells formed tumours in the masseter muscles of nude mice, there were significant differences in the primary tumour size between the two groups (Table 1). Furthermore, shCXCR4-17 tumour-bearing nude mice were significantly heavier than the mock tumour-bearing nude mice at 28 days following cell inoculation (Table 1). These results indicated that suppressing CXCR4 expression contributes to inhibition of lymph node metastases, primary tumour growth and tumour-induced cachexia.

3.4. *Effect of AMD3100, a CXCR4 specific antagonist, on the metastasis of B88 cells to the lymph node*

We examined the effect of AMD3100, a specific antagonist against CXCR4, *in vitro* and *in vivo* to confirm that inhibition of lymph node metastasis by shRNA against CXCR4 was not the result of clonal heterogeneity of the cells or off-target effects of shRNA. Although AMD3100 (1 μg/ml) did not alter the growth of B88 cells (data not shown), the same concentration of AMD3100 significantly inhibited the enhanced-motility of B88 cells that occurred in response to SDF-1 (Fig. 4A). Thus, we examined the effect of AMD3100 on lymph node metastasis of B88 cells that constitutively express firefly luciferase. AMD3100 significantly inhibited the weight of cervical lymph nodes (Fig. 4B). Furthermore, luciferase activities in the lymph nodes were significantly impaired by AMD3100 treatment (Fig. 4C).

3.5. *Involvement of cytokines in the inhibition of tumour growth and tumour-induced cachexia by CXCR4 blockade in the tumour-bearing nude mice*

In order to investigate the mechanism of inhibition of tumour growth and tumour-induced cachexia by CXCR4 shRNA, we examined the production of angiogenic factors, including VEGF, IL-6 and IL-8, in addition to cachexia-related factors, including TNF-α, IL-6 and LIF. IL-6 (Fig. 5A) and IL-8 (Fig. 5B) production was significantly increased by treatment with SDF-1. There was a slight, but significant, induction of VEGF in response to SDF-1 (Fig. 5C). TNF-α and LIF could not be detected in the absence or presence of SDF-1α treatment (data not shown). IL-6 and IL-8 production were impaired by shRNA against CXCR4 and by the AMD3100 CXCR4 antagonist. Next, we examined the involvement of the MAP/ERK kinase (MEK)-extracellular-regulated kinase (ERK)1/2 and the PI3K (phosphatidylinositol 3 kinase)-Akt pathways on the induction of these cytokines. We previously found that these pathways play a role in the SDF-1/CXCR4 system.[12] Induction of IL-6 and IL-8 was inhibited by treatment with the U0126 MEK inhibitor, but not with the PI3K inhibitor (wortmannin), indicating that induction of these cytokines is mainly regulated by a MEK-ERK1/2 pathway (Fig. 5A and B). The serum concentrations of IL-6 and IL-8 in the mice inoculated with shCXCR4-17 cells were significantly reduced compared to mice inoculated with mock cells (Fig. 5D). These results indicate that tumour growth and tumour-induced cachexia mediated by the SDF-1/CXCR4 system are primarily involved in the production of IL-6 and IL-8 via a MEK-ERK1/2 pathway.

4. Discussion

RNA interference (RNAi) can be induced by a synthetic-siRNA method or an expression vector method.[22] When synthetic-siRNA is directly introduced into the cell, the suppression produces a transient response that is not suitable for *in vivo* experiments. When siRNA is expressed from a vector, a continuous RNAi effect can be expected once the vector is introduced into the cells. Thus, in the present study, we isolated three stable cell lines that contained stable knockdown of

Table 1 – Effect of inhibition of lymph node metastasis by shRNA against CXCR4 following orthotopic inoculation of nude mice.

No.	mLNs no.	mLNs weight	Tumour vol.	Body weight
shCXCR4				
1	1	13.8	406.3	20
2	0	0	405.0	20
3	0	0	392.9	19
4	1	9.5	208.3	22
Mean ± SD	0.50 ± 0.58*	5.83 ± 6.95*	353 ± 96.8*	20.3 ± 1.26*
Mock				
1	1	12.4	515.0	19
2	2	18.8	814.1	18
3	2	23.1	595.4	15
4	3	18.0	550.0	18
5	2	19.1	526.5	15
Mean ± SD	2.00 ± 0.71	18.3 ± 3.84	600 ± 124	17.0 ± 1.87

mLNs, metastatic cervical lymph nodes.
* $p < 0.05$ when compared to mock cells.

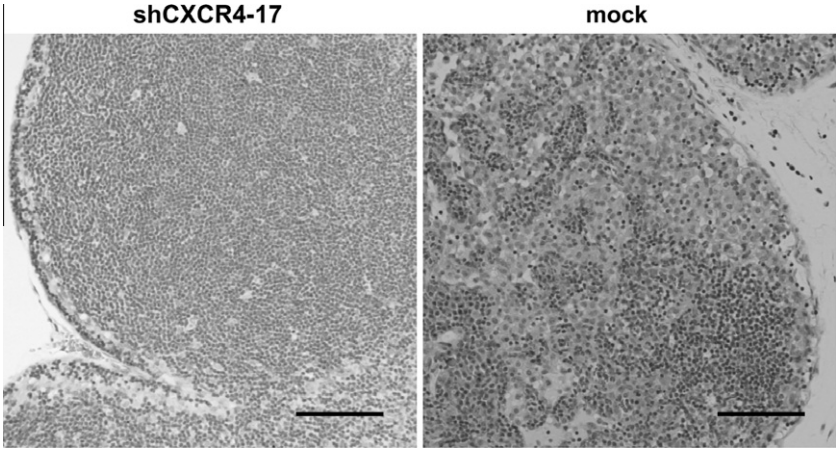

Fig. 3 – Inhibition of lymph node metastasis in the CXCR4-targeted shRNA transfectants. Mock and shCXCR4-17 cells (2×10^6) were orthotopically inoculated into the masseter muscle of nude mice. Mice were sacrificed at day 30. Lymph nodes were fixed, embedded, and stained by haematoxylin–eosin. Scale bar, 50 μm.

CXCR4, using an expression vector method. These cells were used to study lymph node metastasis *in vivo* following down-regulation of CXCR4. CXCR4 expression in these cells was only suppressed by approximately 50%; however, the metastatic function of CXCR4 was significantly regressed. This weak suppression is considered to be due to the target sequence of CXCR4 shRNA. Actually, Wang and colleagues demonstrated similar data in prostate cancer cell lines, which express similar level of CXCR4, by use of a same vector that we used in this study.[21] Moreover, we recently demonstrated that vesnarinone, a quinolinone derivative, significantly inhibits lymph node metastasis of oral cancer cells via down-regulation of CXCR4 expression; however, CXCR4 was only inhibited 50-60% following vesnarinone treatment compared to vehicle control.[23] These results indicate that lymph node metastasis that involves the SDF-1/CXCR4 system requires a high level of CXCR4 expression in OSCC cells.

In the present study, the size of tumours formed by CXCR4 knockdown cells was significantly smaller than tumours from control cells even though no differences in *in vitro* growth between knockdown cells and control cells were observed. Numerous reports have described the production of angiogenic cytokines, such as IL-6, IL-8 and VEGF, by the SDF-1/CXCR4 system in cancer cells.[21,24–26] Thus, we examined the production of angiogenic cytokines in our cancer cells. The production of IL-6 and IL-8 following treatment with SDF-1 was significantly inhibited in the CXCR4 knockdown cells *in vitro* and *in vivo*. Although we did not examine blood vessel density in the cell-derived tumours, a reduction in serum concentrations of IL-6 and IL-8 revealed that inhibition of IL-6 and IL-8 production may cause an inhibition of tumour growth in the mice inoculated with CXCR4 knockdown cells. Several investigations have demonstrated VEGF induction by the activation of Akt.[21,27] Only a faint induction of VEGF by the SDF-1/

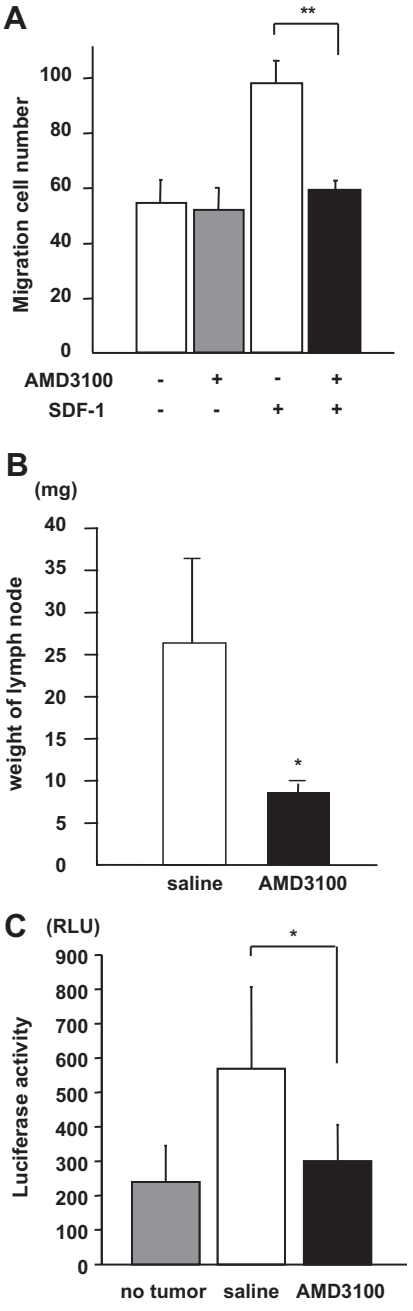

Fig. 4 – Inhibition of SDF-1-induced migration and lymph node metastasis *in vivo* by AMD3100, a specific antagonist of CXCR4. (A) The motility of B88-SDF-1 cells in the presence or absence of AMD3100 was examined by transwell assay. $^{**}p < 0.01$. (B, C) B88 cells constitutively expressing firefly luciferase (2×10^6) were orthotopically inoculated into the masseter muscle of nude mice. The mice were treated daily with AMD3100 (2.5 mg/kg) or with the same volume of saline at 24 h after cell inoculation. Mice were sacrificed at day 30. The weight (B) and luciferase activity (C) of lymph nodes were measured. The results show the means ± SD. $^{*}p < 0.05$ (statistically significant by one-way ANOVA).

CXCR4 system was observed but it was not impaired by shRNA against CXCR4 or treatment with AMD3100. One possible explanation for this effect is due to CXCR7, a recently identified chemokine receptor that binds the CXCL11 and SDF-1 chemokines.[28] SDF-1 stimulation dramatically increases VEGF levels in prostate cancer cells that overexpress CXCR7 and the levels of secreted VEGF are decreased when CXCR7 is reduced.[29] Because B88 cells express CXCR7 mRNA,[23] the induction of VEGF by SDF-1 stimulation may be involved.

Mice inoculated with CXCR4 knockdown cells were significantly heavier than mice inoculated with control cells. Additionally, the production of the cachexia-induced cytokine IL-6 was impaired in the CXCR4 knockdown cells *in vitro* and *in vivo*. Several investigators have reported the importance of IL-6 in cancer cachexia and anorexia in many types of cancer.[30] In patients with head and neck SCC, it has been suggested that the most important cytokine in cancer cachexia is IL-6.[31] Our data indicate that the suppression of CXCR4 may also inhibit cancer cachexia via the suppression of IL-6 production in CXCR4-related oral cancer.

A variety of growth factors stimulate the expression of these angiogenic cytokines via several signal transduction pathways, including ERK1/2, p38 MAPK, JNK, NF-κB and Akt, among others.[32] We have previously shown that the SDF-1/CXCR4 system in B88 cells dominantly activates both ERK1/2 and Akt pathways.[12] In the present study, the SDF-1-directed IL-6 and IL-8 production was effectively inhibited by the treatment with the U0126 MEK inhibitor, but not with the PI3K inhibitor (wortmannin), indicating that induction of these cytokines was regulated by the MEK-ERK1/2 pathway. Tang and colleagues demonstrated that the dominant-negative mutant of ERK, but not the dominant-negative mutants of p38, JNK or Akt, inhibited the SDF-1-induced production of IL-6 in oral cancer cells.[24] However, Wang and colleagues observed differential activation of MEK-ERK and PI3K-Akt pathways in different cell lines, and this activation resulted in altered secretion of proangiogenic signals, such as IL-6, IL-8, TIMP-2 and VEGF by two different pathways.[21] While it is possible that the PI3K-Akt pathway is involved in SDF-1/CXCR4 mediated-angiogenesis in other OSCC cells, the MEK-ERK pathway plays an important role in the SDF-1/CXCR4-induced expression of IL-6 and IL-8 in OSCC cells.

AMD3100, a specific antagonist of CXCR4, was used *in vitro* and *in vivo* to confirm that the inhibition of lymph node metastasis by shRNA against CXCR4 was not the result of clonal heterogeneity of the cells or off-target effects of shRNA. AMD3100 significantly inhibited lymph node metastasis of B88 cells, indicating that the blockade of CXCR4 inhibits lymph node metastasis in these cells. The mechanism of inhibition of lymph node metastasis by CXCR4 blockade is considered to be primarily due to the impaired migration of OSCC cells by the suppression of the SDF-1/CXCR4 gradient. However, a recent investigation suggests an association between the SDF-1/CXCR4 system and VEGF-C, which is a marker for lymphangiogenesis.[33] Furthermore, the SDF-1/CXCR4 system also contributes to cellular adhesion and growth potential in the ectopic sites of cancer cells.[10] Thus, blockade of CXCR4 may also inhibit lymphangiogenesis or adhesion and growth of OSCC cells in the lymph node. Blockade of CXCR4, using agents such as AMD3100, may be effective at preventing metastasis in patients with CXCR4-related OSCC and may also be a useful palliative drug for patients with advanced OSCC.

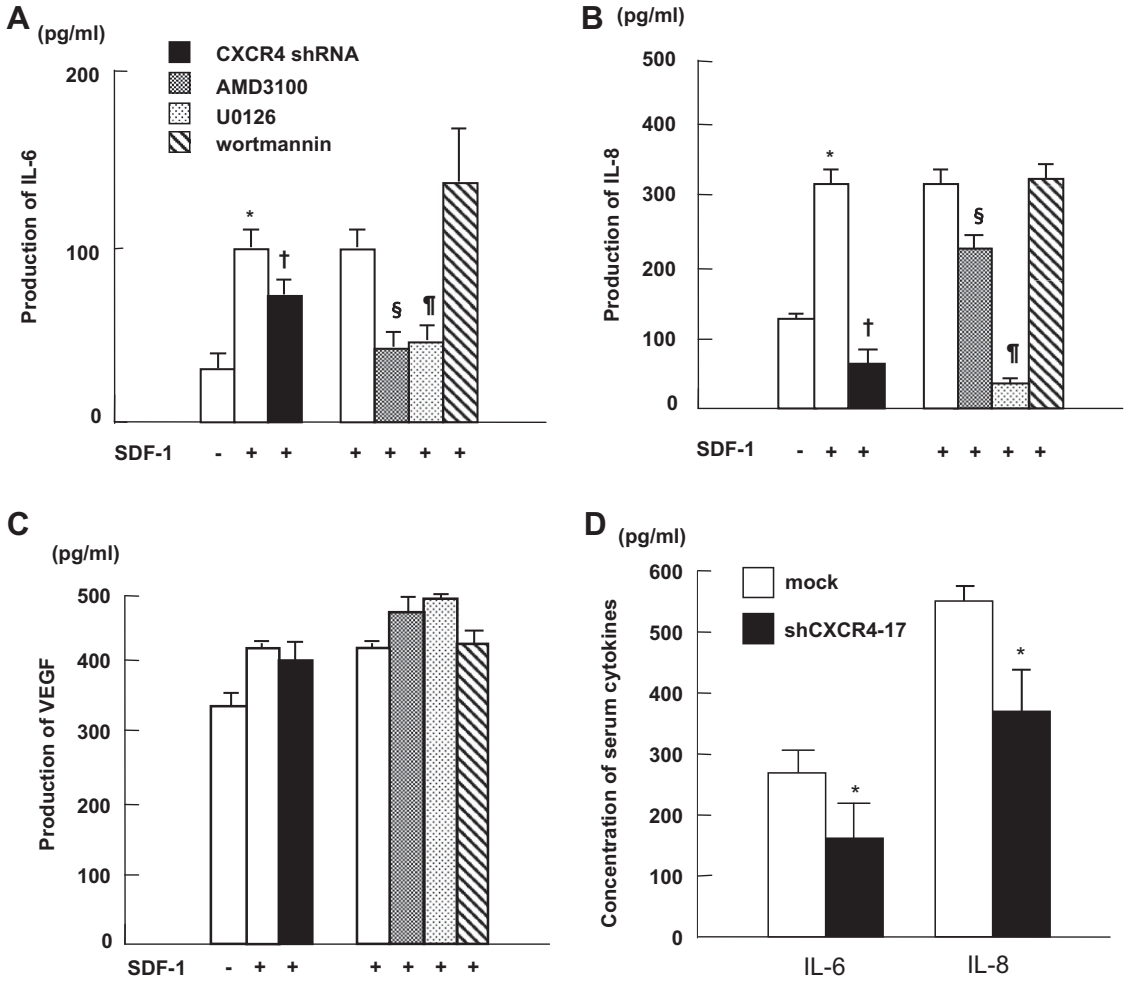

Fig. 5 – Induction of IL-6 and IL-8 production by the SDF-1/CXCR4 system and their inhibitory mechanism by CXCR4 knockdown. Mock and shCXCR4 cells were treated with SDF-1 for 24 h. Mock cells were treated with AMD3100 (1 μg/ml), U0126 (20 μM) or wortmannin (10 μM) for 45 min before addition of SDF-1 (100 ng/ml). The conditioned media were collected and subjected to an ELISA assay for IL-6 (A), IL-8 (B) and VEGF (C). $^{*}p < 0.05$ when compared to mock cells without SDF-1. $^{\dagger\S\P}p < 0.05$ when compared to mock cells with SDF-1. (D) The mice inoculated with mock or shCXCR4-17 cells were sacrificed at day 30 and the sera were subjected to ELISA. $^{*}p < 0.05$ when compared to the sera inoculated with mock cells. The results show the means ± SD.

Conflict of interest statement

None declared.

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