CXCL12/CXCR4 promotes laryngeal and hypopharyngeal squamous cell carcinoma metastasis through MMP-13-dependent invasion via the ERK1/2/AP-1 pathway

Ching-Ting Tan1, Chia-Yu Chu2,3, Ying-Chang Lu1,3, Cheng-Chi Chang3, Been-Ren Lin4, Hsiao-Hui Wu1, Hsin-Ling Liu1, Shih-Ting Cha3, Ekambaramellure Prakash3, Jeno-Yuh Ko1,5,6 and Min-Liang Kuo3,1

1Department of Otolaryngology, 2Department of Dermatology, National Taiwan University Hospital and National Taiwan University, College of Medicine, Taipei 100, Taiwan and 3Laboratory of Molecular and Cellular Toxicology, Institute of Toxicology, College of Medicine and Antitoxiology Research Center, National Taiwan University, Taipei 100, Taiwan

Introduction
Cancers of the larynx and hypopharynx are common head and neck malignancies, constituting ~20% of all tumors. Among them, squamous cell carcinomas (SCCs) are the most common, accounting for over 90% of cancers in this region (1). Lymph node (LN) metastasis is frequently seen in laryngeal and hypopharyngeal squamous cell carcinoma (LHSCC) directly affecting the prognosis of patients (2) and also reduces the probability of regional control and survival significantly (3). Despite numerous recent advances in surgery and cancer therapy, there has been no significant improvement in survival rates for head and neck squamous cell carcinoma (HNSCC) patients (4). Therefore, understanding the details of LHSCC metastasis in local LNs is of primary concern.

Recent studies have indicated that dissemination of cancer metastasis occurs in a discrete orientation to specific organs, much similar to the leukocyte trafficking, rather than a random process (5). Also known is the fact that the tumor cell invasion is regulated by specific chemokines, a large family of proinflammatory polypeptide cytokines that function as regulatory molecules in leukocyte maturation, trafficking and targeting of T and B lymphocytes. They also help regulate lymphoid tissue development and dendritic cell maturation (6,7). Numerous reports have suggested that many cancers, such as breast, ovary, prostate, kidney, brain, lung and thyroid, express chemokine receptors and use chemokines to metastasize in specific target organs, such as the hematopoietic cells (5,8–13).

Chemokine stromal cell-derived factor-1 (SDF-1/CXCL12), a CXC chemokine, is expressed by stromal marrow cells. CXCR4, a CXCL12 receptor, is pivotal in the targeting of hematopoietic stem cells within the narrow microenvironment (14). Muller et al. (5) has demonstrated that primary breast cancer tumors and all their cell lines express CXCR4 receptors. Moreover, treatment of breast cancer cells with an anti-CXCR4 monoclonal antibodies effectively inhibits their metastasis to the lungs and LNs. There is growing evidence that CXCR4 plays a crucial role in promoting metastasis of cancer cells to tissues that strongly express CXCL12 (5,9,15–18). LNs strongly express CXCL12, which could explain why oral SCC cells often disseminate in them (17,19). Laryngeal and hypopharyngeal cancer frequently metastasizes in the lymphatic system and hence this study investigates the relationship between the CXCL12/CXCR4 system and the said metastasis.

Results indicate that LHSCCs strongly express CXCR4. CXCL12 treatment enhances LHSCC cell migration and chemoinvasion. Reverse transcription–polymerase chain reaction (PCR) and western blot analysis show matrix metalloproteinase (MMP)-13 to be a downstream effector gene of CXCL12/CXCR4 signaling. In addition, MMP-13 upregulation is mediated by the extracellular signal-regulated kinase (ERK)1/2 pathway and its downstream transcription factor, activator protein (AP)-1.

Materials and methods

Cell culture
HEp-2 (ATCC CCL-23, laryngeal SCC) and FaDu (ATCC HTB-43, hypopharyngeal SCC) cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown and maintained in Eagle’s minimal essential medium with Eagle’s basic salt solution and 2 mM t-glutamine (Eagle’s minimal essential medium) supplemented 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator with 5% CO2.

Immunohistochemistry
Paraffin sections were dewaxed and pretreated in 0.01 M sodium citrate buffer (pH 6.0) for 20 min at 95°C to unmask tissue antigens. These sections were then incubated with 1% hydrogen peroxide in methanol for 15 min at room temperature to block endogenous peroxidase; they were then incubated with phosphate-buffered saline containing 5% normal goat serum (Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator with 5% CO2.

Abbreviations: AP, activator protein; ERK, extracellular signal-regulated kinase; HNSCC, head and neck squamous cell carcinoma; LHSCC, laryngeal and hypopharyngeal squamous cell carcinoma; LN, lymph node; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; mRNA, messenger RNA; OSCC, oral squamous cell carcinoma; PCR, polymerase chain reaction; PI3K, phosphatidylinositol-3 kinase; SCC, squamous cell carcinoma.

These authors contributed equally to this work.

Advance Access publication May 16, 2008

Carcinogenesis vol.29 no.8 pp.1519–1527, 2008

doi:10.1093/carcin/bgn108

Advance Access publication May 16, 2008

© The Author 2008. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org
simultaneously performed by two investigators who were unaware of clinico-pathological features of the patients. Specimens were regarded as positive when staining intensity was either moderate (focal expression in 5–20% of tumor cells) or strong (diffuse expression in >20% of tumor cells) and negative when the intensity was negative or weak (focal expression in <5% of tumor cells). Several serial specimens that are immunopositive for CXCR4 were stained with anti-CD34 antibody (R&D Systems) to identify vascular endothelial cells. We exposed several tissue specimens to non-specific mouse IgG primary antibody to confirm the specificity of the immunohistochemistry results.

Reverse transcription–PCR
Total RNA extraction from homogenized pieces of fresh, frozen cancer tissue specimens and cultured cells were performed with Trizol (Life Technologies, Eggenstein, Germany) following the acid guanidium thiocyanate-phenol-chloroform method. The cDNA was synthesized through random priming from 1 mg of total RNA with the aid of a First-Strand cDNA synthesis kit (Pharmacia Biotech, Uppsala, Sweden), according to the manufacturer’s instructions. For the PCR, 2 μl of cDNA solution was mixed with 2 μl of a specific primer (20 μM each), 5 μl of 103 reaction buffer, 10 μl of 1 μM deoxyribonucleotide triphosphate mix, 0.5 μl of Taq DNA polymerase and 28.5 μl of double distilled water for a total volume of 50 μl. PCR was performed in a Perkin-Elmer Thermal Cycler (Norwalk, CT); primers used for the amplification of CXCL12, CXCR4 and MMPs are specified below (20,21). Amplification consisted of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 30 s (33 cycles). Ten microliter of PCR products were separated on 2.5% wt/vol agarose gels and stained with ethidium bromide. The messenger RNA (mRNA) levels were quantified by densitometry and corrected for the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA in the same RNA samples. Primer sequences used for the amplification of CXCL12; CXCR4 and MMPs, with antisense oligonucleotide used in this study was 5’-GGCCGCTCCTGCTACGAGGACAGGAAAGG-3’; MMP-2, 5’-CTGACCCCCAGTCCTATCTGCC-3’; MMP-9, 5’-TGCCCCGACAAGGAGTACAG-3’; MMP-13, 5’-TTGTTGTGCGCATATGGTCG-3’.

Quantitative real-time PCR
Real-time quantitative PCR was performed using the Real-Time PCR System Quantitative real-time PCR reactions at 50°C for 15 min, followed by an initial denaturation at 95°C for 15 min, and then 33 cycles consisted of denaturation at 94°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 1 min. The thermal cycle conditions included maintaining the reactions at 50°C for 2 min and at 95°C for 10 min and then alternating for 50 cycles between 95°C for 15 s and 60°C for 1 min. The CXCR4 and CXCL12 probe and primer pairs were obtained from Applied Biosystems. The thermal cycle conditions included maintaining the reactions at 50°C for 2 min and then alternating for 50 cycles between 95°C for 15 s and 60°C for 1 min.

Western blot analysis
The expression of CXCL12, CXCR4 and MMP-13 by LHSCC cells was determined by western blot analysis. Equal aliquots of the conditioned cell media were fractionated on 10% sodium dodecyl sulfate ± polyacrylamide gel electrophoresis and transferred to Hybond ECL filter (Amersham, Little Chalfont, England) following the acid–guanidinium thiocyanate-pH 7.5 buffer containing 20 mmol/l Tris, pH 7.5, 1 mmol/l ethylenediaminetetraacetic acid, 140 mmol/l NaCl, 1% NP-40, 1 mmol/l orthovanadate, 1 mmol/l phenylmethylsulfonyl fluoride and 5 μmol/l aprotinin and 1 mmol/l phenylmethylsulfonyl fluoride. Protein samples (5 μg) from the conditioned media and 100 μg from LN extract were assayed.

Results

CXCL12/CXCR4 expression and clinicopathological LHSCC relationship
Expression of CXCR4 and CXCL12 proteins in LHSCC tissue was examined by immunohistochemical analysis of 30 LHSCC tissue samples. Positive staining of CXCR4 and CXCL12 was observed in 16 (53.5%) and 12 (40.0%) samples, respectively. Cancerous and non-cancerous LHSCC tissue regions positively stained with CXCR4 and CXCL12 are shown in Figure 1A. CXCR4 proteins were located in the cytoplasm and/or cell membranes of cancer cells, but not in the normal stromal cells of non-cancerous LHSCC tissue (Figure 1A, I and II). Negative or weak CXCR4 protein staining was observed in the majority of infiltrating inflammatory cells, whereas strong CXCL12 protein staining was identified not only in the cytoplasm of stromal cells and lymphocytes adjacent to cancer cells
CXCL12/CXCR4 promotes LHSCC metastasis

Table 1. Relationship between CXCL12/CXCR4 expression and clinicopathological features

<table>
<thead>
<tr>
<th>Variables</th>
<th>n</th>
<th>CXCR4 positive (%)</th>
<th>P-value</th>
<th>CXCL12 positive (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN metastases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>11</td>
<td>2 (18.2)</td>
<td></td>
<td>2 (18.2)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>19</td>
<td>14 (73.7)</td>
<td>0.003</td>
<td>10 (52.6)</td>
<td>0.06</td>
</tr>
<tr>
<td>Distant metastases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>24</td>
<td>10 (41.7)</td>
<td></td>
<td>8 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>6</td>
<td>6 (100)</td>
<td>0.01</td>
<td>4 (66.7)</td>
<td>0.14</td>
</tr>
<tr>
<td>Recurrence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>22</td>
<td>12 (54.5)</td>
<td></td>
<td>9 (40.9)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>8</td>
<td>4 (50.0)</td>
<td>0.82</td>
<td>3 (37.5)</td>
<td>0.87</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>16 (53.5)</td>
<td></td>
<td>12 (40.0)</td>
<td></td>
</tr>
</tbody>
</table>

CXCL12-enhanced migration and invasion of CXCR4-positive LHSCC cells

In compliance with the above data, we clearly detected CXCR4, but not its ligand, mRNA or protein in both LHSCC cell lines (HEp-2 and FaDu), either in serum-free or normal culture conditions (Figure 2A). We treated both the cell lines with CXCL12 and examined their changes in migration and invasiveness using modified Boyden chamber assay coating with Matrigel. CXCL12 treatment resulted in a dose-dependent increase in migration and extracellular matrix invasion of both LHSCC cell lines (Figure 2B and C). Pretreatment with a CXCR4 inhibitor, AMD3100, significantly blocked CXCL12-induced migration and invasion of LHSCC cells (Figure 2D and E). To exclude the possibility that CXCL12-enhanced migration and invasion stemmed from increased cell proliferation, we counted the number of viable cells at different times during CXCL12 incubation (up to 72 h). Figure 2F shows that treatment with exogenous CXCL12 did not influence the proliferation rates of HEp-2 and FaDu cells.

The ERK1/2 pathway is essential to CXCL12/CXCR4 functions in LHSCC

Mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase/Akt are involved in CXCL12 signaling in SCC cells (25–29). To explore the signaling pathway involved in CXCL12-induced enhancement of migration/invasion in LHSCC cells, we examined the activation of ERK1/2 and AKT by western blot analysis using antibodies specific for their phosphorylated forms. Exposure of HEp-2 cells to CXCL12 (100 ng/ml) rapidly and transiently activated ERK1/2, with a peak at 5 min (Figure 3A). Western blots showed ERK1/2 to be extensively phosphorylated in FaDu cells after 5 min of CXCL12 exposure (Figure 3A). ERK1/2 levels returned to baselines after 60 min of CXCL12 treatment in both cell lines. Said activation was effectively inhibited by treatment with U0126, a specific inhibitor of MEK1 (30). (Figure 3B) However, phosphorylated AKT levels did not change during CXCL12 treatment. U0126, but not LY294002, effectively blocked CXCL12-induced migration and invasion of LHSCC cells (Figure 3C and D), indicating that ERK1/2 activation by CXCL12 is functionally involved in increased cell migration and invasion of LHSCC cells.

CXCL-involved upregulation of MMP-13 in the invasion of LHSCC cells

SCC tumor cells in the tongue express MMP-2, while MMP-13 mRNA has been observed in 57% of laryngeal SCC tissues (31,32).
CXCL12 stimulates an increase in active MMP-9 but not MMP-2 secretion of HNSCC cells (33). Since MMPs have a vital link to extracellular matrix protein degradation and invasion, we determined whether or not these enzymes are crucial to CXCL12/CXCR4-mediated invasion of LHSCCs (34). CXCL12 enhanced the expression levels of both MMP-13 mRNA (Figure 4A) and protein (Figure 4B) in HEp-2 cells, whereas that of MMP-2 and MMP-9 were unaffected. Enzyme-linked immunosorbent assay confirmed that CXCL12-stimulated HEp-2 cells produce significantly more MMP-13 than untreated cells (Figure 4C). CXCL12-stimulated invasion was effectively reduced by a selective MMP-13 inhibitor (CL82198, 10, 25 μM) (Figure 4D). Results indicate that MMP-13 is a downstream effector of CXCL12/CXCR4 axis-mediated effects in LHSCCs.

**Fig. 2.** Expression of CXCR4, CXCL12 mRNA and protein in LHSCC cell lines and their influences on cell proliferation, migration and invasion. CXCR4 mRNA was identified using reverse transcription–PCR; glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. CXCR4 and CXCL12 proteins were identified by western blot. (A) CXCR4 mRNA and protein detected in both FaDu and HEp-2 cell lines under complete medium (CM) or serum-free (SF) medium. Both cell lines did not express CXCL12. Data were drawn from three independent experiments. CTL: positive control, MDA-MB-231 cells. (B and C) HEp-2 and FaDu cells were allowed to migrate or invade for 24 h toward either SF medium or SF medium supplemented with recombinant human (rh) CXCL12 (1, 10, 100 ng/ml); 10 and 100 ng/ml rhCXCL12 significantly enhances migration and invasiveness for both HEp-2 and FaDu cells. (D and E) HEp-2 and FaDu cells were preincubated in the presence or absence of the CXCR4 inhibitor (AMD3100, 1, 2 μM) for 1 h. Cells were then allowed to migrate for 24 h toward either SF medium or SF medium supplemented with rhCXCL12 (100 ng/ml). AMD3100 effectively blocked the CXCL12-induced migration and invasion of LHSCC cells. Data for (B–E) represent the mean numbers of migratory or invasive cells from two wells (four fields per well). Error bars indicate SD. Asterisks indicate significant differences (P < 0.05) in migration or invasion ability between treated and untreated cells. (F) Treatment with rhCXCL12 (10, 20, 100 ng/ml) for 3 days and counted the number of viable cells. Results were drawn from three independent experiments.
ERK1/2/AP-1 kinase pathway involvement in CXCL12 modulation of MMP-13-mediated LHSCC cell invasion

Because the MMP-13 promoter region contains a conserved AP-1 transcription factor-binding site, activation of MAPK pathways results in downstream activation of c-Fos (ERK) and c-Jun (c-jun N-terminal kinase). We investigated the role of ERK1/2 in MMP-13 expression (35). To examine the necessity of ERK1/2 activity in CXCL12-elicited expression of MMP-13, we blocked the ERK1/2 pathway by adding U0126 to HEp-2 cells prior to CXCL12 treatment. As shown in Figure 5A, CXCL12 alone induced MMP-13 mRNA and protein expression, whereas U0126 blocked CXCL12-elicited expression of MMP-13 mRNA and protein, indicating that ERK1/2 activation is important to induction of MMP-13 expression by CXCL12. Pretreatment with LY294002 did not block CXCL12 induction of MMP-13 expression.

The presence of CXCL12-activated c-Jun was evidenced by the accumulation of its phosphorylated form (p-c-Jun) in the nucleus, starting at 10 min and persisting to 30 min after CXCL12 treatment (Figure 5B). To further clarify the involvement of MAPK/ERK1/2-signaling pathway in p-c-Jun induction, we pretreated U0126, the MEK1 specific inhibitor, in HEp-2 cells. As Figure 5C showed, U0126 treatment significantly blocked CXCL12-induced c-Jun phosphorylation. We also demonstrated the essential role of p-c-Jun in the CXCL12-induced MMP-13 mRNA and protein expression in Figure 5D. Transfection with c-jun antisense oligonucleotide, but not sense, can effectively block CXCL12-induced MMP-13 expression. To determine the functional outcome of CXCL12 and c-Jun in human laryngeal and hypopharyngeal cancers, we performed the invasion assay and examined the results in Figure 5E. The data show that transfection with c-jun antisense oligonucleotide could effectively block CXCL12-induced chemoinvasion. Together, these data indicated that knocking down c-Jun inhibited the expression of MMP-13 and CXCL12-induced invasion of HEp-2 cells.

Discussion

Although local LHSCCs can be effectively controlled by surgical excision and radiotherapy, metastasis to the LNs and lungs significantly decreases the survival rate (2,36). CXCR4 may play an important role in promoting organ-selective metastasis by stimulating tumor

Fig. 3. MAPK signaling in CXCR4/CXCL12 treatment in LHSCC cells. LHSCC cells were incubated in serum-free medium for 24 h and treated with rhCXCL12 (100 ng/ml) for the indicated periods of time. Cell lysates were analyzed by western blotting for activated ERK1/2 (p-ERK1/2) and AKT (p-AKT). (A) Exposure of HEp-2 cells to rhCXCL12 (100 ng/ml) rapidly activated ERK1/2 at 5 min, and this effect lasted till 60 min. Exposure of FaDu cells to rhCXCL12 (100 ng/ml) rapidly and transiently activated ERK1/2 at 5, 15 and 30 min. AKT and phosphor-AKT were also detected in these cell lysates. (B) LHSCC cells were preincubated with/without MEK inhibitor, U0126 (10, 20 μM), or PI3K inhibitor LY294002 (20 μM) for 1 h before stimulation with rhCXCL12 (100 ng/ml) for 5 min. After indicated time interval, cell lysates were collected and analyzed with the indicated antibodies. The experiment was repeated at least thrice. (C and D) Pretreatment with U0126 (20 μM) for 1 h and analyzed with the CXCL12-induced migration and invasion abilities in both HEp-2 and FaDu cells. Error bars indicate SD. Asterisks indicate significant differences between the two groups (P < 0.05). Data were drawn from three independent experiments.

CXCL12/CXCR4 promotes LHSCC metastasis

1523
adhesion to microvascular endothelial cells and by enhancing the growth of tumor cells under stress (37). Recently, it has been proposed that chemokine receptors are critical in determining the metastatic destination of tumor cells (5,9,16,18,38,39). Uchida et al. demonstrated a possible role for CXCR4 in mediating the dissemination of oral SCCs to the LNs and the ability of CXCL12 for inducing oral SCC migration and stimulating multiple intracellular signaling pathways. CXCR4 was also found to be expressed on the surfaces of soft palatal cancer cells (33).

LHSCC is characterized by an easily detectable LN and lung metastasis and the latter is responsible for the poor survival of LHSCC patients. This study indicates that functional CXCR4 chemokine receptors are expressed in a significant number of LHSCC tissues and that this expression is strongly associated with increased LN and distant metastasis of tumors. To our knowledge, this is the first report to indicate that CXCR4 might be critical to LHSCC metastasis. The data show that CXCR4 expression on the primary tumor is significantly correlated with the incidence of LN and distant metastasis. Patients prone to metastasis can be easily identified by biopsy staining with a CXCR4 antibody. Combining the anti-CXCR4 regimen (such as AMD3100) with cytotoxic anticancer drugs may prolong the survival of the said patients.

Our results show that stimulation of LHSCC cells by CXCL12 triggers migration and invasion, suggesting that the CXCL12/CXCR4 axis is an important mediator of the migration and chemoinvasion of LHSCC cells. AMD3100, a bicyclam, is a novel agent that uniquely inhibits the entry of human immunodeficiency virus type 1 into CD4+ T cells by selectively blocking the chemokine CXCR-4 receptor (40). The CXCL12-induced effect can be antagonized by AMD3100. Although previous observations have shown that CXCL12 enhances proliferation of some cells in culture, we did not detect any changes in LHSCC cell proliferation when incubated with CXCL12 (up to 72 h) (20–22,41,42). CXCL12 has a specific effect on migration and chemoinvasion of LHSCC cells that does not stem from enhanced proliferation.

In vivo, CXCL12 is secreted by stromal cells within destination tissues for LHSCC cell metastasis, such as the lung and LNs (5,19). Therefore, stromal cell-derived CXCL12 can activate CXCR4 chemokine receptors on LHSCC cells. Interestingly, this study shows that 40% of our tumor cells were also positively stained by anti-CXCL12 antibodies. Unlike oral SCC cells, which do not express CXCR4 on LHSCC tumor cells suggests that there are not only paracrine but also possible autocrine effects by CXCL12 on the LHSCC metastasis.

This study found CXCL12 treatment to induce a robust, transient phosphorylation of ERK1/2/MAPK. Inhibition of ERK1/2 phosphorylation reduces CXCL12-induced migration and chemoinvasion, suggesting that activation of ERK1/2 is an important step in the signal that leads to increased migration and chemoinvasion. These findings are similar to those already found in oral and oropharyngeal SCC (19,33). However, unlike the oral squamous cell carcinoma results, the phosphorylated form of Akt/PKB was not upregulated by CXCL12 in LHSCC cells. Moreover, this study shows that PI3K is probably not involved in the CXCL12-mediated effects on migration and invasion of LHSCC cells.

MMPs have been implicated to facilitate cancer cell invasion and metastasis through degradation of surrounding ECM proteins. Expression of MMPs and other extracellular proteinases has been shown to positively correlate with the progression of cancer in patients (23,43).

Fig. 4. The role of MMPs in CXCL12-induced invasion of LHSCC cells. HEp-2 cells were incubated in serum-free medium for 24 h and treated with rhCXCL12 (100 ng/ml) for the indicated periods of time. (A and B) Expression of MMP-13 mRNA and protein in HEp-2 cells stimulated by rhCXCL12. MMP-2 and MMP-9 were also detected. (C) Secreted MMP-13 was assessed using an enzyme-linked immunosorbent assay kit (R&D Systems) according to the manufacturer’s protocols and increased by rhCXCL12 (100 ng/ml) in HEp-2 cells. (D) Invasiveness of HEp-2 cells in Matrigel toward serum-free medium, containing either a control mouse IgG1 (10 μg/ml) or a selective MMP-13 inhibitor (CL82198, 10, 25 μM), was determined in a 24 h invasion assay. MMP-13 inhibitor blocked the CXCL12-induced cell invasion in HEp-2 cells. Error bars indicate SD. Asterisks indicate significant differences between the two groups (P < 0.05). Similar results were observed in three independent experiments.
Incubation of oropharyngeal SCC cells with CXCL12 stimulates an increase in active MMP-9 secretion (33). Kawamata et al. (44) reported that a large amount of matrix degrading enzymes, produced by oral SCC cells, contributes to tumor invasion and that the net activity of matrix metalloproteinase (MMP-2) (active MMP-2/tissue inhibitor of MMP-2) contributes to LN metastasis in a nude mouse orthotopic inoculation model. Bogusiewicz et al. (45) found that MMP-2 and MMP-9 may be involved in the expansion of laryngeal cancer, whereas MMP-2 may also play an important role in the lymphatic spread of some laryngeal tumors. MMP-13 has also been found in laryngeal SCC and its expression is correlated with local invasiveness (32).

Although LHSCC and all oral and oropharyngeal SCC are all HNSCCs, they have different MMP expression responses to CXCL12 stimulation. Our results demonstrate that MMP-13, but not MMP-2 or MMP-9, is activated in CXCL12-induced migration and chemoinvasion of LHSCC cells. Cells cannot migrate through native type I collagen without degrading it first by collagenolytic MMPs (MMP-13 or MMP-1) (46). Therefore, CXCL12 enhancement of MMP-13 expression in HEp-2 cells can induce the invasion of cancer cells through collagen gel. MMP-13 also degrades type IV collagen, so increased MMP-13 production may also enhance LHSCC invasiveness through basement membranes (47). The blocking of ERK1/2 activation also inhibits the induction of MMP-13 expression by

Fig. 5. The role of ERK1/2/AP-1 in the CXCL12-induced expression of MMP-13 and invasion. (A) The effect of MEK inhibitor (U0126) and PI3K inhibitor (LY294002) on the CXCL12-induced expression of MMP-13 mRNA and protein in HEp-2 cells. Pretreatment with 20 μM U0126 effectively blocked the CXCL12-induced expression of MMP-13 mRNA and protein in HEp-2 cells. PI3K inhibitor, LY294002, could not effectively block the MMP-13 expression in HEp-2 cells. (B) Nuclear and cytosolic extracts of CXCL12-treated LHSCC cells were extracted as described in Materials and Methods. Phospho-c-Jun (p-c-Jun), SP-1 and α-tubulin were detected of the nuclei and cytosolic extract in CXCL12-treated cells. (C) HEp-2 cell line was pretreated with U0126 for 30 min and stimulated with CXCL12 (100 ng/ml) for the indicated time (10 min) and nucleic extracts were collected and analyzed with the indicated antibodies. (D) c-jun antisense oligonucleotide blocked the CXCL12-enhanced MMP-13 mRNA and protein production. (E) c-jun antisense oligonucleotide was transfected in HEp-2 cells and detected the CXCL12-induced chemoinvasion abilities. Data are representative of three independent experiments.
CXCL12, indicating that the ERK1/2 MAPK pathway is important in MMP-13 expression. MMP-13 is expressed by HNSCC tumor cells and its expression correlates with their invasion capacity (32,48–51). Our results suggest that CXCL12-induced, ERK1/2-mediated MMP-13 expression is involved in LHSCC cellular invasiveness. The interesting observation is the fact that the primary LHSCC tumor cells expressed CXCL12, which is indicative of locally induced CXCL12-mediated MMP-13 expression and its importance in both local invasiveness and metastasis.

Previous studies have revealed that the triggering of CXCL12 signaling results in activation of AP-1 through phosphorylation of Ser-727 by ERK1/2 (52); our data demonstrate that CXCL12 activates ERK1/2 in LHSCC cells. Because our previous study of basal cell carcinomas confirmed the involvement of the AP-1 site in CXCL12-upregulated MMP-13 transcription (52), we checked the role of c-Jun in the CXCL-induced chemoinvasion and found that antisense treatment of c-Jun results in reduction of CXCL12-induced MMP-13 expression and chemoinvasion. Our results indicate that CXCL12/CXCR4 pathway is important in the metastasis of LHSCC. Signals from CXCR4 receptors may induce MMP-13 activation, resulting in chemoinvasion of LHSCC cells. CXCR4 antagonist AMD3100 blocks the CXCL12-induced migration and chemoinvasion. CXCR4 antagonists and MMP-13 blockers seem promising new therapeutic tools in LHSCC treatment.

Funding

National Science Council of Taiwan (NSC 93-2314-B-002-110, NSC 94-2314-B-002-242, NSC 95-2314-B-002-177); National Taiwan University Hospital (NTUH 95M06 to C.-T.T).

Acknowledgements

We thank Dr Din-Lii Lin from TaiGen Biotechnology Company (Taipei, Taiwan) for providing AMD3100.

Conflict of Interest Statement: None declared.

References


Received December 11, 2007; revised April 25, 2008; accepted April 29, 2008