Overexpression of CX3CR1 is associated with cellular metastasis, proliferation and survival in gastric cancer

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Abstract. The CX3CR1/CX3CL1 axis is involved in the metastasis and prognosis of many types of cancer; however, whether CX3CR1 is expressed in gastric cancer cells and whether it participates in gastric cancer metastasis remain unknown. We investigated the expression of CX3CR1 in gastric cancer tissues and non-neoplastic gastric tissues in vivo and in gastric cancer cell lines and a gastric epithelial cell line in vitro, and then the functional roles of CX3CR1 in cellular metastasis, proliferation and survival were explored. We observed that CX3CR1 was highly expressed in gastric cancer tissues in vivo and was related to lymph node metastasis, higher clinical TNM stage and larger tumor size. In vitro, CX3CR1 overexpression promoted gastric cancer cell migration, invasion, proliferation and survival. Additionally, different from several chemokine receptors, CX3CR1 was also expressed in non-neoplastic gastric tissues and in gastric epithelial cells and played a functional role in vitro. Notably, gastric cancer tissues expressed higher CX3CR1 than that in the non-neoplastic gastric tissues in vivo, while in vitro, CX3CR1 expression in the gastric cancer cell lines was equivalent or significantly lower than that in the gastric epithelial cell line, which suggests that the high expression of CX3CR1 in gastric cancer in vivo might be induced, not constitutive. Altogether, our findings suggest that on the one hand overexpression of CX3CR1 promoted gastric cancer cell migration, invasion and proliferation; on the other hand, appropriate expression of CX3CR1 in normal gastric tissues may play a physiological role in tissue remodeling after injury and/or epithelial renewal. Additionally, the tumor microenvironment may play an important role in the high expression of CX3CR1 in gastric cancer cells.

Introduction

Gastric cancer is the fourth most common cancer and is the second leading cause of cancer-related mortality worldwide.

Although there have been marked decreases in the incidence and mortality in some regions of the world, gastric cancer remains an important public health burden, particularly in developing countries, and patient prognosis is generally rather poor, with a 5-year relative survival less than 30% in most countries (1,2). Extragastric lymph node metastasis is a critical independent prognostic factor, which leads to the failure of surgery, chemotherapy or radiotherapy (3,4). Therefore, prevention of metastatic gastric cancer is an important therapeutic goal; however, the underlying mechanism of gastric cancer metastasis remains unclear.

Chemokines are a large subfamily of chemoattractant cytokines which are classified into 4 highly conserved groups: CXC, CC, C and CX3C. Chemokines have been mostly studied for their potent effect on the recruitment of leukocytes at sites of inflammation (5). However, it is now established that migrating malignant cells may exploit chemokine receptors to invade surrounding tissues leading to distant metastasis (6-25).

CX3CL1 is a structurally unique chemokine and is currently the only known member of the CX3C family of chemokines (26,27). Unlike several other chemokines, CX3CL1 has both a membrane-bound and a soluble form. Membrane-bound CX3CL1 can act as an adhesion molecule and can be cleaved by metalloproteinases to create circulating soluble CX3CL1 acting as a chemoattractant (26,28,29). Both transmembrane and soluble forms of CX3CL1 bind to the only known G protein-coupled seven-transmembrane receptor CX3CR1 (30,31). The CX3CR1/CX3CL1 axis has been demonstrated to be involved in the proliferation, survival and metastasis of various malignant tumor types, including clear cell renal cell carcinoma (11), prostate cancer (21), breast cancer (19), pancreatic ductal adenocarcinoma (16) and glioma tumors (32). However, to our knowledge, whether the CX3CR1/CX3CL1 axis is involved in gastric cancer metastasis remains unknown.

In the present study, we investigated CX3CR1 expression in gastric cancer tissues and gastric cancer cell lines compared with non-neoplastic gastric tissues and a gastric epithelial cell line, and further analyzed the functional role of the CX3CR1/CX3CL1 axis in gastric cancer cell lines and a gastric epithelial cell line in vitro. We demonstrated that the CX3CR1/CX3CL1 axis plays an important role in gastric cancer metastasis, proliferation and survival, and additionally might play a physiological role in normal gastric tissue renewal and/or tissue remodeling after injury.
**Materials and methods**

**Patients.** A total of 89 patients diagnosed with gastric cancer as confirmed by pathology at the Peking University People’s Hospital from January 2000 to December 2004 were enrolled in the study. The mean patient age was 61.5±11.6 years. Among the patients, 65 were men and 24 were women. None had previously received radiotherapy, chemotherapy or other medical interventions before surgery. The control group consisted of 30 contemporaneous patients who had chronic superficial gastritis diagnosed by gastroscopy at the Peking University People’s Hospital selected randomly (simple random sampling). The mean age was 59.3±10.4 years. Among the control group, 16 individuals were men and 14 were women. This study was approved by The Ethics Committee of the Peking University, and written informed consent was obtained from all patients at study entry.

**Cell lines and cell culture.** Gastric cancer cell lines MKN-28, SGC-7901, MKN-45 and the immortalized gastric epithelial cell line GES-1 were obtained from the China Center For Type Culture Collection (Wuhan, China). All cells were cultured in RPMI-1640 (Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, USA) in a humid atmosphere of 5% CO₂ and 95% air at 37°C. 

**Quantitative real-time PCR.** Total RNA was isolated using the RNeasy Mini kit (Qiagen, USA), according to the manufacturer’s instructions. The first cDNA strand was synthesized from total RNA with the SuperScript III First-Strand synthesis system for RT-PCR (Life Technologies, USA). The following primers were used for the subsequent PCR: human CX3CR1 sense, 5'-TTGAGTACGATGATTGGCTGTA-3' and antisense, 5'-GGCTTTGGCCTTTGTTGG-3'; human GAPDH sense, 5'-TGTGGGATCAATGACCCCTT-3' and antisense, 5'-CTC CAGACGCTACTACGGG-3'. Quantitative real-time PCR was conducted with RealMasterMix™ (SYBR-Green) (Tiangen, China) and GAPDH to normalize data. PCR conditions were 30 sec at 94°C, 30 sec at 60°C and 1 min at 72°C for 40 cycles.

**Western blot analysis.** The cells were collected and washed with cold PBS three times, and then lysed at 4°C for 30 min in lysis buffer [50 mM Tris (pH 7.4), 100 mM NaCl₂, 1 mM MgCl₂, 2.5 mM NaN₃VO₄, 1 mM PMSF, 2.5 mM ethylenediaminetetraacetic acid, 0.5% Triton X-100, 0.5% NP-40, 5 mg/ml of aprotinin, pepstatin A and leupeptin]. The lysates were centrifuged at 10,000 x g for 20 min at 4°C. The protein concentration was determined using a bicinchoninic acid protein assay reagent kit (Pierce, USA) according to the manufacturer’s protocol. Forty micrograms of total proteins was electrophoresed on a 10% denaturing SDS gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was then incubated with blocking buffer (PBS containing 5% non-fat milk) for 2 h at room temperature, followed by incubation with rabbit polyclonal antibodies against total Akt (1:1,000; Cell Signaling Tech, USA), phospho-Akt (ser473; 1:1,000; Cell Signaling Tech), and CX3CR1 (1:1,000; Origene, USA) overnight with gentle shaking. As a loading control, GAPDH (1:1,000) or β-actin (1:1,000) was detected using a mouse monoclonal antibody (Cell Signaling Tech).

The membrane was washed twice with PBS for 5 min, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit/mouse immunoglobulin G (Cell Signaling Tech) as secondary antibody diluted at 1:2,000 for 2 h at room temperature. The protein bands were detected using a western blotting detection system (Bio-Rad, USA). Experiments were repeated three times.

**Immunohistochemistry.** Paraffin-embedded, formalin-fixed gastric cancer tumor tissues and healthy control tissues of the gastric mucosa were cut into 4-µm sections and placed onto polyllysine-coated slides. Sections were incubated overnight with the diluted rabbit anti-human CX3CR1 antibody (Origene) at 1:100 in PBS in a humidified chamber at 4°C. Tissue sections were counterstained with haematoxylin and permanently mounted. Under an ordinary optical microscope, 5 different perspectives were randomly selected at a x400 magnification. The expression and distribution of CX3CR1 were analyzed systematically and quantitatively by Image-Pro-Plus software 6.0. Integral optical density (IOD) for each perspective was recorded.

**Immunocytochemistry.** After MKN28, SGC-7901, MKN-45 and GES-1 cells were cultured in a 24-well plate (1x10⁴ cells/well) for 24 h, the cell culture was poured and the cells were washed three times with PBS. Following cell fixing with 4% paraformaldehyde for 10 min and washing with PBS, 0.5% Triton X-100 in 10% blocking serum was applied for 20 min at room temperature. Cells were incubated with the diluted rabbit anti-human CX3CR1 antibody at 1:100 in PBS overnight at 4°C, and then the cells were washed three times with PBS for 5 min, and incubated with EnVision™ Detection kit, Peroxidase/DAB, Rabbit/Mouse (Dako, USA) according to the manufacturer’s instructions.

**Cell transfection.** MKN28, SGC-7901, MKN-45 and GES-1 cells were seeded in a 6 well-plate (2x10⁵ cells/well), and were transfected for 4 h with 1 µg of plasmid DNA encoding CX3CR1 short hairpin RNA (pRFP-c-RS; TF313635; Origene) or plasmid DNA encoding irrelevant shRNA with random nucleotides as a control, or plasmid DNA encoding CX3CR1 cDNA (pCMV6-AC-GFP; RG207022; Origene) or empty vector as a control using Lipofectamine 2000 (Invitrogen Life Technologies, USA), according to the manufacturer’s instructions. Stable clones were selected in complete RPMI-1640 medium containing 2 µg/ml puromycin (Merck, Germany) or 500 µg/ml G418 (Merck, Germany) and used for the subsequent experiments. Following transfection of the cells with shRNA or cDNA, the transfected cells were conveniently monitored by fluorescence microscopy for red fluorescent protein or green fluorescent protein expression. Stable transfected cells were identified at the RNA and protein level.

**CCK-8 proliferation assay.** Cellular proliferation was measured using the Cell-Counting Kit-8 (CCK-8; Dojindo, USA) according to the manufacturer’s instructions. Briefly, the cells were treated for 0, 24, 48, 72, or 96 h with complete RPMI-1640 medium containing 200 ng/ml recombinant protein of human CX3CL1 (Origene). At the end of the culture period, 10 µl CCK-8 reagent was added to each well, and the
plates were placed at 37˚C for 3 h. Absorbance was measured at 450 nm using a multiwell spectrophotometer.

Apoptosis assay. Cells were plated in 12-well plates and cultured for 24 h. They were then incubated under apoptosis-inducing conditions (serum deprivation) with 200 ng/ml recombinant protein of human CX3CL1 for 24 h. The cells were collected and resuspended in 100 µl binding buffer, and 5 µl FITC-Annexin-V (eBioscience, USA) was added and incubated in the dark for 15 min at room temperature. Subsequently, 5 µl of 7-AAD (eBioscience, USA) was added and incubation was carried out for 5 min at room temperature in the dark. Annexin-V-positive cells were considered to be apoptotic cells.

Chemotaxis and invasion assay. Migration and invasion assays were performed in 24-well cell culture chambers using inserts with 8-µm pore size (Becton Dickinson, USA). For the invasion assays, the inserts were coated with Matrigel (100 µg/cm²; Becton Dickinson, USA). Gastric cancer cells were suspended in the chemotaxis buffer (RPMI-1640, 0.1% BSA and 12 mM HEPES) at 5x10⁴/ml and added to the inserts, which were transferred to wells containing buffer with recombinant protein of human CX3CL1. After incubation for 6 or 24 h for the chemotaxis or the chemoinvasion assay, respectively, cells on the lower surface of the membrane were stained and counted under a light microscope in five different fields (x200). Assays were performed in triplicate.

Statistical analysis. All data are expressed as mean ± SD. Statistical comparisons between groups were performed using one-way ANOVA or the two-tailed Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

**CX3CR1 is expressed in gastric cancer and non-neoplastic gastric tissues.** Immunohistochemical staining and quantitative real-time PCR showed that both gastric cancer and non-neoplastic gastric tissues expressed CX3CR1, and compared with the non-neoplastic gastric tissues, CX3CR1 expression was significantly increased in the gastric cancer tissues (P<0.01, Fig. 1A and B). We then analyzed the relationship between the CX3CR1 expression level in the primary tumor and the clinicopathological characteristics by comparing the counted IOD (integrated optical density) in five fields at a x400 magnification. Increased CX3CR1 expression was significantly related to lymph node metastasis (P=0.029), higher clinical TNM stage (P=0.021) and larger tumor size (P=0.011); however, the CX3CR1 protein expression level had no association with age, gender, tumor differentiation or tumor location (Table I).

**CX3CR1 is expressed in several different human gastric cancer cell lines and in a gastric epithelial cell line.** As the results from the clinical tissues showed that CX3CR1 was expressed in both gastric cancer tissues and non-neoplastic gastric tissues, we aimed to ascertain whether the expression of CX3CR1 in non-neoplastic gastric epithelial cells and/or gastric cancer cells was constitutive or inducible. We selected three gastric cancer cell lines and one immortalized gastric epithelial cell line to investigate CX3CR1 expression in vitro. Fig. 2 shows that, in line with the clinical results, although the expression level in all cell lines was evidently lower compared with the GAPDH expression level, the gastric epithelial cell line (GES-1) and the three gastric cancer cell lines (MKN-28, SGC-7901 and MKN-45) expressed CX3CR1. In contrast to the results in vivo, MKN-28 and MKN-45 cells produced less CX3CR1 protein and MKN-45 cells produced an equal level of CX3CR1 when compared with the GES-1 cells. We speculated that there possibly unknown mechanisms existing in the tumor microenvironment in vivo, which could increase the expression of CX3CR1 in malignant cells, while gastric cancer cell lines in vitro lacked this tumor microenvironment. According to these results, we hypothesized that CX3CR1 is constitutively expressed in normal gastric epithelial cells at a low level and is induced to express in gastric cancer cells at a higher level in the tumor microenvironment.

**Identification of stable CX3CR1-overexpressing or -knockdown cells.** To simulate the high expression of CX3CR1 in gastric cancer tissues in vivo and clarify the functional role...
of CX3CR1 in gastric normal epithelial and gastric cancer cells, we transfected three gastric cancer cell lines and one gastric epithelial cell line with CX3CR1 cDNA or CX3CR1 short hairpin (sh)RNA to obtain cDNA-mediated CX3CR1-overexpressing or shRNA-mediated CX3CR1-knockdown cell lines. The stable transfected cells were monitored by fluorescence microscopy for red fluorescent protein (a marker for plasmid pRFP-c-RS) or green fluorescent protein (a marker for plasmid pCMV6-AC-GFP) expression (Fig. 3A). In order to determine whether shRNA knockdown and cDNA overexpression are correlated with a change in RNA and protein, we measured the RNA and protein levels of CX3CR1 following transfection by quantitative real-time PCR and western blot analysis. As shown in Fig. 3B and C, cDNA transfection efficiently increased CX3CR1 production compared with cells transfected with the empty vector (i.e. cDNA control); shRNA transfection decreased the CX3CR1 production compared with the cells transfected with irrelevant shRNA (i.e. shRNA control). Importantly, none of these shRNAs and cDNAs affected the transcription of the housekeeping gene GAPDH. Stable transfected cells were further used in the subsequent functional experiments.

The CX3CR1/CX3CL1 axis stimulates gastric cancer cell migration and invasion. Transwell migration and invasion assays were performed to examine the mobilizing effect of the CX3CR1/CX3CL1 axis on the selected cell lines. Fig. 4 shows the number of cells that migrated or invaded per five different fields in response to CX3CL1. In all gastric cancer cell lines, cells overexpressing CX3CR1 exhibited significant migratory and invasive responses to CX3CL1 compared with the empty vector-transfected cells, while cells with CX3CR1 knockdown showed almost no impact on migratory and invasive responses to the CX3CL1 cytokine except for MKN-28.

Table I. Relationship between the CX3CR1 expression level and clinicopathological features of the gastric cancer patients.

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>Primary tumor IOD for CX3CR1 (mean ± SD)</th>
<th>P-value</th>
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<tr>
<td>Age (years)</td>
<td></td>
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</tr>
<tr>
<td>≤60</td>
<td>30</td>
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<tr>
<td>&gt;60</td>
<td>59</td>
<td>34,613.17±20,400.92</td>
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<td>Gender</td>
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<tr>
<td>Male</td>
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<td>35,782.98±23,382.74</td>
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<tr>
<td>Female</td>
<td>24</td>
<td>29,862.10±10,908.13</td>
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<td>Tumor size (cm)</td>
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<tr>
<td>&lt;4</td>
<td>29</td>
<td>24,362.98±10,226.68</td>
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<tr>
<td>≥4</td>
<td>60</td>
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<td>Tumor location</td>
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<tr>
<td>Cardia</td>
<td>16</td>
<td>29,717.94±16,315.59</td>
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<tr>
<td>Non-cardia</td>
<td>73</td>
<td>33,073.17±19,477.31</td>
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<tr>
<td>Tissue differentiation</td>
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<tr>
<td>High</td>
<td>29</td>
<td>28,243.80±15,216.78</td>
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<tr>
<td>Middle</td>
<td>35</td>
<td>31,870.63±23,488.66</td>
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<tr>
<td>Low</td>
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<td>32,053.73±23,683.80</td>
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<td>TNM stage</td>
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<tr>
<td>I+II</td>
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<td>24,228.20±10,866.49</td>
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<tr>
<td>III+IV</td>
<td>56</td>
<td>39,486.33±14,841.06</td>
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<td>Lymph node metastasis</td>
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<tr>
<td>Absent</td>
<td>27</td>
<td>20,233.44±8,167.99</td>
</tr>
<tr>
<td>Present</td>
<td>62</td>
<td>34,065.75±18,939.28</td>
</tr>
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IOD, integrated optical density.
Figure 3. Identification of stable CX3CR1-overexpressing or -knockdown cells. Three gastric cancer cell lines (MKN-28, SGC-7901 and MKN-45) and a gastric epithelial cell line (GES-1) were transfected with CX3CR1 cDNA or CX3CR1 short hairpin (sh)RNA, and then the CX3CR1 level was assessed in the stable transfected cells as detected by (A) fluorescence microscopy, (B) western blotting and (C) quantitative real-time PCR. *P<0.05; **P<0.01.
We proposed that the minor change in CX3CR1 expression at the protein level after shRNA transfection was due to the fact that the CX3CR1 expression level in the parental SGC-7901 and MKN-45 cells was already quite low. In addition, an important finding was that CX3CR1 expressed in the GES-1 cells also stimulated GES-1 cell migration although the number of migrated cells was evidently fewer than that noted in the SGC-7901 and MKN-45 cells. Altogether, these findings suggest that on the one hand, increased expression of CX3CR1 in gastric cancer cells might play a role in migration and invasion; on the other hand, the CX3CR1-CX3CL1 axis also stimulated GES-1 cell migration.

The CX3CR1/CX3CL1 axis stimulates both gastric cancer and GES-1 cell proliferation. The effects of the CX3CR1/CX3CL1 axis on MKN-28, SGC-7901, MKN-45 and GES-1 cell proliferation were assessed by the CCK-8 assay. Under optimal culture conditions (in the presence of 10% FBS), addition of CX3CL1 (200 ng/ml) significantly increased the proliferation of CX3CR1-overexpressing cells compared with that of the empty vector-transfected cells. Whereas, we found that knockdown of CX3CR1 production had no obvious impact on the proliferation of gastric cancer cells, and only shRNA-transfected GES-1 cells showed an inhibited proliferation compared with the irrelevant shRNA-transfected GES-1 cells (Fig. 5). In addition to our findings on CX3CR1, CCR7 has also been proven to promote the growth of gastric carcinoma (20,33).

The CX3CR1/CX3CL1 axis promotes gastric cancer and GES-1 cell survival. An important feature of metastatic cells is the ability to regulate their survival. We, therefore, tested whether the CX3CR1/CX3CL1 axis rescues gastric cancer and GES-1 cells from serum deprivation-induced death. All cells were cultured in serum-free medium with CX3CL1 (200 ng/ml) for 24 h before flow cytometric analysis. Fig. 6 shows that CX3CR1 overexpressed in gastric cancer and GES-1 cells significantly decreased the percentage of Annexin V-positive cells. The data suggest that the CX3CR1/CX3CL1 axis plays an antiapoptotic role in gastric cancer and GES-1 cells.

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The CX3CR1/CX3CL1 axis activates Akt kinase in gastric cancer and gastric epithelial cells in vitro. To investigate the mechanisms underlying the CX3CR1/CX3CL1 axis-induced proliferation and survival of gastric cancer and gastric epithelial cells, signal transduction experiments were next performed. Phosphorylated (p)-Akt (Ser-473) kinase has been found to be an important intermediary in the control of proliferation and apoptosis in many types of cells (21,34,35). We, therefore, studied the effect of the CX3CR1/CX3CL1 axis on Akt activation by measuring the levels of p-Akt (Ser-473) in protein extracts from cells incubated with CX3CL1 for 24 h. Quantitative analysis of the bands was performed by densitometry. As shown in Fig. 7, Akt was significantly phosphorylated in the CX3CR1-overexpressing cells compared with the empty vector-transfected cells. The CX3CR1/CX3CL1 axis has been previously identified as a proliferative factor activating Akt in epithelial ovarian cancer (36), endothelial cells (37) and neurons (34). In conclusion, these results strongly suggest that the proliferation and survival effect of the CX3CL1/CX3CR1 axis in gastric cancer and gastric epithelial cells is associated with Akt activation.
Discussion

In the present study we demonstrated that CX3CR1 was expressed not only in gastric carcinoma, but also in non-neoplastic gastric epithelium, and upregulated expression of CX3CR1 was associated with the metastasis, proliferation and survival of gastric cancer, and a parallel increase was observed in p-Akt levels. In addition to CCR7 (12,23), CXCR4 (33,38)
and CCR4 (14), we demonstrated that the chemokine receptor CX3CR1 is involved in metastasis and growth of gastric cancer. These findings suggest that gastric cancer metastasis is a complex and synergistic process involving multiple chemokine receptors and chemokines.

An intriguing finding was that CX3CR1 was expressed in non-neoplastic gastric tissues in vivo and GES-1 cells in vitro, and played a functional role in stimulating migration, promoting proliferation and inhibiting apoptosis of GES-1 cells in vitro. In addition to CX3CR1, other chemokine receptors were demonstrated to be expressed in normal tissue cells and play a physiological role. Murdoch and colleagues demonstrated that colon epithelium expresses CX3CR1 which regulates epithelial maintenance and renewal (39). Banas et al also found that CCR7 expression in mesangial cells (MC) promoted MC proliferation, migration and survival and enhanced ‘wound healing’ in vitro (40). Therefore we hypothesized that CX3CR1 expressed in normal gastric epithelial cells may have some biological function. Further studies are required to confirm the biological function of CX3CR1 in vivo.

In addition, contradictory CX3CR1 expression in gastric cancer cells in vivo and in vitro was found, that is, gastric cancer cells expressed a higher level of CX3CR1 than that in the non-neoplastic gastric epithelial cells in vitro, but expressed a lower or equal CX3CR1 protein level compared with the gastric epithelial cells in vitro. We hypothesized that there was some unknown mechanisms which could upregulate the expression of CX3CR1 in gastric cancer cells in vivo. Gaudin et al found...
that a decreased concentration of FBS in culture medium led to increased membrane expression of CX3CR1 in epithelial ovarian carcinoma BG1 cells (36). What is more, hypoxia enhanced CXCR4 expression, which was demonstrated in melanoma and oral squamous cell carcinoma (41,42). Thus, we speculated that hypoxia and the lack of nutrients in the tumor microenvironment might increase the expression of CX3CR1 in gastric cancer cells, thus promoting gastric cancer metastasis, proliferation and survival. Further studies are needed to confirm this hypothesis.

In summary, we demonstrated that CX3CR1 was expressed not only in gastric cancer tissues and gastric cancer cell lines, but also in non-neoplastic gastric tissues and a gastric epithelial cell line. Increased expression of CX3CR1 in gastric cancer cells promoted cancer cell metastasis, proliferation and survival, and an appropriate expression level of CX3CR1 in gastric tissues might be beneficial to cell renewal and/or tissue remodeling after injury. In addition, the tumor microenvironment may play an important role in the increased expression of CX3CR1 in gastric cancer cells. Further studies are warranted to clarify the mechanisms responsible for inducing overexpression of CX3CR1 in gastric cancer cells.

Acknowledgements

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References


