

Role of the CXCL12/CXCR4 Axis in Peritoneal Carcinomatosis of Gastric Cancer

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Abstract

Peritoneal carcinomatosis is a frequent cause of death in patients with advanced gastric carcinoma. Because chemokines are now considered to play an important role in the metastasis of various malignancies, we hypothesized that they may be involved in the development of peritoneal carcinomatosis by gastric carcinoma. Human gastric carcinoma cell lines, which were all highly efficient in generating malignant ascites in nude mice upon i.p. inoculation, selectively expressed CXCR4 mRNA and protein. In particular, NUGC4 cells expressed CXCR4 mRNA at high levels and showed vigorous migratory responses to its ligand CXCL12. CXCL12 enhanced proliferation and rapid increases in phosphorylation of protein kinase B/Akt and extracellular signal-regulated kinase of NUGC4 cells. We also showed that AMD3100 (a specific CXCR4 antagonist) effectively reduced tumor growth and ascitic fluid formation in nude mice inoculated with NUGC4 cells. Additionally, we examined human clinical samples. Malignant ascitic fluids from patients with peritoneal carcinomatosis contained high concentrations of CXCL12 (4.67 ng/mL). Moreover, immunohistochemical analysis showed that 22 of 33 primary gastric tumors with peritoneal metastasis were positive for CXCR4 expression (67%), whereas only 4 of 16 with other distant metastasis were positive (25%). Notably, 22 of 26 CXCR4-expressing primary tumors developed peritoneal metastases (85%). CXCR4 positivity of primary gastric carcinomas significantly correlated with the development of peritoneal carcinomatosis ($P < 0.001$). Collectively, our results strongly suggest that the CXCR4/CXCL12 axis plays an important role in the development of peritoneal carcinomatosis from gastric carcinoma. Thus, CXCR4 may be a potential therapeutic target for peritoneal carcinomatosis of gastric carcinoma. (Cancer Res 2006; 66(4): 2181-7)

Introduction

Peritoneal carcinomatosis is a frequent cause of death in patients with advanced gastric carcinoma and often occurs after surgery (1-3). Practically, there is no effective therapy for this condition. The 5-year survival rate of patients with peritoneal carcinomatosis is only 2% even including patients with i.p. free

cancer cells without macroscopic peritoneal carcinomatosis (4). Peritoneal carcinomatosis of gastric carcinoma may develop from direct cancer cell dissemination into the abdominal cavity. To design a new and effective treatment for peritoneal carcinomatosis, it is important to understand the molecular mechanisms that promote the development of this condition.

Chemokines are small secretory proteins that control migration and activation of leukocytes and other types of cell through interactions with a group of seven-transmembrane G protein-coupled receptors (5). It is now known that chemokines may also promote growth/survival and metastasis of several malignancies (6-9). Thus, the expression of chemokine receptors, especially CXCR4 and CCR7, by tumor cells can be an important factor in organ-specific metastases (10-15). For example, tumor cells from the breast express CXCR4, whereas high concentrations of CXCL12 (also called stromal-derived factor-1 α) are present at common metastasis sites of breast cancer (7). The interaction between CXCL12 and CXCR4 has also been implicated in the bone metastasis of prostate cancer (15). Furthermore, the CXCL12/CXCR4 axis has been shown to be involved in the metastasis of non-small-cell lung cancer cells, particularly in their dissemination into the pleural space (16). Here, we report the evidence that the CXCR4/CXCL12 axis also plays a role in the development of peritoneal carcinomatosis from gastric carcinoma.

Materials and Methods

Cell lines. The human gastric cancer cell lines MKN28, TMK-1, MKN45, NKPS, KATO III (17), and NUGC3/NUGC4 (18) were kindly provided by Dr. Yoshio Endo (Division of Experimental Therapeutics, Cancer Research Institute, Kanazawa University, Kanazawa, Japan). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, and 10 μ g/mL streptomycin (Invitrogen Corp., Carlsbad, CA). NKPS, KATO III, and NUGC4 were gastric carcinoma cell lines derived from malignant ascites or pleural effusions of patients with advanced gastric carcinoma. NUGC4 disseminates early after inoculation into the abdominal cavity of nude mice and forms bloody ascitic fluid (19).

Tissue samples. Tissue samples of primary tumors were taken during gastrectomy from untreated patients with gastric carcinomas. Histologic diagnosis was confirmed for each specimen. All surgical samples were obtained from the Division of Surgical Oncology, Cancer Research Institute, Kanazawa University. A written informed consent for molecular analysis of surgical sample was obtained from each patient.

RNA preparation and reverse transcription-PCR. Total RNA was isolated from frozen tissues and cell pellets using acid guanidinium thiocyanate (RNA-Bee; Tel-Test, Friendswood, TX) and phenol-chloroform. cDNA was generated from 1 μ g total RNA by reverse transcription using a Reverse Transcription System kit (Promega, Madison, WI). Reverse transcription-PCR (RT-PCR) was done as described previously (20) using the

Note: K. Yasumoto and K. Koizumi contributed equally to this work.

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primer sequences shown in Table 1. All PCR products were subjected to electrophoresis through native 10% polyacrylamide gels and were visualized by staining with SYBER green 1 (Nippon Gene, Tokyo, Japan).

For semiquantitative PCR, preliminary experiments were carried out to determine the linear range of PCR amplification using representative cases. After choosing 24 as a proper number of cycles, we did amplifications using protocols of denaturation at 96°C for 1 minute; annealing at 58°C, 60°C, 62°C, 64°C, 60°C, or 64°C for 1 minute; and extension at 72°C for 1 minute. The integrity of each RNA preparation was confirmed by amplifying each cDNA sample with primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Table 1) and by changing the number of cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute. Using NIH Image Program software, we did densitometric analysis and compared the levels of the GAPDH PCR products amplified by varying numbers of cycles. We determined the number of PCR cycles that would permit us to quantify the chemokine receptor products within a linear range.

Immunocytochemistry. Indirect immunocytochemistry was done for either CXCR4 or CXCL12 on cells grown in 24-well tissues slides (Costar Corp., Corning, NY). Cells were fixed in 4% paraformaldehyde at 25°C for 30 minutes and incubated with 10 µg/mL murine anti-human CXCR4 monoclonal antibody (DakoCytomation, Kyoto, Japan) or 10 µg/mL murine anti-human CXCL12 monoclonal antibody (IgG1, R&D Systems, Minneapolis, MN), followed by incubation with a 1:200 dilution of goat anti-murine FITC-conjugated serum (Jackson ImmunoResearch, Baltimore, MD).

Chemotaxis assay. Migration assays were done in 24-well Transwell plates (Costar) using inserts with 8 µm pore size membranes as described (21). Briefly, the lower surfaces of the membranes were precoated with 50 µL per filter fibronectin (Iwaki Glass, Tokyo, Japan) in PBS (25 µg/mL), allowed to dry at room temperature, and washed in PBS. Gastric cancer cells were suspended at 1×10^6 cells/mL in chemotaxis buffer [RPMI 1640/0.1% bovine serum albumin (BSA)] and placed in the upper chambers of the membrane; where indicated, the cells were preincubated with 10 µg/mL antihuman CXCR4 monoclonal antibody (IgG2b, DakoCytomation). After incubation for 24 hours, the cells on the lower surface of the membrane were stained and counted under a light microscope in at least five different fields (original magnification, $\times 200$). All assays were done in triplicate.

Proliferation assay. Gastric cancer cells (5×10^3) in RPMI 1640 supplemented with 1% BSA were added to each well of a 96-well cell culture plate and cultured for 18 hours. Where indicated, 10 µg/mL anti-CXCR4 monoclonal antibody (DakoCytomation) was added. The cells were

stimulated with various concentrations of CXCL12 and cultured for 72 hours. Two hours before the end of the assay, 10 µL of WST-8 (WST-8 Cell Counting kit, Wako Pure Chemical, Osaka, Japan) was added to each well, and the absorbance at 450 nm in each well was measured using an ELISA plate reader (Labsystems, Helsinki, Finland).

Western blot analysis. Cells were cultured in RPMI 1640 supplemented with 0.5% FBS for 24 hours. After indicated treatments, cell lysates were prepared with sample buffer [25 mmol/L Tris-HCl (pH 6.8), 5% w/v glycerol, 1% w/v SDS, and 0.05% w/v bromophenol blue] and were subjected to SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Blots were probed by primary antibodies and then treated with horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO) and enhanced chemiluminescence (ECL) kit (Amersham, Piscataway, NJ). Antibodies against phospho-extracellular signal-regulated kinase (ERK) and phospho-protein kinase B/Akt were purchased from Cell Signaling (Beverly, MA).

Animal studies. Pathogen-free, 6-week-old, female KSN Slc (*nu/nu*) mice were obtained from Japan SLC (Hamamatsu, Japan). Animals were quarantined for 1 week under pathogen-free conditions at the Advanced Science Research Center of Kanazawa University to confirm the absence of diseases before use. Mice weighing between 18 and 22 g were used for the experiments, complying with the standards set out in the Guidelines for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University. Animals were injected i.p. with 5×10^6 NUGC4 cells in 250 µL PBS. NUGC4 cells can rapidly disseminate in the abdominal cavities of nude mice upon i.p. inoculation and form bloody ascitic fluid (19). Mice were given i.p. injections of 7.5 mg/kg AMD3100 (Sigma, St. Louis, MO), a small-molecule CXCR4 antagonist, or PBS daily simultaneously beginning i.p. injection of NUGC4 cells. No toxicity of AMD3100 was detected in *in vivo* assays. To monitor the extent of the development of peritoneal carcinomatosis, the body weights were routinely measured. Mice were sacrificed 40 days after tumor injection. The ascitic fluid was collected and the volume was recorded for each mouse. We also examined the macroscopic peritoneal tumor dissemination and the size and number of tumors in the abdomen.

ELISA. The concentration of CXCL12 in each sample of ascitic fluid was measured using Quantikine ELISA kits (R&D Systems, Minneapolis, MN) according to the protocol of the manufacturer; this system had a lower detection limit of 18 pg/mL CXCL12. The absorbance at 450 nm was measured using an ELISA plate reader (Integrated EIA Management System, Labsystems).

Immunohistochemistry. Surgical specimens of primary gastric tumor were routinely fixed in formalin and embedded in paraffin. Following antigen retrieval, the sections were stained with a 1:500 dilution of polyclonal goat anti-human CXCR4 antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) using a standard indirect avidin-biotin HRP method. The sections were developed with diaminobenzidine, counterstained with Mayers hematoxylin, and mounted. The sections were regarded as positive when the intensity of staining was $\geq 10\%$.

Statistical analysis. The mean and SD or SE were calculated for all variables. Between-group statistical significance was determined using the χ^2 test or Student's *t* test where appropriate. *P* < 0.05 was considered as statistically significant.

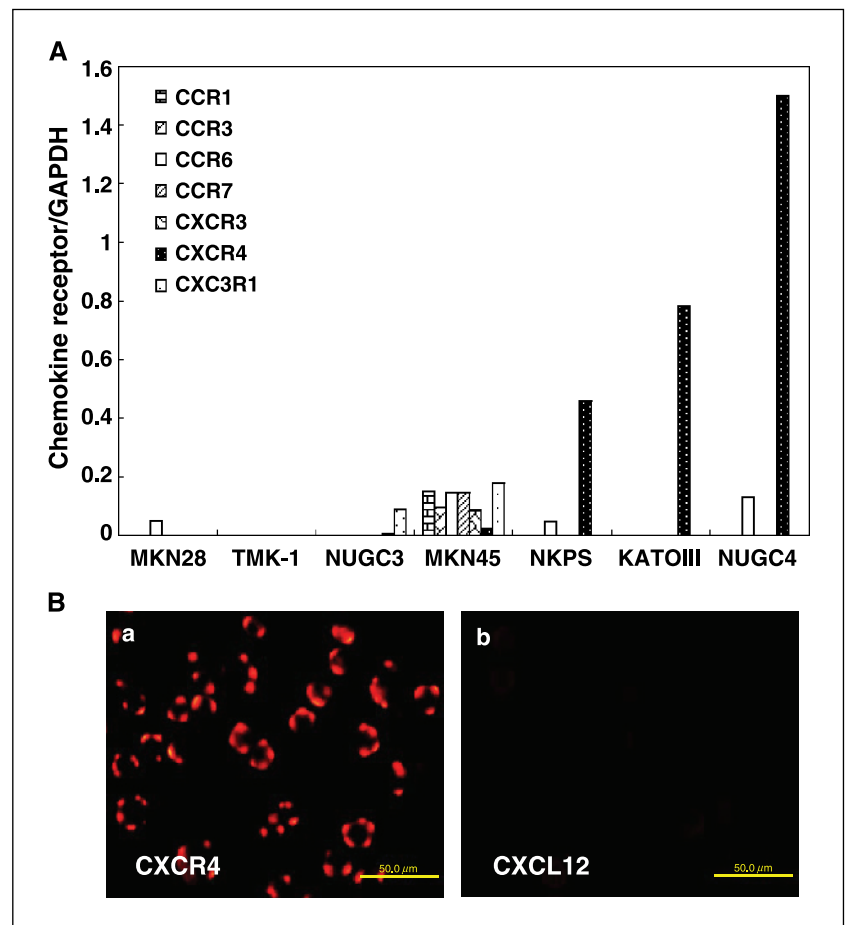
Results

Selective expression of CXCR4 in human gastric carcinoma cell lines. By using RT-PCR, we first examined mRNA expression of chemokine receptors—CCR1, CCR3, CCR6, CCR7, CXCR3, CXCR4, and CX3CR1—in seven human gastric carcinoma cell lines. As shown in Fig. 1A, the three gastric carcinoma cell lines (NKPS, NUGC4, and KATO III) selectively expressed CXCR4 mRNA at high levels. In particular, NUGC4, which rapidly disseminates in the abdominal cavity of nude mice upon i.p. inoculation (19), expressed CXCR4 mRNA at high levels. NKPS and NUGC4 also expressed CCR6 at low levels. On the other hand, the other four gastric carcinoma cell lines—MKN28, TMK-1, NUGC3, and MKN45—hardly expressed

Table 1. Primer sequences in RT-PCR analysis of human chemokine receptors

Gene	Primers
<i>CCR1</i>	5'-CAACTCCGTGCCAGAAGGTGAA-3' (forward) 5'-GCCAGGCCCAATGATGAT-3' (reverse)
<i>CCR3</i>	5'-GAGCCCGGACTGTCACTTTTG-3' (forward) 5'-CAGATGCTTGCTCCGCTCACAG-3' (reverse)
<i>CCR6</i>	5'-CCTGGGGAATATTCTGGTGGTGA-3' (forward) 5'-CATCGCTGCCTTGGGTTGTGAT-3' (reverse)
<i>CCR7</i>	5'-GTGCCCGCGTCTTCTCATCAG-3' (forward) 5'-GGCCAGGACCACCCATTTGTAG-3' (reverse)
<i>CXCR3</i>	5'-CAACGCCACCCACTGCCAATACAA-3' (forward) 5'-CAGGCGCAAGAGCAGCATCCACA-3' (reverse)
<i>CXCR4</i>	5'-ATCTTCTGCCACCATCTACTCCATCATC-3' (forward) 5'-ATCCAGACGCCAACATAGACCACCTTTCA-3' (reverse)
<i>CX3CR1</i>	5'-TGGCCTTGCTGATCTGCTGTTTG-3' (forward) 5'-CTTGGTGTCTCTCGGTTTCGGTA-3' (reverse)
<i>CXCL12</i>	5'-CCGCGCTCTGCTCAGCGACGGGAAG-3' (forward) 5'-CTTGTTTAAAGCTTTCTCCAGGTACT-3' (reverse)
<i>GAPDH</i>	5'-CCACCCATGGCAAATCCCATGGCA-3' (forward) 5'-TCTAGACGGCAGGTCAGGTCCACC-3' (reverse)

Figure 1. RT-PCR analysis for expression of chemokine receptors and immunocytochemical staining of CXCR4 and CXCL12. *A*, semiquantitative RT-PCR analyses on the expression of seven chemokine receptors (CCR1, CCR3, CCR6, CCR7, CXCR3, CXCR4, and CXCR3R1) in seven gastric carcinoma cell lines (MKN28, TMK-1, NUGC3, MKN45, NKPS, KATO III, and NUGC4). The relative expression level of each chemokine receptor mRNA to GAPDH mRNA is shown. *B*, NUGC4 cells were immunocytochemically stained with specific antibodies to CXCR4 (*a*) and CXCL12 (*b*). CXCR4 signals are localized strongly in the membrane and weakly in the cytoplasm, whereas CXCL12 signals are not detected. Original magnification, $\times 200$. Representative from one of three independent experiments.



CXCR4 mRNA. Only MKN45 expressed mRNA of various chemokine receptors at low levels. We also examined mRNA expression of CXCL12, the ligand of CXCR4, by RT-PCR. We found no expression of CXCL12 mRNA in any of the gastric carcinoma cell lines (data not shown).

We also examined protein expression of CXCR4 and CXCL12 by immunocytochemistry using specific antibodies. As shown in Fig. 1*B*, NUGC4 cells were strongly positive for CXCR4 but not for CXCL12. The results from other six cell lines were also in good agreement with our RT-PCR results (data not shown). Because NUGC4 cells exhibited the highest levels of CXCR4 expression, we used this cell line as our model of CXCR4-expressing gastric carcinoma in the subsequent experiments.

Functional significance of CXCR4 in gastric cancer cells.

To determine whether CXCR4 expressed by NUGC4 cells was functional, we first examined chemotactic responses of NUGC4 cells to CXCL12. As shown in Fig. 2*A*, NUGC4 cells showed significant chemotactic response to CXCL12 with a typical bell-shaped dose-response curve and an optimal concentration of 10 to 100 ng/mL. Furthermore, the chemotactic responses of NUGC4 cells to CXCL12 were significantly blocked by neutralizing anti-CXCR4 antibody (Fig. 2*B*).

We next examined effects of CXCL12 on cell proliferation in NUGC4 cells. As shown in Fig. 3*A*, CXCL12 significantly and dose-dependently enhanced cell proliferation of NUGC4 cells when the cells were maintained in suboptimal serum-free growth conditions for 72 hours. We also confirmed that neutralizing anti-CXCR4

antibody significantly blocked the enhancing effects of CXCL12 on proliferation of NUGC4 cells (Fig. 3*B*).

By using Western blotting, we further examined phosphorylation of Akt and ERK, the molecules involved in cell survival signals (22). As shown in Fig. 3*C*, we observed rapid and strong increases in

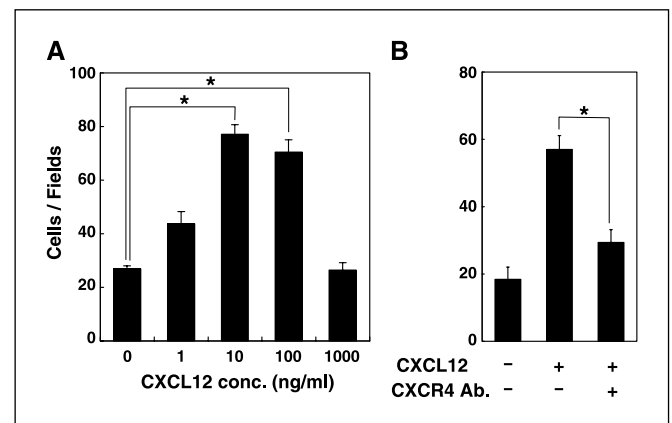


Figure 2. Induction of cell migration in CXCR4-expressing NUGC4 cells by CXCL12. NUGC4 cells expressing CXCR4 mRNA at high levels were examined for cell migration to CXCL12 using Transwell plates. *A*, dose-dependent chemotactic responses of NUGC4 cells to CXCL12. *B*, effect of neutralizing anti-CXCR4 antibody on CXCL12-mediated chemotaxis in NUGC4 cells. Columns, mean number of migrated cells per field from a representative experiment done in triplicate and repeated thrice; bars, SD. *, $P < 0.05$.

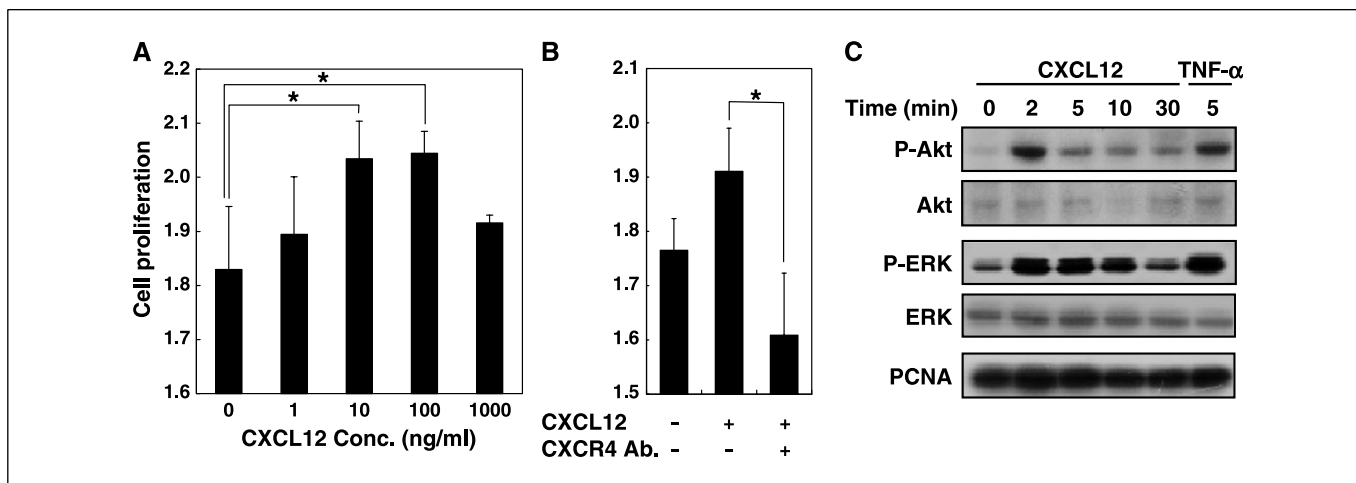


Figure 3. CXCL12 enhances proliferation of NUGC4 cells and induces rapid phosphorylation of Akt and ERK. **A**, NUGC4 cells were grown in serum-free medium with or without indicated doses of CXCL12. CXCL12 significantly increased the cell number of NUGC4 cells. **B**, NUGC4 cells were grown as above without or with 10 µg/mL anti-CXCR4 antibody. Neutralizing anti-CXCR4 antibody significantly blocked CXCL12-induced cell proliferation. Representative of three independent experiments. Bars, SD. *, $P < 0.05$. **C**, immunoblotting analysis for phosphorylation of Akt and ERK. NUGC4 cells were seeded in the 6.0 cm dish (1×10^6 per dish), and were stimulated with CXCL12 (100 ng/mL) or TNF- α (100 ng/mL) for the indicated time periods. Whole cell lysates were electrophoretically fractionated, blotted onto a filter membrane, and probed with primary antibodies against phospho-Akt, Akt, phospho-ERK, and ERK. The bound primary antibodies were detected by HRP-conjugated anti-rabbit IgG and visualized with the ECL system. Representative from three separate experiments.

phosphorylation of Akt and ERK in NUGC4 cells upon stimulation with CXCL12. The enhanced phosphorylation of Akt and ERK by CXCL12 was already seen in 2 minutes and at levels similar to those induced by tumor necrosis factor (TNF- α).

Collectively, CXCR4 expressed on NUGC4 cells was functional and capable of mediating CXCL12-induced chemotactic responses, cell proliferation, and cell survival in NUGC4 cells.

AMD3100, a CXCR4 antagonist, inhibits ascites formation by NUGC4 cells in nude mice. Upon i.p. inoculation of nude mice, NUGC4 cells were known to efficiently produce peritoneal carcinomatosis (19). We took advantage of this model and tested the effect of a CXCR4 antagonist AMD3100 (23) on the development of experimental peritoneal carcinomatosis in mice. Figure 4 shows the representative macroscopic results of mice 40 days after tumor inoculation. The sizes of disseminated tumors on the greater omentum and mesenterium in the abdominal cavity of mice daily treated with AMD3100 were consistently less than half of those in mice daily treated with PBS alone. Furthermore, the treatment with AMD3100 significantly ($P < 0.05$) decreased the volumes of ascitic fluid (PBS alone, mean \pm SE = 8.2 ± 2.68 mL; AMD3100, mean \pm SE = 2.1 ± 4.20 mL; Fig. 5A) and the body weight gains (PBS alone, mean \pm SE = 27.2 ± 2.58 g; AMD3100, mean \pm SE = 23.5 ± 1.26 g; Fig. 5B).

Production of CXCL12 by peritoneal mesothelial cells and presence of CXCL12 in malignant ascitic fluid. To determine the clinical relevance of CXCR4 in gastric carcinoma, we next examined expression of CXCL12 in peritoneum, liver, lymph nodes, and gastric mucosa representing the main sites of gastric cancer metastasis. As shown in Fig. 6A, CXCL12 mRNA was strongly expressed in peritoneum but only weakly in other tissues. We also did immunohistochemical staining of CXCL12 protein in the representative target tissues of gastric cancer metastasis. In accordance with the results from RT-PCR, we observed strong staining of CXCL12 in peritoneal mesothelial cells (Fig. 6B) but not in liver or lymph node tissues (data not shown). The contents of CXCL12 protein were also much higher in 19 malignant ascitic fluids from patients with gastric carcinoma (mean, 4,667 pg/mL;

range, 2,530-7,320 pg/mL) than in six nonmalignant peritoneal exudates fluids (mean, <2,000 pg/mL).

Expression of CXCR4 in primary gastric tumors with or without peritoneal carcinomatosis. To analyze the correlation of CXCR4 expression by primary gastric carcinoma cells and development of peritoneal carcinomatosis, we did immunohistochemical

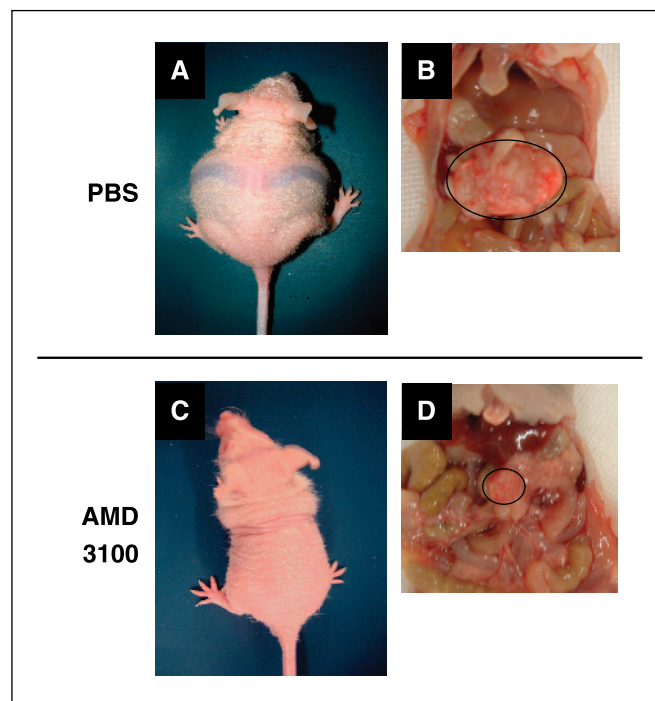


Figure 4. The CXCR4 antagonist AMD3100 prevents experimental peritoneal carcinomatosis. Representative results of PBS-treated mice (**A** and **B**) and AMD3100-treated mice (**C** and **D**) on 40 days after i.p. inoculation of CXCR4-expressing NUGC4 cells (5×10^6 per mouse). Treatment with PBS or AMD3100 was done daily starting from the day of tumor inoculation. **B** and **D**, omental tumors in abdominal cavity.

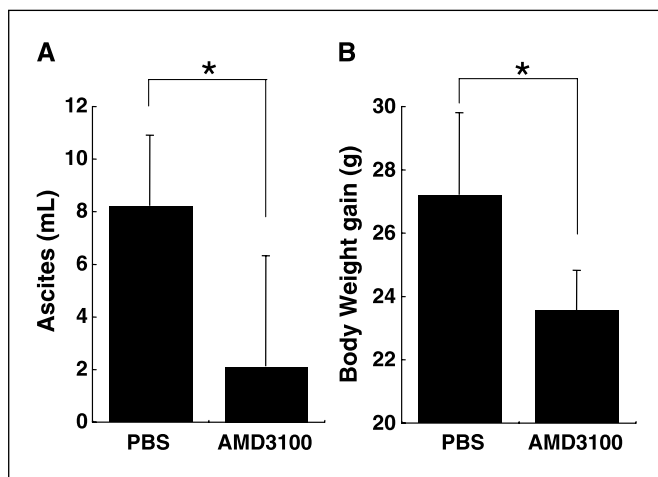


Figure 5. Reduction of ascitic fluid formation and body weight gain by the CXCR4 antagonist AMD3100. Ascitic fluid volumes (A) and body weights (B). Mice were i.p. injected with CXCR4-expressing NUGC4 cells and daily treated with AMD3100 (6 mg/mouse) or PBS alone starting from the day of tumor inoculation. On day 40, autopsy was done. Representative data from two experiments (four or five animals per group per experiment). Bars, SD. *, $P < 0.05$.

staining of CXCR4 in 49 primary tumors of stage IV gastric carcinoma. These samples were derived from 33 patients with peritoneal metastases and 16 patients with distant metastases but not peritoneal metastases. Representative results are shown in Fig. 7. As summarized in Table 2, CXCR4 expression in primary gastric tumors is significantly correlated with the development of peritoneal carcinomatosis ($P < 0.01$). Among the 33 primary gastric tumors with peritoneal metastasis, 22 tumors were scored positive for CXCR4 expression (67%). On the other hand, only 4 of 16 primary tumors with other distant metastases were scored positive for CXCR4 (25%). Furthermore, 22 of 26 CXCR4-expressing primary tumors developed peritoneal metastases (85%), whereas 11 of 23 CXCR4-negative primary tumors developed peritoneal metastases (48%). These results support the notion that CXCR4 expression of primary gastric carcinoma promotes the development of peritoneal carcinomatosis.

Discussion

Peritoneal carcinomatosis is a frequent cause of death in patients with advanced gastric carcinoma and often associated with malignant ascites (1–3). The molecular mechanisms by which gastric carcinoma undergoes peritoneal carcinomatosis remain to be clarified. Here, we have shown that human gastric carcinoma cell lines derived from malignant ascites (NUGC4) or pleural effusions (NKPS, KATOIII) selectively express CXCR4 mRNA and proteins at high levels (Fig. 1). We have also shown that its ligand CXCL12 induces chemotactic responses, cell proliferation, and a rapid phosphorylation of Akt and ERK in NUGC4 cells expressing CXCR4 at high levels (Figs. 2 and 3). Furthermore, we have shown that the treatment with a CXCR4 antagonist AMD3100 (23) significantly suppresses the development of peritoneal carcinomatosis in a xenograft model of NUGC4 cells in nude mice (Fig. 4). The inhibition of the experimental peritoneal carcinomatosis by AMD3100 was clearly shown by the reduction of ascitic fluids and the inhibition of growth of disseminated tumors in the AMD3100-treated mice in comparison with the PBS-treated control mice (Fig. 5).

To verify the important role of CXCR4 in promotion of peritoneal carcinomatosis by gastric carcinoma, we extended our investigations into human clinical samples. We have shown that peritoneal mesothelial cells strongly express its ligand CXCL12 (Fig. 6). In contrast, CXCL12 is barely expressed in liver or lymph nodes, the other main sites of distant gastric cancer metastasis (data not shown). Furthermore, we have shown that malignant ascitic fluids from patients with peritoneal carcinomatosis of gastric cancer contained CXCL12 at concentrations much higher than normal fluids in the peritoneal cavity. Most importantly, we have shown that CXCR4 expression in primary tumors of patients with advanced gastric carcinomas significantly correlates with the occurrence of peritoneal carcinomatosis (Fig. 7; Table 2). Collectively, these results strongly suggest that CXCR4-expressing gastric carcinoma cells are preferentially attracted to the peritoneum cavity where its ligand CXCL12 is abundantly produced.

Besides migration of cancer cells, their local growth and/or survival are also important for cancer metastasis. In this context, growth factors, such as vascular endothelial growth factor (VEGF) and VEGF-C, have been associated with the development of peritoneal metastasis (24–26). In particular, VEGF, also called vascular permeability factor, is considered a potent mediator of peritoneal fluid accumulation and a primary stimulant of vascularization in ascites tumors. VEGF is markedly elevated in malignant ascites and is one of the essential elements in the development of peritoneal metastasis (27). VEGF is produced by several types of tumor cells and peritoneal mesothelial cells (26). In our experiments, VEGF was produced by human gastric carcinoma cells and elevated in malignant ascitic fluids from patients with gastric carcinoma (data not

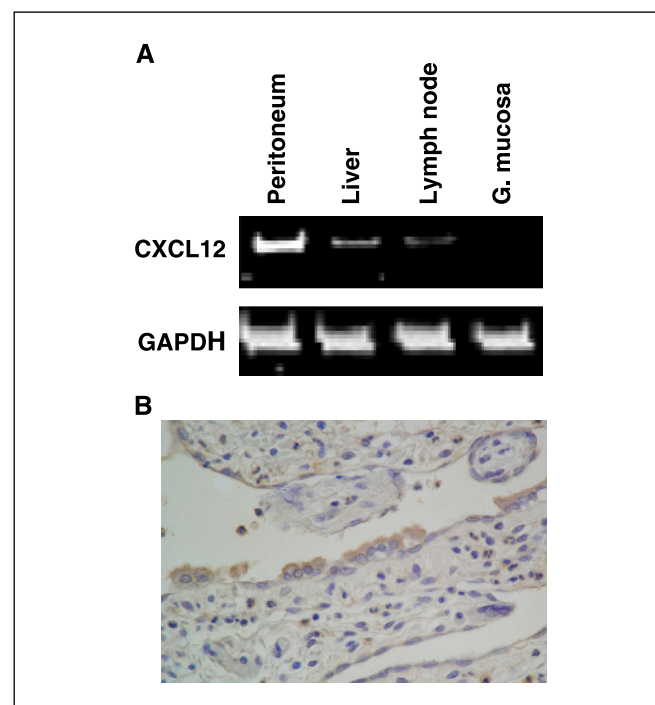


Figure 6. High CXCL12 expression by mesothelial cells in human normal peritoneum. A, RT-PCR analysis for expression of CXCL12 in peritoneum, liver, and lymph node, representing the main sites of gastric cancer metastasis and gastric mucosa. B, immunohistochemistry of peritoneum. Mesothelial cells in the normal peritoneum are strongly positive for CXCL12. Magnification, $\times 160$. G., gastric.

shown). VEGF also has a direct effect on tumor cell proliferation and invasion (28). However, VEGF production is not always associated with the metastatic potential in peritoneal cavity (26). We have shown here evidence that the CXCR4/CXCL12 axis plays a role in the development of peritoneal carcinomatosis with malignant ascites from gastric cancer. Notably, CXCL12 was initially identified as a growth-stimulating factor for B-cell progenitors (29). Given that CXCL12 was not expressed by any human gastric carcinoma cell lines (Fig. 1B), CXCL12 produced by peritoneal mesothelial cells may act on proliferation and/or survival of CXCR4-expressing gastric carcinoma cells in the peritoneal cavity by a paracrine manner, leading to survival and peritoneal dissemination of gastric carcinoma. The production of CXCL12 is also markedly elevated in malignant ascites. It remains to be seen how CXCL12 production is regulated in the peritoneal cavity. CXCL12 is produced by several tumors, including ovarian cancer (9) and pancreatic cancer (30), and is also produced by stromal cells such as peritoneal mesothelial cells (16, 26), vascular endothelial cells, and fibroblasts. In particular, carcinoma-associated stromal fibroblasts have the capacity to efficiently secrete CXCL12 and promote tumor growth through elevated CXCL12 secretion (31). Moreover, the interaction between VEGF, CXCR4, and CXCL12 has been recently reported to be relevant to the development of peritoneal metastasis (32, 33). Tumor-derived CXCL12 synergizes VEGF-mediated neovascularization of multifocal i.p. dissemination in human ovarian carcinomas, and VEGF also up-regulates CXCR4 expression on vascular endothelial cells and tumor cells (33). CXCR4 and CXCL12 are up-regulated by VEGF and contribute to glioma cell invasion (28). Therefore, activation of CXCR4 in malignant glioma cells promotes the production of VEGF (34). Additionally, inflammatory cytokines, such as TNF- α and interleukin-1 β , which are potent modulators

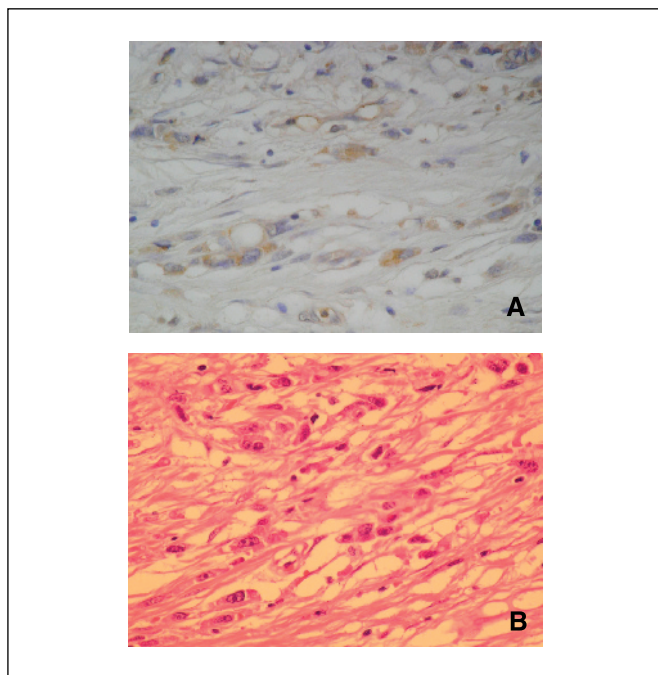


Figure 7. Immunohistochemical staining of CXCR4 in human primary gastric tumors. Anti-CXCR4 staining (A) and H&E staining (B) of a representative CXCR4-positive gastric cancer. CXCR4 is immunolocalized strongly in the membranes and weakly in the cytoplasm of these cells. Original magnification, $\times 160$.

Table 2. Significant correlations between CXCR4 expression in primary gastric carcinomas and peritoneal carcinomatosis

Peritoneal carcinomatosis	CXCR4	
	+ (n = 26)	- (n = 23)
Positive	22*	11
Negative	4	12

NOTE: Immunohistochemical staining of CXCR4 was done in 46 samples of primary gastric cancer with (n = 26) or without (n = 23) peritoneal carcinomatosis.

* $P < 0.01$, χ^2 test, two-sided.

of chemokine expression and frequently present in the tumor microenvironment, fail to modify the CXCR4 expression on tumor cells. In contrast, IFN- γ , which is well known as a crucial factor in immune resistance against tumors, consistently reduced CXCR4 expression (35). Moreover, hypoxia synchronously induces CXCL12 and VEGF production by ovarian cancers (33) and also induces CXCR4 expression in cancer cells (36). It would also be interesting to determine whether other cytokines/growth factors and/or the hypoxia condition present in ascitic fluid synergize with CXCL12 in the dissemination promoting effects on gastric carcinoma cells.

We have shown that CXCL12 stimulates cell proliferation and also induces a rapid phosphorylation of Akt and ERK in a CXCR4-expressing gastric carcinoma cell line NUGC4 (Fig. 3). Phosphorylation of survival kinase Akt suppresses various types of apoptotic molecules, including caspase-9, caspase-3, and Bcl-2 (37). The activation of Akt induced by CXCL12 has been shown to be associated with promotion of survival and prevention of apoptosis in glioma cells (38). Furthermore, CXCL12-dependent proliferation correlates with phosphorylation and activation of ERK in ovarian cancer cells (9).

In conclusion, our results suggest that the expression of CXCR4 in biopsy specimen from primary gastric tumors may be useful for preoperative evaluation of risks for the occurrence of peritoneal carcinomatosis. Evaluation of CXCL12 levels in intraoperative lavage fluids of abdominal cavity in patients with advanced gastric carcinomas may also be useful as a predictive molecular marker for the risk of peritoneal carcinomatosis. Furthermore, CXCR4 antagonists may be useful for the treatment of peritoneal carcinomatosis, an incurable complication of gastric tumor, and especially beneficial for patients with i.p. free cancer cells without macroscopic peritoneal metastasis. Targeting CXCL12 signal may also be a novel, efficient strategy for treating human gastric cancers.

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