Article

Roles of Chemokine Receptor 4 (CXCR4) and Chemokine Ligand 12 (CXCL12) in Metastasis of Hepatocellular Carcinoma Cells

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Chemokines are involved in human hepatocellular carcinoma (HCC) carcinogenesis. However, the exact mechanism of chemokines in HCC carcinogenesis remains unknown. Here we investigated the roles of chemokine receptor 4 (CXCR4) and chemokine ligand 12 (CXCL12) in the metastasis of HCC. We found that the expression levels of CXCR4 mRNA in HCC tissues, MHCC97 cells, and HUVEC cells were 2.52 ± 1.13, 2.34 ± 1.16 and 1.63 ± 1.26, respectively and that the CXCR4 protein levels were 1.38 ± 0.13, 1.96 ± 0.32 and 1.86 ± 0.21, respectively. In contrast, CXCR4 was not detected in normal hepatic tissues. In 78 HCC patients, we also found that the concentration of CXCL12 in cancerous ascitic fluid was 783-8,364 pg/ml and that *CXCL12* mRNA level in HCC metastasis portal lymph nodes was 1.21 ± 0.87 but undetectable in normal hepatic tissues. Finally we discovered that recombinant human CXCL12 could induce MHCC97 cells and HUVEC cells to migrate with chemotactic indexes (CI) of 3.9 ± 1.1 and 4.1 ± 1.6, respectively. Cancerous ascitic fluid could also induce the migration of MHCC97 cells with a CI of 1.9 ± 0.8. Thus, our data suggest that CXCR4 and CXCL12 may play an important role in the metastasis of HCC by promoting the migration of tumor cells. *Cellular & Molecular Immunology*. 2008;5(5):373-378.

Key Words: CXCR4, CXCL12, hepatocellular carcinoma, migration, metastasis

Introduction

Chemokines are a superfamily of small secreted molecules. There are as many as 40 chemokine ligands and at least 20 corresponding receptors (1, 2). Recent studies have found that the chemokines and their receptors play important roles in inflammatory responses, angiogenesis, tumor growth and metastasis (3-5). Chemokines attract and activate specific subsets of leucocytes and thus mediate chemotactic responses. Some chemokines and their receptors regulate the migration of certain cells in the lymphoid system and also control the migration of tumor cells such as breast cancer cells (6, 7) and melanoma (8). Several chemokine/receptor pairs have been identified to control tumor cell migration (9). The chemokine ligands can be separated into four categories depending on the amino acid motif in their N termini, which include C, CC, CXC, CX3C. CXCL12 (stromal cell-derived factor-1, SDF-1) is a member of the CXC chemokine subfamily and binds to CXC chemokine receptor 4 (CXCR4). The expressions of CXCL12 and CXCR4 have been detected in several cancer cells indicating that they may be important in metastasis of tumor cells (10-14).

Hepatocellular carcinoma (HCC) is an aggressive and rapidly fatal malignancy representing the fifth most common cancer worldwide (7) and the second leading cause of death in China (8). Although surgical techniques and perioperative care have improved in recent years, the long-term prognosis of HCC remains unsatisfactory because of its high capacity of invasiveness and metastasis. Chemokine receptors have been indicated to be involved in HCC carcinogenesis (15-19). However, the exact mechanism of CXCL12-CXCR4 in HCC carcinogenesis remains unknown.

In this study, we investigated the expression patterns of CXCR4 and CXCL12 in human HCC specimens, metastatic lymph nodes and HCC cell line MHCC97 by RT-PCR and Western blot. Furthermore, we studied their effects on the migration of tumor cells.

Materials and Methods

Samples and cells

Following informed consent, 25 cancerous ascitic fluid and 43 metastasis portal lymph nodes obtained from individuals with histologically confirmed HCC were collected from the Department of Hepatic Surgery, Eastern Hepatobiliary

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Surgery Hospital, Second Military Medical University. Seventy-eight HCCs was biopsy-proven in all poorly differentiated adenocarcinoma type, including 61 males and 17 females, with ages ranging from 25 to 76 years old (median 49 ± 6.4 years). HUVECs, which expressed the CXCR4, as the positive control, were cultured as described previously (20).

RT-PCR

Total RNA from HCC cell line MHCC97 and HUVECs was extracted using a Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The RNA was treated with DNase, and 2.5 μg of total RNA was used for cDNA synthesis using random hexamers. Full-length open reading frames of CXCR4 and CXCL12 were amplified by PCR from cDNA samples of MHCC97 or HUVECs. For human CXCR4, the sense primer, 5'-ATG CAA GGC AGT CCA TGT CAT-3' and the reverse primer 5'-ATG AAT GTC CAC CTC GCT TT-3' yield a 692 bp product. For human CXCL12, the sense primer 5'-GAG CCA ACG TCA AGC ATC TG-3' and the reverse primer 5'-CGG GTC AAT GCA CAC TTG TC-3' yield a 227 bp product. The primers used for glyceraldehyde-3-phosphate dehydrogenase (internal control) were 5'-AAT CCC ATC ACC ATC TTC CA-3' and 5'-CCT GCT TCA CCA CCT TCT TG-3'. The PCR products were analyzed by agarose gel electrophoresis and confirmed by appropriate size and/or sequencing.

Western blot

Supplemented with 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM sodium orthovanadate, 5 μg/ml leupeptin, 2 μg/ml aprotonin, and one Complete Protease Inhibitor Cocktail tablet (Roche Diagnostics Corporation, Indianapolis, IN) per 50 ml buffer, crude protein extracts were used directly for Western blot analysis. Protein concentration was determined using Bio-Rad protein assay kit (BioRad). Twenty micrograms of total protein from each sample was separated on a 10% Tris-glycine polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Invitrogen, San Diego, CA). Membranes were blocked for 2 h at room temperature in PBS containing 5% non-fat dry milk and 0.1% Tween-20. The membranes were then incubated overnight at 4°C in primary antibody (anti-CXCR4 or anti-CXCL12, Santa Cruz, CA) diluted 1:1,000 in PBS containing 5% non-fat dry milk and 0.1% Tween-20. The membranes were washed in PBST (PBS containing 0.1% Tween-20) three times and then incubated for 1 h with secondary antibody (goat anti-mouse-HRP, Santa Cruz, CA). The blots were washed in PBST three times and the proteins were detected using the ECL Plus Western Blotting Kit (Amersham Biosciences UK Limited, Buckinghamshire, UK) and X-MAT AR Film (Eastman Kodak, Rochester, NY).

Enzyme-linked immunosorbent assay (ELISA)

Cancerous ascitic fluid samples were collected from patients with hepatocellular carcinoma. After centrifugation, sera were divided into aliquots and stored at -80°C. The amounts of CXCL12 in these cancerous ascitic fluids were determined using ELISA (Ferment, United States).

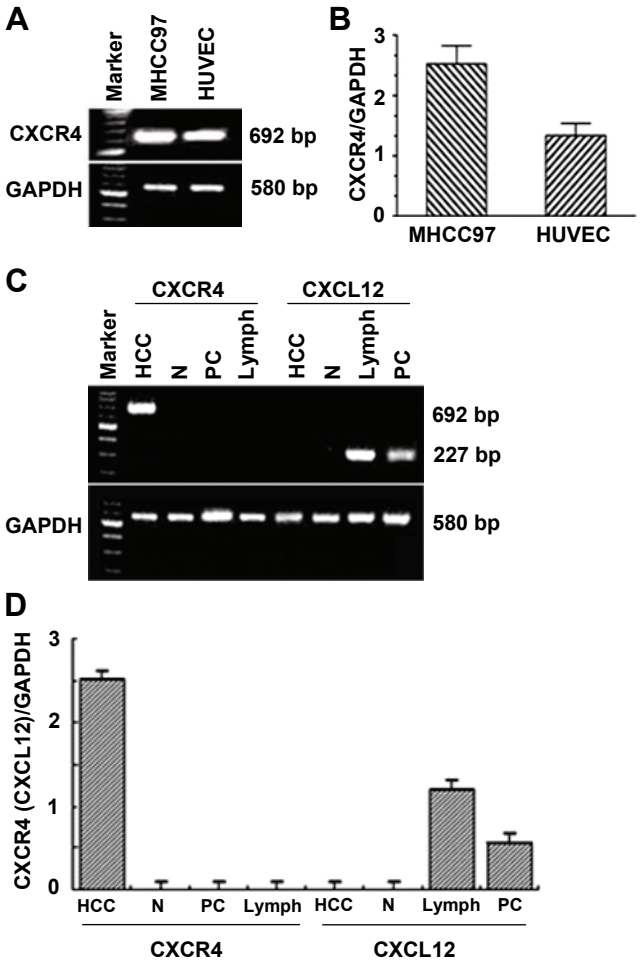

Figure 1. Expressions of CXCR4 and CXCL12 genes in HCC cells or tissues. (A) Electrophoretic analysis of RT-PCR products with primers specific to CXCR4 in MHCC97 and HUVECs. (B) Quantification of the expression levels of CXCR4 in cells by Grey Scanning. (C) Electrophoretic analysis of RT-PCR products with primers specific to CXCR4 and CXCL12 in different tissues. (D) Comparison of expression levels of CXCR4 and CXCL12 across different tissues. HCC, hepatocellular carcinoma tissues; N, normal hepatic tissues; PC, tissues surrounding HCC; Lymph, HCC metastasis portal lymph nodes. Data presented the representative of three independent experiments.

Chemotaxis assay

Chemotaxis assays were done using 48-well chemotaxis chambers (Neuro Probe, Cabin John, MD) as described previously (21). Aliquots of 27 to 29-μl assay medium (RPMI 1640 containing 1% bovine serum albumin, 30 mmol/L HEPES) containing different concentrations of CXCL12 were placed in the lower wells of the chamber. Cell suspension (50 μl, 1×10^6 cells/ml) was placed in the upper wells. The upper and lower wells were separated by a polycarbonate filter (Osmonics, Livermore, CA; 10-μm pore size) which was precoated with 50 μg/ml collagen type I

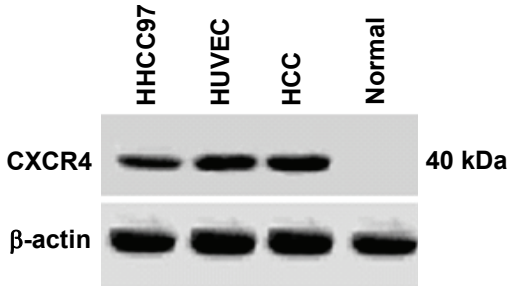

Figure 2. Expression pattern of CXCR4 protein in MHCC97, HUVECs, HCC tissues and normal hepatocellular tissues. The expression levels of CXCR4 protein in MHCC97 cells, HUVEC cells and HCC tissues were 1.96 ± 0.32, 1.86 ± 0.21 and 1.38 ± 0.13, respectively. But CXCR4 was not detectable in normal hepatocellular tissues.

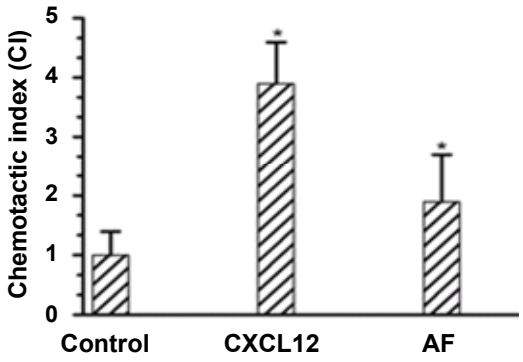

Figure 3. CXCL12 and cancerous ascitic fluid induced metastatic migration. The migration of MHCC97 cells in response to CXCL12 (100 ng/ml) and cancerous ascitic fluid (AF) (contained CXCL12 with a concentration of 8,364 pg/ml) was measured using chemotaxis chambers. Chemotactic index was calculated as the ratio of the number of MHCC97 cells migrating toward CXCL12 or ascitic fluid to the number of MHCC97 cells migrating toward negative control. Columns indicate the mean value of 3 independent experiments and error bars indicate standard deviations.

(Collaborative Biomedical Products, Bedford, MA). After incubation at 37°C for 5 h, the filter was removed, stained, and the cells migrating across the filter were counted under light microscope. The results were presented as chemotaxis index (CI), which represents the fold increase in the number of migrating cells in response to chemoattractants over that in response to control medium.

Statistical analysis

Data were reported as mean values ± SD (standard deviation) and analyzed by Student's *t* test. The *p* values < 0.05 were considered statistically significant.

Results

CXCR4 and CXCL12 gene expression

Firstly the CXCR4 gene expression in MHCC97 and HUVECs cells was examined (Figure 1A). The expression levels of CXCR4 mRNA in MHCC97 and HUVEC cells were 2.34 ± 1.16 and 1.63 ± 1.26, respectively (Figure 1B). In 61 out of 78 HCC tissues CXCR4 expression was detected by RT-PCR with an expression level of 2.52 ± 1.13 (Figure 1C). Meanwhile, no CXCR4 expression was detected in HCC metastasis portal lymph nodes or normal hepatic tissues. On the other hand, CXCL12 expression was detected in 32 out of 43 portal lymph nodes and in 73 out of 78 tissues surrounding HCC. The expression levels of CXCL12 were 1.21 ± 0.87 and 0.58 ± 0.11 (Figure 1D). In addition, CXCL12 mRNA expression was not detectable in HCC and normal hepatic tissues (Figure 1C).

Table 1. The concentration of CXCL12 in 25 peritoneal fluids of HCC patients

Patient	Concentration of CXCL12 (pg/ml)				
HCC	5965	7562	6957	5296	4686
	6763	5642	6634	5932	783
	6871	7246	6934	7425	5964
	8364	4452	7241	5553	7574
	7124	6132	8143	7746	7853

CXCR4 protein expression

CXCR4 protein expression levels in MHCC97 and HUVECs were analyzed by Western blot. As shown in Figure 2, the levels of CXCR4 protein in MHCC97 and HUVEC cells were 1.96 ± 0.32 and 1.86 ± 0.21, respectively. CXCR4 protein was detected in all HCC tissues examined and the expression level was 1.38 ± 0.13. Consistent with the RT-PCR results, CXCR4 protein was undetectable in normal hepatic tissues.

Concentration of CXCL12 in cancerous ascitic fluid

We examined the concentration of CXCL12 in 25 peritoneal fluids of HCC patients by ELISA. The results showed that all samples contained CXCL12 with a concentration of 783-8,364 pg/ml (the median was 6,871 pg/ml) (Table 1).

Both recombinant human CXCL12 and cancerous ascitic fluid induced metastatic migration

To test the function of CXCL12 in HCC metastasis, we found that both recombination human CXCL12 and cancerous ascitic fluid from HCC patients could induce MHCC97 cell migration (Figure 3). Cancerous ascitic fluid could induce the migration of MHCC97 cells with a CI of 1.9 ± 0.8 (Figure 3). CXCL12 could also induce MHCC97 or HUVEC cells to migration (Figures 4). The chemotaxis activity of MHCC97 or HUVEC cells depended on the concentrations of recombination human CXCL12 used for the induction. And the chemotaxis activity was the highest when the recombinant human CXCL12 was at 100 ng/ml (Figure 5). Under this optimal condition, the CI values for MHCC97 and HUVECs were 3.9 ± 1.1 and 4.1 ± 1.6, respectively, which were

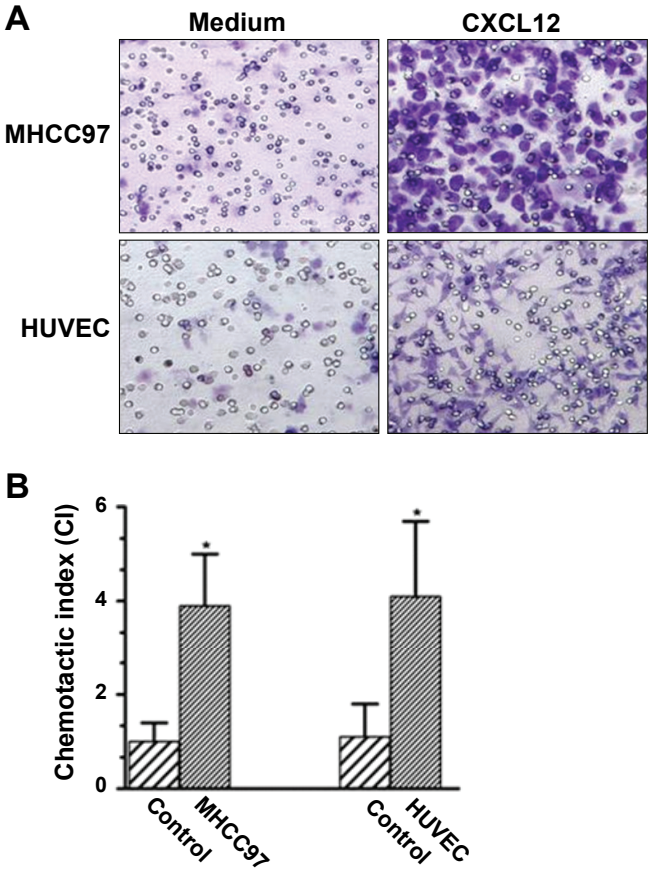

Figure 4. Chemotactic activity of CXCL12 for MHCC97 and HUVEC cells. The migration of MHCC97 and HUVEC cells in response to CXCL12 was measured using chemotaxis chambers. Cells migrating across polycarbonate filters were photographed. (A) MHCC97 or HUVEC cells migration in response to medium alone or 100 ng/ml CXCL12, respectively (× 200). (B) Chemotactic index was calculated as the ratio of the number of cells migrating toward CXCL12 to the number of cells migrating toward negative control.

significantly higher than those of the controls (1.0 ± 0.4 and 1.1 ± 0.7) ($p < 0.05$, Figure 4B).

Discussion

Many studies suggest that tumor cell migration and organ-specific metastasis are critically regulated by chemokines and their receptors (21-29). The axis of CXCR4/CXCL12 plays an important role in metastasis of many tumors (9-14, 22-25). Abnormal expression of CXCR4 or CXCL12 has been observed in solid tumors such as ovarian cancer (30), rhabdomysarcoma (26), nasopharyngeal carcinoma (21), melanoma (27), colorectal cancer (28), pancreatic cancer (24), and breast cancer (7). Muller et al. showed that CXCR4 was highly expressed in some malignant breast cancer cells but not in normal mammary tissues and that CXCL12 was expressed in organs where breast cancer metastases were frequently found (BM, lymph nodes, lung, and liver) (29).

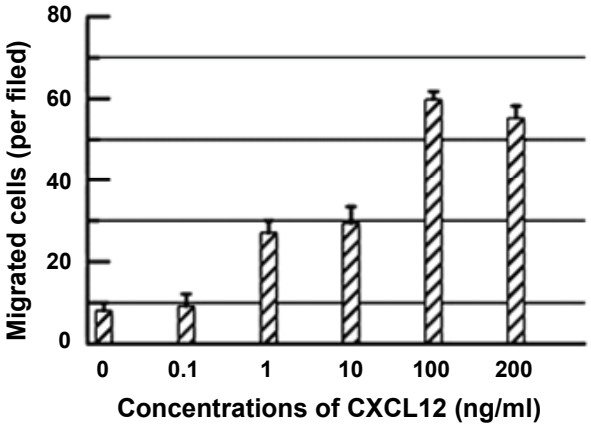

Figure 5. The migration of MHCC97 cells in response to CXCL12 was dosage-dependent. The migration of MHCC97 cells in response to CXCL12 was measured using chemotaxis chambers. The number of migrating MHCC97 cells was represented by the mean value in 7 observation fields. Columns indicated the mean value of 3 experiments and error bars indicated standard deviations.

Interestingly, Ottaiano et al. found that anti-CXCR4 antibody treatment could efficiently reduce the tumor load of a human colon cancer cell line in the lungs and lymph nodes of SCID mice (31).

HCC metastasis was originally characterized by Paget (32), who demonstrated that this cancer had a distinct metastatic pattern preferentially involving the regional lymph nodes, bone marrow, and lung. Müller and colleagues have provided evidence that organ-specific metastases of breast cancer cells directly related to a CXC chemokine (29). They found that CXCR4 was the most highly expressed chemokine receptor in human breast cancer cells and that CXCL12, the ligand of CXCR4, was abundant in organs that were preferential destinations of breast cancer metastasis. Moreover, *in vivo* CXCR4 inhibition significantly reduced metastases of breast cancer in an organ-specific manner.

Although the predominant function of CXCL12-CXCR4 in tumorigenesis is to induce metastases, studies have also indicated that this chemokine ligand-receptor pair is important in promoting angiogenesis (33, 34). CXCL12 may be involved in up-regulating expression levels of vascular endothelial growth factor and basic fibroblast growth factor in that subcutaneous injection of CXCL12 into mice induced formation of local small blood vessels (35, 36). Endogenous CXCL12 together with CXCR4 mediated a significant portion of primary tumor angiogenesis and angiogenesis-dependent tumor growth (37, 38).

In this study we detected *CXCR4* expression in 88.33% (65/78) HCC tissues but not in HCC metastasis portal lymph nodes. These results are not in line with the previous reports (18, 19) where CXCR4 receptor is frequently expressed in HCC metastases. Notably, the metastatic cell lines MHCC97 migrated in response to CXCL12 *in vitro*, but the nonmetastatic cell lines did not migrate toward CXCL12 despite the factor that CXCR4 were expressed in these cells.

Whether cells possess the machinery that controls chemotaxis to CXCL12 is currently under investigation. It should be mentioned that in HCC sections and cell lines, not all tumor cells were stained with anti-CXCR4 antibody (data not shown). In addition, 11 out of 43 HCC portal lymph node metastases examined in this study were CXCR4 negative. These observations suggest that tumor cell populations are heterogeneous and factors other than CXCR4 may also contribute to tumor cell metastasis. In this context, studies of more homogeneous single HCC cell clones may provide valuable information.

In conclusion, the chemokine receptor CXCR4 was associated with the migration of HCC cells both *in vitro* and *in vivo*. The interaction between the chemokine ligand CXCL12 and its receptor CXCR4 played an important role during HCC metastasis. These data indicated a potential therapeutic application of CXCR4 inhibitors to treat HCC.

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