Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Possible Regulation of Migration of Intrahepatic Cholangiocarcinoma Cells by Interaction of CXCR4 Expressed in Carcinoma Cells with Tumor Necrosis Factor- α and Stromal-Derived Factor-1 Released in Stroma

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Intrahepatic cholangiocarcinoma (ICC) is highly fatal because of early invasion, widespread metastasis, and lack of an effective therapy. We examined roles of CXCR4 and its ligand, stromal cell-derived factor (SDF)-1, in migration of ICC with respect to tumor-stromal interaction by using two ICC cell lines, a fibroblast cell line (WI-38), and 28 human ICC tissues. The two ICC cell lines expressed CXCR4 mRNA and protein, and WI-38 fibroblasts expressed SDF-1 mRNA and protein. Migration of cultured ICC cells in Matrigel was induced by co-culture with WI-38 fibroblasts and by incubation with SDF-1. Anti-SDF-1 antibody suppressed migration, demonstrating that SDF-1 released from WI-38 fibroblasts was responsible for this migration. Tumor necrosis factor (TNF)- α pretreatment of ICC cells up-regulated CXCR4 mRNA and protein expression in a concentration-dependent manner. Administration of SDF-1 and TNF- α increased synergistically ICC cell migration, which was suppressed by the CXCR4 antagonist AMD3100. In ICC tissue, TNF- α was mainly expressed in infiltrated macrophages, CXCR4 in ICC cells, and SDF-1 in stromal fibroblasts. In conclusion, the interaction of SDF-1 released from fibroblasts and CXCR4 expressed on ICC cells may be actively involved in ICC migration, and TNF- α may enhance ICC cell migration by increasing CXCR4 expression. CXCR4 could be a therapeutic target to prevent ICC invasion.

apeutic target to prevent ICC invasion. (Am J Pathol 2006, 168:1155–1168; DOI: 10.2353/ajpath.2006.050204)

Intrahepatic cholangiocarcinoma (ICC) is the most frequent primary malignant liver tumor next to hepatocellular carcinoma and is highly fatal because of early invasion, widespread metastasis, and the lack of an effective therapy.^{1,2} Whereas several molecules and histological features of ICC are reported to relate to the prognosis of the patients and to other features such as metastasis,^{3,4} the genetic and molecular aspects of its biological behavior, particularly information regarding the mechanisms regulating invasion or migration, remain poor.

Although the stroma had been thought to passively support tumor development and progression, there is increasing evidence that the stroma actively contributes to the growth and invasion of malignant tumors.^{5–8} That is, stromal cells are reported to influence the malignant progression in adjacent epithelia,^{9,10} and the specific paracrine factors or molecules and signaling pathways involved in the progression of malignant tumors are now being extensively studied.^{11–14}

Recently, there has been evidence of a role for chemokines in tumor biology in addition to the control of the migration of leukocytes.^{11–14} Marchesi and colleagues¹⁵ reported that chemokine receptors expressed on tumor cells are involved in the migration of malignant cells and are associated with distant metastasis, suggesting that chemokines may control tumor dissemination. Chemokines may also favor tumor growth by directly promoting cell proliferation or neovascularization in tumor tissue.^{15,16} Among chemokines, CXC chemokine, stromal

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Table 1. Main Clinicopathological Features of Intrahepatic Cholangiocarcinoma (ICC) and Control Livers Used in This Study

	ICC cases (28 cases)	Control cases (6 cases)
Age (years)	Average, 69.4 (range, 49 to 85)	Average, 70 (range, 55 to 86)
Gender (M:F)	18:10	3:3
Tissue specimen	Surgically resected, 21; autopsy, 7	Autopsy, 6
Location of ICC in the liver	Right hepatic lobe, 14; left hepatic lobe, 14	
Histological grading	Well differentiated, 2; moderately differentiated, 15; poorly differentiated, 11	
Tumor size (cm in diameter)	5.7 (range, 2 to 14)	
Background liver histology	Nonspecific reactive change	Almost normal

cell-derived factor-1 (SDF-1) (CXCL12), and its specific receptor CXCR4 have gained considerable interest because of their roles in carcinogenesis, invasion, the metastasis and proliferation of malignant cells, and tumor recurrence.^{16–19} For example, in breast cancer and oral squamous cell carcinoma, carcinoma cells expressing CXCR4 are able to metastasize to bone marrow or lymph nodes.^{17,19–22} Sehgal and colleagues²³ concluded that CXCR4 plays an important role in determining the tumorigenic properties of brain, breast, and other tumor types. CXCR4 is also involved in the migration and spread of ovarian carcinoma cells.¹³

Cytokines secreted from malignant cells and mesenchymal/inflammatory cells are also known to regulate the biological activities of malignant cells.^{11,12,14,17,22} Among them, tumor necrosis factor (TNF)- α released from tumor-associated macrophages and also from malignant cells themselves, has been shown to promote expression of chemokines/cytokines and their receptors and intercellular adhesion molecule-1 (ICAM-1), thereby contributing to the growth and metastasis of malignant tumors.^{14,24–27} In fact, an increased serum level of TNF- α reflects a poor prognosis among patients with malignant tumors.²⁸ However, the exact role of TNF- α as a cross-talk molecule in the tumor-stroma interaction remains unexplored.^{14,24–26}

In nonneoplastic, inflamed intrahepatic bile ducts, SDF-1 is expressed in biliary epithelial cells (BECs).^{29,30} BECs are also known to secrete cytokines such as TNF- α and interleukin (IL)-6.³¹ There have been several studies on TNF- α and its apoptotic role in ICC cells.³² Recently, Park and colleagues³³ reported that ICC cell lines increased IL-6 secretion in response to TNF- α , and IL-6 is known to induce the proliferation of ICC cells and is a marker of poor prognosis among ICC patients.³⁴ So far, specific paracrine effects of the CXCR4/SDF-1 system and TNF- α in the biological activities of ICC have not been identified. In this study, we examined the roles of the CXCR4/SDF-1 system in ICC during migration with respect to tumor-stromal interactions by using two ICC cell lines, one fibroblast cell line, and 28 human ICC tissues.

Materials and Methods

Patients and Preparation of Tissue Specimens

A total of 28 ICC specimens with enough marginal non-tumoral liver tissue were obtained from 28 patients (Table

1). All of these tumors were peripheral ICCs and presented grossly as mass-forming type.^{1,2} More than three tissue sections containing both the ICC and surrounding nonneoplastic liver were obtained in each case. As a control, six normal autopsied livers with minimal autolytic changes were used, and more than three sections were obtained from each liver. The age and sex distribution were comparable with those of ICC patients. All of these specimens were obtained from the Liver Disease File of the Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan, and were fixed in 10% buffered formalin and embedded in paraffin. More than 20 serial sections, 3 μ m in thickness, were cut from each paraffin block.

Antibodies and Immunological Reagents

Monoclonal and polyclonal antibodies and other immunological reagents and their sources are shown in Table 2. We used a goat polyclonal antibody against SDF-1 (Santa Cruz Biotechnology, Santa Cruz, CA) for immunohistochemistry. Mouse monoclonal antibody against SDF-1 (clone 79014; R&D Systems, Minneapolis, MN) was used as a neutralization antibody for SDF-1.

Cell Culture

Two ICC cell lines (HuCCT-1, obtained from Cell Resource Center for Biochemical Research, Tohoku University, Sendai, Japan; and CCKS-1, established in our laboratory),^{34,35} one line of nonneoplastic human intrahepatic biliary epithelial cells (HIBECS) from an explanted liver with hepatitis C virus-related cirrhosis,³⁶ and the embryonic lung fibroblast cell line WI-38 (Cell Resource Center for Biochemical Research) were used. Cultured HuCCT-1, CCKS-1, and WI-38 cells were maintained in RPMI 1640 medium, whereas HIBECS were maintained in Dulbecco's modified Eagle's medium/F12, containing 10% fetal calf serum (Life Technologies, Inc., Grand Island, NY), and penicillin-streptomycin-glutamine (Life Technologies, Inc.).

SDF-1 was used at concentration of 0.1, 1, 10, and 100 ng/ml, according to previous reports,^{15,17} and TNF- α was used at 100 U/ml and 1000 U/ml. Two receptors for TNF- α , tumor necrosis factor receptors (TNFR) 1 and 2, are well known. For the experiment on the inhibition of TNF- α , TNF- α neutralization antibody and anti-TNFR1

Table 2. Antibodies, Antigens, and Other Immunohistological Materials Used in This Study

	Source	Clone	Type of antibody	Animals immunized	Optimal dilution
Anti-TNF- α *	Santa Cruz Biotechnology		Poly	Goat (IgG)	1:200
Anti-SDF-1	Santa Cruz Biotechnology		Poly	Goat (IgG)	1:50
Anti-CXCR4	Santa Cruz Biotechnology		Poly	Goat (IgG)	1:50
Anti-CD68	DAKO	KP1	Mono	Mouse (IgG)	1:600
Anti-mast cell tryptase	DAKO	AA1	Mono	Mouse (IgG)	1:100
Anti-TNF- α [†]	R&D Systems		Poly	Goat (IgG)	2 μ g/ml
Anti-SDF-1	R&D Systems	79014	Mono	Mouse (IgG)	50 μ g/ml
Anti-TNFR1	R&D Systems	16805.21	Mono	Mouse (IgG)	10 μ g/ml
Anti-TNFR2	R&D Systems	22221	Mono	Mouse (IgG)	10 μ g/ml
TNF- α	PeproTech, London, UK				100, 1000 U/ml (=5, 50 ng/ml)
CXCR4	Santa Cruz Biotechnology				40 μ g/ml
SDF-1	R&D systems				100 ng/ml
IL-1 β	PeproTech				1000 U/ml (=100 ng/ml)
IL-4	PeproTech				1000 U/ml (=200 ng/ml)
IL-6	PeproTech				1000 U/ml (=100 ng/ml)
INF- γ	PeproTech				1000 U/ml (=50 ng/ml)
goat IgG	DAKO				
Mouse IgG	DAKO				
AMD3100	Sigma, St. Louis, MO				1 μ g/ml

* Used for immunohistochemical staining.

[†] Used for neutralization study.

and anti-TNFR2 neutralization antibodies (Table 2) were used. Furthermore, the effects of other inflammatory cytokines (IL-1 β , IL-4, IL-6, and interferon (IFN)- γ ; each at 1000 U/ml) on the expression of CXCR4 mRNA in ICC cell lines were examined by adding each cytokine in the culture medium and by comparing them with the effect of TNF- α .

Immunohistochemistry

The expression of TNF- α , SDF-1, CXCR4, mast cell tryptase, and CD68 in ICC and control specimens was examined immunohistochemically using each primary antibody. To unmask the antigen in the tissue, deparaffinized sections were pretreated in a microwave oven in ethylenediaminetetraacetic acid buffer (pH 8.0) at 95°C for detection of SDF-1, CXCR4, and CD68 or in 0.1% trypsin buffer at 37°C for detection of TNF- α . After the blockage of endogenous peroxidase in 1% H₂O₂ in methanol for 20 minutes and pretreatment with protein block serum (DakoCytomation, Kyoto, Japan) for 15 minutes to block nonspecific reaction, the sections were incubated with each primary antibody at 4°C overnight. The Envision+ solution (DakoCytomation) was then applied for 60 minutes. The reaction products were visualized via a benzidine reaction. The sections were then lightly counterstained with hematoxylin.

Negative controls included substituting the primary antibody with similarly diluted goat normal IgG. Our preliminary study showed that in the immunostaining of SDF-1, TNF- α , and CXCR4, mononuclear cells resembling mast cells were clearly positive. These cells were positive when control immunoglobulin was used for the primary antibody and were actually positive for mast cell tryptase. Because mast cells are known to show nonspecific binding to immunoglobulin,³⁷ mast cell-like cells positive for SDF-1, TNF- α , and CXCR4 were not evaluated by immu-

nohistochemistry. Preincubation of anti-SDF-1, anti-TNF- α , or anti-CXCR4 antibodies with SDF-1 or TNF- α or CXCR4 (Table 2) during the immunostaining of SDF-1, TNF- α , or CXCR4 in ICC tissues resulted in a marked reduction in the immunostaining of each protein. To confirm further the specificity of anti-TNF- α and anti-CXCR4 antibodies, the expression of TNF- α or CXCR4 was immunohistochemically examined in the formalin-fixed, paraffin-embedded sections of the ICC cell lines (HuCC-T1 and CCKS-1) cultured in plastic bottles for 3 days, using the same antibodies. It was found that these cultured ICC cells were positive for TNF- α and CXCR4, and the positive staining was diminished by preincubation of each of these antibodies with TNF- α or CXCR4, respectively.

Staining of CXCR4 in ICC was classified into two types according to Kato and colleagues³⁸: CXCR4 was expressed homogeneously in almost all ICC cells (diffuse-type) but expressed heterogeneously in carcinoma cells of ICC (focal-type). The latter was found in the background of diffuse immunostaining of CXCR4 of varying degrees. In this sense, focal-type seems to express more CXCR4 than diffuse-type.

Extraction of RNA and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA was isolated from HuCC-T1, CCKS-1, and HIBEC cell lines using the Qiagen RNAeasy kit (Qiagen, Tokyo, Japan). RNA was also isolated from carcinoma tissues from central parts of three surgically resected ICC tissues (ICC 1, 73-year-old male; ICC 2, 71-year-old female; and ICC 3, 72-year-old female) that were included among the ICC cases described above. Then, 2 μ g of RNA was used to synthesize the first-strand cDNA with the superscript system (Life Technologies, Inc., Rockville, MD), according to the manufacturer's instructions. RT-PCR re-

Table 3. Primer Sequences Used in this Study

	Forward	Reverse	PCR product	Annealing	Cycle no.
Human					
TNF- α	5'-GCTGTACCTCATCTACTCCCA-3'	55'-GCAATTTCTAGGTGAGGTCTTC-3'	660 bp	55°C	21
TNFR1	5'-GAGAGGCCATAGCTGTCTGG-3'	5'-GTTTTCTGAAGCGGTGAAGG-3'	303 bp	60°C	28
TNFR2	5'-GGATGAAGCCCACTTAACCA-3'	5'-GTGTCCTGTCTTCATGGGTGA-3'	501 bp	55°C	32
CXCR4	5'-TTCTACCCCAATGACTTGTG-3'	5'-ATGTAGTAAGGCAGCCAACA-3'	260 bp	56°C	33
SDF-1	5'-CCGCGCTCTGCCTCAGCGACGGGAAG-3'	5'-CTTGTTTAAAGCTTTCTCCAGGTACT-3'	227 bp	56°C	33
β -Actin	5'-CAAGAGATGGCCACGGCTGCT-3'	5'-TCCTTCTGCATCCTGTCGGCA-3'	275 bp	60°C	18

actions for TNF- α , TNFR1, TNFR2, CXCR4, SDF-1, and β -actin were performed as described previously.³⁹ The oligonucleotide sequences, numbers of cycles, and annealing temperatures of these primers are shown in Table 3. As a quantitative control, primers for the β -actin gene, a housekeeping gene that is considered to be constitutively expressed, were used. After PCR, 5- μ l aliquots of the products were subjected to 1.5% or 2.0% agarose gel electrophoresis and stained with ethidium bromide.

Real-Time Quantitative PCR for CXCR4 mRNA

ICC cells from both cell lines were cultured in the presence of 100 or 1000 U/ml TNF- α for 48 hours. Multiplex real-time analysis was performed using premade CXCR4 (FAM)- and β -actin (VIC)-specific primers and probes with the ABI Prism 7700 sequence detection system (PE Applied Biosystems, Warrington, UK). RT-PCR was done with the TaqMan Universal PCR Master Mix (PE Applied Biosystems) using 5 μ l of cDNA in a 25- μ l final reaction mixture. Cycling conditions were as follows: incubation at 50°C for 2 minutes, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. CXCR4 was normalized (Δ Ct) to β -actin by subtracting the cycle threshold (Ct) value of β -actin from the Ct value of CXCR4. Each experiment was performed in triplicate, and the mean was adopted in each experiment. Fold difference compared with control was calculated.

Extraction of Protein and Western Blot Analysis for CXCR4 and TNF- α

Proteins were extracted from cultured cells and three surgically resected ICC tissues (the same specimens for mRNA study in ICC tissues, see above) using T-PER tissue protein extraction reagent (Pierce Chemical Co., Rockford, IL). Total protein was measured by a spectrophotometer. Extracted protein was used for Western blot analysis. TNF- α protein was specifically concentrated by the immunoprecipitation using primary antibody to TNF- α and protein G-agarose beads (Roche, Indianapolis, IN). In this analysis, 20 μ g of protein was used as a sample, and the analysis of CXCR4 and TNF- α was performed on 10% and 15% sodium dodecyl sulfate-polyacrylamide gel, respectively. The protein in the gel was electrophoretically transferred onto a nitrocellulose membrane. The membrane was incubated with primary antibody to CXCR4 and TNF- α , respectively. The protein was de-

tected using secondary antibody conjugated to peroxidase-labeled polymer Histofine Simple Stain MAX PO (G) (Nichirei, Tokyo, Japan) and benzidine reaction.

Enzyme-Linked Immunosorbent Assay (ELISA) of SDF-1

The baseline level of SDF-1 production by WI-38 fibroblasts and ICC cells from two cell lines was determined. Each of these three cell lines was seeded on 6-cm dishes at a density of 1×10^5 /ml and cultured for 24 hours. After the medium was replaced with fresh RPMI 1640 medium, the cells were cultured for another 48 hours. The concentration of SDF-1 in the supernatant was measured by ELISA using a human SDF-1 antibody and enzyme immunoassay kit (R&D Systems), according to the manufacturer's instructions.

Migration Assays of Cultured ICC Cells

The migration of cultured ICC cells of both lines was assayed using a BD BioCoat Matrigel invasion chamber (24-well plate, 8- μ m pore) (BD Biosciences, Bedford, MA). This Matrigel invasion chamber is a growth factor-reduced type. Medium (0.5 ml) containing 5×10^5 ICC cells was added to the upper chamber, and 0.5 ml of either medium alone or medium supplemented with 0.1, 1, 10, or 100 ng/ml of SDF-1 was added to the lower chamber. TNF- α at 100 or 1000 U/ml was added in the upper chamber. AMD3100, an antagonist of CXCR4, was used at 1 μ g/ml (Table 2). For the migration assay of ICC cells co-cultured with WI-38 fibroblasts, medium (0.5 μ l) containing 5×10^5 CC cells was added to the upper chamber, and 0.5 ml of either medium alone or medium containing 1 to 2×10^4 WI-38 cells was added to the lower chamber. In some wells with WI-38, we administered 50 μ g/ml of anti-SDF-1 antibody. As a negative control, 50 μ g/ml of mouse IgG (IgG) (DAKO, Glostrup, Denmark) was used.

To document the efficiency of the neutralization of SDF-1 by anti-SDF-1 antibody (50 μ g/ml) in the migration assay, we incubated 1 ml of the supernatant (the culture with WI-38 for 48 hours) with anti-SDF-1 antibody (5 or 50 μ g/ml) conjugated with protein G-agarose beads (Roche) for 2 hours. Fifty μ g/ml of mouse IgG (IgG) (DAKO) conjugated with protein-G agarose beads (Roche) was used as a negative control. After spin down,

Figure 1. **A** and **B** are serial sections and correspond to almost the same area of ICC. **A:** There are many CD68-positive macrophages infiltrating at the interface of the ICC (C) and surrounding liver (H). Kupffer cells in the surrounding liver are also strongly positive. Immunostaining for CD68 counterstained by hematoxylin. **B:** There are many TNF- α -positive mononuclear cells infiltrating at the interface of the ICC (C) and surrounding liver (H). A majority of them correspond to infiltrating macrophages. Kupffer cells in the surrounding liver are also strongly positive. Immunostaining for TNF- α counterstained by hematoxylin. Original magnifications, $\times 200$.

the concentration of SDF-1 in the supernatant was measured by ELISA as described above.

Chambers were incubated for 48 hours at 37°C and 5% CO₂. ICC cells on the upper surface of the filter were removed using a cotton wool swab, and the cells that had migrated to the lower surface were stained using 1% toluidine blue after fixation with 100% methanol. The number of migrated cells was counted in 10 medium power fields ($\times 20$). Each experiment was conducted in triplicate. A migration index (the ratio of the number of migrated cells in an experimental group/the number of migrated cells in control groups without chemokine or cytokine) was calculated in each experiment.

Proliferation Assays

Viable cell numbers were measured with a cell counting kit-8 (CCK-8) containing 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) (Dojin Laboratories, Kumamoto, Japan). Briefly, cells from both ICC cell lines ($0.5 \sim 2 \times 10^4$ cells/100 μ l)

Figure 2. Correlation between CD68-positive mononuclear cells and TNF- α -positive mononuclear cells at the interface of ICC and surrounding liver. **Filled circles** indicate a case of ICC.

were plated in 96-well plates and cultured for 24 hours before the medium was replaced with RPMI 1640 medium without fetal calf serum (control). Either 100 ng/ml SDF-1 + 100 U/ml TNF- α , 100 ng/ml SDF-1 + 1000 U/ml TNF- α , or 100 ng/ml SDF-1 + 1000 U/ml TNF- α + AMD3100 was added to the culture medium, and these cells were cultured for 48 hours. At the end of each experiment, the cell proliferation reagent WST-8 (10 μ l) was added to each well and incubated for 3 hours at 37°C. OD ($A_{450 \text{ nm}}$) was measured using an automatic ELISA plate reader. Each experiment was done in octuplicate.

Statistical Analysis

Differences among groups were assessed using the Mann-Whitney *U*-test. The correlation among two groups was assessed with Spearmann's rank-correlation test. When the *P* value was < 0.05 , the difference was regarded as significant.

Results

Immunohistochemistry

TNF- α /CD68

There were always a considerable number of CD68-positive macrophages at the interface of ICC and the surrounding liver (Figure 1A). Kupffer cells in the hepatic parenchyma and macrophages in portal tracts were also strongly positive for CD68 in the hepatic tissue around ICC. TNF- α was expressed in Kupffer cells and macrophages in the surrounding liver. In addition, there were considerable numbers of TNF- α -positive mononuclear cells at the periphery of ICCs (Figure 1B), whereas such cells were rather rare in the central parts of ICC tissues. Serial sections immunostained for TNF- α and CD68 showed that a majority of these TNF- α -positive cells were CD68-positive macrophages. When the cells were semi-

Figure 3. ICC cells are homogeneously positive for CXCR4 at the periphery of ICC (C) (diffuse-type). Lymphoid cells in the surrounding liver are also positive for CXCR4. H, surrounding hepatic lobules. Immunostaining for CXCR4 counterstained by hematoxylin. Original magnification, $\times 200$.

quantitatively classified into three categories (minimum to mild, moderate, and marked), the number of CD68-positive macrophages and that of TNF- α -positive mononuclear cells were well correlated in individual cases (Figure 2). TNF- α was also focally detectable at the cytoplasm and in the luminal surface of ICC cells in 19 (67.9%) ICC cases. In control livers, Kupffer cells were variably positive for CD68 and also for TNF- α .

CXCR4/SDF-1

CXCR4 was mainly expressed diffusely in the cytoplasm of ICC cells. Within the ICC tissue, the expression was diffuse-type in 18 cases (Figure 3) and that in the remaining 10 cases focal-type. Whereas lymph node metastasis and extrahepatic metastasis were rather more frequent in the ICC cases with focal-type of CXCR4 expression, there was no significant correlation between the two types of immunohistochemical expression of CXCR4 and pathological parameters (Table 4). In the surrounding liver, hepatocytes were weakly positive for CXCR4, and lymphoid cells were also positive. Nonneoplastic bile ducts of the background liver and control livers did not express CXCR4. SDF-1 was expressed in the cytoplasm of fibroblast-like stromal cells and also diffusely and weakly in ICC cells (Figure 4). SDF-1 was also weakly positive in the hepatocytes and proliferated bile ductules. In control livers, a few fibroblasts and hepatocytes and bile ducts were positive for SDF-1.

TNF- α and CXCR4 mRNA and Protein Expression in ICC Tissue

CXCR4 and TNF- α mRNA were detected in all three ICC tissues by RT-PCR (Figure 5A), and TNF- α protein (17 kd) and CXCR4 protein (46 kd) were detected in all three ICC tissues (Figure 5, B and C) by Western blotting. These data were compatible with the above-mentioned immunohistochemical expression of CXCR4 and TNF- α . The specificity of the primary antibodies used for the

Table 4. Correlation between Immunohistochemical Expression of CXCR4 and Pathological Parameters of the Intrahepatic Cholangiocarcinoma

	Diffuse	Focal
Tumor size (cm) (n.s.)		
<4	8	3
≥ 4	10	7
Histological grade (n.s.)		
Well	1	1
Moderately	10	5
Poorly	7	4
Intrahepatic metastasis (n.s.)		
Negative	14	4
Positive	4	6
Lymph node metastasis* (n.s.)		
Negative	11	2
Positive	5	6
Extrahepatic metastasis (n.s.)		
Negative	14	7
Positive	4	3

n.s., not significant.

* Twenty-four cases were available.

immunohistochemical detection of TNF- α and CXCR4 was also confirmed by the whole blot without few non-specific bands (Figure 5, B and C).

TNF- α , TNFR1, TNFR2, CXCR4, and SDF-1 mRNA in ICC Cell Lines and HIBECs

RT-PCR using primers specific for TNF- α and CXCR4 demonstrated mRNA in both ICC cell lines but not in HIBECs. TNFR1 and TNFR2 mRNA were detected in all cell lines. SDF-1 mRNA was not detected in either ICC cell line but was detected in HIBECs (Figure 6).

WI-38 Fibroblasts and SDF-1 Up-Regulate Migration of Cultured ICC Cells

Less than 10 cultured cells per a medium power field ($\times 20$) migrated through Matrigel when ICC cells were

Figure 4. Fibroblast-like stroma cells (arrows) are clearly positive for SDF-1 in ICC. ICC cells are also weakly and homogeneously positive for SDF-1. ICC tissue. Immunostaining for SDF-1 counterstained by hematoxylin. Original magnification, $\times 200$.

Figure 5. A: CXCR4 and TNF- α mRNA were detected in carcinoma tissues obtained from central parts of ICC (three surgical specimens: ICC1, ICC2, and ICC3) by RT-PCR. B: TNF- α protein (17 kD) was detected in carcinoma tissues obtained from central part of all of three ICCs by immunoprecipitation using anti-TNF- α antibody and Western blotting. The right lane was a negative control using the second antibody only and the signals at 55 kD and 27 kD indicated goat immunoglobulins used for immunoprecipitation. C: By Western blotting, CXCR4 protein (46 kD) was detected in carcinoma tissues obtained from central part of all of three ICCs.

cultured alone (control). In contrast, more ICC cells (~10 to, at most, 60 cultured cells per a medium power field) migrated when WI-38 fibroblasts were co-cultured in the lower chamber: the migration index was 5.5-fold in HuCCT-1 and 20.8-fold in CCKS-1 when compared to the control (Figure 7A). It seems conceivable that WI-38 fibroblasts produced some factor(s) that promoted the migration of cultured ICC cells. When anti-SDF-1 neutralization antibody (50 μ g/ml) was added to the culture medium of both ICC cells co-cultured with WI-38, the increase in migration of ICC cells was significantly suppressed compared with that in WI-38 fibroblasts alone but not to the control level (Figure 7A), whereas the migration of ICC cells (HuCTT-1 or CCKS-1) co-cultured with WI-38 cells on control IgG was almost similar to that of ICC co-cultured with WI-38 cells, suggesting participation of TNF- α in the increased migration of ICC cells co-cultured with WI-38 fibroblasts. The possibility that SDF-1 derived from WI-38 fibroblasts was the factor enhancing the migration of ICC cells was further tested. When ICC cells were treated with 100 ng/ml of SDF-1 in the lower chamber, the number of migrated HuCCT-1 and CCKS1 cells was increased significantly by 5.3- and 4.0-fold, respectively (Figure 7B). In addition, 1 and 10 ng/ml of SDF-1 increased the number of migrated HuCCT-1 cells significantly (1.4- and 1.5-fold, respectively), whereas 10 ng/ml of SDF-1 increased the number of migrated CCKS1 cells significantly (1.7-fold). However, 0.1 ng/ml of SDF-1 did not increase the migration of either of the two cell lines.

Figure 6. RT-PCR for TNF- α , TNFR1, TNFR2, CXCR4, and SDF-1 mRNA in ICC cell lines (HuCCT-1 and CCKS-1), and HIBECs. In both ICC cell lines, TNF- α and CXCR4 mRNA are detectable. In HIBECs, the expression of TNF- α and CXCR4 mRNA is not detectable. Both TNFR1 and TNFR2 mRNA are detectable in all cell lines including HIBECs. SDF-1 mRNA is not detectable in the two ICC cell lines. But in HIBECs, SDF-1 mRNA is detectable. β -Actin was used as an internal control.

Next, we measured the mRNA and protein expression of SDF-1 in WI-38 fibroblasts by RT-PCR and ELISA, respectively. WI-38 fibroblasts expressed SDF-1 mRNA and protein constitutively, whereas ICC cells of neither cell line expressed SDF-1 mRNA or protein (Figure 7C). ELISA showed that SDF-1 level in the culture medium of WI-38 fibroblasts was 172.3 ± 22.2 pg/ml (Figure 7D). Next, we confirmed whether anti-SDF-1 antibody eliminated SDF-1 in the medium. With the treatment of anti-SDF-1 antibody (5 and 50 μ g/ml) conjugated with protein G-agarose beads, SDF-1 level was significantly reduced in the supernatant of cultured WI-38 fibroblasts (Figure 7D). When control mouse IgG (50 μ g/ml) conjugated with protein G-agarose beads was used, the level of SDF-1 in the supernatant was almost the same as that without immunoprecipitation. These results indicated that SDF-1 secreted by WI-38 in the supernatant was efficiently eliminated by anti-SDF-1 at 5 and also 50 μ g/ml.

TNF- α Treatment Induced Increased Expression of CXCR4 mRNA and Protein in ICC Cell Lines

Next, we examined the effect of TNF- α on CXCR4 expression in cultured ICC cells. CXCR4 mRNA levels measured by quantitative PCR in ICC cell lines treated with 100 U/ml of TNF- α for 48 hours were increased to 1.3-fold in HuCTT-1 and 5.0-fold in CCKS-1, and those in cells treated with 1000 U/ml of TNF- α increased 11.1-fold and 8.2-fold, respectively, compared with the control (Figure

Figure 7. **A:** Migration of cultured ICC cells increased on co-culture with WI-38 fibroblasts in the lower chamber for 48 hours (migration of HuCCT-1 cells increased 5.5-fold and that of CCKS-1 increased 20.8-fold, compared with the control). When anti-SDF-1 neutralization antibody was added to the culture medium of ICC cells co-cultured with WI-38 fibroblasts, the increase in migration of ICC cells was down-regulated (from 5.5- to 1.7-fold in HuCCT-1 and from 20.8- to 34-fold in CCKS-1, respectively). Migration of ICC cells (HuCTT-1 or CCKS-1) co-cultured with WI-38 cells on control IgG was 4.85 ± 0.93 and 19.42 ± 2.14 , almost similar to that of ICC co-cultured with WI-38 cells. * $P < 0.05$ versus control. ** $P < 0.05$ versus HuCCT-1 + WI-38 fibroblasts or CCKS-1 + WI-38 fibroblasts. Migration index is the ratio of the number of migrated cells in an experimental group/the number of migrated cells in control group. The data are provided as the mean $\pm$ SD. The experiments were performed five times. **B:** Migration of cultured ICC cells of HuCCT-1 increased when 1, 10, and 100 ng/ml SDF-1 was added in the lower chamber [migration index is 1.3-, 1.4-, and 5.3-fold, respectively, compared with the control (ICC cells alone are cultured)]. Migration of CCKS-1 increased when 10 and 100 ng/ml of SDF-1 was added (migration index is 1.7- and 4-fold, respectively). * $P < 0.05$ and ** $P < 0.01$ versus control. The number of migrated cells was counted in 10 medium power fields ($\times 20$). The data are provided as the mean $\pm$ SD. Migration assay was performed five times in each experiment. **C:** RT-PCR shows that SDF-1 mRNA is detected in cultured WI-38 fibroblasts. SDF-1 mRNA is not detected in cultured HuCCT-1 or CCKS-1 cells. **D:** ELISA shows that SDF-1 protein is present in the supernatant of cultured WI-38 fibroblasts (172.3 ± 22.2 pg/ml), but it was not detected in the supernatant of cultured HuCCT-1 or CCKS-1 cells. The addition of 5 ng/ml and 50 ng/ml of anti-SDF-1 antibody in the supernatant of the cultured WI-38 fibroblasts almost eliminated SDF-1 (8.8 ± 4.0 pg/ml and 16.3 ± 3.1 pg/ml), whereas SDF-1 level in the supernatant of WI-38 cells treated with control IgG was 141.0 ± 7.0 pg/ml, similar to that of WI-38 cells alone. * $P < 0.01$ versus WI-38 cells alone and WI-38 cells + control IgG. ** $P > 0.05$ versus WI-38 cells. The data are provided as the mean $\pm$ SD, and the SDF-1 level was measured three times in each experiment.

8A). That is, CXCR4 mRNA expression was up-regulated in cultured ICC cells from both cell lines on TNF- α treatment in a concentration-dependent manner, although the increase was rather slight in HuCTT-1 treated with 100 U/ml of TNF- α . Whereas CXCR4 protein was detectable in both cell lines, the amount was evidently increased in the cells treated with 1000 U/ml of TNF- α in the Western blotting analysis (Figure 8B). The effects of other inflammatory cytokines (IL-1 β , IL-4, IL-6, and IFN- γ) on CXCR4 mRNA expression was examined in cultured cells from both ICC cell lines for 48 hours. In HuCCT-1 cells, IL-1 β , IL-4, IL-6, and IFN- γ modified the expression of CXCR4 mRNA only 0.5- ~ 1.5-fold, whereas IL-4 up-regulated the expression of CXCR4 mRNA 1.8-fold (Figure 8C). In CCKS-1 cells, IL-1 β , IL-4, IL-6, and IFN- γ modified the expression of CXCR4 mRNA only 0.5- ~ 1.5-fold.

Then, we examined the receptor(s) of TNF- α involved in the expression of CXCR4 mRNA on TNF- α treatment by real-time quantitative PCR. Up-regulation of CXCR4 mRNA expression in cultured ICC cells after TNF- α treatment was considerably but not completely inhibited by pretreatment with either anti-TNFR1 antibody or anti-

TNFR2 antibody in both ICC cells; CXCR4 mRNA expression was 2.7-fold in HuCCT-1 and 2.3-fold in CCKS-1 by anti-TNFR1 antibody, and 3.3-fold and 1.6-fold, respectively, by anti-TNFR2 antibody, compared with the control, respectively (Figure 9). Neither antibody suppressed CXCR4 expression completely. Treatment with control IgG failed to such suppression in ICC cells treated with TNF- α . The up-regulation of CXCR4 mRNA expression in cultured ICC cells by TNF- α was inhibited more by the pretreatment with simultaneous administration of TNFR1 and TNFR2. Treatment with TNF- α neutralization antibody suppressed CXCR4 mRNA expression significantly in cultured HuCTT-1 and CCKS-1 cells treated with TNF- α .

Effects of TNF- α on Migration of ICC Cells Induced by SDF-1

Next, we examined the influence of increased expression of CXCR4 in ICC cells induced by TNF- α on the migration of the cells by using the Matrigel invasion chamber (Figure 10). In the experiment with 100 ng/ml of SDF-1 in the

Figure 8. A: ICC cells (HuCCT-1 and CCKS-1) were cultured in the presence of 100 or 1000 U/ml of TNF- α for 48 hours. Real-time quantitative PCR shows that CXCR4 mRNA expression in HuCCT-1 and CCKS-1 cells was up-regulated 1.3-fold and 5.0-fold with 100 U/ml TNF- α , respectively, and 11.1-fold and 8.2-fold with 1000 U/ml TNF- α , respectively, compared with the control condition. * $P < 0.05$ versus control. ** $P < 0.05$ versus 100 U/ml TNF- α treatment. B: Western blotting shows that in cultured HuCCT-1 cells, CXCR4 protein was weakly detected without treatment and with 100 U/ml TNF- α , but was clearly detected with 1000 U/ml TNF- α . In CCKS-1 cells, CXCR4 protein is only weakly detected without treatment, but clearly detected with 100 U/ml and especially 1000 U/ml TNF- α . C: Effects of other inflammatory cytokines (IL-1 β , IL-4, IL-6, and IFN- γ) on the expression of CXCR4 mRNA in HuCCT-1 and CCKS-1 cells were examined by addition of each cytokine (1000 U/ml) to the culture medium and compared with the control and the effects of TNF- α . The level of CXCR4 mRNA in response to these cytokines was considerably low, when compared with TNF- α : IL-1 β , IL-4, IL-6, and IFN- γ modified the expression of CXCR4 mRNA only 0.5- ~ 1.8-fold in HuCCT-1 cells and 0.5- ~ 1.5-fold in CCKS-1 cells, compared with the control. The data are provided as the mean $\pm$ SD. Each experiment was performed five times.

lower chamber, CCKS-1 cells treated with 100 U/ml of TNF- α and 1000 U/ml of TNF- α and HuCCT-1 cells treated with 1000 U/ml of TNF- α in the upper chamber showed a significant increase in their migration to 13.7-fold and 14.9-fold, and 9.1-fold, respectively, when compared with SDF-1 alone, suggesting that SDF-1 enhanced the migration of cultured HuCCT-1 and CCKS-1 cells with the assistance of TNF- α . However, CCKS-1 cells did not exhibit a dose-dependent effect with TNF- α . The finding that the migration of HuCCT-1 cells at 100 U/ml of TNF- α was not increased (5.2-fold) when compared with SDF-1 alone (5.3-fold), was explained by the finding that expression of CXCR4 protein in cultured HuCCT-1 cells on addition of 100 U/ml was similar to that without treatment (Figure 8B). Then, we tested whether the increased migration of ICC cells induced by TNF- α

was mediated by CXCR4 up-regulated by TNF- α treatment. When AMD3100, a CXCR4 antagonist, was added with SDF-1 and with SDF-1 and TNF- α (1000 U/ml), the migration of HuCCT-1 and CCKS-1 cells was suppressed completely by 0.9- and 0.9-fold, respectively, suggesting that CXCR4 expressed on ICC cells and up-regulated CXCR4 by TNF- α on ICC cells was important for the increased migration of ICC cells via the interaction with SDF-1.

Effects of SDF-1/CXCR4 Interaction and CXCR4 Expression Induced by TNF- α on the Proliferation of Cultured ICCs

The proliferative activity of cultured HuCCT-1 and CCKS-1 cells did not change after the treatment with 100 ng/ml of SDF-1 alone, or 100 ng/ml of SDF-1 + TNF- α (100 U/ml or 1000 U/ml) (Figure 11). The addition of AMD3100, an antagonist of CXCR4, did not influence the proliferation of these ICC cells either, suggesting that the increased expression of CXCR4 caused by TNF- α and SDF-1/CXCR4 interaction in ICC cells had no influence on cell proliferation.

Discussion

Invasion and metastasis are the most challenging and important aspects of malignant tumors, although their exact molecular mechanisms with respect to tumor-stromal interactions remain to be clarified.⁴⁰ Several factors including hepatocyte growth factor, epidermal growth factor, vascular endothelial growth factor, and SDF-1 are regarded as candidate factors involved in cross-talk in tumor-stromal interactions.^{10,41,42}

First, it was found in this study by using HuCCT-1 and CCKS-1 cells that although the migration of cultured ICC cells alone through Matrigel was minimal, it significantly increased when WI-38 fibroblasts were co-cultured in the lower chamber. Interestingly, when anti-SDF-1 neutralization antibody was added to the Matrigel chamber, the increase in migration of cultured ICC cells induced by WI-38 fibroblasts was significantly attenuated. Because cultured ICC cells of two lines were found to express CXCR4 mRNA and protein and cultured WI-38 fibroblasts expressed SDF-1 mRNA and protein, it seems conceivable that SDF-1 released from WI-38 fibroblasts induced the increase in migration of ICC cells via CXCR4/SDF-1 interaction. In fact, the migration of both cultured ICC cells in the upper chamber was increased significantly, when 100 ng/ml of SDF-1 was added to the lower chamber, and the migration index was dependent on the concentration of SDF-1, suggesting that the interaction of CXCR4/SDF-1 was responsible for the increased migration of cultured ICC cells. As for CXCR4 and the migration of other malignant cells, CXCR4 may also influence cell migration in the peritoneum, a major route for the spread of ovarian cancer.¹³ SDF-1 production in the culture medium with WI-38 fibroblasts was 172.3 ± 22.2 pg/ml (Figure 7C), whereas 100 pg (0.1 ng)/ml of SDF-1 failed

Figure 9. Real-time quantitative PCR analysis for CXCR4 mRNA in two ICC cells (HuCTT-1 and CCKS-1) shows that on administration of TNFR1 or TNFR2 neutralization antibody, CXCR4 mRNA expression decreased 2.7-fold and 3.3-fold in HuCTT-1 and 2.3-fold and 1.6-fold in CCKS-1, respectively, compared to 1000 U/ml TNF- α alone (11.2-fold in HuCTT-1 and 8.2-fold in CCKS-1) but was still higher than the control (without TNF- α treatment). Simultaneous administration of anti-TNFR1 and anti-TNFR2 antibody (anti-TNFR1/2) suppressed CXCR4 mRNA expression 1.0-fold (HuCTT-1) and 0.9-fold (CCKS-1), respectively, relative to the control. TNF- α neutralization antibody suppressed more CXCR4 mRNA expression in HuCTT-1 and CCKS-1 cells treated with TNF- α to 1.2-fold and 0.9-fold, respectively, compared to the control. Control IgG did not suppress CXCR4 mRNA expression. * P < 0.05 versus 1000 U/ml TNF- α alone. ** P < 0.05 versus 1000 U/ml TNF- α + anti-TNFR1 or -TNFR2 antibody.

to increase the migration of ICC cells (Figure 7D). At least 1 ng/ml in HuCTT-1 cells and 10 ng/ml in CCKS-1 cells were necessary to increase the migration. It is plausible that the activity of recombinant SDF-1 used in this study might have been slightly weaker than that of native SDF-1 produced by WI-38 and this difference may explain the gap. However, the migration of cultured ICC cells, particularly CCKS-1 cells, was not decreased to the level of the control by treatment with anti-SDF antibody (Figure 7A), suggesting that SDF-1 is only one of the molecules responsible for the migration of cultured ICC cells, especially CCKS-1 cells, induced by WI-38 fibroblasts. Recent studies showed that several other factors such as syndecan and IFN- γ , influence the SDF-1/CXCR4 signaling system.^{16,43}

Essential physiological and pathological roles of SDF-1/CXCR4 interactions have been increasingly demonstrated in various tissues and culture systems.^{11,16–19,43} SDF-1 is broadly and constitutively expressed in stromal cells and endothelial cells in numerous tissues.⁴⁴ In malignant tumors, SDF-1/CXCR4 may provide paracrine signals promoting malignant progression such as metastasis and invasion, and cell proliferation.^{11,15,17,22,42,45} It was found in this study that SDF-1 was expressed in fibroblast-like stromal cells and CXCR4 was expressed in ICC cells in ICC tissues. CXCR4 was expressed mainly in the cytoplasm, consistent with previous reports.^{38,42} The reason why the expression of CXCR4 was detected in the cytoplasm and not accentuated on the cell membrane remains obscure. These immunohistochemical

findings and the above-mentioned cultural study suggest that SDF-1 released from fibroblast-like stromal cells in ICC and CXCR4 expressed on ICC cells interact and this interaction is at least partly involved in the migration and invasion of ICC.

TNF- α is known to play a role in the growth of malignant tumors by enhancing neovascularization and promoting invasion or metastasis by inducing the production of chemokines/cytokines.^{18,24–26,46,47} It was found in this study that treatment with TNF- α up-regulated CXCR4 mRNA and protein expression in cultured HuCTT-1 and also CCKS-1 cells. However, CXCR4 expression in cultured ICC cells was not significantly up-regulated when other cytokines such as IL-1 β , IL-4, IL-6, and IFN- γ were added to the culture medium, suggesting that TNF- α is a rather unique cytokine in the regulation of CXCR4 expression in cultured ICC cells. Furthermore, TNF- α was immunohistochemically detected rather constantly and clearly in infiltrated CD68-positive macrophages, particularly at the peripheral, invasive fronts of ICCs. TNF- α was also focally expressed in ICC cells in 68% of ICC cases. Immunohistochemically, CXCR4 was homogeneously expressed (diffuse-type) or focally accentuated (focal-type) with a background of diffuse-type in ICC cells. These two types of expression of CXCR4 were described in breast cancer, and the focal-type showed significantly more extensive lymph node metastasis.³⁸ Whereas lymph node metastasis and extrahepatic metastasis were rather frequent in ICC cases of focal-type, this difference was not statistically significant. As for the

Figure 10. Effect of SDF-1 and TNF- α on the migration of ICC cells. TNF- α was added into the upper chamber and SDF-1 was added to the lower chamber. Treatment with SDF-1 alone increased the migration index in both cell lines (5.3-fold in HuCCT-1 and 4.0-fold in CCKS-1). Combined administration of SDF-1 and TNF- α (100 and 1000 U/ml) increased the migration index of cultured ICC cells (13.7-fold and 14.9-fold, respectively) compared with SDF-1 alone in CCKS-1 (4.0-fold), whereas combined administration of TNF- α and SDF-1 (1000 U/ml) increased the migration index of cultured ICC cells (9.1-fold) compared with SDF-1 alone in HuCCT-1 (5.3-fold). There is no difference in the migration index between SDF-1 alone and SDF-1 + 100 ng/ml TNF- α (5.2-fold) in HuCCT-1 cells. Increased migration of cultured ICC cells from both cell lines treated with SDF-1 and TNF- α (1000 U/ml) was completely suppressed by AMD3100, a CXCR4 antagonist (0.9-fold in HuCCT-1 and 0.9-fold in CCKS-1). * P < 0.05 versus SDF-1 alone; ** P < 0.05 versus SDF-1 + TNF- α (100 U/ml); *** P < 0.05 versus SDF-1 + TNF- α (1000 U/ml). Migration index is the ratio of the number of migrated cells in an experimental group/the number of migrated cells in control group. The data are provided as the mean $\pm$ SD. Each experiment was performed five times.

explanation for this correlation, the amount of CXCR4 expressed in ICC could be one reason. In addition, Kato and colleagues³⁸ speculated that the focal expression of CXCR4 might reflect increased heterogeneity in comparison with diffuse-type tumors, which might be related to increased malignant potential. This could also be the case in ICC. Nonneoplastic bile ducts of the background liver and control livers were slightly positive for SDF-1 but failed to express CXCR4, and cultured HIBECs failed to express CXCR4 mRNA. Such neoexpression of CXCR4 in carcinoma cells but not in normal counterparts is also reported in other organs such as the breast.¹¹ Taken together, TNF- α released from infiltrated macrophages, particularly at the periphery of ICC, and to a lesser degree from ICC cells, may act on ICC cells to increase the expression of CXCR4 *in vivo*.

Two types of receptors of TNF- α , TNFR1 and TNFR2, are known, and TNF- α has a fivefold higher affinity for TNFR2 than TNFR1 in mouse cells, suggesting preferential ligation of TNFR2 at physiological TNF- α concentrations.^{48,49} TNFR1 induces growth arrest through sustained mitogen-activated protein kinase activity,⁵⁰ whereas TNF- α stimulates proliferation via TNFR2.⁵¹ It was found in this study that each neutralization antibody against TNFR1 or TNFR2 suppressed considerably but not completely the expression of CXCR4 mRNA. Interestingly, simultaneous treatment with both neutralization an-

tibodies further inhibited this up-regulation, suggesting that TNF- α up-regulates the expression of CXCR4 via both TNFR1 and TNFR2. Neutralization antibody against TNF- α suppressed CXCR4 mRNA expression considerably, supporting that TNF- α functions in the up-regulation of CXCR4 in cultured ICC cells. The absence of difference in the inhibition of increased expression of CXCR4 by anti-TNFR1 and by TNFR2 despite the higher affinity of TNF for TNFR2 than for TNFR1 may be because of the different amounts of the two types of receptors in cultured ICC cells.

Interestingly, the presence of TNF- α in the upper chamber and SDF-1 in the lower chamber significantly increased the migration of ICC cells, when compared with SDF-1 alone, suggesting that SDF-1 further enhanced the migration of cultured ICC cells induced by the high concentration of TNF- α (Figure 7A). As described above, ICC cells treated with TNF- α expressed significantly more CXCR4 mRNA and protein. Therefore, the increased expression of CXCR4 induced by TNF- α might have been responsible for the greater increase in the migration induced by TNF- α + SDF-1 than by TNF- α alone. Interestingly, when AMD3100, an antagonist of CXCR4, was added to the culture medium, the migration of ICC cells of either cell line was completely reduced to the control level, suggesting that CXCR4 induced in ICC cells by TNF- α is significantly involved in the migration of

Figure 11. Effect of SDF-1/CXCR4 interaction on proliferation of cultured ICC cells and the influence of TNF- α were examined by using the proliferative reagent WST-8 and measuring the absorbance at 450 nm. Addition of 100 ng/ml of SDF-1 has no effect on proliferative activities of HuCCT-1 and CCKS-1 cells cultured for 48 hours. Additional treatment with 100 or 1000 U/ml TNF- α in this experiment had no influence on the proliferation of the cultured ICC cells for 48 hours either. Administration of AMD3100, an antagonist of CXCR4, had no effect on the proliferation of the cultured ICC cells. The data are presented as the mean $\pm$ SD. The assay for cell proliferation was performed three times in each experiment.

ICC cells. Taken together, ICC expressing CXCR4 induced by SDF-1 derived from stromal cells and TNF- α released from macrophages and also probably from ICC cells themselves at the periphery of ICC may be an important factor for the migration of ICC.

It was found in this study that CXCR4 and SDF-1 were expressed in ICC cells and fibroblast-like stromal cells and TNF- α was expressed in accumulated macrophages at the peripheral, invasive fronts of ICC, suggesting that SDF-1 and TNF- α might have been secreted into the stroma, and the specific interactions of CXCR4, SDF-1, and TNF- α might have been operative within the tumor, particularly at the invasive front. Recently, the bindings of extracellular matrix macromolecules such as basement-membrane-type heparan sulfate proteoglycan to cytokines, chemokines, and growth factors are biochemically shown, and their interaction with stromal cells and also tumor cells are regarded as important in the biological behaviors of malignant tumors.^{52,53} Our previous study showed that heparan sulfate proteoglycan was abundant in fibrous stroma of ICC, whereas heparan sulfate proteoglycan expression was negligible in the surrounding liver.⁵⁴ Furthermore, Charnaux and colleagues⁴³ reported that a proteoglycan, syndecan-4, behaves as a SDF-1 receptor, and is selectively involved in signal transduction induced by SDF-1. Taken together, it seems possible that the specific interactions of SDF-1 and TNF- α with their receptors expressed on ICC cells are likely to occur in such tumor-stromal microenvironments, and

that these interactions are responsible for the characteristic biological behaviors of ICC such as invasion and migration.

There have been several reports that SDF-1/CXCR4 signaling enhances cell proliferative activity in malignant cells,^{15,16} whereas this proliferative effect is different among the cultured cells examined. It was found in this study that the proliferative activity of HuCCT-1 and CCKS-1 cells was not changed by SDF-1 at any concentration or by the simultaneous addition of SDF-1 and TNF- α , suggesting that CXCR4/SDF-1 signaling is not involved in the cell proliferation of these cultured ICC cells.

In conclusion, SDF-1 and WI-38 fibroblasts promoted the migration of ICC cells expressing CXCR4. TNF- α up-regulated CXCR4 expression in cultured ICC cells, and induced increased migration of cultured ICC cells via SDF-1/CXCR4 interaction. TNF- α expressed evidently in infiltrating macrophages and to a lesser degree in ICC cells at the periphery of ICC may be also involved in the migration or invasion of ICC by inducing CXCR4 expression in ICC cells. CXCR4, SDF-1, and TNF- α are candidates for factors involved in the cross-talk of the tumor-stroma interaction of ICC and may be actively involved in its migration. Because the increase in the migration of cultured ICC cells induced by TNF- α and SDF-1 was completely inhibited by AMD3100, a CXCR4 antagonist, therapeutic strategies that target CXCR4 may be beneficial to ICC patients.

References

- Okuda K, Nakanuma Y, Miyazaki M: Cholangiocarcinoma: recent progress. Part 2: molecular pathology and treatment. *J Gastroenterol Hepatol* 2002, 17:1056–1063
- Okuda K, Nakanuma Y, Miyazaki M: Cholangiocarcinoma: recent progress. Part 1: epidemiology and etiology. *J Gastroenterol Hepatol* 2002, 17:1049–1055
- Harada K, Masuda S, Hirano M, Nakanuma Y: Reduced expression of syndecan-1 correlates with histologic dedifferentiation, lymph node metastasis, and poor prognosis in intrahepatic cholangiocarcinoma. *Hum Pathol* 2003, 34:857–863
- Shimonishi T, Miyazaki K, Kono N, Sabit H, Tuneyama K, Harada K, Hirabayashi J, Kasai K, Nakanuma Y: Expression of endogenous galectin-1 and galectin-3 in intrahepatic cholangiocarcinoma. *Hum Pathol* 2001, 32:302–310
- Chung LW: The role of stromal-epithelial interaction in normal and malignant growth. *Cancer Surv* 1995, 23:33–42
- Liotta LA, Kohn EC: The microenvironment of the tumour-host interface. *Nature* 2001, 411:375–379
- Pupa SM, Menard S, Forti S, Tagliabue E: New insights into the role of extracellular matrix during tumor onset and progression. *J Cell Physiol* 2002, 190:259–267
- Hayashi N, Cunha GR: Mesenchyme-induced changes in the neoplastic characteristics of the Dunning prostatic adenocarcinoma. *Cancer Res* 1991, 51:4924–4930
- Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S, Washington MK, Neilson EG, Moses HL: TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* 2004, 303:848–851
- Nakamura T, Matsumoto K, Kiritoshi A, Tano Y, Nakamura T: Induction of hepatocyte growth factor in fibroblasts by tumor-derived factors affects invasive growth of tumor cells: in vitro analysis of tumor-stromal interactions. *Cancer Res* 1997, 57:3305–3313
- Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A: Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001, 410:50–56
- Wang JM, Deng X, Gong W, Su S: Chemokines and their role in tumor growth and metastasis. *J Immunol Methods* 1998, 220:1–17
- Scotton CJ, Wilson JL, Milliken D, Stamp G, Balkwill FR: Epithelial cancer cell migration: a role for chemokine receptors? *Cancer Res* 2001, 61:4961–4965
- Csiszar A, Szentes T, Haraszi B, Balazs A, Petranyi GG, Pocsik E: The pattern of cytokine gene expression in human colorectal carcinoma. *Pathol Oncol Res* 2004, 10:109–116
- Marchesi F, Monti P, Leone BE, Zerbi A, Vecchi A, Piemonti L, Mantovani A, Allavena P: Increased survival, proliferation, and migration in metastatic human pancreatic tumor cells expressing functional CXCR4. *Cancer Res* 2004, 64:8420–8427
- Katayama A, Ogino T, Bando N, Nonaka S, Harabuchi Y: Expression of CXCR4 and its down-regulation by IFN- γ in head and neck squamous cell carcinoma. *Clin Cancer Res* 2005, 11:2937–2946
- Scotton CJ, Wilson JL, Scott K, Stamp G, Wilbanks GD, Fricker S, Bridger G, Balkwill FR: Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer. *Cancer Res* 2002, 62:5930–5938
- Salvucci O, Basik M, Yao L, Bianchi R, Tosato G: Evidence for the involvement of SDF-1 and CXCR4 in the disruption of endothelial cell-branching morphogenesis and angiogenesis by TNF- α and IFN- γ . *J Leukoc Biol* 2004, 76:217–226
- Uchida D, Begum NM, Tomizuka Y, Bando T, Almofti A, Yoshida H, Sato M: Acquisition of lymph node, but not distant metastatic potentials, by the overexpression of CXCR4 in human oral squamous cell carcinoma. *Lab Invest* 2004, 84:1538–1546
- Almofti A, Uchida D, Begum NM, Tomizuka Y, Iga H, Yoshida H, Sato M: The clinicopathological significance of the expression of CXCR4 protein in oral squamous cell carcinoma. *Int J Oncol* 2004, 25:65–71
- Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C, Guise TA, Massague J: A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* 2003, 3:537–549
- Zhou Y, Larsen PH, Hao C, Yong VW: CXCR4 is a major chemokine receptor on glioma cells and mediates their survival. *J Biol Chem* 2002, 277:49481–49487
- Sehgal A, Ricks S, Boynton AL, Warrick J, Murphy GP: Molecular characterization of CXCR-4: a potential brain tumor-associated gene. *J Surg Oncol* 1998, 69:239–248
- Balkwill F: Tumor necrosis factor or tumor promoting factor? *Cytokine Growth Factor Rev* 2002, 13:135–141
- Rosen EM, Goldberg ID, Liu D, Setter E, Donovan MA, Bhargava M, Reiss M, Kacinski BM: Tumor necrosis factor stimulates epithelial tumor cell motility. *Cancer Res* 1991, 51:5315–5321
- Katerinaki E, Evans GS, Lorigan PC, MacNeil S: TNF- α increases human melanoma cell invasion and migration in vitro: the role of proteolytic enzymes. *Br J Cancer* 2003, 89:1123–1129
- Chen TC, Hinton DR, Apuzzo ML, Hofman FM: Differential effects of tumor necrosis factor- α on proliferation, cell surface antigen expression, and cytokine interactions in malignant gliomas. *Neurosurgery* 1993, 32:85–94
- Michalaki V, Syrigos K, Charles P, Waxman J: Serum levels of IL-6 and TNF- α correlate with clinicopathological features and patient survival in patients with prostate cancer. *Br J Cancer* 2004, 90:2312–2316
- Terada R, Yamamoto K, Hakoda T, Shimada N, Okano N, Baba N, Ninomiya Y, Gershwin ME, Shiratori Y: Stromal cell-derived factor-1 from biliary epithelial cells recruits CXCR4-positive cells: implications for inflammatory liver diseases. *Lab Invest* 2003, 83:665–672
- Coulomb-L'Hermine A, Amara A, Schiff C, Durand-Gasselin I, Foussat A, Delaunay T, Chaouat G, Capron F, Ledee N, Galanaud P, Arenzana-Seisdedos F, Emilie D: Stromal cell-derived factor 1 (SDF-1) and antenatal human B cell lymphopoiesis: expression of SDF-1 by mesothelial cells and biliary ductal plate epithelial cells. *Proc Natl Acad Sci USA* 1999, 96:8585–8590
- Yasoshima M, Kono N, Sugawara H, Katayanagi K, Harada K, Nakanuma Y: Increased expression of interleukin-6 and tumor necrosis factor- α in pathologic biliary epithelial cells: in situ and culture study. *Lab Invest* 1998, 78:89–100
- Taniai M, Grambihler A, Higuchi H, Werneburg N, Bronk SF, Farrugia DJ, Kaufmann SH, Gores GJ: Mcl-1 mediates tumor necrosis factor-related apoptosis-inducing ligand resistance in human cholangiocarcinoma cells. *Cancer Res* 2004, 64:3517–3524
- Park J, Tadlock L, Gores GJ, Patel T: Inhibition of interleukin 6-mediated mitogen-activated protein kinase activation attenuates growth of a cholangiocarcinoma cell line. *Hepatology* 1999, 30:1128–1133
- Sugawara H, Yasoshima M, Katayanagi K, Kono N, Watanabe Y, Harada K, Nakanuma Y: Relationship between interleukin-6 and proliferation and differentiation in cholangiocarcinoma. *Histopathology* 1998, 33:145–153
- Saito K, Minato H, Kono N, Nakanuma Y, Ishida F, Kosugi M: Establishment of the human cholangiocellular carcinoma cell line (CCKS1). *Acta Hepatol Jpn* 1993, 34:122–129
- Kamihira T, Shimoda S, Harada K, Kawano A, Handa M, Baba E, Tsuneyama K, Nakamura M, Ishibashi H, Nakanuma Y, Gershwin ME, Harada M: Distinct costimulation dependent and independent autoreactive T-cell clones in primary biliary cirrhosis. *Gastroenterology* 2003, 125:1379–1387
- Yamashiro M, Kouda W, Kono N, Tsuneyama K, Matsui O, Nakanuma Y: Distribution of intrahepatic mast cells in various hepatobiliary disorders. An immunohistochemical study. *Virchows Arch* 1998, 433:471–479
- Kato M, Kitayama J, Kazama S, Nagawa H: Expression pattern of CXC chemokine receptor-4 is correlated with lymph node metastasis in human invasive ductal carcinoma. *Breast Cancer Res* 2003, 5:R144–R150
- Harada K, Ohira S, Isse K, Ozaki S, Zen Y, Sato Y, Nakanuma Y: Lipopolysaccharide activates nuclear factor- κ B through toll-like receptors and related molecules in cultured biliary epithelial cells. *Lab Invest* 2003, 83:1657–1667
- Wu X, Jin C, Wang F, Yu C, McKeenan WL: Stromal cell heterogeneity in fibroblast growth factor-mediated stromal-epithelial cell cross-talk in premalignant prostate tumors. *Cancer Res* 2003, 63:4936–4944
- Kajita T, Ohta Y, Kimura K, Tamura M, Tanaka Y, Tsunozuka Y, Oda M, Sasaki T, Watanabe G: The expression of vascular endothelial growth factor C and its receptors in non-small cell lung cancer. *Br J Cancer* 2001, 85:255–260
- Koshihara T, Hosotani R, Miyamoto Y, Ida J, Tsuji S, Nakajima S, Kawaguchi M, Kobayashi H, Doi R, Hori T, Fujii N, Imamura M: Expression of stromal cell-derived factor 1 and CXCR4 ligand recep-

- tor system in pancreatic cancer: a possible role for tumor progression. *Clin Cancer Res* 2000, 6:3530–3535
43. Charnaux N, Brule S, Hamon M, Chaigneau T, Saffar L, Prost C, Lievre N, Gattegno L: Syndecan-4 is a signaling molecule for stromal cell-derived factor-1 (SDF-1)/CXCL12. *FEBS J* 2005, 272:1937–1951
 44. Fedyk ER, Jones D, Critchley HO, Phipps RP, Blieden TM, Springer TA: Expression of stromal-derived factor-1 is decreased by IL-1 and TNF and in dermal wound healing. *J Immunol* 2001, 166:5749–5754
 45. Schrader AJ, Lechner O, Templin M, Dittmar KE, Machtens S, Mengel M, Probst-Keppler M, Franzke A, Wollensak T, Gatzlaff P, Atzpodien J, Buer J, Lauber J: CXCR4/CXCL12 expression and signalling in kidney cancer. *Br J Cancer* 2002, 86:1250–1256
 46. Suganuma M, Okabe S, Marino MW, Sakai A, Sueoka E, Fujiki H: Essential role of tumor necrosis factor alpha (TNF-alpha) in tumor promotion as revealed by TNF-alpha-deficient mice. *Cancer Res* 1999, 59:4516–4518
 47. Moore RJ, Owens DM, Stamp G, Arnott C, Burke F, East N, Holdsworth H, Turner L, Rollins B, Pasparakis M, Kollias G, Balkwill F: Mice deficient in tumor necrosis factor-alpha are resistant to skin carcinogenesis. *Nat Med* 1999, 5:828–831
 48. Tartaglia LA, Pennica D, Goeddel DV: Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. *J Biol Chem* 1993, 268:18542–18548
 49. Vandenabeele P, Declercq W, Beyaert R, Fiers W: Two tumour necrosis factor receptors: structure and function. *Trends Cell Biol* 1995, 5:392–399
 50. Kaiser GC, Yan F, Polk DB: Conversion of TNF alpha from antiproliferative to proliferative ligand in mouse intestinal epithelial cells by regulating mitogen-activated protein kinase. *Exp Cell Res* 1999, 249:349–358
 51. Kaiser GC, Polk DB: Tumor necrosis factor alpha regulates proliferation in a mouse intestinal cell line. *Gastroenterology* 1997, 112:1231–1240
 52. Nackaerts K, Verbeken E, Deneffe G, Vanderschueren B, Demedts M, David G: Heparan sulfate proteoglycan expression in human lung-cancer cells. *Int J Cancer* 1997, 74:335–345
 53. Blackhall FH, Merry CL, Davies EJ, Jayson GC: Heparan sulfate proteoglycans and cancer. *Br J Cancer* 2001, 85:1094–1098
 54. Sabit H, Tsuneyama K, Shimonishi T, Harada K, Cheng J, Ida H, Saku T, Saito K, Nakanuma Y: Enhanced expression of basement-membrane-type heparan sulfate proteoglycan in tumor fibro-myxoid stroma of intrahepatic cholangiocarcinoma. *Pathol Int* 2001, 51:248–256