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Original Article

Local balance of transforming growth factor-β1 secreted from cholangiocarcinoma cells and stromal-derived factor-1 secreted from stromal fibroblasts is a factor involved in invasion of cholangiocarcinoma

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Tumor–stromal interactions are important for the progression of malignant tumors. The purpose of the present study was to examine interactions of cholangiocarcinoma (CC) cells and stromal fibroblasts with respect to stromal-derived factor-1 (SDF-1) and transforming growth factor (TGF)-β1. Two cell lines of CC (HuCCT-1 and CCKS-1) and WI-38 fibroblast cell line were used for cell culture, and 12 CC tissue specimens for immunohistochemical studies. Invasion of CC cells was increased significantly by the supernatant from fibroblast cultures, but not by the supernatant from fibroblasts cocultured with CC cells. Expression of SDF-1 in cultured fibroblasts was downregulated by TGF-β1 treatment, and coculture with CC cells and anti-TGF-β1 neutralizing antibody restored the decreased SDF-1 expression, suggesting that TGF-β1 secreted from CC cells might have reduced the expression of SDF-1 by fibroblasts and might have reduced the increased invasion of CC cells induced by the supernatant from fibroblasts. Immunohistochemical expression of TGF-β1 in CC cells was focal or negative and that of SDF-1 was evident in stromal fibroblasts at the invasive front of CC. In conclusion, local mutual influence of TGF-β1 secreted from carcinoma cells and SDF-1 expressed by stromal fibroblasts may be involved in invasion of CC cells.

Key words: cholangiocarcinoma, fibroblasts, invasion, SDF-1, TGFβ-1, tumor stroma

Recently, there has been increasing evidence that tumor stromal cells provide a unique microenvironment that is pivotal for tumor cell growth, invasion and metastasis.[1–6] Crucial in this process are fibroblasts that are located in the vicinity of neoplastic epithelial cells. These fibroblasts can produce an extracellular matrix (ECM) that is used as an anchorage for tumor cell, and also functions as a reservoir of growth factors derived from tumor and stromal cells.[7,8] As the specific paracrine factors in the tumor–stromal interactions, various growth factors and their receptors, including hepatocyte growth factor (HGF)/c-Met, stromal-derived factor-1 (SDF-1)/CXCR4 (a specific receptor of SDF-1), and epidermal growth factor (EGF)/EGF-R, have been reported.[6,9–12] Among them, SDF-1, which is known to be expressed in the stromal fibroblasts and endothelial cells, strongly promotes invasion and directional chemotaxis of tumor cells and also increases the adhesion of tumor cells.[12,13] In addition, CXCR4 is expressed significantly in the malignant tumors and SDF-1–CXCR4 interaction is involved in tumor progression, especially in brain and breast tumors.[14] Our recent study showed that the interaction of SDF-1 released from fibroblasts and CXCR4 expressed on cholangiocarcinoma (CC) cells is actively involved in the invasion of CC, and that tumor necrosis factor-α (TNF-α) expressed at the interface of CC may enhance the invasion of CC cells by increasing CXCR4 expression on CC cells.[12]

Transforming growth factor (TGF)-β, which is involved in the signaling network for several autocrine and paracrine mechanisms, is known as an important mediator of tumor–stromal interactions and is responsible for regulation of cell proliferation, cell motility, and neoplastic transformation.[5,12,15,16] For example, Bhowmick *et al.* have recently reported that stromal cells influence the carcinogenesis and progression process in adjacent epithelia by changing TGF-β signaling in fibroblasts.[5] As for the role of TGF-β in tumor–stromal interactions in CC, overexpressed TGF-β1 and vascular endothelial growth factor (VEGF) and their

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receptors in CC cells and endothelial cells might contribute to the 'angiogenic switch' and malignant transformation of biliary epithelial cells.[17] In CC with hepatolithiasis, TGF-β1 was immunohistochemically expressed in mononuclear cells and mesenchymal cells around the stone-containing bile ducts and invasive CC, and also in hyperplastic and dysplastic biliary epithelial cells.[18] TGF-β receptor (TβR-II) was constantly expressed on biliary epithelial cells irrespective of biliary lesions. A high level of TGF-β1 around CC or its precursors may be involved in development and progression of CC via interaction with TβR-II expressed on these lesions.[18]

As for the relationship between SDF-1 and TGF-β1, TGF-β1 is known to control the proliferation and differentiation of hematopoietic progenitor cells by downregulating SDF-1 expression in the bone marrow stem cell.[19] Salvucci *et al*. reported that basic fibroblast growth factor (bFGF) increases SDF-1 expression in endothelial cells and SDF-1/CXCR-4 identifies bFGF-regulated autocrine signaling systems that are essential regulators of angiogenesis.[20] However, there have been no studies on the interaction of SDF-1 and TGF-β1 in CC, so far.

To clarify the tumor–stromal interactions in CC, and determine whether SDF-1 expressed in the stromal fibroblasts and TGF-β1 expressed in carcinoma cells influence each other, we cultured CC and fibroblast cell lines and carried out immunohistochemistry for human CC tissues.

MATERIALS AND METHODS

Patients selection and tissue processing of CC specimens

Twelve cases of surgically resected CC located at hepatic hilar and/or perihilar regions[21,22] (10 men and two women) were consecutively retrieved from the Biliary Disease files of the Department of Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan, in 2003. The mean age of the patients was 65.5 years (range: 59–80 years). Surgical procedures were partial hepatectomy with extrahepatic bile duct resection in all cases. Specimens were collected as a result of routine operative procedures and prepared for pathological diagnosis. Tissue procurement was approved by the ethics board in Nagoya University Graduate School of Medicine. All cases of CC had histologically determined adenocarcinoma of variable differentiation. Cancerous large bile ducts (CC cells replacing the luminal surface of the original intrahepatic large bile ducts), which were available in five cases, had a micropapillary pattern of adenocarcinoma in their lumen, while at the invasive front areas or border, the CC, which were available in all cases, had a tubular or cord-like pattern of adenocarcinoma with significant desmoplastic reaction. All tissue specimens were fixed in neutral formalin, and embedded in paraffin. More than 20 serial sections, 3 μm thick, were cut from each paraffin block, and were used for routine histological stainings including HE and also for the immunohistochemistry.

Antibodies and immunological reagents

We used a goat polyclonal antibody against SDF-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and a mouse monoclonal antibody against TGF-β1 (clone TB21; Chemicon, Temecula, CA, USA) for immunohistochemistry. Preincubation of anti-SDF-1 with SDF-1 resulted in a marked reduction. The specificity of anti-TGF-β1 was confirmed by western blotting. We also used recombinant human TGF-β1 (R&D systems, Minneapolis, MN, USA).

Immunohistochemical staining of SDF-1 and TGF-β1

Expression of SDF-1 and TGF-β1 in CC cells and stromal cells within CC tissue was immunohistochemically examined by using the Envision+ system (Dako, Santa Barbara, CA, USA). Briefly, deparaffinized sections were pretreated in a microwave oven in EDTA buffer at 95°C, 20 min for SDF-1. After blocking endogenous peroxidase in 1% H_2O_2 in methanol for 20 min at room temperature and then non-specific reaction in Proteinblock Serum (Dako) for 15 min at room temperature, deparaffinized sections were incubated with each primary antibody at 1:50 dilution for SDF-1, and 1:1000 dilution for TGF-β1 at 4°C overnight. The Envision+ solution for mouse or goat (Dako) was then applied for 60 min at room temperature. The reaction products were visualized by 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical, St Louis, MO, USA), and the sections were then lightly counterstained with hematoxylin. Negative controls included substituting the primary antibody with similarly diluted mouse or goat control immunoglobulin G (Dako).

Expression of SDF-1 and TGF-β1 was mainly examined in two areas of CC: at the border or invasive front areas of CC and around the carcinomatous large bile ducts in which papillary-shaped or stratified carcinoma remained or was identifiable on the luminal surface. The former area was available in all 12 cases, while the latter was available in five of 12 cases.

Cell culture

Two cell lines of CC (HuCCT-1, Cell Resource Center for Biochemical Research, Tohoku University; and CCKS-1, established in our laboratory at Kanazawa University Graduate School of Medicine[23,24]) and one cell line of fibroblasts

(WI-38, embryonic lung fibroblasts, Cell Resource Center for Biochemical Research, Tohoku University) were used. These cells were maintained in RPMI-1640 medium containing 10% fetal calf serum (FCS) and penicillin–streptomycin–glutamine (Gibco-BRL, Grand Island, NY, USA).

Invasion assays of cultured CC cells

Migration of CC cells was assayed using Matrigel Invasion Chambers (24-well format, 8 μm pore; BD Bioscience, Bedford, MA, USA). Medium (0.5 μL) containing 5×10^5 CC cells (HuCTT-1 and CCKS-1) was added to the upper chamber, and 0.5 mL of either supernatant with WI-38 alone or WI-38 cultured with CC cells, was added to the lower chamber. Chambers were incubated for 48 h at 37°C and 5% CO_2. The CC cells in the upper surface of the filter were then removed using a cotton wool swab. Migrated cells on the lower surface were stained using 1% toluidine blue after fixing with 100% methanol. The number of migrated cells in 10 medium-power fields (×20) was counted, and the experiments were conducted in triplicate. The data were compared and evaluated by calculating the invasion index (no. migrating cells in an experimental group/no. migrating cells in control groups without coculture, in culture fluid, or in other reagent).

RNA extraction and reverse transcription–polymerase chain reaction

RNA was isolated from cultured WI-38 fibroblasts using the Qiagen RNAeasy kit (Qiagen, Santa Clarita, CA, USA). RNA was then used for first-strand cDNA synthesis with the superscript reamplification system (Gibco-BRL), according to the manufacturer's instructions. Reverse transcription–polymerase chain reaction (RT-PCR) for TGF-β receptor type I (TβRI), TGF-β receptor type II (TβRII) and β-actin were then performed. The following primers were designed complementary to the published nucleotide sequence: β-actin (forward, 5′-CAA-GAG-ATG-GCC-ACG-GCT-GCT-3′; reverse, 5′-TCC-TTC-TGC-ATC-CTG-TCG-GCA-3′; PCR product size, 275 bp); TβRI (forward, 5′-AAC-CGC-ACT-GTC-ATT-CAC-3′; reverse, 5′-TTC-CTC-TCC-AAA-CTT-CTC-C-3′; PCR product size, 220 bp); TβRII forward, 5′-ATG-GAG-TTC-AGC-GAG- CAC-3′; reverse, 5′-CAC-AGG-CAG-CAG-GTT-AGG-3′; PCR product size, 560 bp).[21,22]

The PCR conditions consisted of a denaturing step at 94°C for 30 s, an annealing step at 60°C (β-actin), 58°C (TβRI), or 61°C (TβRII) for 1 min, and an extension step at 72°C for 1 min for one cycle, followed by 18 cycles (β-actin) or 29 cycles (TβRI and TβRII). After PCR, 5 μL aliquots of the products were subjected to 1.5% or 2.0% agarose gel electrophoresis and stained with ethidium bromide.

Protein extraction and western blot analysis for TGF-β1

Proteins were extracted from cultured CC cells (HuCCT-1 and CCKS-1) using T-PER Tissue Protein Extraction Reagent (Pierce Chemical, Rockford, IL, USA). Total protein was measured by a spectrophotometer. A total of 20 μg of extracted protein was used for western blot analysis.

The analysis was performed on 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis for TGF-β1. The proteins in the gel were electrophoretically transferred onto nitrocellulose membrane. The membranes were incubated with primary antibodies to TGF-β1. These proteins were detected using second antibodies conjugated to peroxidase-labeled polymer Envision+ solution (Dako) for mouse. 3-3′-Diaminobenzidine tetrahydrochloride was used as the chromogen.

Real-time PCR for SDF-1 mRNA in WI-38 fibroblasts

WI-38 fibroblasts were cultured alone or were cocultured with CC cells (HuCCT-1 or CCKS-1) in Transwell 4 μm-pore tissue culture dish (Corning, Corning, NY, USA) for 48 h. RNA was isolated from these cultured WI-38 fibroblasts, and multiplex real-time analysis was performed using premade SDF-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers and probes with the ABI Prism 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Warrington, UK). RT-PCR was carried out with the TaqMan Universal RT-PCR Master Mix (PE Applied Biosystems) using 5 μL cDNA in a 25 μL final reaction mixture. Cycling conditions were incubation at 50°C for 2 min, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. Experiments were performed in duplicate for each sample, and SDF-1 was normalized (ΔCt) to GAPDH by subtracting the cycle threshold (Ct) value of GAPDH from the Ct value of SDF-1. Fold difference compared with control was calculated. Our preliminary study showed that CC cells of the two cell lines failed to express SDF-1 mRNA.

Measurement of SDF-1 production

First, the baseline SDF-1 production from cultured WI-38 fibroblasts and CC cells (HuCCT-1 and CCKS-1) was determined. Either of these three cell lines was seeded on 6 cm culture dishes at a density of 1×10^5/mL and cultured for 24 h. After replacing the media with fresh RPMI1640, the cells were cultured for another 48 h. Concentration of SDF-1 in the supernatant was measured by enzyme-linked immunosorbent assay (ELISA) using a human SDF-1 and Enzyme Immunoassay kit (R&D systems), according to the manufacturer's instructions.

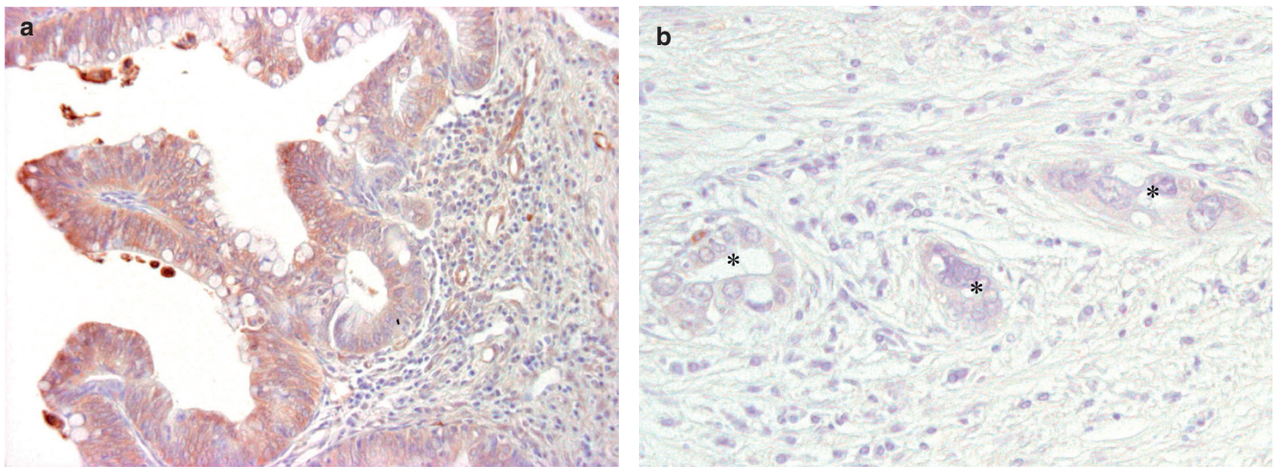

Figure 1 Expression of tumor growth factor (TGF)-β1. (**a**) Expression of TGF-β1 is evident in carcinoma cells of cancerous large bile duct (the lumen of original intrahepatic large bile duct is replaced by micropapillary carcinoma). Several stromal cells, particularly endothelial cells, are also positive for TGF-β1. (**b**) TGF-β1 expression in carcinoma cell (*) is vague or negative at the invasive front of cholangiocarcinoma (CC). Several stromal cells, particularly endothelial cells, are strongly positive for TGF-β1. (**a**) immunohistochemistry for TGF-β1 and hematoxylin; (**b**) immunohistochemistry for TGF-β1 and hematoxylin.

For the analysis of influence of cultured CC cells on SDF-1 expression by WI-38 fibroblasts, the coculture of CC cells with WI-38 fibroblasts in the following combination was done. That is, at the density of WI-38 fibroblasts to CC cells of 1:10 and 1:100 both CC and WI-38 cells were cocultured for 48 h. As controls, WI-38 fibroblasts alone or either of the two CC cell lines alone were cultured. Then, the conditioned culture media were harvested from each coculture system, and SDF-1 protein was evaluated by ELISA. Preliminary studies showed that CC cells of the two cell lines did not express SDF-1 protein.

Statistical analysis

The difference among two or three groups was assessed by Mann–Whitney *U*-test. Significance was taken for $P < 0.05$.

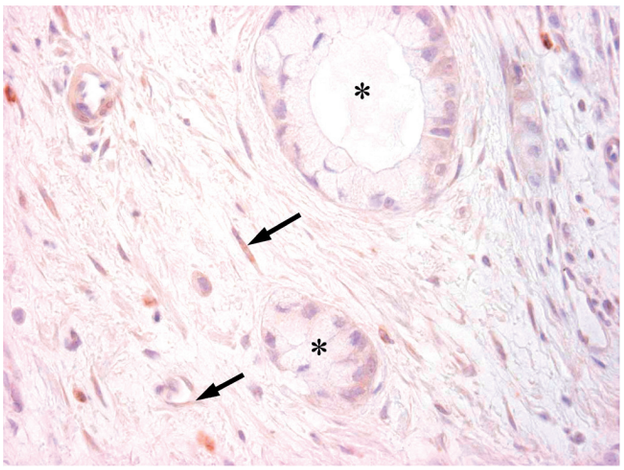

Figure 2 Expression of stromal-derived factor (SDF)-1. SDF-1 expression is evident in several fibroblast-like stromal cells and endothelial cells (arrows) at the invasive front of cholangiocarcinoma. Carcinoma cells (*) are negative for SDF-1. Immunohistochemistry for SDF-1 and hematoxylin.

RESULTS

Expression of SDF-1 and TGF-β1 in human CC tissues

TGF-β1 was strongly expressed in the cytoplasm of endothelial cells and also in fibroblast-like stromal cells within CC. It was also positive diffusely in the cytoplasm of a variable number of CC cells of all CC cases; expression of TGF-β1 in CC cells was variable according to the location in CC. That is, TGF-β1 was frequently and strongly expressed in carcinoma growing within the lumen of cancerous large bile ducts in five cases examined (Fig. 1a), while at invasive areas of CC, the expression of TGF-β1 in CC cells was negative or minimal in nine cases and focally positive in the remaining three cases (Fig. 1b).

SDF-1 was expressed in the cytoplasm of fibroblast-like stromal cells and also endothelial cells within CC, although the distribution of positive cells and their staining intensity were variable in individual cases. Around the cancerous large bile ducts, expression of SDF-1 in fibroblasts was minimal or absent. At the border of CC reflecting invasive fronts, SDF-1-positive fibroblast-like stromal cells were usually observed

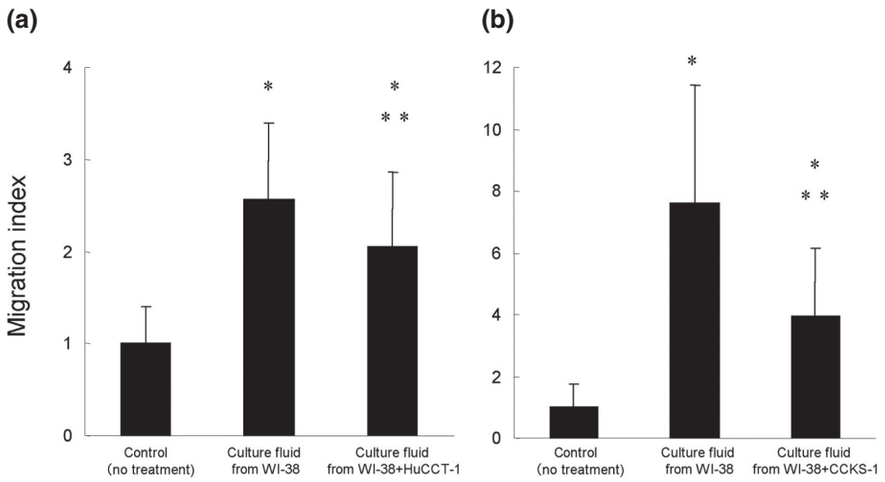

Figure 3 Invasion assay of cholangiocarcinoma (CC) cells in Matrigel invasion chamber. Invasion of cultured CC cells from either (**a**) HuCCT-1 or (**b**) CCKS-1 is increased when the cells are incubated with the supernatant from culture dishes in which WI-38 fibroblasts alones are cultured for 48 h. The invasion of HuCCT-1 cells is increased by 2.6 ± 0.8-fold, and that of CCKS-1 by 7.6 ± 3.8-fold, compared with the control (only CC cells cultured; $*P < 0.05$). When CC cell lines are cultured with addition of the cultured fluid from WI-38 fibroblasts cocultured with HuCCT-1 CC cells or CCKS-1 CC cells, the increase in invasion of CC cells is limited to 2.2 ± 0.8-fold and 4.0 ± 2.2 fold, respectively ($**P < 0.05$ vs WI-38 fibroblasts alone).

(Fig. 2). Expression of SDF-1 was evident in fibroblasts at the invasive front zones in nine cases. In the remaining three cases, SDF-1 expression in fibroblasts was minimal or absent.

Influence of cultured WI-38 fibroblasts on invasion of cultured CC cells

Next, we examined the influence of cultured WI-38 fibroblasts on the migration of CC cells (HuCTT-1 and CCKS-1), by using migration assay. Migration assay of cultured CC cells was measured by addition of the supernatant without cellular elements, which was obtained from the culture dishes in which (i) only WI-38 fibroblasts were cultured, and from those in which WI-38 fibroblasts were cocultured with either of (ii) HuCCT-1 or (iii) CCKS-1 cells, for 48 h. As shown in Fig. 3, invasion of cultured CC cells was significantly increased by addition of the supernatant from (i) (2.6 ± 0.8-fold in HuCCT-1 and 7.6 ± 3.8-fold in CCKS-1), while this increased invasion was significantly reduced to 2.0 ± 0.8-fold in HuCCT-1 cells and to 4.0 ± 2.2-fold in CCKS-1, respectively, when the supernatant obtained from (ii) or (iii) were added (each, $P < 0.05$). These data suggest that cultured WI-38 fibroblasts secreted molecule(s) that increased invasion of cultured CC cells and that coculture of WI-38 fibroblasts with CC cells secreted molecule(s) that partially inhibited the increased invasion of CC cells induced by WI-38.

Influence of cultured CC cells on the expression of SDF-1 mRNA and protein in WI-38 fibroblasts

Real-time PCR and ELISA showed that SDF-1 mRNA was detectable in cultured WI-38 fibroblasts, and that cultured WI-38 fibroblasts secreted 172 ± 22.2 pg/mL SDF-1 in the culture fluid (Fig. 4a,b). When WI-38 fibroblasts were cultured in a cell ratio of 1:1.5, SDF-1 in the culture fluid increased to 1.3-fold (221 ± 10.1 pg/mL). These data indicate that the increased cell density up to 1.5-fold does not suppress the secretion of SDF-1. Interestingly, the level of SDF-1 mRNA in WI-38 fibroblasts was significantly decreased when they were cocultured with CC cells of either of the two cell lines (to 0.28 ± 0.01-fold, HuCCT-1; to 0.15 ± 0.08 fold, CCKS-1; $P < 0.05$, respectively). Next, WI-38 fibroblasts were cocultured with a variable number of CC cells (Fig. 4c): when the number of cocultured CC cells was increased stepwise, secretion of SDF-1 protein was decreased stepwise (from 98 ± 22.2 pg/mL to 66 ± 22.2 pg/mL in HuCCT-1 and from 169 ± 40.4 pg/mL to 80 ± 38.5 pg/mL in CCKS-1). These results indicate that cultured CC cells influenced the expression of mRNA and the secretion of SDF-1 protein in WI-38 fibroblasts, probably by secretion of soluble molecule(s).

TGF-β1 secreted by CC cells downregulates expression of SDF-1 in WI-38 fibroblasts

Carcinoma cells of several organs are well-known to express and secrete TGF-β1, and it was found by immunohistochemistry in the present study that TGF-β1 was expressed in CC cells (Fig. 2). Western blotting showed that TGF-β1 was produced by cultured HuCCT-1 cells and also by CCKS-1 cells (Fig. 5). Therefore, we tested the possibility that TGF-β1 secreted from cultured CC cells reduced SDF-1 mRNA expression and protein secretion by cultured WI-38 fibroblasts. SDF-1 protein secreted by WI-38 fibroblasts (172 ± 22.2 pg/mL) was reduced to 70 ± 37 pg/mL by treatment with 10 ng/mL TGF-b1 ($P < 0.05$; Fig. 6a). The expres-

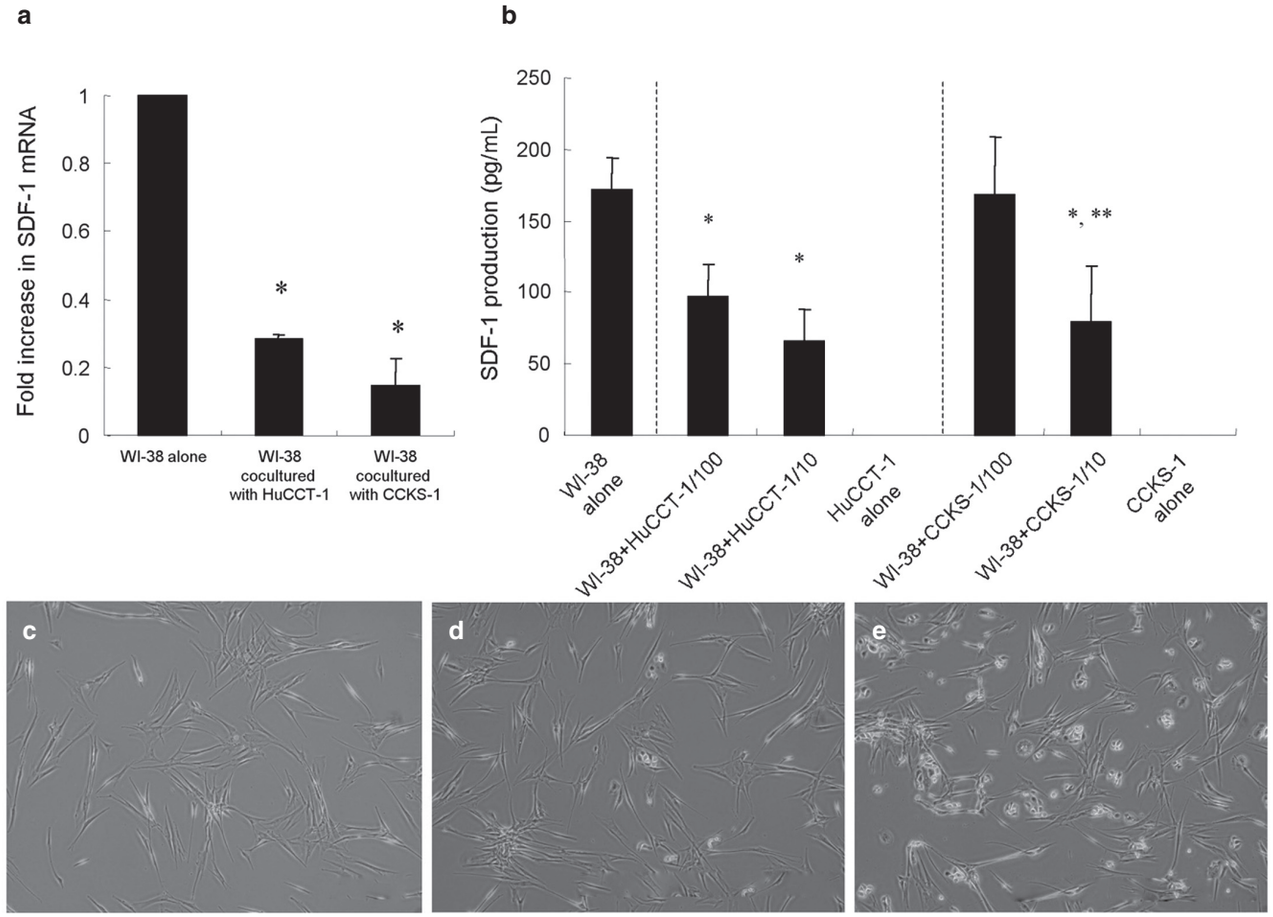

Figure 4 Effects of coculture of cholangiocarcinoma (CC) cell lines on the expression of stromal-derived factor-1 (SDF-1) in WI-38 fibroblasts. (**a**) Real-time polymerase chain reaction shows that SDF-1 mRNA expression in WI-38 fibroblasts is decreased to 0.28 ± 0.01-fold in the coculture with HuCCT-1 and to 0.15 ± 0.08-fold in the coculture with CCKS-1, compared with control (WI-38 fibroblasts cultured alone). (**b**) WI-38 fibroblasts cultured alone secrete 172 ± 22.2 pg/mL of SDF-1. Secretion of SDF-1 protein is decreased to 98 ± 22.2 pg/mL and further to 66 ± 22.2 pg/mL when WI-38 cells are cocultured with increasing amounts of HuCCT-1 cells, and secretion of SDF-1 protein is decreased to 169 ± 40.4 pg/mL and further to 80 ± 38.5 pg/mL when WI-38 cells are cocultured with increasing amounts of CCKS-1 cells. HuCCT-1 cells or CCKS-1 cells alone do not secrete SDF-1 protein. $^*P < 0.05$ to WI-38 cells; $^{**}P < 0.05$ to WI-38 cells + CCKS-1/100. (**c**–**e**) Phase-contrast photographs of representative examples of the cocultured HuCCT1 cells and WI-38 fibroblasts. (**c**) Only 1×10^5 cells/mL WI-38 fibroblasts are seeded and cultured. (**d**) 1×10^5 cells/mL WI-38 fibroblasts and 1×10^3 cells/mL HuCCT-1 cells (WI-38 cells : HuCCT-1 cells, 100:1) are seeded and cocultured. (**e**) 1×10^5 cells/mL WI-38 fibroblasts and 1×10^4 cells/mL HuCCT-1 cells (WI-38 cells : HuCCT-1 cells, 10:1) are seeded and cocultured.

sion of SDF-1 mRNA in WI-38 fibroblasts was also reduced significantly to 0.30 ± 0.18-fold by 10 ng/mL TGF-β1 treatment, compared to control (without TGF-β1 treatment; $P < 0.05$; Fig. 6b). These data strongly suggest that SDF-1 mRNA expression and protein secretion in cultured WI-38 fibroblasts was downregulated by TGF-β1. As for receptors for TGF-β1, RT-PCR showed that WI-38 fibroblasts expressed both TβRI and TβRII mRNA (Fig. 7), suggesting that WI-38 fibroblasts reacted with TGF-β1 via these receptors.

Interestingly, reduced expression of SDF-1 mRNA in WI-38 fibroblasts cocultured with CC cell lines (0.28 ± 0.01-fold in HuCCT-1 cells and 0.14 ± 0.08-fold in CCKS-1 cells, respectively) was partially recovered when 5 μg/mL anti-TGF-β1 neutralization antibody was added to the culture medium (0.33 ± 0.10-fold in HuCCT-1 cells and 0.47 ± 0.03-fold in CCKS-1 cells, respectively; Fig. 8). These data support the aforementioned suggestion. However, this recovery was statistically significant in CCKS-1 cells but not in HuCCT-1 cells. Because the recovery was not complete even in CCKS-1, it is suggested that other factors may be acting with TGF-β1 for the suppression of SDF-1 expression. Such factors might be responsible for the lower effect of TGF-β1 antibody neutralization in HuCCT-1 cells.

Taken together, it seems likely that TGF-β1 secreted from cultured CC cells downregulated SDF-1 mRNA and protein expression in WI-38 fibroblasts.

DISCUSSION

Recently, several molecules such as HGF, SDF-1, and EGF have been reported as effective molecules involved in the cross-talk in the tumor–stromal interactions in malignant tumors.[6,9–11] Among them, the SDF-1–CXCR4 axis is important in the progression of malignant tumors. SDF-1 is known to be produced in stromal fibroblasts and CXCR4 is reportedly expressed on tumor cells in some malignant neoplasms.[11,25–29] Our previous study showed that the invasion of cultured CC cells in Matrigel was increased by SDF-1 secreted from cultured WI-38 fibroblasts, and SDF-1–CXCR4 interaction was involved in this increased invasion.[12]

In the present study we further examined the interaction between stromal fibroblasts and CC cells with respect to TGF-β1 and SDF-1, using culture studies of CC cell lines and immunohistochemistry for human CC tissues. First, it was found that the invasion of cultured CC cells (HuCCT-1 and CCKS-1) was increased significantly when these CC cells were treated with the supernatant from the culture of WI-38 fibroblasts for 48 h, confirming our previous study.[12] Interestingly, when these CC cells were treated with the supernatant obtained from the coculture of WI-38 fibroblasts and CC cells, the increased invasion of CC cells was significantly reduced, suggesting that the cultured CC cells might have secreted a soluble factor that inhibited the increased invasion of CC cells induced by the supernatant from cultured WI-38 fibroblasts. It is noteworthy that SDF-1 mRNA and protein expression in WI-38 fibroblasts were decreased when they were cocultured with either of two CC cell lines, and this is compatible with the aforementioned data that SDF-1 secreted from WI-38 fibroblasts mediates the increased invasion of CC cells.[12]

Wright *et al*. reported that in the bone marrow, TGF-β1 downregulates the expression of SDF-1 in bone marrow stromal cells.[19] It was found in the present study that TGF-β1 was expressed in cultured CC cells (HuCCT-1 and CCKS-1) by western blotting. Therefore, we tested the possibility that

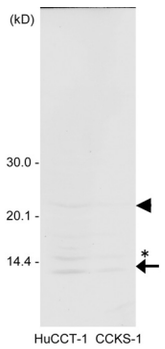

Figure 5 Detection of tumor growth factor (TGF)-β1 in cultured cholangiocarcinoma (CC) cells (HuCCT-1 and CCKS-1) by western blotting. TGF-β1 (12.5 kDa) was detected in both CC cell lines (HuCCT-1 and CCKS-1; arrow). In HuCCT-1, a dimeric form of TGF-β1 (25 kDa) was also detected (arrowhead). A protein (15–17 kDa) was also detected (*) in HuCCT-1, but the details of this protein remain unclear. This might be a precursor of TGF-β1 truncated irregularly.

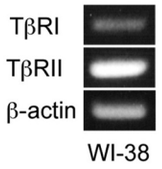

Figure 7 Expression of mRNA for two transforming growth factor (TGF)-β1 receptor, TGF-β receptor type I (TβRI) and TGF-β receptor type II (TβRII) mRNA in WI-38 fibroblasts detected by reverse transcription–polymerase chain reaction.

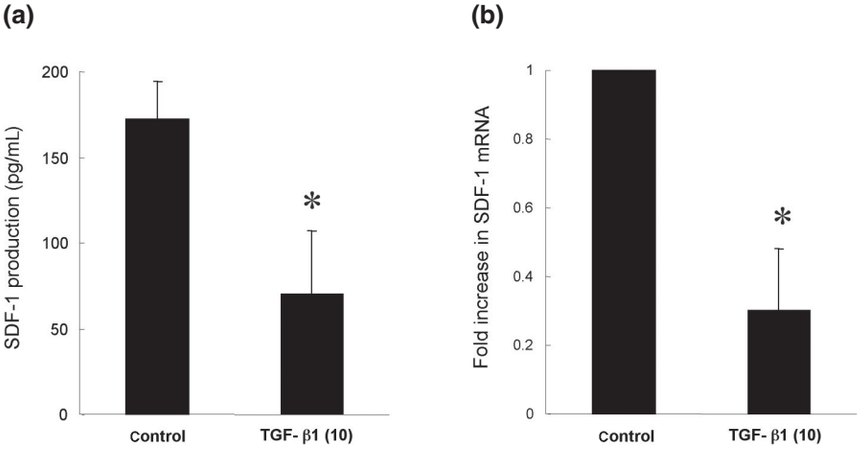

Figure 6 Influence of transforming growth factor (TGF)-β1 on expression of stromal-derived factor (SDF)-1 mRNA and protein in cultured WI-38 fibroblasts. (**a**) ELISA showed that SDF-1 protein secreted by WI-38 cells is 172 ± 22.2 pg/mL without TGF-β1 treatment and this is reduced to 70 ± 37 pg/mL on treatment with 10 ng/mL TGF-β1 (*$P < 0.05$). (**b**). Real-time quantitative polymerase chain reaction shows that SDF-1 mRNA expression in WI-38 fibroblasts without treatment is reduced to 0.30 ± 0.18-fold by treatment with 10 ng/mL TGF-β1 (*$P < 0.05$).

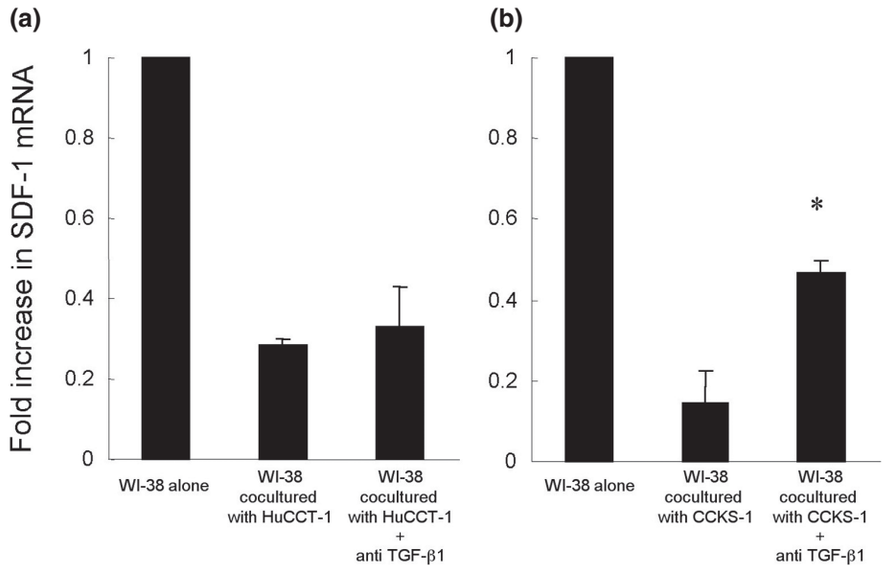

Figure 8 Effects of transforming growth factor (TGF)-β1 neutralization antibody. When TGF-β1 neutralization antibody is added to the medium of WI-38 fibroblasts cocultured with cholangiocarcinoma (CC) cell lines in Transwell 4 μm-pore tissue culture dish, stromal-derived factor (SDF)-1 mRNA expression is partially restored in (**a**) HuCCT-1 cells to 0.33 ± 0.10-fold and (**b**) CCKS-1 cells to 0.47 ±0.0.03-fold, when compared to that without addition of this antibody (HuCCT-1 cells, 0.28 ± 0.01-folds; CCKS-1 cells, 0.14 ± 0.08-fold). This restoration is statistically significant for CCKS-1 (*$P < 0.05$). These data suggest that TGF-β1 produced in CC cells contributes to downregulation of SDF-1 mRNA expression in WI-38 fibroblasts.

TGF-β1 secreted by CC cells downregulated the expression of SDF-1 in WI-38 fibroblasts, and then inhibited the invasion of CC cells. Interestingly, when TGF-β1 was added to the medium of WI-38 fibroblasts culture, the production of SDF-1 by WI-38 fibroblasts was decreased. RT-PCR showed that WI-38 fibroblasts expressed both TGF-β type I and II receptors, suggesting that TGF-β1 secreted from CC cells might have downregulated the expression of SDF-1mRNA in WI-38 fibroblasts via these receptors. In fact, administration of anti-TGF-β1 neutralization antibody tended to restore the decreased SDF-1 expression in cultured WI-38 fibroblasts by coculture with either of the CC cells, and this restoration was statistically significant in CCKS-1, suggesting that the downregulation of SDF-1 expression in WI-38 fibroblasts cocultured with CC cells might have been mediated via TGF-β1. Taken together, our results show that TGF-β1 secreted from cultured CC cells downregulated SDF-1 expression in fibroblasts, and that reduced expression of TGF-β1 in carcinoma cells downregulated the invasion-promoting SDF-1–CXCR4 axis of CC at invasive fronts of CC.

We also immunohistochemically examined the correlation between expression of SDF-1 in stromal cells and TGF-β1 in carcinoma cells within human CC tissue specimens. We found that TGF-β1 was expressed in CC cells of all CC cases, although the expression was quite variable. CC cells expressing TGF-β1 were focal or scarce at the invasive fronts of CC, while the expression of TGF-β1 was diffuse and strong around CC growing in the lumen of cancerous large bile ducts. We also found that although SDF-1 was expressed heterogeneously in several fibroblast-like stromal cells and endothelial cells in CC, its expression was pronounced at invasive areas or fronts of CC. These findings suggest that decreased expression of TGF-β1 in CC cells is likely to mediate the increased expression of SDF-1 in stromal fibroblasts at the invasive fronts, where carcinoma cells are likely to invade or migrate actively into the surrounding tissue.[21,22] In preliminary data, the clinical stage of three patients with CC in whom the expression of TGF-β1 was positive in CC cells at the invasive front appeared to be low when compared with the remaining nine patients in whom the expression of TGF-β1 was absent at the invasive front. However, the number of patients was limited and there was no difference statistically between the two groups. Further study using a large number of patients is needed to confirm this point.

There were TGF-β1-positive cells such as endothelial cells, other than CC cells, in CC tissue, suggesting that the tumor–stroma interactions via TGF-β1 and SDF-1 expressed in cells and processes other than those discussed in the present study, may also contribute to the progression of CC.

In conclusion, the data obtained here suggest that SDF-1 secreted from cultured WI-38 fibroblasts increased invasion of CC cells and that TGF-β1 secreted from CC cells inhibited expression of SDF-1 by stromal fibroblasts, and then reduced the increased migration of CC cells induced by SDF-1. This suggests that the reduced secretion of TGF-β1 from CC cells may induce increased expression of SDF-1 in stromal fibroblasts and then lead to increased invasion of CC cells. Immunohistochemical findings suggesting expression of SDF-1 in stromal fibroblasts and lowered or absent expression of TGF-β1 on CC cells at the invasive fronts of CC may represent the action of a local factor favorable to the invasion of CC cells. Therefore, augmentation therapy of TGF-β1 expression in CC cells could downregulate the expression of SDF-1 in stromal fibroblasts and inhibit the invasion of CC.

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