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*BASIC RESEARCH*

# Effect of transforming growth factor-β1 on human intrahepatic cholangiocarcinoma cell growth

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## Abstract

**AIM:** To elucidate the biological effects of transforming growth factor-β1 (TGF-β1) on intrahepatic cholangiocarcinoma (ICC).

**METHODS:** We investigated the effects of TGF-β1 on human ICC cell lines (HuCCT1, MEC, and HuH-28) by monitoring the influence of TGF-β1 on tumor growth and interleukin-6 (IL-6) expression in ICC cells.

**RESULTS:** All three human ICC cell lines produced TGF-β1 and demonstrated accelerated growth in the presence of TGF-β1 with no apoptotic effect. Studies on HuCCT1 revealed a TGF-β1-induced stimulation of the expression of TGF-β1, as well as a decrease in TGF-β1 mRNA expression induced by neutralizing anti-TGF-β1 antibody. These results indicate that TGF-β1 stimulates the production and function of TGF-β1 in an autocrine fashion. Further, IL-6 secretion was observed in all three cell lines and exhibited an inhibitory response to neutralizing anti-TGF-β1 antibody. Experiments using HuCCT1 revealed a TGF-β1-induced acceleration of IL-6 protein expression and mRNA levels. These findings demonstrate a functional interaction between TGF-β1 and IL-6. All three cell lines proliferated in the presence of IL-6. In contrast, TGF-β1 induced no growth effect in HuCCT1 in the presence of small interfering RNA against a specific cell surface receptor of IL-6 and signal transducer and activator of transcription-3.

**CONCLUSION:** ICC cells produce TGF-β1 and confer a TGF-β1-induced growth effect in an autocrine fashion. TGF-β1 activates IL-6 production, and the functional interaction between TGF-β1 and IL-6 contributes to ICC cell growth by TGF-β1.



**Key words:** Transforming growth factor-β1; Interleukin-6; Intrahepatic cholangiocarcinoma

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## INTRODUCTION

Intrahepatic cholangiocarcinoma (ICC) is the second most common form of primary liver cancer after hepatocellular carcinoma. Despite advances in diagnosis and treatment, the prognosis of ICC has not yet been resolved[1-3]. When compared with other malignancies, ICC is generally characterized by strong proliferation, invasion, and early metastasis. To improve the prognosis, we require a fuller understanding of the molecular mechanisms behind its proliferation and progression.

Transforming growth factor-β1 (TGF-β1) is a multifunctional polypeptide with potent effects as a growth inhibitor for most epithelial cells[4]. TGF-β1 has been shown to induce cell arrest and fibrosis in hepatocytes[5-8]. While TGF-β1 expression is normally low in normal intrahepatic biliary cells, it has been found to markedly increase in inflammatory and obstructive lesions of the bile duct[9,10]. There are also reports suggesting that the TGF-β1 signaling system plays a role in carcinogenesis and cancer progression[11,12]. Patients with breast cancer[13,14] and hepatocellular carcinoma[15] have shown elevated concentrations of TGF-β1 in serum. TGF-β1 and its receptor mRNA were confirmed to be expressed at elevated levels in an animal model of ICC[15,16], and the former has also been found to be elevated in human ICC[17,18]. These data seem to conflict with the well-known mitoinhibitory effect of TGF-β1, and the mechanism and function of TGF-β1 in carcinogenesis remain poorly

understood. In this study we investigated the biological effects of TGF-β1 on ICC by monitoring the influences of TGF-β1 on tumor growth and interleukin-6 (IL-6) expression in human ICC cells.

## MATERIALS AND METHODS

### Cells and culture conditions

Three human intrahepatic cholangiocarcinoma cell lines, HuCCT1, MEC, and HuH-28, and a mink lung epithelial cell line, Mv1Lu, were kindly provided by the Cell Resource Center for Biomedical Research, Tohoku University, and used for the various experiments in this study. HuCCT1 and MEC were cultured in RPMI1640 (Sigma-Aldrich Co., St. Louis, MO), HuH-28 was cultured in MEM (GIBCO, Rockville, MD), and Mv1Lu was cultured in EMEM-NEAA (GIBCO). All medium was supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin (GIBCO), and 10% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria). Cells were grown as subconfluent mono-layers in a humidified atmosphere containing 5% $CO_2$ at 37℃ and passaged using a treatment with 0.25% trypsin every 7 d.

### Immunofluorescence study

HuCCT1 cells grown to 70% confluency on slide glass were fixed in Zamboni solution at room temperature for 30 min. After a specific antibody against TGF-β1 (rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), TGF-β receptor Ⅱ (rabbit polyclonal, Santa Cruz Biotechnology), and IL-6 (mouse monoclonal, Genzyme, Cambridge, MA) were added for 45 min at 37℃, Alexa Fluor 488-conjugated anti-rabbit antibody (Invitrogen Corp., Carlsbad, CA) was used as a second antibody for 30 min at 37℃. Finally, cells were stained for 30 min at room temperature with 4, 6-diamidino-2-phenylindole (DAPI). Pictures were taken using a cooled CCD camera and MetaMorph Imaging Software (Universal Imaging Corporation, Downingtown, PA) with equal exposure times.

### ELISA for TGF-β1 and IL-6 in cell culture supernatants

Conditioned media were generated by plating approximately $3.0 \times 10^5$ ICC cells in 6-well dishes in growth medium overnight. The cells were washed 3 times with PBS, switched to serum-free medium (SFM). After incubation for indicated times, the supernatants were collected, and stored frozen at -80℃. TGF-β1 and IL-6 concentrations were assessed by ELISA (DuoSet; R&D systems, Minneapolis, MN) according to the manufacturer's instructions. The plates were read on an ELNX96 microplate reader (SCETI Co., Ltd., Tokyo, Japan) at 450 nm.

Experiments were also performed to assess the effects of recombinant human TGF-β1 (rhTGF-β1, R&D systems) and neutralizing anti-TGF-β1 antibody on IL-6 production by the cell lines. Cells were cultured as describe above. SFM supplemented with rhTGF-β1 (0, 1, 10 ng/mL, R&D systems), 5 μg/mL neutralizing anti-TGF-β1 antibody (mouse monoclonal, R&D systems), or nonimmune control IgG (mouse monoclonal $IgG_{2B}$, R&D systems) was collected after indicated times. IL-6 concentrations were measured by ELISA by the method described above.

### Western blotting

Approximately $3.0 \times 10^5$ HuCCT1 cells were cultured in growth medium overnight to determine the expression of the Smad2 phosphorylation (phospho-Smad2), $p15^{Ink4B}$, and plasminogen activator inhibitor-I (PAI-1). After 3 washes with PBS, the cells were switched to SFM with additional rhTGF-β1, stimulated for indicated times, and lysed by M-PER protein extraction reagent (PIERCE Biotechnology, Rockford, IL) with Halt protease inhibitor cocktail (PIERCE Biotechnology) for protein extraction. Total cell lysates were electrophoresed by SDS-PAGE and transferred to membranes. Anti-Phospho-Smad2 (1:400; rabbit polyclonal, CHEMICON International, Temecula, CA), anti-$p15^{Ink4B}$ antibody (1:200; rabbit polyclonal, Santa Cruz Biotechnology) and anti-PAI-1 antibody (1:500; rabbit polyclonal, Santa Cruz Biotechnology) was used together with a secondary horseradish peroxidase-conjugated polyclonal goat anti-rabbit antibody (1:1000; Medical & Biological Laboratories Co., Nagoya, Japan). Immunodetection was performed using an enhanced chemiluminescence kit for Western blotting detection (Amersham Biosciences, Buckinghamshire, UK). X-ray film was exposed for about 1 min for visualization. Autoradiograms of the immunoblots were scanned using Adobe Photoshop (Adobe System, Inc., San Jose, CA). The blots were adjusted for brightness and contrast for minimum background, and the mean density for each band was analyzed.

### Real-time semi-quantitative PCR

Studies with cell lines were conducted to investigate the effects of rhTGF-β1 and anti-TGF-β1 antibodies on the expression of TGF-β1, IL-6, $p15^{Ink4B}$, and PAI-1 mRNA. Conditioned media were generated by plating $3.0 \times 10^5$ ICC cells in 6-well dishes in growth medium overnight. The cells were washed 3 times with PBS, switched to SFM supplemented with rhTGF-β1 (0, 0.1, 1, 10 ng/mL, 5 μg/mL neutralizing anti-TGF-β1 antibody, or 5 μg/mL nonimmune control IgG, and incubated for indicated times. RNA was isolated using ISOGEN reagent (Nippon Gene Co., Toyama, Japan) according to the manufacturer's instructions. Total RNA (1 μg per sample) was reverse transcribed in a final volume of 20 μL using a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). TaqMan probes for human TGF-β1, IL-6, $p15^{Ink4B}$, and PAI-1 RNA gene (TaqMan Gene Expression Assays; Applied Biosystems) were obtained. The samples were amplified in a total volume of 20 μL containing 1 μL of each specific probe and 10 μL of the TaqMan PCR Core Reagent Kit (Universal PCR Master Mix; Applied Biosystems). The amplifications were performed in 40 cycles using a GeneAmp 5700 Sequence Detection system (Applied Biosystems), and the expression levels were calculated using the comparable cycle number method as recommended by the manufacturer. Semi-quantitative mRNA levels were evaluated based on the ratios of the mRNA levels of the target to those of the mRNA levels of GAPDH.

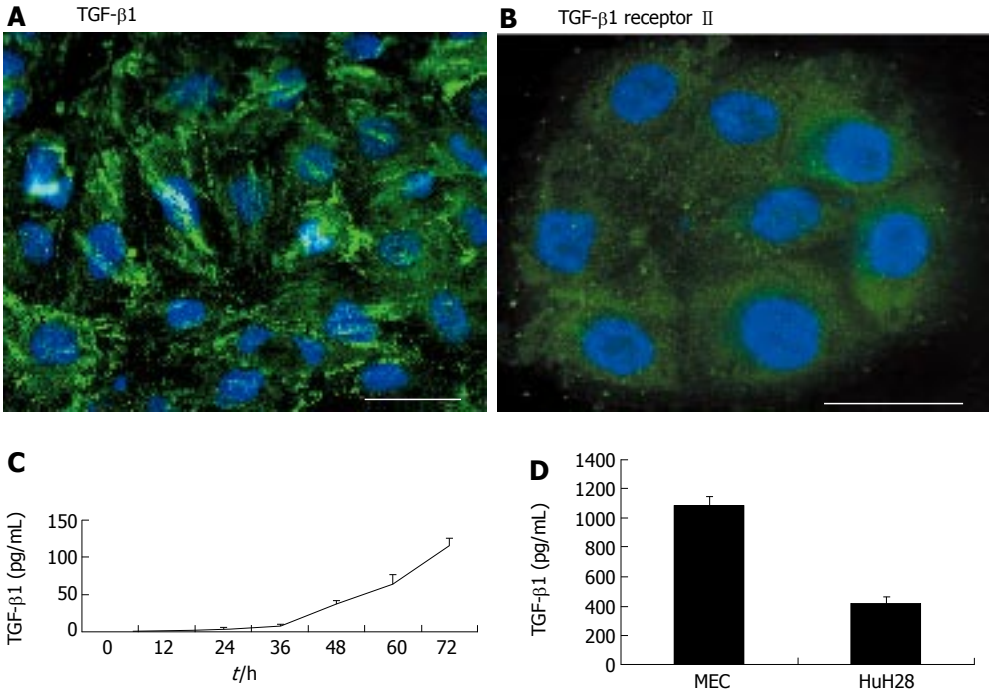


**Figure 1** Expression of TGF-β1 and TGF-β receptor Ⅱ in intrahepatic cholangiocarcinoma cell lines. Immunofluorescence showed staining of TGF-β1 (**A**) and TGF-β receptor Ⅱ (**B**) in HuCCT1 cells. Scale bar = 20 μm. **C**: Detection of TGF-β1 in supernatant of HuCCT1 by ELISA. TGF-β1 levels in the supernatant of HuCCT1 showed a time-dependent increase; **D**: The other ICC cell lines were incubated for 48 h and assayed by ELISA to determine the levels of TGF-β1 in the supernatants. MEC and HuH-28 cells also secreted TGF-β1 into the supernatants.

### $^3$H-thymidine incorporation

$1 \times 10^4$ cells were plated in a 96-well flat-bottomed plate in growth medium and changed to SFM 24 h later. The indicated concentrations of rhTGF-β1 and rhIL-6 (R&D systems) were added the following day for 12 h. For the measurement of DNA synthesis, 1 μCi/mL of $^3$H-thymidine (Perkin Elmer, Boston, MA) was added for 3 h. The $^3$H-thymidine incorporation was determined using a liquid scintillation counter (LSC-3000, Aloca, Tokyo, Japan) after detaching the cells from the plates with 0.25% trypsin.

### Apoptosis assay

HuCCT1 and Mv1Lu cells were seeded on slide glass, and incubated in SFM in the absence or presence of rhTGF-β1 for 48 h. Apoptotic cells were identified by a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method. TUNEL assay was performed using an in situ Apoptosis Detection Kit (TAKARA BIO INC., Shiga, Japan) according to the manufacturer's instruction. Positive staining was detected by diaminobenzine. Finally, slides were restained slightly with 1.0% methyl green. The apoptotic effect was determined by counting the number of positively labeled nuclei per 300 cells. The apoptotic index was calculated as follows: (number of apoptotic cells/total number counted) × 100%[19].

### RNA interference to IL-6Rα and STAT3

The mRNA expressions of interleukin-6 receptor (IL-6Rα or gp80) and the signal transducer and activator of transcription-3 (STAT3) were silenced by small interfering RNA (siRNA) using the SureSilencing siRNA kit (SuperArray Bioscience Co., Frederick, MD). One day before transfection, $5.0 \times 10^4$ cells were seeded in each well of a 24-well plate. The cells were transfected for 24 h at 37℃ in a $CO_2$ incubator using transfection reagents (Lipofectamine2000; Invitogen Corp., Carlsbad, CA) and Opti-MEM Reduced-Serum Medium (GIBCO) according to the manufacturer's instructions. After transfection, the cells were incubated in SFM with or without 1 ng/mL TGF-β1 for 12 h, detached from the plates with 0.25% trypsin, and tested to determine DNA synthesis based on the method of $^3$H-thymidine incorporation described above. Reverse transcription-PCR (RT-PCR) was used to check the silencing effects of gene expression. The RT$^2$ real-time gene expression assay kit (SuperArray Bioscience Co.) and TaqMan probe (Applied Biosystems) were used for human IL-6Rα and STAT3, respectively.

### Statistical analysis

The data were expressed as mean ± SD ($n$ = 4, independent experiments) and analyzed for significance using the Mann-Whitney $U$-test. A $P$ value of less than .05 was considered statistically significant.

## RESULTS

### Expressions of TGF-β1 and TGF-β receptor Ⅱ in human ICC

We began our study by confirming the expression of TGF-β1 and its receptors in the HuCCT1 cell line. Immunofluorescent staining with specific antibody confirmed the expression of TGF-β1 and TGF-β receptor Ⅱ in HuCCT1 (Figure 1A and 1B). Next, we used the ELISA technique to examine the secretion of TGF-β1 from HuCCT1 into the medium. As shown in Figure 1C, HuCCT1 secreted TGF-β1 in a time-dependent fashion. The mean concentration of TGF-β1 in the supernatants after 72 h of incubation was 115.1 pg/mL. We also measured TGF-β1 production in two other ICC cell lines, MEC and HuH-28. TGF-β1 was secreted into the supernatants of the MEC and HuH-28 cell lines at mean concentrations of 1094.4 and 419.9 pg/mL at 48 h, respectively (Figure 1D).

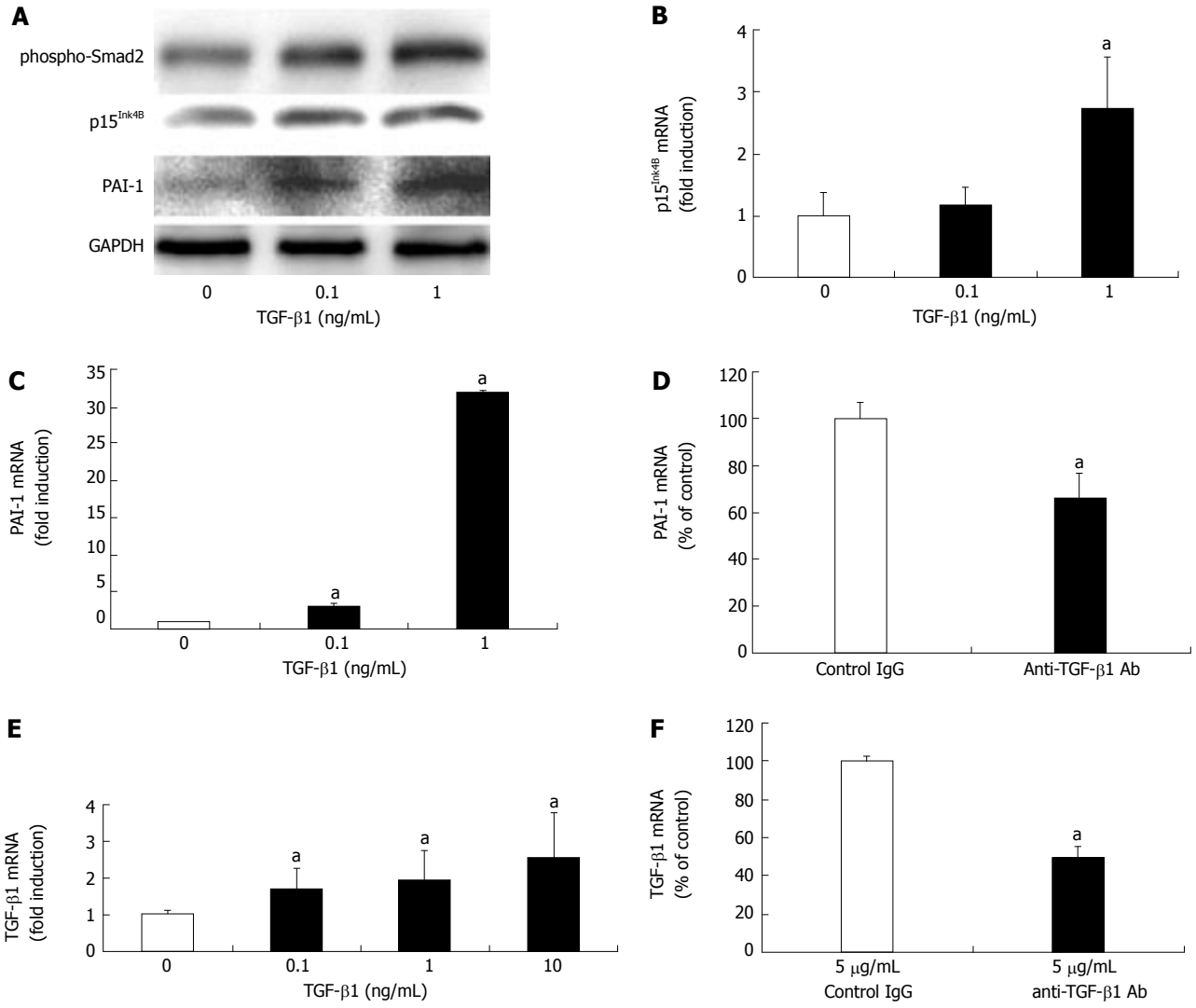


**Figure 2** TGF-β1 stimulates the TGF-β signaling pathway. **A**: The addition of 0.1 and 1 ng/mL TGF-β1 augmented Smad2 phosphorylation (phospho-Smad2), $p15^{Ink4B}$, and PAI-1 proteins in Western blotting of HuCCT1 cells; **B**: TGF-β1 stimulation for 48 h enhanced $p15^{Ink4B}$ mRNA expression in HuCCT1 cells in RT-PCR: $^aP < 0.05$ *vs* absence of TGF-β1; **C**: TGF-β1 stimulation for 48 h also augmented PAI-1 mRNA expression in HuCCT1 cells in RT-PCR: $^aP < 0.05$ *vs* absence of TGF-β1; **D**: PAI-1 mRNA was determined by RT-PCR after incubating the HuCCT1 cells with 5 ng/mL neutralizing anti-TGF-β1 antibody or nonimmune control IgG for 96 h. The addition of anti-TGF-β1 antibody attenuated the level of PAI-1 mRNA: $^aP < 0.05$ *vs* control IgG; **E**: The addition of TGF-β1 to HuCCT1 for 6 h led to a concentration-dependent increase in TGF-β1 mRNA expression by RT-PCR: $^aP < 0.05$ *vs* absence of TGF-β1; **F**: TGF-β1 mRNA in HuCCT1 was determined by RT-PCR after incubating the cells with 5 ng/mL neutralizing anti-TGF-β1 antibody or nonimmune control IgG for 96 h. The addition of anti-TGF-β1 antibody attenuated the level of TGF-β1 mRNA: $^aP < 0.05$ *vs* control IgG.

### *TGF-β1 stimulates the TGF-β signaling pathway*

TGF-β1 has been reported to induce the expressions of $p15^{Ink4B}$ and PAI-1 in a variety of different cells[12,20]. Accordingly, we confirmed Smad2 phosphorylation (phospho-Smad2) and expression of the target genes $p15^{Ink4B}$ and PAI-1 as markers of TGF-β1 and the status of Smad-dependent signaling. Figure 2A demonstrates the effects of rhTGF-β1 on endogenous expression of the phospho-Smad2, $p15^{Ink4B}$, and PAI-1 proteins in the HuCCT1 cell line. Western blotting of HuCCT1 cells after 48 h of stimulation by 0, 0.1, and 1 ng/mL rhTGF-β1 revealed induction of phospho-Smad2, $p15^{Ink4B}$, and PAI-1 proteins. Similar results were obtained by measurements of the mRNA levels of $p15^{Ink4B}$ and PAI-1. The expression of $p15^{Ink4B}$ mRNA induced by 48 h of incubation with 1 ng/mL rhTGF-β1 (Figure 2B) was significantly greater than that measured in the absence of rhTGF-β1 stimulation. Forty-eight hours of incubation with rhTGF-β1 at concentrations of 0.1 and 1 ng/mL also induced significant increases in PAI-1 mRNA (3.0- and 32.0-fold increases, respectively) (Figure 2C). Furthermore, the addition of a neutralizing anti-TGF-β1 antibody reduced the PAI-1 mRNA level. The addition of neutralizing TGF-β1 antibody for 96 h significantly reduced PAI-1 mRNA to 66.1% of the level measured in the presence of nonimmune control IgG (Figure 2D). These findings demonstrate that secreted TGF-β1 can stimulate the TGF-β signaling pathway mediated by Smads in an autocrine fashion without disrupting TGF-β signaling.

### *TGF-β1 stimulates endogenous TGF-β1 mRNA expression in an autocrine fashion*

Next, we studied the effect of TGF-β1 on endogenous TGF-β1 mRNA expression in HuCCT1 with RT-PCR. Additional rhTGF-β1 stimulation for 6 h resulted in a significant dose-dependent increase of TGF-β1 mRNA

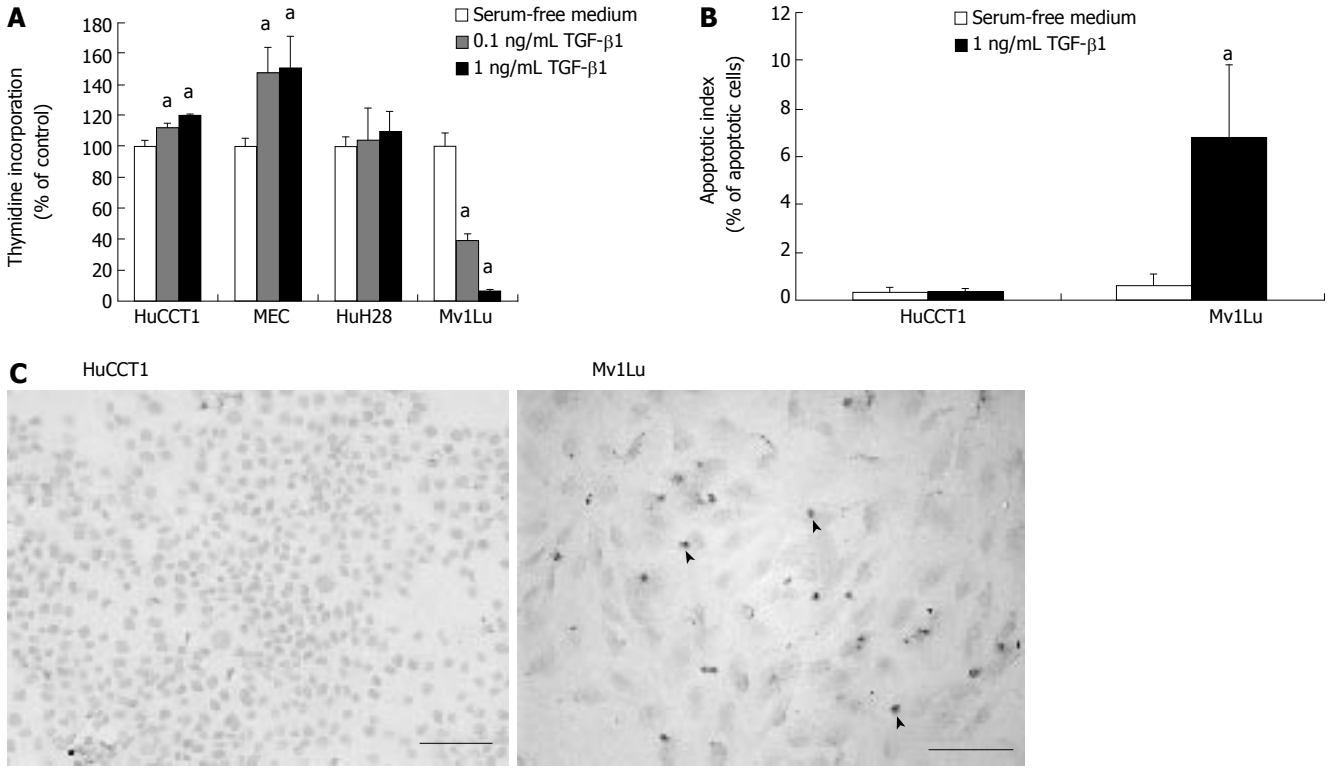


**Figure 3** Effect of TGF-β1 on the cell growth and apoptosis of ICC cell lines. **A**: The cells were subjected to a $^3$H-thymidine incorporation assay in the absence or presence of TGF-β1. No mitoinhibitory effect of TGF-β1 was observed in the ICC cells. TGF-β1 stimulation augmented the DNA synthesis of TGF-β1 in the HuCCT1 and MEC cells. In contrast, TGF-β1 treatment elicited a strong mitoinhibitory effect in Mv1Lu; **B**: Stimulation with 1 ng/mL TGF-β1 for 48 h did not induce apoptosis of HuCCT1 cells in a TUNEL assay. In the experiment using Mv1Lu, however, stimulation with TGF-β1 at the same concentration for the same duration did elicit an apoptotic effect: $^aP < 0.05$ *vs* absence of TGF-β1; **C**: HuCCT1 and Mv1Lu cells were treated with 1 ng/mL TGF-β1 for 48 h. The arrows denote apoptotic cells identified by the TUNEL method. Scale bar = 100 μm.

expression in HuCCT1 cells (Figure 2E). Moreover, the addition of neutralizing TGF-β1 antibody for 96 h resulted in a significant decrease of TGF-β1 mRNA to 49.1% of the level measured in the presence of nonimmune control IgG (Figure 2F).

### *TGF-β1 accelerates ICC cell proliferation*

To further confirm the effect of TGF-β1 on ICC cells, we investigated whether TGF-β1 influenced the proliferation and apoptosis of ICC cells. The HuCCT1 and MEC cell lines showed significant increases in $^3$H-thymidine incorporation in response to 0.1 and 1 ng/mL rhTGF-β1 stimulation for 12 h (Figure 3A). No mitoinhibitory effect of TGF-β1 was observed in any of the ICC cell lines in this study. In contrast, the additions of 0.1 and 1 ng/mL rhTGF-β1 to Mv1Lu resulted in 61.1% and 95.8% reductions in DNA synthesis, respectively.

Next, we applied the TUNEL method to determine whether we could observe apoptosis of the ICC cells. In an experiment using HuCCT1 cells, no significant differences in the levels of apoptosis were observed between cells incubated in SFM for 48 h and cells incubated with 1 ng/mL rhTGF-β1 for 48 h. However, the TUNEL assay demonstrated that 1 ng/mL rhTGF-β1 induced evident apoptosis of Mv1Lu cells, and the apoptotic index at 48 h after rhTGF-β1 treatment was significantly higher than that of the control group (Figure 3B). Almost no apoptotic cells were observed in the HuCCT1 cells stimulated with 1 ng/mL rhTGF-β1 for 48 h (Figure 3C). However, the TUNEL assay using Mv1Lu indicated an increase of apoptotic cells in response to 1 ng/mL rhTGF-β1 for 48 h. On the basis of these results, we can conclude that TGF-β1 inhibits cell proliferation and induces apoptosis in normal epithelial cells, whereas ICC cells are equipped with a mechanism to resist the growth-inhibitory response and apoptotic effect induced by TGF-β1.

### *TGF-β1 stimulates the secretion of IL-6*

Previous studies have shown that IL-6 can induce the proliferation of ICC cells[21,22]. To elucidate the mechanism further, we measured the level of IL-6 production by ICC cells and explored the relationship between TGF-β1 and IL-6. A time-dependent increase of IL-6 was observed in the HuCCT1 supernatants unexposed to TGF-β1. To investigate the potential interaction between TGF-β1 and IL-6, we evaluated the levels of IL-6 in the HuCCT1 supernatants stimulated with rhTGF-β1. As a result, we found a time-dependent increase of IL-6 concentrations in response to rhTGF-β1 throughout the 72-h observation period (Figure 4A). The increases in IL-6 induced by 1 and 10 ng/mL rhTGF-β1 after 72 h of incubation (2.2- and 5.7-fold increases, respectively) were significantly greater than the increases observed in the supernatants from cells untreated by rhTGF-β1. Similar results were obtained in IL-6 mRNA quantification after 6 h of stimulation by rhTGF-β1. A significant 2.7-fold increase of IL-6 mRNA was observed in the HuCCT1 cells stimulated with 10

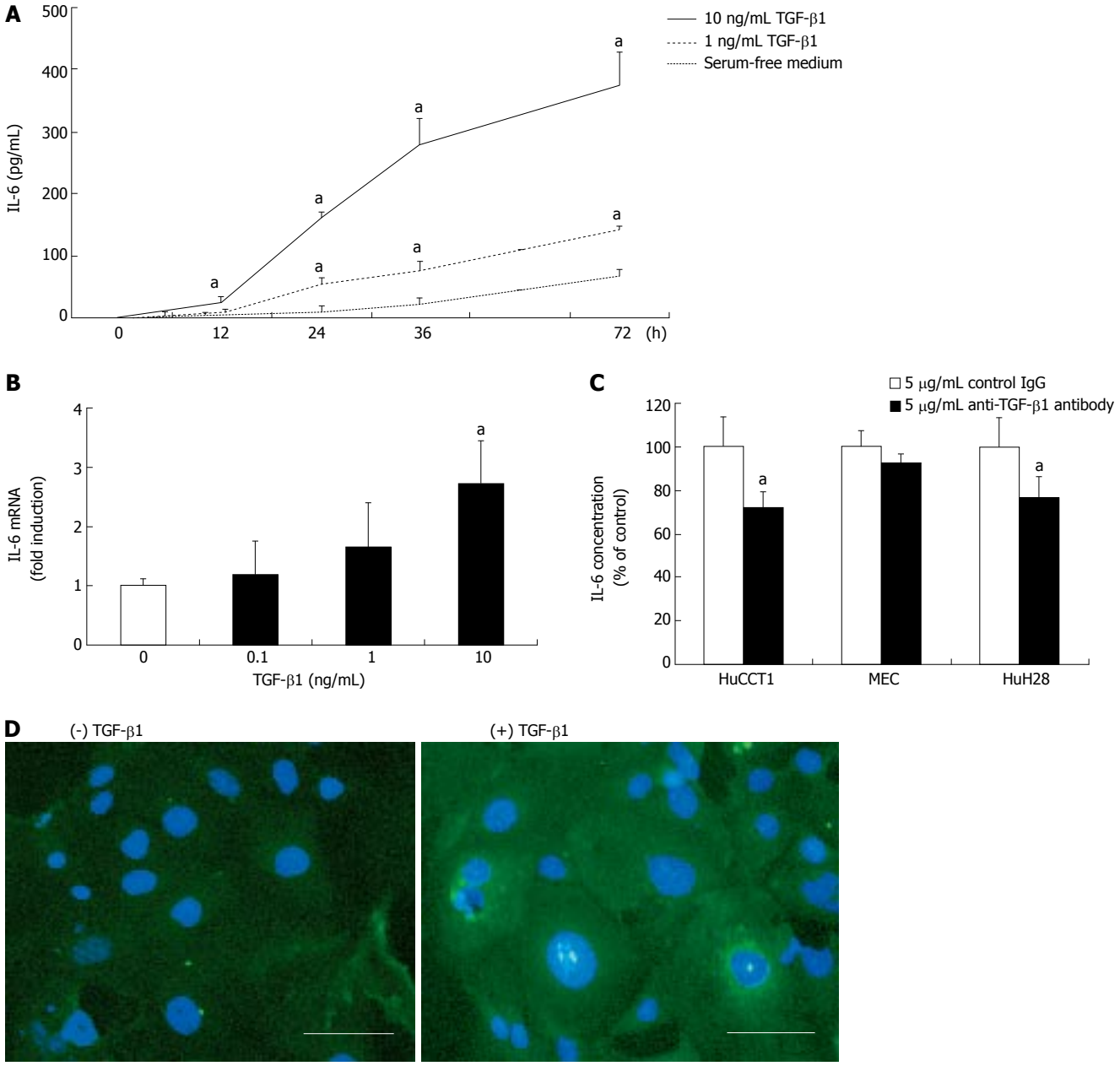


**Figure 4** Effect of TGF-β1 on the IL-6 expression of ICC cells. **A**: ELISA revealed the secretion of IL-6 by HuCCT1 into the serum-free medium. HuCCT1 also showed a time-dependent increase of IL-6 concentrations in response to 1 and 10 ng/mL TGF-β1 stimulation: $^{a}P < 0.05$ *vs* absence of TGF-β1; **B**: The addition of TGF-β1 to HuCCT1 for 6 h resulted in a dose-dependent increase of IL-6 mRNA expression by RT-PCR: $^{a}P < 0.05$ *vs* absence of TGF-β1; **C**: IL-6 levels in the supernatants were determined by ELISA after incubating ICC cells with 5 ng/mL neutralizing anti-TGF-β1 antibody or nonimmune control IgG for 96 h. The addition of neutralizing anti-TGF-β1 antibody resulted in a decrease of IL-6 production in HuCCT1 and HuH28: $^{a}P < 0.05$ *vs* control IgG; **D**: An immunofluorescence study using HuCCT1 cells demonstrated an increase in IL-6 production in response to TGF-β1. Immunoreactivity with a specific anti-IL6 antibody was weak, with no stimulation of TGF-β1, whereas 10 ng/mL TGF-β1 induced IL-6 expression in the cytoplasm of the HuCCT1 cells. Scale bar = 20 μm.

ng/mL TGF-β1 (Figure 4B). IL-6 production was also confirmed in the MEC and HuH-28 ICC cell lines. After 72 h of incubation in SFM, the mean IL-6 concentrations in the supernatants of MEC and HuH-28 cells were 307.4 and 6.93 ng/mL, respectively. We next investigated the effects of a neutralizing anti-TGF-β1 antibody on IL-6 production in ICC cell supernatants. Compared with nonimmune control IgG, the addition of anti-TGF-β1 antibody resulted in a significant inhibition of IL-6 concentrations in the HuCCT1 and HuH28 supernatants (Figure 4C). Next, we investigated the IL-6 localization in response to rhTGF-β1 by immunofluorescence. As shown in Figure 4D, the cytoplasm of HuCCT1 cells untreated by rhTGF-β1 was only weakly immunoreactive to the anti-IL-6 antibody. In contrast, we identified a dominant localization of IL-6 in the cytoplasm of the HuCCT1 cells stimulated with 10 ng/mL rhTGF-β1 for 48 h.

### *Effects of TGF-β1 on ICC cell growth under inhibition of IL-6 functions via RNA interference with IL-6Rα (gp80) and STAT3*

We measured thymidine incorporation in the ICC cells after the addition of 0.1 and 1 ng/mL rhIL-6 to clarify whether IL-6 accelerates the cell growth of ICC cells.

As seen in Figure 5A, rhIL-6 brought about significant increases in thymidine incorporation in all of the ICC cells studied. This, taken together with the results of the other experiments described in this study, indicates that TGF-β1 and IL-6 act as ICC growth factors with interrelated functions.

To further elucidate the mechanism of TGF-β1-induced proliferation of ICC, we examined whether TGF-β1 functioned as a growth factor while IL-6 functions were inhibited with the use of small interfering RNA (siRNA) in order to silence the expression of IL-6Rα and STAT3 mRNA. As shown in Figure 5B, siRNA treatment suppressed IL-6Rα and STAT3 mRNA expressions in the HuCCT1 cells to 10.6% and 41.9% of the control siRNA level, respectively. Figure 5C shows the effects of rhTGF-β1 on DNA synthesis in HuCCT1 cells with siRNA against IL-6Rα and STAT3. HuCCT1 stimulated by 1 ng/mL rhTGF-β1 exhibited a 37.4% increase of DNA synthesis under the control siRNA condition, whereas HuCCT1 stimulated by rhTGF-β1 during siRNA silencing of IL-6Rα brought about no increase after TGF-β1 stimulation. Furthermore, HuCCT1 stimulated by TGF-β1 during siRNA silencing of STAT3 exhibited a 19.4% decrease of DNA synthesis. These findings indicate that the TGF-β1-induced production of IL-6 confers resistance to the growth inhibition by TGF-β1 and plays a role in the mechanism of TGF-β1-induced ICC proliferation.

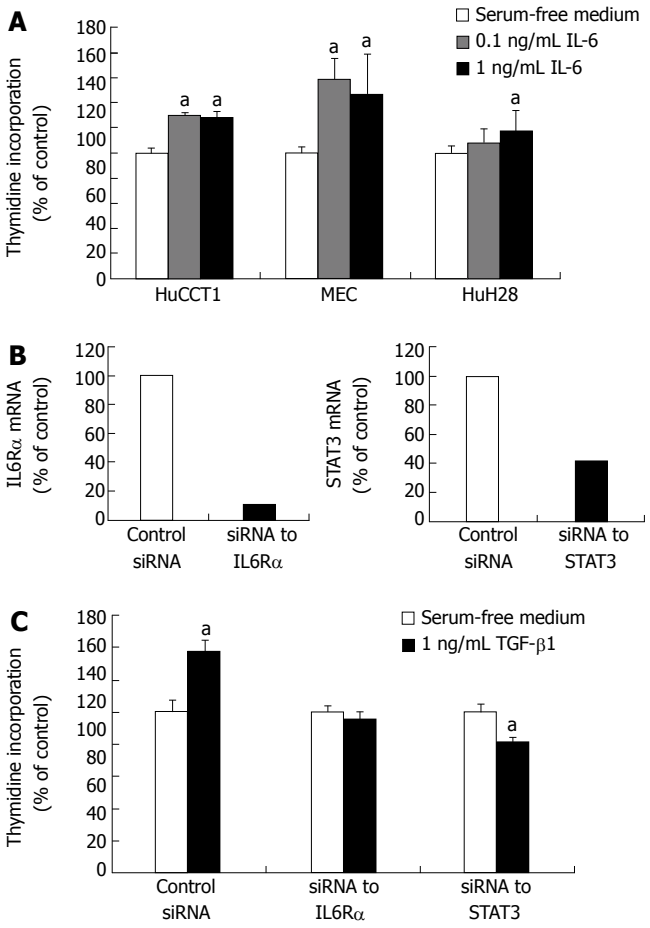


**Figure 5** Effects of TGF-β1 on DNA synthesis during inhibition of IL-6 functions. **A**: All of the ICC cells demonstrated enhanced DNA synthesis in response to IL-6: $^{a}P < 0.05$ *vs* absence of IL-6. We next investigated whether TGF-β1 promoted ICC cell growth during the inhibition of IL-6 functions by small interfering RNA (siRNA); **B**: RT-PCR was conducted to assess the effects of siRNA in suppressing the mRNA expressions of IL-6Rα and STAT3. IL-6Rα and STAT3 mRNA expression was suppressed by 10.6% and 41.9%, relative to control siRNA; **C**: The silencing of IL-6Rα and STAT3 mRNA expressions by siRNA in HuCCT1 cells led to a suppression in the TGF-β1-induced DNA synthesis relative to that observed under the control siRNA condition: $^{a}P < 0.05$ *vs* absence of rhTGF-β1.

## DISCUSSION

In previous studies, ICC cells were found to resist the growth inhibitory effect of TGF-β1[18,22]. Our current study, in contrast, demonstrated that TGF-β1 stimulation in ICC resulted in cellular proliferation rather than resistance to the innate mitoinhibition. The molecular mechanism by which some malignant cells evade the mitoinhibitory effect of TGF-β1 remains obscure. Recent studies on malignancies have demonstrated the disruption of TGF-β signaling, including lack or mutations of the TGF-β receptor[23-26] and the Smad family[26-29]. This study, in contrast, revealed no disruption of TGF-β signaling. In fact, TGF-β1 was found to activate the expression of PAI-1 as a target gene via Smad2 phosphorylation. These results suggest that ICC cells are equipped with a mechanism to resist the innate mitoinhibitory response and encourage cell activation by TGF-β1. Given that TGF-β1 upregulation has been identified in both pancreatic cancer and hepatocellular carcinoma, we know that it may also contribute to disease progression in patients with some malignancies[12,30,31].

The results from our current study demonstrate that the growth effect of TGF-β1 in ICC cells is closely associated with IL-6 production and function. IL-6 plays a key role in the hepatic response to inflammation, as well as in the regulation of bile duct growth[32-35]. ICC is generally characterized by strong cellular proliferation and inflammation around the tumor. From these points of view, we decided to perform several studies on IL-6, a reported inducer of ICC growth[21,22,36,37]. IL-6 is elevated in the serum of patients with ICC[38] and expressed in ICC surgical specimens and cell lines[21,36,39]. All of the ICC cell lines used in our study expressed IL-6. This IL-6 expression was augmented by TGF-β1 and attenuated by the neutralizing anti-TGF-β1 antibody. A similar mechanism of TGF-β1-induced IL-6 expression was also reported in prostate cancer cells[40]. Stimulation by IL-6 augmented DNA synthesis in all cell lines. In contrast, TGF-β1 stimulation during siRNA silencing of IL-6Rα and STAT3 mRNA expression led decreased levels of DNA synthesis in HuCCT1 cells. On this basis, we know that the two cytokines share a functional relation, and further, that IL-6 function is very important for the TGF-β1-based growth effect in ICC. Recent studies on ICC cells have also demonstrated effects of TGF-β1 on the induction of prostaglandin $E_2$[41] and vascular epithelial growth factor (VEGF)[17], two factors closely related to tumor proliferation and angiogenesis. This may shed light on the oncogenic switch to resist the innate growth inhibitory effect of TGF-β1. As one step in this switch, the malignant cells might acquire a mechanism to produce

cell growth inducers via the stimulation of TGF-β1.

IL-6 has also been reported to participate in the chemoresistance of IL-6-induced inhibitors of apoptosis protein against conventional chemotherapeutic agents[42]. In addition to facilitating ICC cell growth, IL-6 aggravates the difficulties in treating malignancy. Effective regulators of these cytokines might be attractive therapeutic targets to encourage tumor cell apoptosis and cell growth inhibition.

In summary, we have provided evidence in support of the autocrine growth effect of TGF-β1 in human ICC. We also demonstrated that the TGF-β1-induced growth effect is closely associated with IL-6 function. ICC has acquired these mechanisms to resist the well-known growth inhibitory response of TGF-β1. Further elucidation of the TGF-β1 and IL-6 function in cholangiocarcinogenesis may afford an important opportunity to define a novel molecular target for ICC prevention and treatment.

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