Angiotensin II induces tumor progression and fibrosis in intrahepatic cholangiocarcinoma through an interaction with hepatic stellate cells

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Abstract. Intrahepatic cholangiocarcinoma (ICC) is characterized as a highly fatal tumor with poor prognosis because of its strong progression, early invasion, widespread metastasis and rich cancerous stroma. Although it is widely accepted that fibroblasts facilitate stromal fibrosis and tumor progression, the mechanisms of the interaction between cancer cells and activated fibroblasts have not been fully elucidated thus far. In this study, we demonstrate the presence of angiotensin II (*Ang*II) in ICC tissues and explore the interaction between hepatic stellate cells (HSCs) and ICC cells as one of the sources of stromal fibrosis and tumor progression through the interaction of the *Ang*II/*Ang*II type 1 receptor (AT-1) axis. The concentrations of *Ang*II in ICC tissues were significantly higher than those of HCC and normal liver. Two human ICC cell lines (HuCCT-1, CCKS-1) and a human HSC cell line (LI-90) expressed AT-1 mRNA and protein. The proliferative activity of ICC cells and HSCs to which *Ang*II was added dose-dependently increased and AT-1 antagonist inhibited the proliferative effects. HSCs to which *Ang*II was added showed a higher expression of α-smooth muscle actin (α-SMA, a marker of activated HSCs and myofibroblasts), glial fibrillary acidic protein (GFAP, a specific marker of HSCs) and collagen type I than control cells. AT-1 antagonist also inhibited the activation and transformation of HSCs stimulated by *Ang*II. These findings suggested that locally formed *Ang*II in ICC tissues plays a role in the proliferation and activation of ICC cells and HSCs expressing AT-1 as a growth factor in autocrine and paracrine fashions. Our mechanistic findings provide the first insight into an autocrine and paracrine *Ang*II-initiated signaling pathway that regulates ICC proliferation and fibrosis.

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Introduction

Although both hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC) are primary liver tumors, the amount of collagen tissue in each tumor is completely different. It is well known that the characteristics of ICC are the hypovascularity and richness of cancerous stroma as determined by diagnostic imaging compared with those of HCC. Although it is widely accepted that fibroblasts facilitate stromal fibrosis and tumor progression, the mechanisms and origins of such activated fibroblasts have not been fully elucidated until now (1).

Terada *et al* demonstrated that ICC tissues had a greater tendency to highly express trypsinogen/trypsin and cathepsin B and that no HCC tissues had any expression of trypsinogen (2). Human pancreatic cancer cells expressed and secreted pancreatic cationic-type trypsinogen *in vitro*, which is spontaneously converted into active trypsin at acidic pH (pH 4.5-5.5), in contrast to anionic-type trypsinogen, which is not. Cationic-type trypsinogen could increase the invasive ability and cell proliferation of pancreatic cancer cells (3). In previous reports, it was described that the high expression of a specific G-protein-coupled receptor, protease-activated receptor-2 (PAR-2), was observed in pancreatic cancer cells and fibroblasts around tumor tissue (4,5). PAR-2 activated by its agonists, including trypsin and tryptase, plays an important role in promoting the proliferation of pancreatic cancer (6).

Meanwhile, Arakawa *et al* (7) demonstrated that trypsin generated angiotensin II (*Ang*II) from human plasma protein in the absence of angiotensin converting enzyme (ACE)

at weakly acidic pH 7.0). Our previous report showed that pancreatic cancer tissues had higher trypsinogen expression and higher *Ang*II concentrations than other solid tumors, such as HCC and colorectal cancer (8). We demonstrated that angiotensinogen in the blood was directly converted to *Ang*II and *in vivo* evidence suggested a renin-angiotensin system (RAS)-independent cascade in pancreatic cancer tissues. Locally formed *Ang*II was thought to act on pre-existing pancreatic arteries around the tumor, leading to the formation of hypoperfusive regions, so-called hypovascular or avascular regions. Some reports have described the expression of specific G-protein-coupled receptor, *Ang*II type 1 receptor (AT-1), in cancer cells and the role of *Ang*II/AT-1 axis in cancer proliferation, as well as the influence of the environment surrounding cancer cells as a potent mitogen (9).

Meanwhile, it has been reported that pancreatic cancer cells induce fibrosis by stimulating pancreatic stellate cells to proliferate and synthesize matrix (11,12). Hepatic stellate cells (HSCs) are well known as an important component of the background liver associated with chronic liver diseases and liver tumors. The proliferation and activation of HSCs are the dominant events in liver injury, inflammation and fibrosis that render the cells responsive to cytokines and other local stimuli. Following liver injury or stimulation by various attractants, including monocyte chemoattractant protein (MCP)-1, transforming growth factor (TGF)-ß1, vascular endothelial growth factor (VEGF) or *Ang*II, HSCs undergo a response known as activation, which is the transformation of quiescent cells into proliferative and fibrogenic myofibroblasts (activated HSCs), thereby modulating inflammatory cell infiltration and subsequent hepatic fibrosis (13-16). HSCs are similar to pancreatic stellate cells and may contribute to the desmoplastic reaction associated with primary and metastatic liver tumors (17,18). Recently, Okabe *et al* reported that HSCs may differentiate into myofibroblasts in the cancerous stroma of ICC tissues and promote the invasiveness and cell proliferation of cancer cells on coculturing with ICC cells in a paracrine fashion (19).

We hypothesize that activated HSCs are one of the resources of stromal fibrosis especially in collagen-rich liver tumors, such as ICC, which has a higher level of *Ang*II through the co-interaction between cancer cells and HSCs. The aim of the present work was to investigate the presence of a RAS-independent *Ang*II-generating system in ICC tissues and to clarify the role of *Ang*II in ICC progression in an autocrine fashion. The interaction between fibrosis-associated cells and cancer cells may induce the characteristic differences between collagen-poor HCC and collagen-rich ICC, which have fundamental differences in the amounts of tumor-derived trypsin and locally formed *Ang*II. The hypoperfusive and fibrotic environment may make ICC tissues more malignant and resistant to various therapies. We also explored the paracrine interaction between ICC cells and HSCs in tumor fibrosis through the *Ang*II/AT-1 axis.

Materials and methods

Human tissue samples. The current study included ten specimens of primary ICC (well to poorly differentiated adenocarcinoma) that were surgically resected between 1998 and 2009. The average age of ICC patients was 67 years (range 50-84 years). All patients had stage I-IVB disease on the basis of the general rules for the clinical and pathological study of primary liver cancer (20). Histologically, normal liver tissues were obtained from non-tumoral portions of resected ICC specimens. In addition, 11 cases of HCC were examined for comparative study.

Immediately following surgical removal, the tissue samples were frozen in liquid nitrogen and stored at -80˚C until the time of the assay for measuring *Ang*II concentration. For immunohistochemical examination, the materials employed in this study, 20% formalin-fixed and paraffin-embedded specimens, were retrieved from the surgical pathology files of the Pathology Section of Kanazawa University Hospital (School of Medicine, Kanazawa University, Kanazawa, Japan).

Cell culture. Two human ICC cell lines [HuCCT-1, obtained from the Cell Resource Center for Biochemical Research, Tohoku University, Sendai, Japan (21) and CCKS-1, obtained from the Department of Human Pathology, Kanazawa University Graduate School of Medicine (22)], and a human hepatic stellate cell line [LI-90, obtained from the Human Science Cell Bank, Saitama, Japan (23)] were used. ICC cell lines were maintained at 37˚C in a 5% CO_2 incubator and grown in RPMI-1640 medium supplemented with 2 mM glutamine, 10% fetal bovine serum (FBS), 100 U/l penicillin and 100 μg/ml streptomycin. LI-90 cell lines were maintained at 37˚C in a 5% CO_2 incubator and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 10% FBS, 100 U/l penicillin and 100 μg/ml streptomycin.

Reagents and antibodies. *Ang*II was used at concentrations of 1, 10, 100 or 1000 nM (9). An active compound, telmisartan, which is a novel, long-acting, selective AT-1 receptor antagonist, was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used at concentrations of 100 and 1000 nM, according to a previous report (24). Transforming growth factor (TGF)-ß1 was purchased from Sigma-Aldrich and was used at a concentration of 10 ng/ml, according to a previous report (25). For primary antibodies, we obtained rabbit polyclonal AT-1 antibody (Santa Cruz Biotechnology, CA, USA), mouse monoclonal collagen type I antibody (Santa Cruz), mouse monoclonal α-smooth muscle actin (α-SMA) antibody (Sigma-Aldrich), mouse monoclonal ß-actin antibody (Sigma-Aldrich) and rabbit polyclonal glial fibrillary acidic protein (GFAP) antibody (Dako Cytomation, Glostrup, Denmark).

Measurement of angiotensin II in tissues. The determination of *Ang*II content was performed as follows. Briefly, tissue samples were homogenized at 4˚C in saline containing 0.1 N HCl and 5% urinastatin. The homogenate was sedimented at 10,000 x g for 30 min at 4˚C, and the supernatant was used for radioimmunoassay of *Ang*II using the florisil method (florisil absorption and elution with acetone-hydrochloric acid solution) as described previously (26). This method is more sensitive, specific and useful for routine clinical

investigation. Parallel frozen tissue samples were homogenized in phosphate-buffered saline (PBS).

Immunohistochemistry. The expression of AT-1 in ICC specimens was examined immunohistochemically using each primary antibody. To identify the antigen in the tissue, deparaffinized sections were pretreated by autoclaving in 10% citric acid buffer (pH 8.0) at 120˚C for 15 min. After pretreatment with protein block serum (Dako Cytomation, Kyoto, Japan) for 10 min and in 2% skim milk for 20 min to block non-specific reactions, the sections were incubated with each primary antibody at 4˚C overnight. The Envision+ polymer solution (horseradish peroxidase, HRP, secondary antibody, Dako Cytomation) was then applied for 1 h. The reaction products were developed in 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution containing 0.1% H_2O_2. The sections were then lightly counterstained with hematoxylin. The slides were examined under a fluorescence microscope (Olympus, Tokyo, Japan). Specimens were classified as positive when >10% of cancer cells were stained.

Immunocytochemistry. The expressions of AT-1 in ICC cells and AT-1, α-SMA and GFAP in HSCs were examined immunocytochemically using respective primary antibodies. The suspensions of each cancer cell were seeded on Lab Tek chamber slides (Nunc) and incubated for 24-48 h at 37˚C in a humid atmosphere of 5% CO_2/95% air. The coverslips with cells were then fixed with methanol and acetone 1:1 (v/v). After pretreatment with protein blocking serum for 10 min to block non-specific binding, immunostaining was performed using Envision+ System. Briefly, the slides were incubated with each primary antibody (1:50) at 4˚C overnight. After washing, the Envision+ polymer solution was applied for 1 h. The reaction products were visualized via a DAB reaction. The cells were then lightly counterstained with hematoxylin and examined under a fluorescence microscope.

Western blot analysis. AT-1 was recovered from subconfluent HuCCT-1, CCKS-1 and LI-90 cells, which were harvested, washed in cold PBS and lysed in ice-cold lysis buffer [10 mM PBS (pH 7.4), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA] supplemented with 1% phenylmethylsulfonyl fluoride and a protease inhibitor cocktail for 30 min at 4˚C. The lysate was sonicated and centrifuged at 15,000 rpm for 30 min at 4˚C to remove debris, and the protein concentration was measured using a BCA Protein Assay Kit (Thermo Scientific, USA). Total protein was measured using a spectrophotometer. Extracted protein was used for Western blot analysis. In this analysis, 20 μg of protein from each sample was loaded onto 12.5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by the semi-dry blotting method. The membrane was washed for 10 min with blocking solution (0.1% Tween-20; Ez Block, ATTO Corporation, Japan), blocked at room temperature for 30 min with blocking solution, and washed with washing solution (0.1% Tween-20, Ez Wash; ATTO Corporation). The blots were incubated for 2 h at room temperature with each primary antibody against AT-1 diluted at 1:500 with washing solution. The blots were incubated for 1 h with an HRP-conjugated anti-mouse antibody (against anti-AT-1 antibody diluted at 1:5000 with washing solution as a second antibody). Chemiluminescence was detected with the ECL Plus Western blotting detection system (GE Healthcare Bioscience, Japan) according to the supplier's recommendations. We also used two antibodies, α-SMA and GFAP, for Western blot analysis as fibrous markers to measure the up- or down-regulation of the expressions of α-SMA and GFAP in LI-90 incubated in medium with added *Ang*II (100 nM) or TGF-ß1 (10 ng/ml).

Extraction of RNA and reverse transcriptase polymerase chain reaction (RT-PCR) for AT-1 and GAPDH mRNA. mRNA was isolated from HuCCT-1, CCKS-1 and LI-90 cell lines using TRI Reagent (Sigma-Aldrich). Then, 1 μg of mRNA was used to synthesize first-strand cDNA with an RNeasy Mini Kit (Qiagen, USA) and QIA shredder (Qiagen), according to the manufacturer's instructions. PCR for AT-1 and GAPDH was performed with a Taq Man PCR Kit (Takara, Japan), according to the manufacturer's instructions. The AT-1 forward primer sequence was 5'- TCAACAAAAAT GAGCACGCTTT-3'; and the reverse was 5'-AAACATGGT GCAGGCTTCTTG-3' (the pair generated a 485-bp fragment) (27). The GAPDH oligonucleotide primer set (forward 5'-ACC ACAGTCCATGCCATCAC-3', reverse 5'-TCCACCACCCT GTTGCTGTA-3'; the pair generated a 452-bp fragment) was used as an internal standard. PCR was performed for 35 cycles (denaturation at 98˚C for 15 sec, annealing at 58˚C for 30 sec and extension at 74˚C for 45 sec). After PCR, 5 μl samples of the products were subjected to 2.0% agarose gel electrophoresis and stained with ethidium bromide.

Cell proliferation assay. The proliferative effect of *Ang*II on ICC and HSC cell lines was quantified using an MTT colorimetric assay with Cell Proliferation Kit I (Roche), according to the manufacturer's instructions. In brief, each cancer cell line ($5x10^3$ cells/well) was grown in 96-well flat-bottom microtiter plates in 100 μl of medium including 1% FBS and incubated for 48-96 h at 37˚C in a humidified atmosphere (e.g. 37˚C, 5% CO_2). The medium contained different concentrations (1-1000 nM) of *Ang*II or 10 ng/ml TGF-ß1. In some experiments, telmisartan was added to *Ang*II treatment to make sure that the proliferative effect caused by *Ang*II occurred through the AT-1 receptor. After the incubation period, 10 μl of the MTT labeling reagent (final concentration 0.5 mg/ml) was added to each well, the microplate was incubated for 4 h in a humidified atmosphere (e.g. 37˚C, 5% CO_2) and then 100 μl of the solubilization solution was added to each well. We allowed the plate to stand overnight in the incubator in a humidified atmosphere (e.g. 37˚C, 5% CO_2), then checked for complete solubilization of the purple formazan crystals and measured the spectrophotometric absorbance value of the samples using a microplate reader. The wavelength to measure absorbance of the formazan product is 595 nm. The experiments were repeated in triplicate wells. Cell viability was calculated as follows: Cell number (% of control) = (absorbance of experimental wells) / (absorbance of control wells) x 100 (%).

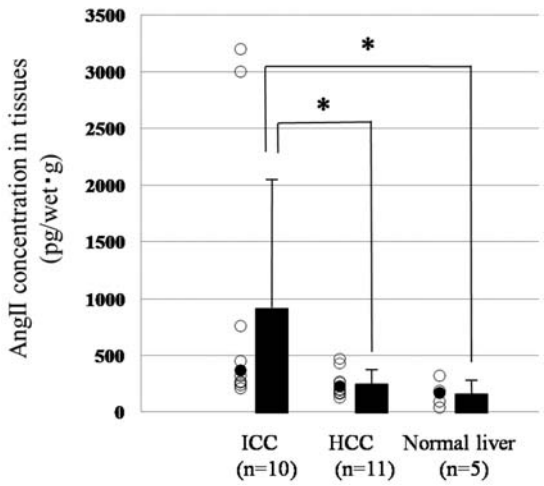

Figure 1. *Ang*II concentration in tissue extracts of ICC, HCC and normal liver. *Ang*II concentrations in ICC tissues were significantly higher than those of HCC and normal liver. *P<0.05.

Fluorescent immunocytochemistry. LI-90 cells were grown on Lab-Tek chamber slides to 40-60% confluence, serum-deprived overnight (DMEM + 1% FBS) and then treated with *Ang*II (100 nM) or TGF-ß1 (10 ng/ml). In some experiments, telmisartan (1000 nM) was added to *Ang*II treatment to make sure that the effect caused by *Ang*II occurred through the *Ang*II/AT1 axis. After fixation with 3.7% formalin, cells were blocked with protein blocking serum. Cells were incubated with the primary antibodies (α-SMA and GFAP) for 1 h at room temperature. Slides were then washed and incubated with the appropriate Alexa Fluor 488 and 592 nm phalloidin-conjugated (Molecular Probes Inc., Eugene, OR) specific secondary antibodies for double staining for 1 h at room temperature. Cells were then incubated with Hoechst 33258 for nuclear staining for 5 min and mounted with propyl gallate containing phenylenediamine under glass coverslips. Cells were then visualized for immunofluorescence with a laser scanning Olympus microscope at x10, x20 and x40 magnification.

Statistical analysis. Statistical analyses were carried out using an unpaired t-test except for the measurement of *Ang*II concentration in tissues. In the measurement of *Ang*II concentration in tissues, analysis was carried out using the Mann-Whitney U test. P<0.05 was considered significant.

Results

Angiotensin II concentration in tissues. Tissue *Ang*II concentration was determined radioimmunologically in tissue extracts of ICC (n=10), HCC (n=11) and normal liver (n=5). The concentration was 908±1167 pg/g wet tissue in ICC, 244±111 pg/g wet tissue in HCC and 162±107 pg/g wet tissue in normal liver. Thus, *Ang*II concentrations in ICC tissues were significantly higher than those of HCC and normal liver (Fig. 1).

Immunohistochemistry and immunocytochemistry. Immunohistochemistry for AT-1 was expressed in 14 of 16 (87.5%)

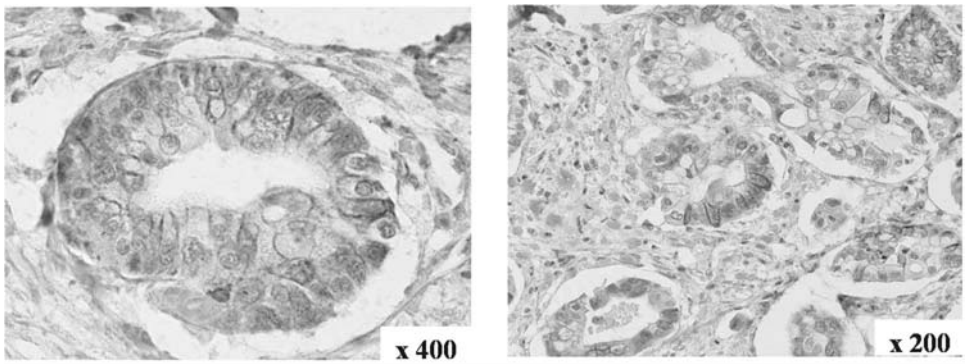

Figure 2. Expression of AT-1 in ICC tissues. AT-1 receptor immunoreactivity was evident in membranous and granular cytoplasmic patterns and was predominantly expressed in the center of the tumor. In addition, vascular endothelium, smooth muscle cells, fibroblasts and tumor-associated macrophages were strongly positive for AT-1 receptor.

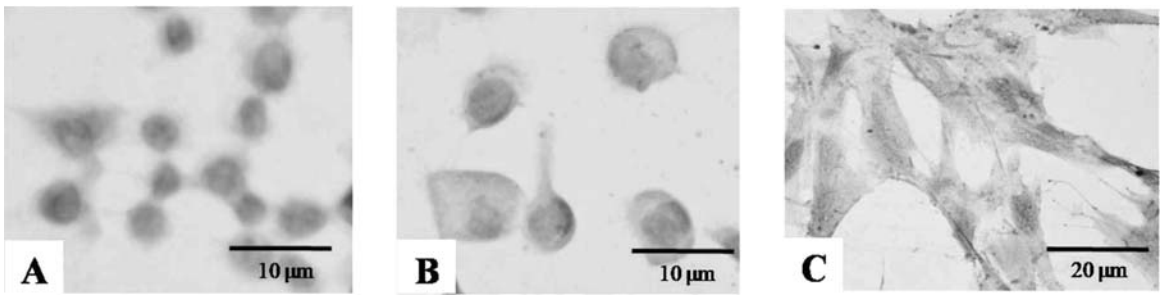

Figure 3. Immunocytochemistry for AT-1 in ICC cells and HSCs. (A, HuCCT-1; B, CCKS-1; C, LI-90) AT-1 expression was intense in HuCCT-1, CCKS-1 and LI-90 cells. AT-1 receptor immunoreactivity was evident in membranous and granular cytoplasmic patterns.

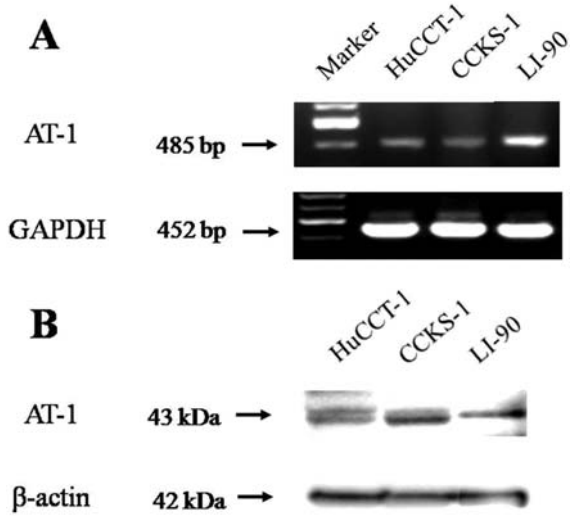

Figure 4. Expression of AT-1 mRNA and protein in ICC cells and HSCs. AT-1 expression at the mRNA level was detected in HuCCT-1, CCKS-1 and LI-90 cells (A). Western blot analysis also demonstrated AT-1 protein expression in all three cells types (B).

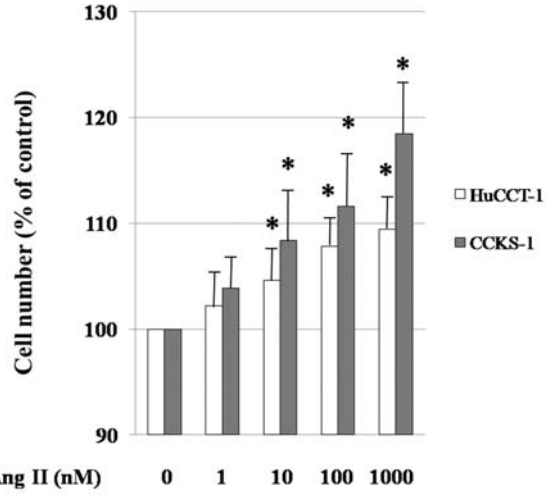

Figure 5. Proliferative effects of *Ang*II in ICC cells. ICC cells were treated with different concentrations (1-1000 nM) of *Ang*II, and their proliferative effects were quantified by MTT assay. *Ang*II induced a dose-dependent increase in proliferative activity at concentrations of *Ang*II from 10 to 1000 nM for both HuCCT-1 and CCKS-1 cells. Results are the means ± SD of triplicate experiments. *P<0.05 compared with control (untreated) cells by unpaired t-test.

surgical specimens of human ICC. AT-1 receptor immunoreactivity was evident in membranous and granular cytoplasmic patterns and was predominantly expressed in the center of the tumor (Fig. 2). In addition, vascular endothelium, smooth muscle cells, fibroblasts and tumor-associated macrophages were strongly positive for AT-1 receptor. In immunocytochemistry, AT-1 expression was intense in HuCCT-1, CCKS-1 and LI-90 cells. AT-1 receptor immunoreactivity was evident in membranous and granular cytoplasmic patterns (Fig. 3).

RT-PCR and Western blot analysis of AT-1 in human ICC and HSC cells. AT-1 expression at the mRNA level was detected in HuCCT-1, CCKS-1 and LI-90 cells (485 bp,

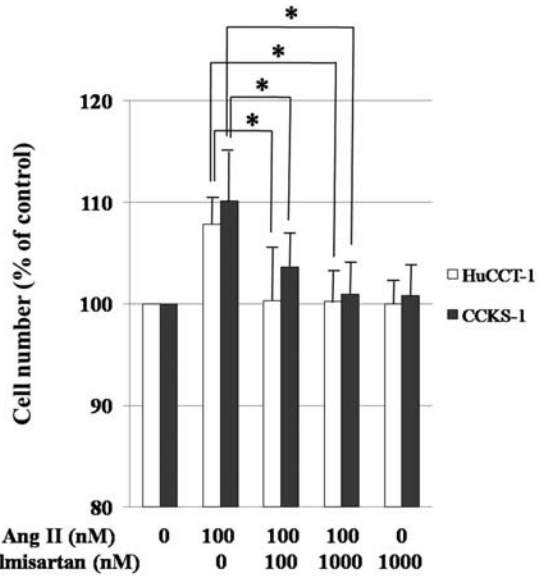

Figure 6. Inhibitory effect of AT-1 antagonist, telmisartan, against proliferating ICC cells to which *Ang*II was added. *Ang*II-induced proliferative response was significantly inhibited by 100 and 1000 nM telmisartan. A decrease in proliferation was not observed in the telmisartan alone group compared with that of vehicle-treated cells. Results are the means ± SD of triplicate experiments. *P<0.05 versus 100 nM *Ang*II.

Fig. 4A). Western blot analysis also demonstrated AT-1 protein expression in all three cells (43 kDa) and AT-1 protein expression mirrored that of mRNA (Fig. 4B).

Effect of angiotensin II on ICC and HSC cell proliferation. To examine the effect of *Ang*II, AT-1 receptor agonist, on the proliferation of ICC and HSC cells, HuCCT-1, CCKS-1 and LI-90 were treated with *Ang*II or TGF-ß1 and the proliferative effects were quantified by MTT assay. After incubation for 48 h, cell proliferation was significantly and dose-dependently increased by *Ang*II at concentrations from 1 to 1000 nM in HuCCT-1 and CCKS-1 cells (Fig. 5). In addition, we determined whether the *Ang*II-induced proliferative response was inhibited by a specific AT-1 receptor antagonist, telmisartan. Telmisartan significantly inhibited the 100 nM *Ang*II-induced proliferative response at a concentration of 1000 nM in both HuCCT-1 and CCKS-1 cells (Fig. 6). A decrease in proliferation was not observed in the telmisartan alone group compared with that of the control cells (vehicle-treated cells). Therefore, these results demonstrate that the suppressive effect of telmisartan on cell growth resulted from the inhibition of *Ang*II as a specific AT-1 receptor antagonist.

In LI-90 cells with each concentration of *Ang*II, cell numbers dose-dependently increased at concentrations from 1 to 100 nM. Telmisartan significantly inhibited the 100 nM *Ang*II-induced proliferative response at a concentration of 100 nM (Fig. 7).

Effect of AngII on activation and transformation in HSCs. It has been reported that GFAP is expressed in quiescent or partially activated HSCs and is known as a cell-specific marker of HSCs. α-SMA is also known as a cell-specific marker of activated HSCs and myofibroblasts. HSCs transform into

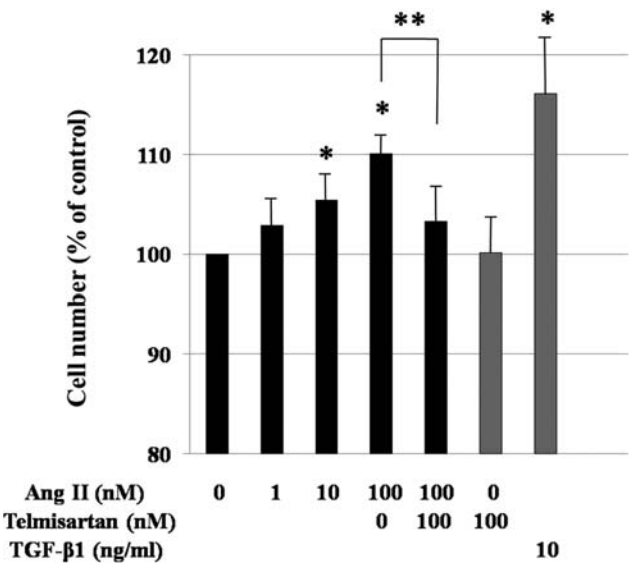

Figure 7. Proliferative effects of *Ang*II in HSCs. LI-90 cells treated with each concentration of *Ang*II (1-100 nM) dose-dependently increased in number at concentrations of *Ang*II from 10 to 100 nM. Telmisartan completely inhibited the 100 nM *Ang*II-induced proliferative response at a concentration of 100 nM. Results are the means ± SD of triplicate experiments. *P<0.05 versus control. **P<0.05 versus 100 nM *Ang*II.

myofibroblasts and the expression of α-SMA in the cytoplasm of activated HSCs is enhanced when quiescent HSCs are stimulated by attractants. In immunocytochemistry, the expression of GFAP was ubiquitously found in the cytoplasm of almost all HSCs, but the expression of α-SMA was only partially found in the cytoplasm of cultured HSCs. In fluorescent immunocytochemistry, when LI-90 cells were added to 100 nM *Ang*II, the number of α-SMA-positive LI-90 cells was increased (Fig. 8). Telmisartan at 1000 nM inhibited the activation of LI-90 cells and the transformation to myofibroblasts cells. When 10 ng/ml TGF-ß1 was added to LI-90 cells, the cells were activated and transformed into myofibroblasts. In Western blot analysis, when LI-90 cells were added to 100 nM *Ang*II, the protein expression of α-SMA, GFAP and collagen type I significantly increased (Fig. 9).

Discussion

Classically, RAS has been considered a hormonal circulating system. This circulating RAS plays an important role in the maintenance of blood pressure, electrolyte balance and fluid homeostasis (28). Meanwhile, recent reports have suggested that *Ang*II is strongly associated with inflammatory diseases and cancer progression in various organs (29-31). Locally expressed tissue RAS components, including angiotensinogen, renin and *Ang*II receptors, may be involved in the regulation of individual tissue functions, independent of circulating RAS.

AT-1 activation by *Ang*II is proposed to signal mitogen-activated protein kinases (MAPKs), primarily via G proteins,

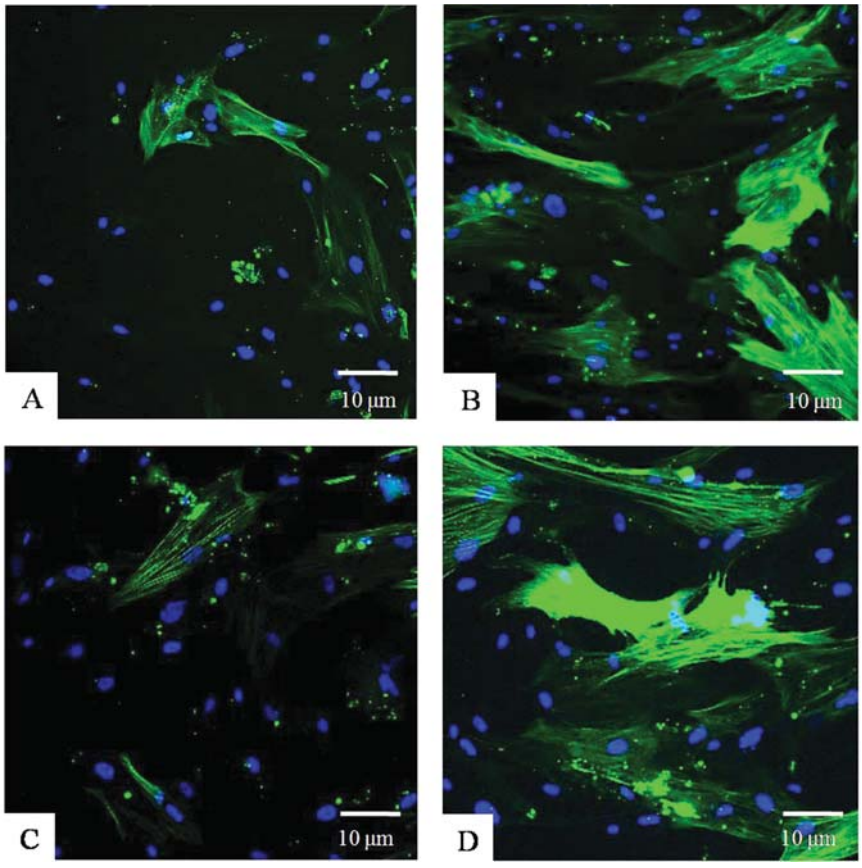

Figure 8. Fluorescent immunocytochemistry of *Ang*II-induced activation and transformation of LI-90 cells (green, α-SMA; blue, nuclei). When LI-90 cells were added to *Ang*II, the number of α-SMA-positive cells increased (A, control cells; B, 100 nM *Ang*II-treated cells). Telmisartan inhibited the activation of cells (C, *Ang*II 100 nM + Telmisartan 100 nM). Addition of 10 ng/ml TGF-ß1 also increased the number of α-SMA-positive cells (D).

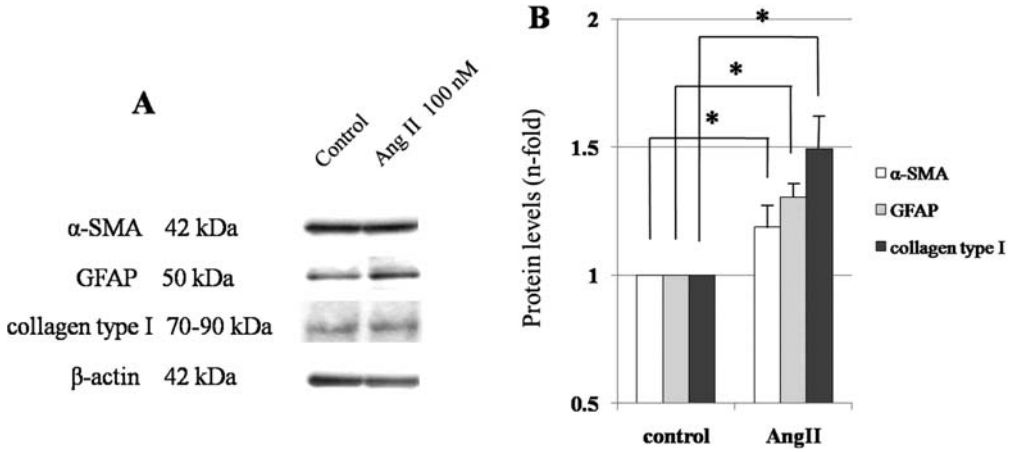

Figure 9. LI-90 cells were incubated with 100 nM *Ang*II. Representative Western blot results (A). Data are the mean ± SD of three independent experiments (B). *P<0.05 versus control.

in multiple ways. AT-1 activates focal adhesion kinase (FAK) and this enzyme activates Src, Ras and Raf, which in turn activate MAPKs resulting in the final activation of extracellular signal-regulated kinase (ERK) that participates in the phosphorylation of signal transducers and activators of transcription (STATs). Phosphorylated STATs are translocated to the nucleus, where they promote the transcription of genes that participate in cell hypertrophy and hyperplasia, as well as interferon-α, VEGF, fibroblast growth factor (FGFb), platelet-derived growth factor (PDGF), Bcl-2 and Bcl-xL, which block the caspase cascade, inhibiting apoptosis (30). Nuclear factor-κB (NF-κB) plays a critical role in the regulation of cell survival. NF-κB activation leads to anti-apoptosis-related gene transcription including the inhibition of apoptosis protein (IAP) and Bcl-2 families, thereby providing a survival-promoting signal and inhibiting cell apoptosis (32). Survivin, a member of the IAP family, is expressed in the most common cancer cells, and plays an important role in resistance to radiotherapy (33,34). Amaya *et al* have demonstrated that ERK1/2 and NF-κB activated by *Ang*II enhance the expression of anti-apoptotic proteins, including survivin and Bcl-XL, and significantly prevent cancer cell apoptosis in pancreatic cancer cells *in vitro*. Their study suggested that *Ang*II enhances cell proliferation and chemoresistance of cancer cells through the activation of ERK and NF-κB via transactivation of epidermal growth factor (EGF) receptor 9).

*Ang*II is occasionally reported to be a stimulating factor in HSC activation and the production of various cytokines and chemokines. Activated HSCs to which *Ang*II was added promoted the production of TGF-ß and were an important source of extracellular matrix (ECM) including collagens (types I, III and IV), fibronectin, undulin, elastin, laminin, hyaluronan and proteoglycans (13-16). We demonstrated that the activation-dependent up-regulation of α-SMA, GFAP and collagen I expression occurred in LI-90 cells stimulated by *Ang*II. It was also demonstrated that *Ang*II receptor blocker (ARB) inhibited HSC proliferation, activation and transdifferentiation into myofibroblasts. Our study specifically addressed the *in vitro* behavior of HSCs to which *Ang*II was added; therefore, we monitored cell-specific markers for HSC activation, namely, α-SMA and GFAP. In liver tissue, GFAP is expressed in quiescent or partially activated HSCs but not in perivascular myofibroblasts. Therefore, the increased expression levels of GFAP in *Ang*II-treated cells reveal the increase in the overall number of HSCs and can therefore be utilized as a specific HSC proliferation marker. α-SMA is well known to be a marker for activated HSCs and myofibroblasts. The increased expression of α-SMA, positive correlation to collagen expression and the fibrosis-dependent enhanced expression of α-SMA emphasize the importance of HSCs in *Ang*II-induced activation (36,37).

Myofibroblasts can arise from activated HSCs, hepatocytes, bone-marrow-derived cells and possibly endothelial cells. Many studies suggest that myofibroblasts facilitate tumor fibrosis, tumor growth and cancer progression (17-19,38-40). The source of myofibroblasts and other activated fibroblasts within tumors is still debated, but HSCs have become recognized as one of the sources of cancerous stroma of liver tumors.

Tumor progression and fibrosis are pivotal aspects of malignant tumors, although their exact molecular mechanisms with respect to tumor-stromal interactions remain to be clarified. Several factors, including TGF-ß, hepatocyte growth factor (HGF), EGF, VEGF, trypsinogen and *Ang*II, are regarded as candidate factors involved in cross-talk in tumor-stromal interactions (9,10,38). Recent reports have demonstrated the roles of stromal-cell-derived factor-1 (SDF-1) and its specific receptor, CXCR4, in tumor fibrosis and cell proliferation, migration, survival and metastasis of cancer cells (41-43). It has been demonstrated that SDF-1 released from stromal fibroblasts was responsible for the migration of cancer cells expressing CXCR4. It has also been reported that bone-marrow-derived fibroblasts can contribute to the tumor stromal reaction and tissue fibrosis. This suggests that bone marrow may be a source of tissue fibroblasts, which are one of the components of cancer-induced stroma, and bone-marrow-derived activated fibroblasts were recruited into cancer-induced stroma in late stages of cancer (39). Carcinoma-associated fibroblasts (CAFs) are thought to promote the growth of cancer cells, angiogenesis and stromal fibrosis by recruiting endothelial progenitor cells through the

interaction between SDF-1 secreted from CAFs and CXCR4 (40).

In conclusion, the present study demonstrated that ICC tissues had high *Ang*II concentrations and that this locally formed *Ang*II was associated with the tumor microenvironment. The interaction between ICC cells and HSCs through the *Ang*II/AT-1 axis was suggested to have autocrine and paracrine synergistic effects on tumor progression and cancer fibrogenesis, and to make ICC highly malignant. A better understanding of the interplay between ICC cells and cancerous stroma will be important in developing strategies for improved tumor therapy that take into account the influence of the tumor microenvironment on tumor survival and growth. Additionally, we showed that some important effects of *Ang*II antagonist are involved in tumor progression and cancerous stroma in ICC tissues. Targeting the *Ang*II signaling pathway may not only affect the vascular dynamic state in cancer tissues, but also be a novel, efficient strategy for treating high-grade neoplasms with rich cancerous stroma.

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