Cancer Letters 355 (2014) 46–53

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

The Angiotensin II type 1 receptor blocker candesartan suppresses proliferation and fibrosis in gastric cancer

Mitsuyoshi Okazaki [a], Sachio Fushida [a,*], Shinichi Harada [b], Tomoya Tsukada [a], Jun Kinoshita [a], Katsunobu Oyama [a], Hidehiro Tajima [a], Itasu Ninomiya [a], Takashi Fujimura [a], Tetsuo Ohta [a]

[a] Department of Gastroenterologic Surgery, Division of Cancer Medicine, Graduate School of Medical Science, Kanazawa University, Kanazawa Ishikawa 920-8641, Japan
[b] Center for Biomedical Research and Education, School of Medicine, Kanazawa University, Kanazawa Ishikawa 920-8641, Japan

ARTICLE INFO

Article history:
Received 26 April 2014
Received in revised form 28 August 2014
Accepted 10 September 2014

Keywords:
Angiotensin II
Gastric cancer
Fibrosis
TGF-β
Peritoneal dissemination

ABSTRACT

Gastric cancer with peritoneal dissemination has poor clinical prognosis because of the presence of rich stromal fibrosis and acquired drug resistance. Recently, Angiotensin II type I receptor blockers such as candesartan have attracted attention for their potential anti-fibrotic activity. We examined whether candesartan could attenuate tumor proliferation and fibrosis through the interaction between gastric cancer cell line (MKN45) cells and human peritoneal mesothelial cells. Candesartan significantly reduced TGF-β1 expression and epithelial-to-mesenchymal transition-like change, while tumor proliferation and stromal fibrosis were impaired. Targeting the Angiotensin II signaling pathway may therefore be an efficient strategy for treatment of tumor proliferation and fibrosis.

Introduction

Gastric cancer is a major global health concern, with an estimated 989,600 new cases and more than 738,000 attributable deaths in 2011 [1]. A critical indicator for poor prognosis and the most frequent metastatic pattern in gastric cancer is peritoneal dissemination [2–4]. While clinical outcomes for gastric cancer patients with peritoneal dissemination have improved with advances in systemic and/or intraperitoneal chemotherapy, desirable outcomes remain elusive [5–10]. Peritoneal dissemination is characterized by cancer cell infiltration of and proliferation within the peritoneal cavity, with accompanying extensive stromal fibrosis [11,12]. This leads to disorders including ileus, obstructive jaundice, and hydronephrosis. Therefore, new strategies for the treatment of tumor proliferation and fibrosis in peritoneal dissemination of gastric cancer are required.

Many studies suggest that the volume and composition of fibrous tissue in various organs are influenced by epithelial–mesenchymal transition (EMT), which is characterized by a loss of epithelial cell characteristics and gain of extracellular matrix-producing myofibroblast characteristics [13–15]. TGF-β1 signals play an important role in the progression of EMT and contribute to the metastatic spread of cancer cells, influencing migration and invasion [16,17]. Activated TGF-β1 can be found in tumor cells, fibroblasts, and peritoneal lavage fluid, and previous studies have demonstrated that TGF-β1 serum and tissue levels are significantly correlated with survival in gastric cancer patients [18–20]. Therefore, targeting the effects of TGF-β1-induced EMT is important to attenuate both metastasis and fibrosis in gastric cancer.

Recent developments have shown that tumor progression results from interactions between cancer cells and various stromal cells, including endothelial cells, immune cells, and fibroblasts in the tumor microenvironment [21–23]. Bone marrow-derived fibroblasts have been shown to contribute to the tumor stromal environment and influence tissue fibrosis, being dubbed cancer-associated fibroblasts (CAF) [24]. We have previously reported that TGF-β1-mediated activation of human peritoneal mesothelial cells (HPMCs) induces an EMT-like process whereby these cells adopt a fibroblast or myofibroblast-like phenotype process [25]. Furthermore, activated HPMCs function as a source of CAFs, and drive the process of fibrosis [25]. HPMCs, which are classified as epithelial in the broadest sense of the term, form a monolayer of squamous epithelial cells that cover the peritoneal cavity to form a serosal membrane which serves as a protective anatomical barrier [26]. Previous studies have suggested that CAFs produce stroma-modulating growth factors including TGF-β1, VEGF, and HGF [27–29]. VEGF is a potent and selective endothelial mitogen capable of inducing a rapid and complete angiogenic response [27,28]. HGF may also be involved in promoting the growth of various epithelial cells that express c-met,

* Corresponding author. Tel.: +762652362; fax: +76-234-4260.
E-mail address: fushida@staff.kanazawa-u.ac.jp (S. Fushida).

http://dx.doi.org/10.1016/j.canlet.2014.09.019

which is the HGF receptor [29]. Therefore, CAFs stimulate tumor proliferation and induce fibrosis [25]. Additionally, fibrotic tumors demonstrating advanced progression can be established in a subcutaneous xenograft model using the gastric cancer cell line MKN45 in co-culture with HPMCs [25]. Therefore, the interaction between cancer cells and HPMCs in the tumor microenvironment contributes to tumor proliferation and fibrotic change.

Previously, several studies have identified that Angiotensin II can promote cell proliferation during cancer development, and Angiotensin II type 1 (AT1) blockers (ARBs) may suppress this effect by antagonizing the AT1 receptor [30–32]. Furthermore, ARBs have recently attracted attention for their direct anti-fibrotic activity. In particular, ARBs may have the potential to inhibit fibrotic change in chronic kidney disease by reducing TGF-β1 expression [33–35]. Additionally, ARB treatment in patients with Marfan's syndrome significantly slowed the rate of progressive aortic-root dilation, which is caused by excessive TGF-β1 signaling [36]. These findings led us to hypothesize that ARBs may effectively suppress tissue fibrosis during peritoneal dissemination of gastric cancer.

Therefore, this study aimed to assess whether ARBs attenuate proliferation and fibrosis in our established fibrotic tumor model, and also to clarify the mechanisms of Angiotensin II–AT1 receptor–TGF-β1 molecular interactions in gastric cancer.

Materials and methods

Cell lines and cell culture

HPMCs were isolated from surgical specimens of human omentum as previously described [37]. Briefly, small pieces of omentum were surgically resected under sterile conditions and were incubated in pre-warmed phosphate-buffered solution (PBS) containing 0.125% trypsin/EDTA (Gibco/Invitrogen, USA) for 30 min at 37 °C. The suspension was centrifuged at 1500 × g for 5 min. Collected cells were cultured in RPMI-1640 medium (Gibco/Invitrogen) supplemented with 20% heat-inactivated fetal bovine serum (FBS; Nichirei Bioscience Inc., Japan). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO^2 in air. For the following experiments, cells were used during the second or third passage after primary culture. HPMCs possibly contaminated with endothelial cells or fibroblasts at the time of harvest were not used. Donors were not subjected to chemotherapy or radiation treatment prior to surgery, and had no evidence of peritoneal inflammation and/or malignancy. We used homogeneous HPMCs from a different donor for each experiment. All patients provided written informed consent prior to participation in the study. The gastric cancer cell lines used in this study were MKN7, MKN74, and MKN45 which were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% FBS.

Chemicals

Angiotensin II and the AT1 receptor blocker, candesartan (CV-11974), and telmisartan were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Mouse xenograft model

All animal experiments were performed according to Kanazawa University's standard guidelines. Female immunocompromised BALB/c-*nu*/*nu* mice (Charles River Laboratories Inc. Japan) at 4–6 weeks of age were maintained in a sterile environment. HPMCs were first stained using a red fluorescent dye PKH26 cell linker kit (Sigma) according to the manufacturer's instructions; the concentration of PKH26 during incubation was 4 μM. MKN45 cells were co-cultured with an equivalent number of HPMCs for five days, and a total of 5×10^6 cells in 100 μL of RPMI-1640 were then subcutaneously injected into the dorsal side of each mouse on day 0. Three groups of 10 mice each were established: MKN45 cells alone (a total of 5×10^6 cells) without candesartan, and MKN45cells co-cultured with HPMCs with or without candesartan. Beginning on day 7, mice were administered 10 mg/kg of candesartan daily by gavage. Animals were carefully monitored, tumors were measured every 4 days. The tumor volume (V) was calculated according to the formula $V = AB^2/2$, where A is the length of the major axis, and B is the length of minor axis.

Immunohistochemistry

Tumor specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) and Azan stain for assessment of fibrosis, while the expression levels of E-cadherin (H-108, rabbit polyclonal IgG, diluted 1:100; Santa Cruz Biotechnology, Inc.) and α-smooth muscle actin (α-SMA; 1A4, mouse monoclonal IgG, diluted 1:100; Dako Cytomation, Denmark) were assessed immunohistochemically. Deparaffinized sections were pretreated by autoclaving in 10% citric acid buffer (pH 8.0) at 120 °C for 15 min. Following treatment with protein block serum (Dako Cytomation, Kyoto, Japan) for 10 min and incubation with 2% skim milk for 30 min to block non-specific reactions, sections were incubated with primary antibody at 4 °C overnight. The Envision-polymer solution (horseradish peroxidase, HRP, Dako Cytomation) was then applied for 1 h. Signals were developed in 0.02% 3,3′-diaminobenzidinetetrahydrochloride (DAB) solution containing 0.1%. Sections were then lightly counter stained with hematoxylin and examined using a fluorescence microscope (Olympus, Tokyo, Japan). The degree of fibrosis was calculated as a percentage of fibrosis within the whole section in all samples using a BZ-9000 BZII microscope (Keyence, Osaka, Japan).

Preparation of serum-free conditioned media (SF-CM)

SF-CM was prepared from MKN45 and MKN7 cells as previously reported [12]. Briefly, 1.0×10^6 cells were seeded into 100-mm tissue culture dishes with 10-mL RPMI, supplemented with 10% FBS and incubated at 37 °C for 3 days. To obtain SF-CM, the cells were washed twice with PBS and then incubated for 2 days with 5 mL of serum-free RPMI with or without 100 nM of Angiotensin II. Cells were pretreated for 1 h with 1000 nM of candesartan prior to treatment with Angiotensin II where required. The SF-CM was harvested, centrifuged at 1500 × g for 5 min, passed through a filter (pore size: 0.45 μm) and stored at –80 °C until used.

Phase contrast microscopy

Briefly, HPMCs were seeded into 100-mm tissue culture dishes at 5.0×10^4 cells in RPMI growth medium with 10% FBS. The HPMCs in cultures were treated with SF-CM or left untreated (control) for 24 h and morphological changes were visualized by phase contrast microscopy. The images were collected using a Nikon inverted microscope (Nikon Corp., Japan).

MTT assay

The effect of Angiotensin II and ARB on the proliferative capacity of MKN45 cells was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, MKN45 cells were seeded in 96-well plates at 4×10^3 cells per well in RPMI growth medium with 10% FBS and incubated overnight at 37 °C in a humidified environment containing 5% CO_2. Following incubation, the supernatant was discarded and replaced with fresh serum-free medium containing different concentrations (1–100 nM) of Angiotensin II. When required, candesartan (1–1000 nM) was added 1 h before Angiotensin II treatment to ensure that the proliferative effect caused by Angiotensin II occurred via the AT1 receptor. At 48 h post-treatment, the supernatant was discarded and MTT solution was added to each well (final concentration, 500 μg/mL) and incubated at 37 °C for 3 h. The supernatant was then removed and 150 μL of DMSO (Wako, Japan) was added. The absorbance of the solution was read at 535 nm with a microplate reader (Bio-Rad 550; Bio-Rad, Japan). Cell viability was calculated as: viability = (absorbance of experimental wells)/(absorbance of control wells). All experiments were repeated at least three times.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of TGF-β1 in the SF-CM was measured using an ELISA assay (Quantikine, R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

Western blotting

Approximately 5×10^6 cells were lysed in RIPA buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich, Inc.). Protein from each sample was loaded onto 12.5% SDS-PAGE gels and subjected to electrophoresis. Proteins were transferred to a PVDF membrane (Bio-Rad, USA) and blocked with blocking solution (0.1% Tween-20; EZ Block ATTO Corporation, Japan) at room temperature for 30 min. Blots were incubated overnight at 4 °C with each primary antibody (see below). The blots were then incubated for 1 h with appropriate HRP-conjugated secondary antibodies. The immunoblots were visualized using an ECL Plus Western blotting detection system (GE Healthcare Japan Ltd., Japan) and the Light-Capture system (ATTO). To ensure equal protein loading, β-actin levels were measured using an anti-β-actin monoclonal antibody (AC-15, mouse monoclonal IgG, diluted 1:10,000; Sigma). The following primary antibodies were used: AT-1 (N-10, rabbit polyclonal IgG, diluted 1:500; Santa Cruz Biotechnology, Inc.), E-cadherin (H-108, rabbit polyclonal IgG, diluted 1:1000; Santa Cruz Biotechnology, Inc.), α-SMA (1A4, mouse monoclonal IgG, diluted 1:5000; Dako Cytomation, Denmark), TGF-β1 (V, mouse polyclonal IgG, diluted 1:500; Santa Cruz Biotechnology, Inc.) and anti-β-actin.

Immunofluorescence

For visualization of E-cadherin and α-SMA in HPMCs, cells were grown on 4-well collagen type I-coated culture slides (BD BioCoat) at 1.0×10^3 cells, incubated overnight, fixed in a mixture of methanol and acetone (1:1) for 10 min and then incubated with E-cadherin and α-SMA antibodies (each diluted 1:100) at 4 °C overnight. Following three PBS washes, slides were incubated with anti-mouse IgG-Alexa Fluor® 488 and anti-rabbit IgG-Alexa Fluor® 546 (1:400 dilution; Molecular Probes/Invitrogen, USA) for 1 h at room temperature. Slides were then incubated with Hoechst 33258 for 5 min to aid nuclear visualization and mounted with propyl gallate containing phenylenediamine under glass coverslips. The slides were observed with an immunofluorescence microscope (BX50/BX-FLA; Olympus, Japan).

Statistical analysis

All data are expressed as mean ± SD. Statistical analyses were conducted using the SPSS statistical software, version 11.0 (SPSS). Comparisons of drug effects were made using one-way analysis of variance (ANOVA) or Student's t-test. A p-value < .05 was considered significant.

Results

Effect of ARB in subcutaneous xenograft models

To determine whether ARB could be evaluated for anti-proliferative and anti-fibrotic activity in vivo, ARB (candesartan 10 mg/kg) was delivered orally to female nude mice with tumor xenografts (Fig. 1). Tumors derived from MKN45 cells co-cultured with HPMCs without administration of candesartan were significantly larger than those from MKN45 cells alone when measured at day 28 ($p = .003$). This was consistent with results from our previous study [25]. Furthermore, tumors derived from MKN45 cells co-cultured with HPMCs were significantly smaller in the candesartan treatment group compared with those in the untreated group as early as day 20 ($p = .004$). Therefore, candesartan suppressed tumor growth of cells derived from MKN45 and HPMC co-cultures.

Histological examination of xenograft tumors

Fibrotic areas in tumors derived from MKN45 cells co-cultured with HPMCs were significantly larger than those in tumors from MKN45 cells alone (Fig. 2A, B). We confirmed the implantation of the subcutaneous tumors and HPMCs by labeling cells using a PKH26 cell linker kit (Fig. 2C). MKN45 cells co-cultured with HPMCs also possessed increased α-SMA expression and decreased E-cadherin expression (Fig. 2D, E). Conversely, tumors from the candesartan treatment group exhibited increased E-cadherin expression, and decreased α-SMA expression when compared with untreated tumors in the co-culture groups (Fig. 2A–E.). Furthermore, Azan staining revealed that fibrotic areas within tumors of the candesartan group were significantly smaller (Fig. 2F). This suggests that candesartan was responsible for the suppression of EMT and fibrosis in the tumors from treated mice.

AT1 receptor expression in human gastric cancer cell lines

We examined three human gastric cancer cell lines for the presence of the AT1 receptor by Western blot analysis. All gastric cancer cell lines expressed AT1 receptor protein as shown in Fig. 3.

The influence of Angiotensin II and ARB on cellular proliferation

To quantitate the effects of Angiotensin II and ARB on the growth of human gastric cancer cells the MTT assay was employed. Proliferation of MKN45 cells was significantly increased following

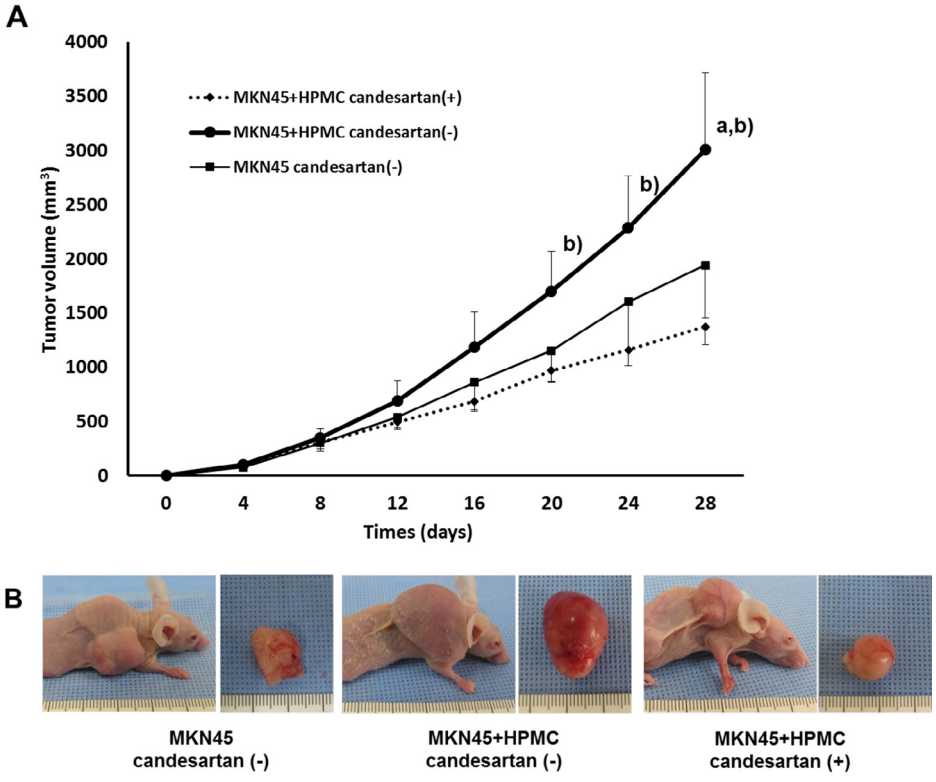

Fig. 1. (A) Candesartan inhibits growth of tumors derived from MKN45 and HPMC co-cultures. Tumor volume was measured every fourth day. Results are expressed as the means ± SD (n = 10). (B) Representative images depict the macroscopic appearance of the tumors at day 28. MKN45 ARB(−): MKN45 without candesartan, MKN45+HPMC candesartan(−): co-culture of MKN45 and HPMCs without candesartan, MKN45+HPMC candesartan(+): co-culture of MKN45+HPMCs with 10 mg/kg candesartan, a) $p < .01$ vs. MKN45 candesartan(−) b) $p < .01$ vs. MKN45+HPMC candesartan(+).

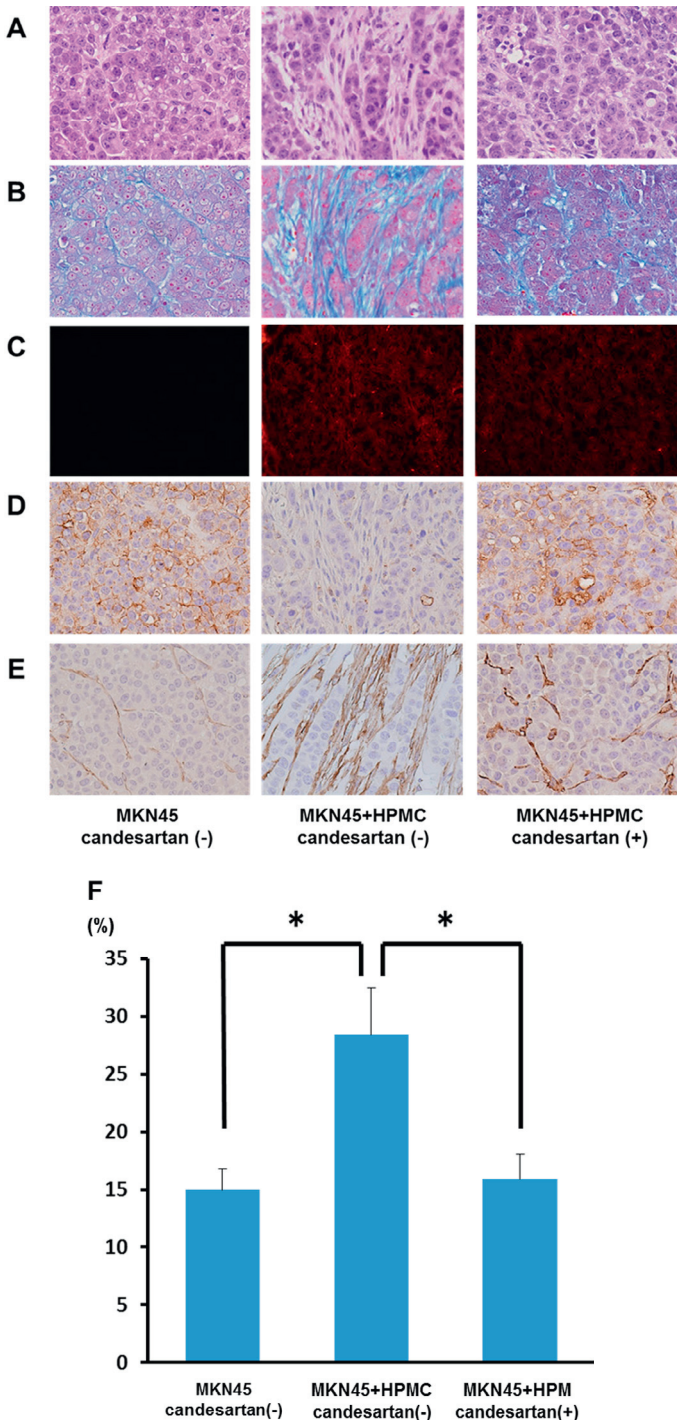

Fig. 2. Growth and EMT is inhibited in MKN45-HPMC co-culture-derived tumors on mice treated with candesartan. (A) Histological examination using H&E staining. (B) Fibrotic tissue as determined by Azan staining of subcutaneous xenograft tumors 28 days after inoculation. (C) Fluorescence microscopy investigating implantation of HPMCs. Red indicates labeled HPMCs. Immunohistochemical examination of (D) E-cadherin and (E) α-SMA in subcutaneous xenograft tumors (original magnifications ×200). (F) The fibrotic area was measured and shown as a percentage (fibrotic area/whole section area) of that in (B) $^{*}p < .01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

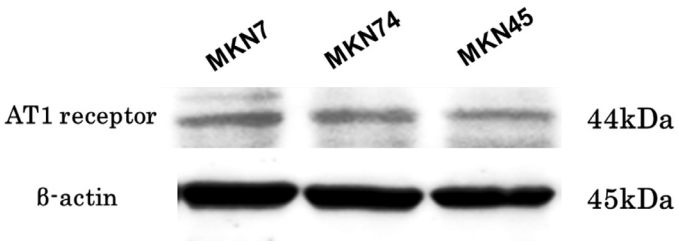

Fig. 3. Three gastric cancer cell lines express AT1 receptor. Western blot analysis demonstrating expression of the AT1 receptor in all three gastric cancer cell lines.

treatment with 100 nM Angiotensin II ($p = .016$, Fig. 4A). Furthermore, pretreatment of cells with 1000 nM of candesartan or 500 nM of telmisartan for 1 h completely inhibited the Angiotensin II-induced proliferative response (candesartan; $p = .001$, telmisartan; $p = .036$, Fig. 4B). As neither candesartan nor telmisartan (0.1–1000 nM) had a cytotoxic effect on MKN45 cells as determined by MTT assay (data not shown), these findings demonstrate that the inhibition of Angiotensin II by a specific AT1 receptor antagonist could reduce cellular proliferation.

The effect of SF-CM from MKN45 cells on HPMC cell morphology

Control HPMCs without SF-CM treatment grew as a monolayer of polygonal and cobblestone-like cells (Fig. 5A). In contrast, HPMCs treated with SF-CM of MKN45 cells demonstrated the elongated spindle-shaped morphology characteristic of fibroblasts (Fig. 5B). Similar phenotypic changes were observed with treatment using SF-CM from cells treated with Angiotensin II (Fig. 5C). HPMCs with

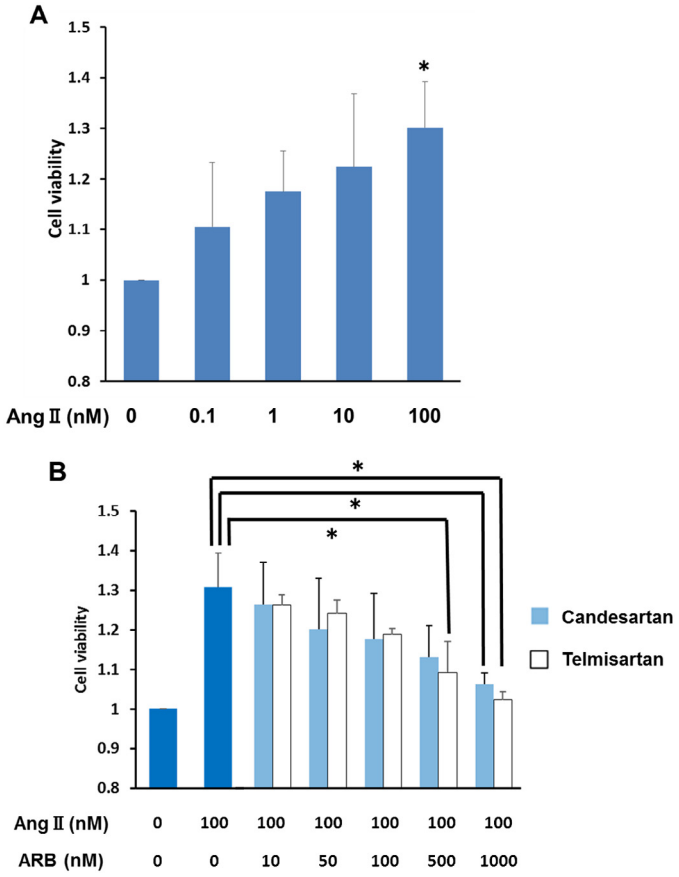

Fig. 4. Angiotensin II-induced proliferation of MKN45 cells is inhibited by ARBs. (A) The proliferation of MKN45 cells treated with increasing doses of Angiotensin II was measured by MTT assay. (B) Cells were then pretreated with increasing doses of candesartan prior to addition of Angiotensin II and measurement of proliferation by MTT assay at 48 hours. Results are mean ± SD of three experiments.$^{*}p < .05$.

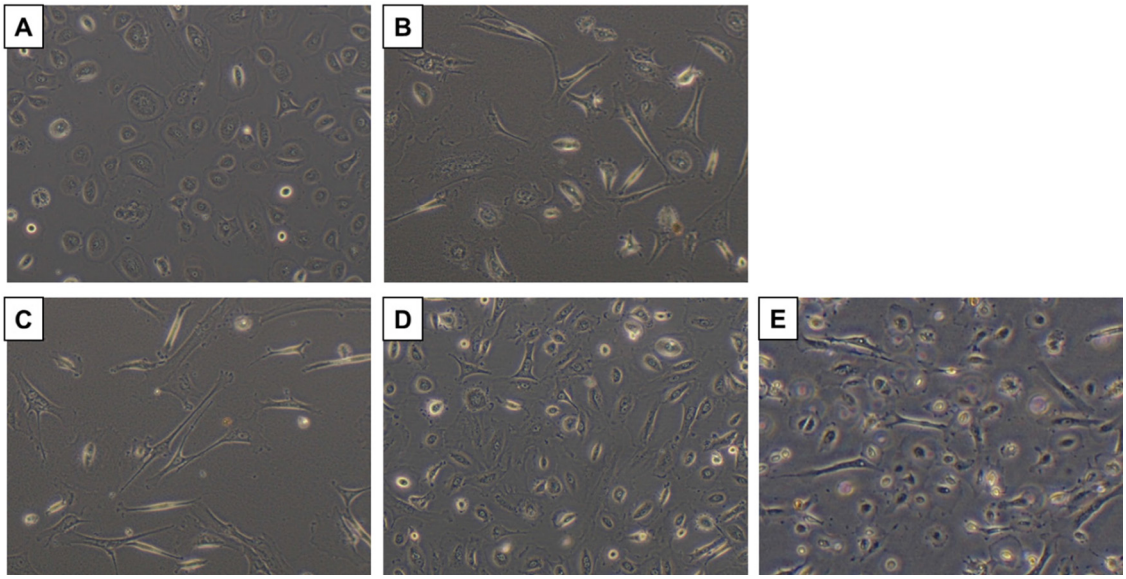

Fig. 5. SF-CM from MKN45 cells induces morphological changes in HPMC cells that are impaired by pretreatment of cells with candesartan and telmisartan. Representative images of morphological changes induced after culture of HMPCs with SF-CM HPMCs cultured in control medium (A), SF-CM of MKN45 (B), SF-CM from MKN cells treated with Angiotensin II (C), or SF-CM from MKN45 cells pretreated with candesartan (D) and telmisartan (E) prior to Angiotensin II. Images were obtained by phase contrast microscopy at magnification, × 40.

SF-CM of these phenotype changes were attenuated by pretreatment of MKN45 cells with 1000 nM candesartan and 1000 nM telmisartan prior to harvesting SF-CM (Fig. 5D, E). We selected candesartan for the following experiments because it is a more selective ARB than telmisartan, which also affects peroxisome proliferator-activated receptor-γ (PPAR-γ) [38,39].

TGF-β1 expression

To investigate whether Angiotensin II and candesartan influence the expression of TGF-β1, we used an ELISA and Western blotting to measure levels of TGF-β1 in the culture media. Treatment of MKN45 and MKN7 with Angiotensin II elevated the level of TGF-β1, while pretreatment of cells with candesartan effectively inhibited this response (Fig. 6).

Examination of EMT marker expression in HPMCs

Immunofluorescence staining of HPMCs cultured with SF-CM from MKN45 cells treated with Angiotensin II demonstrated expression of the mesenchymal marker α-SMA in the cell membrane and cytoplasm, with no nuclear staining observed (Fig. 7A). Conversely, HPMCs cultured with SF-CM from MKN45 cells pretreated with candesartan had a markedly diminished presence of α-SMA signal in the cytoplasm (Fig. 7A). Western blotting confirmed the immunofluorescence study, with increased α-SMA expression and decreased E-cadherin expression found in HPMCs cultured with SF-CM from MKN45 cells treated with Angiotensin II (Fig. 7B). Western blotting also detected diminished α-SMA and increased E-cadherin expression was identified in HPMCs cultured with SF-CM from MKN45 cells pretreated with candesartan (Fig. 7B). Blots were re-probed for β-actin to ensure equal protein loading in each lane. Results are representative data from three separate experiments.

Discussion

We used candesartan and telmisartan as ARBs in this study. However, we chiefly used candesartan because telmisartan is also a partial agonist of PPAR-γ [38,39]. Our data reveal that the candesartan can significantly suppress tumor proliferation and fibrotic changes in a subcutaneous xenograft model. Furthermore, we have demonstrated that Angiotensin II induces TGF-β1 expression and that ARB suppresses this stimulation in the gastric cancer cell line MKN45. SF-CM from MKN45 cells and exposure to Angiotensin II induced an EMT-like change in HPMCs, while pretreatment with candesartan impaired this change.

Angiotensin II is a main effector peptide in the renin–angiotensin system (RAS) and also plays a fundamental role as a vasoconstrictor in controlling cardiovascular function and renal homeostasis [40]. Recent studies have shown that a local RAS can exist in malignant tumor tissue, with Angiotensin II potentially acting as a key factor for promotion of tumor growth and metastasis via the AT1 receptor [41]. Arakawa and Maruta have demonstrated that trypsin generates Angiotensin II from circulating angiotensinogen in the absence of angiotensin converting enzyme (ACE) at a weakly acidic pH of 5.5 [42]. In acidic tissues, such as those found in gastric cancer, tryptase and trypsinogen derived from migrating mast cells may convert angiotensinogen to Angiotensin II. We have further suggested that circulating angiotensinogen in the blood is converted directly to Angiotensin II by trypsin in the tumor microenvironment at the weakly acidic pH found with anaerobic glycolysis [43]. We have also previously demonstrated that gastric cancer and intrahepatic cholangiocarcinoma tissue have higher levels of Angiotensin II than normal tissues [30,31]. Furthermore, gastric cancer tissue and cell lines alike have been found to express the AT1 receptor in both the current and previous studies [30,44]. Local tissue RAS signaling is potentially able to up-regulate the Angiotensin II/AT1 receptor pathway in an autocrine fashion, resulting in Angiotensin II-induced progression of gastric cancer malignancy [44].

Lever and colleagues reported the first clinical evidence that a long-term Angiotensin II blockade may be protective against carcinogenesis [45]. We have previously reported that Angiotensin II has the potential to enhance cell proliferation and impair apoptosis by promoting ERK1/2 and NF-κB activation and overexpression [30]. ERK1/2 transduces extracellular signals to the nucleus and increases the expression of genes involved in cellular proliferation [46]. Meanwhile, the transcription factor NF-κB regulates the expression of numerous genes, including survival factors and cell growth

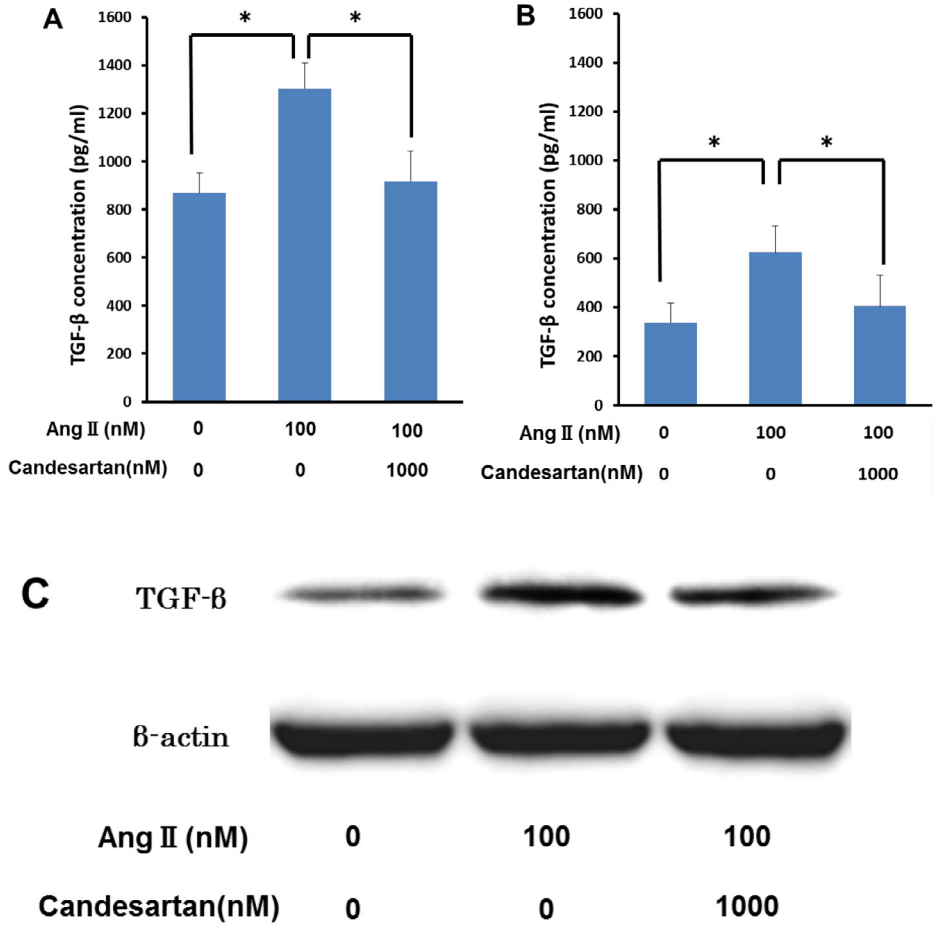

Fig. 6. Angiotensin II-induced elevation of TGF-β1 levels in MKN45 and MKN7 cell media is wholly inhibited by pretreatment of cells with candesartan. Levels of TGF-β1 were measured in both the culture media and cells from MKN45 and MKN7 cultures treated with Angiotensin II or pretreated with candesartan as indicated. Results are mean ± SD of three experiments. (A) MKN45 and (B) MKN7 TGF-β1 levels by ELISA, $^{*}p < .01$ and (C) Western blot.

regulatory molecules [47,48] Hence, the blockade of Angiotensin II has been considered a potential target for anti-proliferative therapy in tumorigenesis. Our finding that ARB suppresses Angiotensin II-induced tumor growth in vitro and in vivo supports this.

Fibrosis is a common feature of both peritoneal dissemination and chronic renal disease. Numerous studies have demonstrated that the Angiotensin II/AT1 receptor axis contributes to renal fibrosis through endogenous production of TGF-β1 [49,50]. Angiotensin II induces expression of the TGF-β1 activator thrombospondin-1 via the AT1 receptor, thereby mediating activation of latent TGF-β1 [51,52]. TGF-β1 then signals to the nuclei through activation of the Smad pathway, and promotes EMT in tubuloepithelial cells [50].

The development of peritoneal dissemination is a multistep process. Cancer cells within the peritoneal cavity can attach to HPMCs and induce an EMT-like change by release of TGF-β1 [17,53]. Such spindle-shaped HPMCs can then facilitate adhesion of cancer cells to the submesothelial basement membrane [26]. Furthermore, activated HPMCs are a source of CAFs through transformation to a myofibroblast-like phenotype. These CAFs can infiltrate the submesothelial basement membrane together with cancer cells and promote the processes of proliferation and fibrosis [25]. Thereafter, locally synthesized Angiotensin II stimulates cellular proliferation and fibrosis through AT1 receptor activation and TGF-β1 signals. In this cascade, TGF-β1 is produced by both cancer cells and CAFs [21]. This interaction between gastric cancer cells and CAFs through the Angiotensin II/AT1 receptor axis likely has autocrine and paracrine synergistic effects on tumor progression and fibrosis. As a result, peritoneal dissemination progresses rapidly with extensive stromal fibrosis, contributing to the poor prognosis associated with disseminated gastric cancer.

Inhibiting the effects of Angiotensin II and TGF-β1 described above is important for the attenuation of tumor proliferation and fibrosis. Previous reports have shown that TGF-β1 neutralizing antibodies and TGF-β1 receptor kinase inhibitors can suppress EMT and reduce stromal fibrosis [17,54]. However, these agents cannot be administrated to patients with various cancer and fibrotic diseases because TGF-β1 and its receptors are almost ubiquitously expressed in normal tissues. This is a major conceptual problem with the long-term clinical use of these agents as there is a high likelihood of adverse side effects because of disruption of the many important roles played by TGF-β1 in normal tissues [55]. However, as candesartan has been widely used as a clinical antihypertensive agent without serious side effects [56], it could potentially be safely used as an anticancer agent. This current study supports the hypothesis that candesartan offers a new strategy for peritoneal dissemination through suppression of both tumor proliferation and fibrosis.

In conclusion, we have shown that candesartan can significantly reduce TGF-β1 expression, and suppress tumor cell proliferation and stromal fibrosis in a mice xenograft tumor model. Candesartan was effective at a dose rate of 10 mg/kg/day, which is close to the maximal clinical dose [57]. Targeting the Angiotensin II signaling pathway may not only impair tumor proliferation, but may also be a novel, efficient strategy for treating associated tissue fibrosis. Therefore, combination therapy using ARBs and cytotoxic

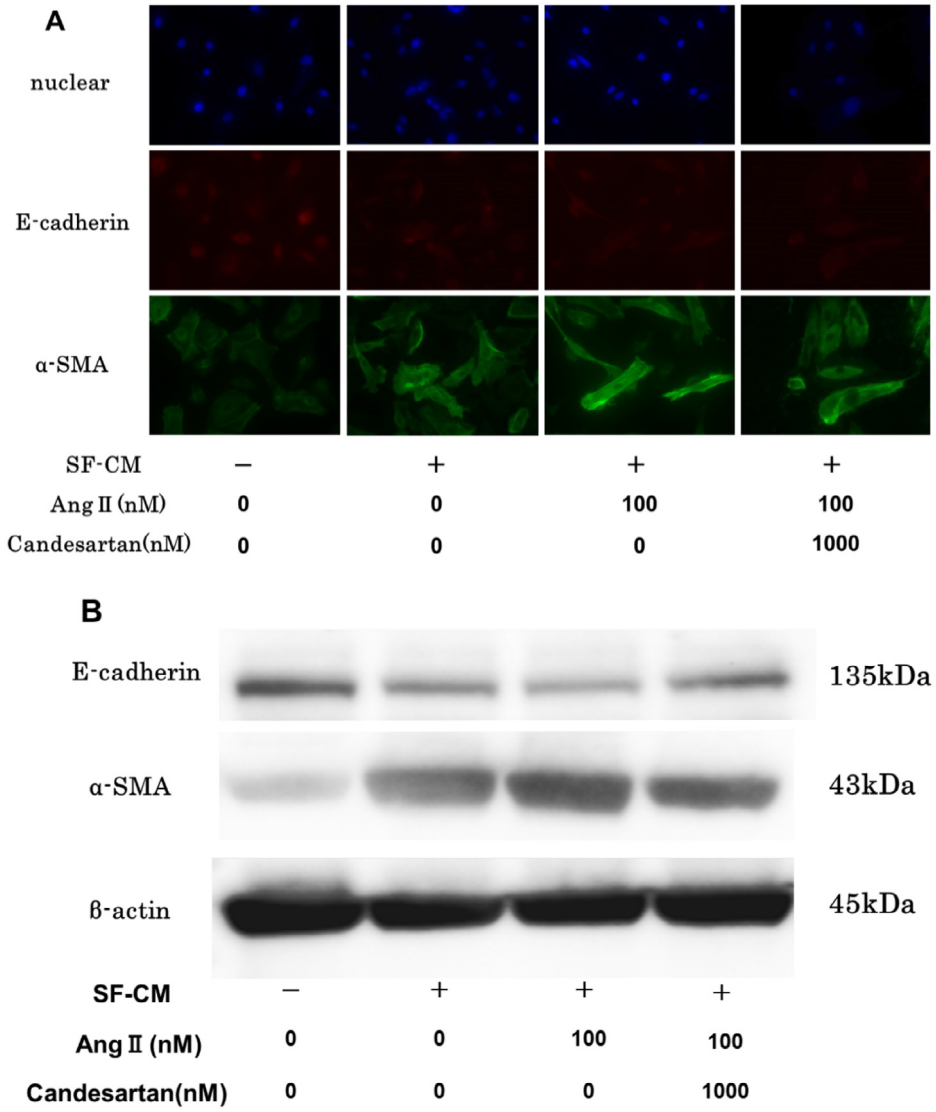

Fig. 7. Elevated expression of α-SMA and decreased expression of E-cadherin in HPMCs cultured with SF-CM from MKN45 cells are blocked by pretreatment with candesartan. (A) Representative photomicrographs of HPMCs labeled with antibodies to E-cadherin (red) and α-SMA (green). SF-CM from MKN45 cells alone, treated with Angiotensin II or pretreated with candesartan was used as indicated. Original magnification ×400. (B) Western blot analysis of E-cadherin and α-SMA protein levels in HPMCs cultured with SF-CM from MKN45 cells alone, treated with Angiotensin II or pretreated with candesartan as indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

antineoplastic agents could potentially improve the prognosis for patients with peritoneal dissemination of gastric cancer.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

We are grateful to members of the Department of Gastroenterologic Surgery of Kanazawa University for their helpful suggestions.

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