Local angiotensin II-generation in human gastric cancer: Correlation with tumor progression through the activation of ERK1/2, NF-κB and survivin

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Abstract. Angiotensin II is a main effector peptide in renin-angiotensin system, acting as a growth promoter via angiotensin II type 1 (AT1) receptor. The present study examined intrinsic angiotensin II generating system in gastric cancer and potential roles of angiotensin II in cellular proliferation and survival. The expression of AT1 receptor was examined in gastric cancer cell lines and tissues. In addition, we measured angiotensin II concentration in tissues from twenty gastric cancer and corresponding normal region using the florisil method. *In vitro*, we investigated the potential roles of angiotensin II in cellular proliferation and cell survival in cultured human gastric cancer cell line. The effects of AT1 receptor blocker candesartan were evaluated in a mouse model of peritoneal carcinomatosis. AT1 receptor protein was expressed in gastric cancer cell lines and tissues. Angiotensin II concentration in tumor region (1447±624 pg/g wet) was significantly higher than those of normal region (775±320 pg/g wet) ($p<0.05$). Angiotensin II stimulates the cell proliferation in the AT1 receptor-positive OCUM2MD3 gastric cancer cell line and this proliferative effect of angiotensin II was inhibited by a specific AT1 receptor antagonist, candesartan. We also confirmed the angiotensin II stimulated ERK1/2, nuclear transcript factor-κB (NF-κB) and surviving activation, which are central molecules of cellular proliferation and survival in OCUM2MD3 cells. Candesartan significantly prolonged survival time in a mouse model of peritoneal carcinomatosis compared with control group ($p=0.0.197$, log-rank test). Our data provide *in vivo* evidence of intrinsic angiotensin II generating system in gastric cancer and indicate locally formed angiotensin II is involved in cellular proliferation and survival.

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Introduction

Gastric cancer remains a major health problem being the second most common malignancy in the world (1). Despite the significant search and development of even more effective chemotherapies for advanced gastric cancer, only a proportion of patients with advanced disease will respond to therapy. Therefore, considerable attention has been focused on novel strategies required in addition to conventional surgery and chemotherapy.

Angiotensin II, a multifunctional bioactive octapeptide of the renin-angiotensin system (RAS), plays a fundamental role as a vasoconstrictor in controlling cardiovascular function and renal homeostasis. Previously, it has been thought that RAS exists only in the circulatory system (circulating RAS). However, a number of reports exist on the concept of a localized RAS (tissue RAS) in various tissues. Angiotensin II type 1 (AT1) receptor has been also detected in carcinomas of the larynx, lung, liver, pancreas, kidney, bladder, prostate gland, breast, ovary, cervix, in malignant melanoma, and in sarcomas (2-14). Lever *et al* reported the first clinical evidence that a long-term angiotensin II blockade might have a protective effect against carcinogenesis (17). There is increasing evidence that angiotensin II is involved in tumor biology and the potential anti-tumor effect of angiotensin II type 1 (AT1) receptor antagonist in tumors expressing AT1 receptor (8-16). Hence, the blockade of angiotensin II has been considered a noteworthy molecular targeted therapy in recent years.

With respect to gastric cancer, several studies have been reported on the local expression of AT1 receptor and have suggested that angiotensin II could play a key factor in tumor

growth and metastatic spread via the AT1 receptor (18-21). These data might indicate that a local tissue RAS is present and is potentially able to supply angiotensin II to the receptors in gastric cancer tissues. However, none of the previous studies proved the presence of angiotensin II itself in gastric cancer tissues. To confirm the intrinsic angiotensin II generating system in gastric cancer tissues, we measured angiotesin II concentration in tissues, for the first time, in this study.

We further investigated the potential roles of angiotensin II in cultured human gastric cancer cell lines *in vitro*. It has been reported that angiotensin II induces tumor-angiogenesis via up-regulation of vascular endothelial growth factor (VEGF), and acts as a potent growth factor in some malignant tumors, including gastric cancer (11-14,19). Although these findings suggest that angiotensin II could act as a key factor for tumor growth, the molecular mechanisms remain to be fully elucidated.

In this study, we dissected the underlying molecular mechanisms by examining the effects of angiotensin II on cellular proliferation and survival. The signaling molecules included AT1 receptor, ERK1/2 which is the best characterized of the mitogen-activated protein kinase (MAPK) pathways, and nuclear transcript factor (NF-κB). We further investigated whether angiotensin II enhanced the expression of survivin, a member of the inhibitor of apoptosis (IAP) family.

Furthermore, we assessed whether AT1 receptor antagonist could suppress the progression of gastric cancer *in vivo* with the use of murine model of peritoneal carinomatosis and evaluated the potential effectivity of this agent as a novel targeted therapy.

Materials and methods

Antibody. For primary antibodies, we obtained rabbit polyclonal AT1 antibody (Santa Cruz Biochemistry, CA, USA), mouse monoclonal phospho-ERK1/2 antibody (Santa Cruz), rabbit polyclonal ERK1/2 antibody (Santa Cruz), mouse monoclonal surviving 6E4 antibody (Cell Signaling Technology, Beverly, MA, USA).

Chemicals. Angiotensin II was obtained from Sigma (St. Louis, MO, USA). AT1 receptor antagonist, candesartan (CV-11974) was generously donated by Takeda Chemical Industries (Osaka, Japan).

Cancer cell line and culture conditions. The gastric cancer cell lines used in this study were human gastric cancer cell line, OCUM2MD3 which was kindly supplied by Department of Surgical Oncology, Osaka City University Graduate School of Medicine, Osaka, Japan, and MKN28, AZ521, TMK1, MKN45 which were purchased from American Type Culture Collection (Rockville, MD, USA). OUUM2MD3 is a human scirrhous gastric cancer cell line and it has a strong ability to metastasize into the peritoneum in nude mice (22,23). This cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, USA) supplemented with 10% fetal bovine serum (FBS; Iwaki, Japan), 2 mM glutamine (Nissui Pharmaceutical, Tokyo, Japan), 10 U/ml penicillin-streptomycin (Gibco-BRL) and the other cell lines were maintained in RPMI-1640 medium (Nissui Pharmaceutical) supplemented with the same chemicals. Cells were grown at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. All experiments were performed within 30 passages of the frozen stocks from which the cells were periodically recovered.

Human gastric cancer specimens. The current study included 20 patients with adenocarcinoma of the stomach. The group consisted of twelve men and eight women whose mean age was 61.5 (range 33-75) years.

The tumor samples were obtained from patients who underwent surgical treatment with regional lymph node dissection between 2004 and 2006 at the Department of Gastroenterologic Surgery of Kanazawa University Hospital. Normal tissues located far from the tumor site were also obtained. None of the patients received preoperative treatment, such as radiation or chemotherapy. Histological findings, including cancer gross classification, histological type, lymph node metastasis, cancer-stroma relationship and clinical stage were according to Japanese guidelines for gastric cancer (24). Tissue sample were obtained with written patient consent and the Institutional Review Board approved this clinical study.

Immediately following surgical removal, the tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin for histological examination. Three representative sections were used for immunohistochemical staining as described below. In addition, parallel samples were frozen in liquid nitrogen and stored at -80˚C until the measurement of angiotensin II concentration.

Immunohistochemistry. Immunohistochemical analysis for AT1 receptor was performed on blocks of resected gastric tissues per patient. Tumor tissue specimens were fixed with 10% buffered formalin and embedded in paraffin. Briefly, 4-μm thick sections were mounted on poly-L-lysine-coated glass slides, air-dried, and deparaffinized with graded xylene and ethanol solutions. To retrieve the antigen, sections were pretreated in 10 mM citrate buffer, at pH 6.0, and autoclaved for 10 min at 120˚C before the immunohistochemical staining of the primary antibody took place. Endogenous peroxidase was blocked with peroxidase block solution provided in the EnVision kit for 10 min, the slides were rinsed/washed with phosphate-buffered saline (PBS). The primary antibody, a rabbit polyclonal anti-human AT1 receptor antibody (1:100) was incubated overnight at 4˚C. Immunostaining was performed using the EnVision method (Dako, Glostrup, Denmark) according to manufacturer's instructions. The slides were developed with diaminobenzidine and counterstained with hematoxylin.

Cell growth assay. The proliferative effect of angiotensin II on gastric cancer cell line was quantified using an MTT colorimetric assay. In brief, cancer cell lines was seeded at a cell density of $5x10^3$/well in 96-well plates in DMEM growth medium with 10% FBS and incubated 24 h at 37˚C humidified environment containing 5% CO_2. The medium was then changed to serum-free medium containing different concentrations of angiotensin II (0.1 to 100 nM). After incubation for 48 h, MTT solution was added to each well at

250 μg/ml. The cells were incubated for 3 h and lysed in dimethyl sulfoxide and the absorbance value was analyzed using a spectrophotometer (EAR 340 AT, SLT, Vienna, Austria) at a wavelength of 540 nm. In some experiments, candesartan was added 30 min before angiotensin II treatment to confirm that the proliferative effect caused by angiotensin II occurred through the AT1 receptor.

Quantitative analysis of angiotensin II in tissues. The determination of angiotensin II concentration was performed as follows. The tissue samples were homogenized at 4˚C in saline containing 0.1 N HCl and 5% urinastatin. The homogenate was centrifuged at 10,000 x g for 30 min at 4˚C and the supernatant was used for radioimmunoassay of angiotensin II using the florisil method (florisil absorption and elution with acetone-hydrochloric acid solution), as described previously (25). This method is more sensitive and specific and useful for routine clinical investigation.

Western blot analysis. Cell lysates were made by standard methods. The protein concentration of each sample was measured using a Bio-Rad protein assay kit II (Bio-Rad Laboratory, Richmond, CA, USA). For SDS-PAGE, 30 μg of proteins from each sample were subjected to electrophoresis on 10-15% polyacrylamide gels. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes with a tank transfer systems (Bio-Rad Laboratory), then blocked with buffer containing 5% skim-milk and 0.1% Tween-20 in Tris-buffered saline (TBST) at room temperature for 1 h. All primary antibodies were diluted in TBST containing 5% skim-milk. The membranes were incubated with primary antibody overnight at 4˚C, washed (4x10 min) with washing buffer (TBST), followed by incubation with a horseradish peroxidase-conjugated secondary antibody (0.02 μg/ml in TBST) as appropriate for 1 h at room temperature, then washed (4x10 min) with washing buffer. Detection of chemiluminescence was performed with ECL Western blot detection kits (Amersham, Little Chalfont, UK) following the manufacturer's instructions.

Electrophoretic mobility shift assay. Electophoretic mobility shift assay was performed, as described previously (26). In brief, nuclear proteins of OCUM2MD3 were extracted with Nuclear and Cytoplasmic Extraction Reagents (NE-PER; Pierce Biotechnology, Rockford, IL, USA), according to the supplier's recommendation. Protein concentration was determined using a protein assay reagent (Advanced Protein Assay Reagent; Cytoskeleton, Denver, CO) with bovine serum albumin as a reference standard. Double-stranded NF-κB consensus oligonucleotides (5'-AGTGAGGGGACTTTCCC AGGC-3',5'-TCAACTCCCCTGAAAGGGTCCG-3', Promega, Madison, WI, USA) were end-labeled with [γ-^{32}P]-adenosine triphosphate (3000 Ci/mmol at 10 mCi/ml; NEN Life Science Products, Boston, MA, USA) using T4 polynucleotide kinase. Binding reactions that contained 10 μg of nuclear protein extracts and 10^5 cpm of oligonucleotides were performed for 30 min at room temperature in binding buffer [10 mM HEPES, pH 7.6, 50 mM KCl, 1 mM EDTA, 0.4% Ficoll, 1 mM DTT, 0.125 mM PMSF and 0.05 mg/ml poly-(dI-dC)]. Reaction products were separated on a 4% polyacrylamide gel and analyzed by autoradiography. Specificity of the DNA and protein complex was confirmed by competition with 50-fold excess of unlabeled NF-κB oligonucleotides. For supershift analysis, 1 μg of anti-NF-κB antibodies (p50 and p65; Santa Curz Biotechnology Inc.) was added to reaction mixtures for 30 min at room temperature before the addition of a radiolabeled probe.

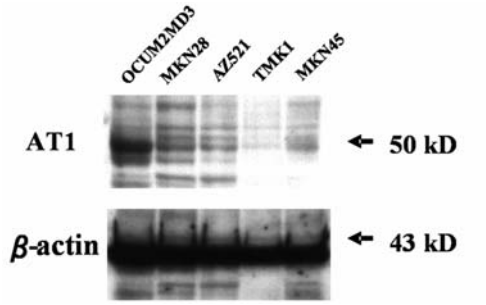

Figure 1. Western blot analysis of the AT1 receptor expression in gastric cancer cell lines. Four of five (80%) gastric cell lines, OCUM2MD3, MKN28, AZ52 and MKN45 showed strong bands of ~50 kD.

In vivo studies using a mouse model of peritoneal carcinomatosis. An *in vivo* model of peritoneal carcinomatosis was used, as described previously (22). Four-week-old female-immunocompromised BALB/c-*nu/nu* mice (Nihon CLEA, Japan) were maintained under sterile conditions and used for all *in vivo* experiments. Procedures involving animals and their care were conducted in conformity with national and international laws and policies and were approved by our Institutional Review Board. OCUM2MD3 cells ($1x10^7$/1.0 ml medium/mouse) were injected intra-peritoneally (i.p.) into nude mice on day 0. Beginning on day 7, they were then randomly assigned to receive either the angiotensin II receptor antagonist (n=5), candesartan (10 mg/kg/day) by gavage or vehicle (control group, n=5). Animals were carefully monitored daily for body weight, behavior and survival. As soon as the mice were dead, an autopsy was performed on all mice that received i.p. injections of OCUM2MD3 to determine the cause of death.

Statistical analysis. The statistical analysis was done using the Mann-Whitney U test and Student's t-test. Survival curves were drawn according to the Kaplan-Meier method and the log-rank test was applied to compare the survival curves. Statistical significance was set at ≤0.05.

Results

AT1 expression in human gastric cancer cell lines and tissues. We first analyzed five human gastric cancer cell lines for the presence of the AT1 receptor in protein level by Western blot analysis. All gastric cell lines expressed AT1 receptor protein as shown in Fig. 1. OCUM2MD3, MKN28, AZ521 and MKN45 showed strong bands of ~50 kDa which is the known molecular weight of the AT1 receptor. TMK1 showed a weak band in comparison with the other cell lines.

Next, we examined 20 surgically resected specimens by immunohistochemistry. Sixteen out of 20 (80%) specimens

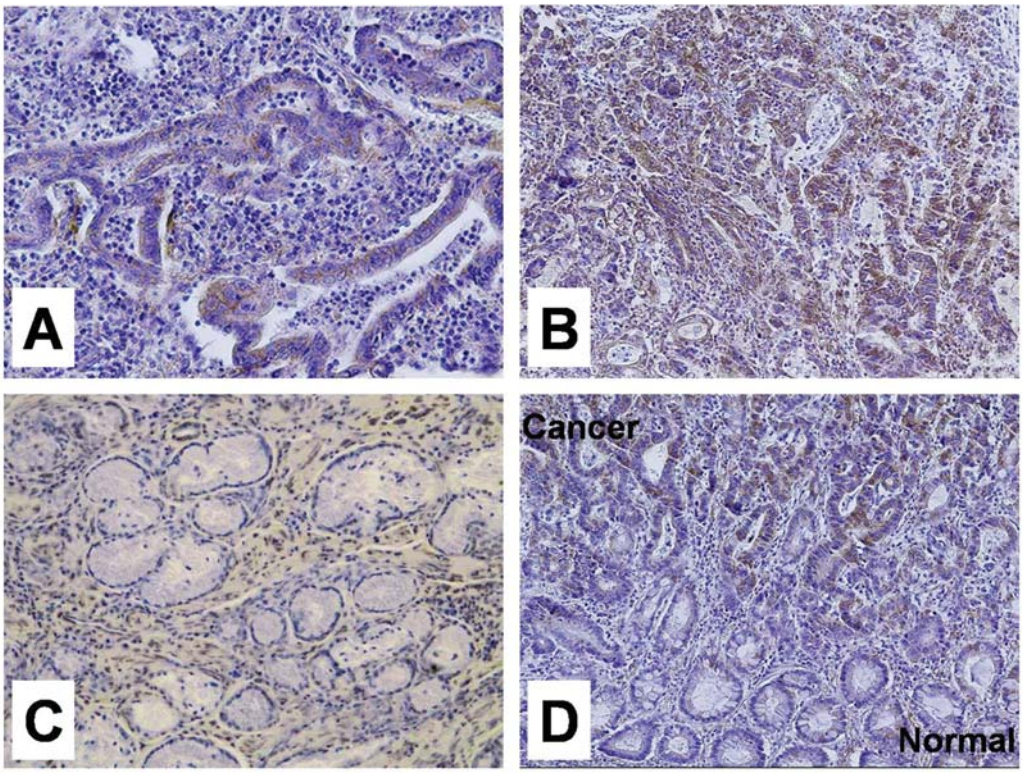

Figure 2. A representative gastric cancer case exhibited the AT1 receptor protein expression in cancer cells (A) differentiated type, (B) undifferentiated type. The expression was positive in membranous and granular cytoplasmic patterns. However, negative or weak staining was observed in corresponding normal mucosal cells (C). Typical contrast of AT1 receptor expression in the specimens with normal and gastric cancer regions is shown (D). Original magnification x200 (A-C) and x100 (D).

of gastric cancer expressed AT1 receptor protein in >50% of carcinoma cells (Fig. 2A and B). In contrast, a negative or weak immuno-staining for AT1 receptor was observed in corresponding normal gastric mucosa (Fig. 2C and D). Regarding histologic localization of the AT1 receptor, immunoreactivity was evident in membranous and cytoplasmic patterns. This is in accordance with findings described by Huang *et al* (18).

Angiotensin II concentration in tissues. Angiotensin II concentration was determined radioimmunologically in the extracts of normal and gastric cancer regions (n=20). The concentration was 775±320 pg/g wet tissue in normal region, 1447±624 pg/g wet tissue in tumor regions (Fig. 3). Tissue angiotensin II consentration in tumor regions was significantly higher than those of normal regions. This result indicated the presence of an angiotensin II generating system in gastric cancer. Next, we compared the angiotensin II concentration in tumor regions with various clinicopathological variables as detailed in Table I. However, there was no statistically significant relationship between the angiotensin II concentration and the clinicopathological findings (Table I).

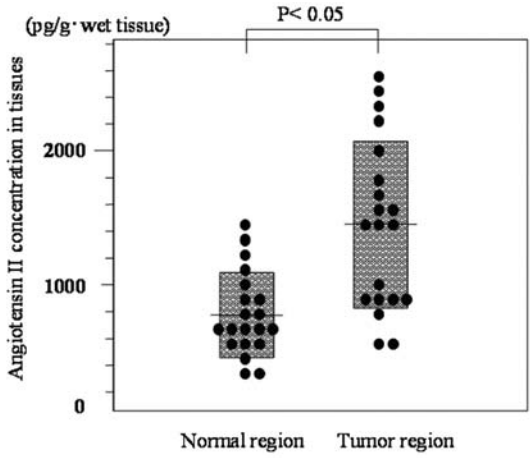

Figure 3. Angiotensin II concentration in tissue extracts of gastric cancers and normal regions. Each homogenate was sedimented and supernatant was used for radioimmunoassay of angiotensin II using the florisil method (florisil absorption and elution with acetone-hydrochloric acid solution).

Effect of angiotensin II on the growth of human gastric cancer cells. To investigate the effect of angiotensin II on the growth of human gastric cancer cells, the proliferative effect was quantified by MTT assay, using OCUM2MD3 cells which showed strong expression of AT1 receptor protein in Western blot analysis. The cell line was treated with angiotensin II at a range of doses (0.1-100 nM) for 24 h. The proliferative activity of OCUM2MD3 was significantly increased in a dose-dependent manner (Fig. 4).

Meanwhile, angiotensin II had no significant effect upon the proliferation of TMK1 which showed extremely weak expression of AT1 receptor in comparison with the other cell lines in Western blot analysis (data not shown).

Next, we examined whether the proliferative effect of angiotensin II was inhibited by a specific AT1 receptor

Table I. Relationship between angiotensin II concentration and clinicopathological features.

Variables	No. of patients	Angiotensin II concentration in tumor tissues (pg/g wet tissue)	P-value
Gross type			
2	6	1110±444	N.S.
3	7	1787±657	
4	7	1400±620	
Histological type			
Differentiated	5	1714±731	N.S.
Undifferentiated	15	1360±585	
Serosal invasion			
Absent	5	1466±610	N.S.
Present	15	1442±649	
Lymph node metastasis			
Absent	8	1408±616	N.S.
Present	12	1510±672	
Amount of cancer stroma			
Med	4	1068±364	N.S.
Int	6	1583±728	
Sci	10	1520±633	
Stage of disease			
I	6	1283±645	N.S.
II	6	1666±432	
III	6	1346±601	
IV	2	1595±1421	

By histological type, differentiated type included papillary adenocarcinoma (pap) and tubular adenocarcinoma (tub 1 and 2). Undifferentiated type included poorly differentiated adenocarcinoma (por 1 and 2), mucinous adenocarcinoma (muc) and signet ring cell carcinoma (sig). Med, medullary type (stroma is scanty); int, intermediate type (the quantity stroma is intermediate between those of the scirrhous type and medullary type); sci, scirrhous type (stroma is scanty). N.S., not statistically significant.

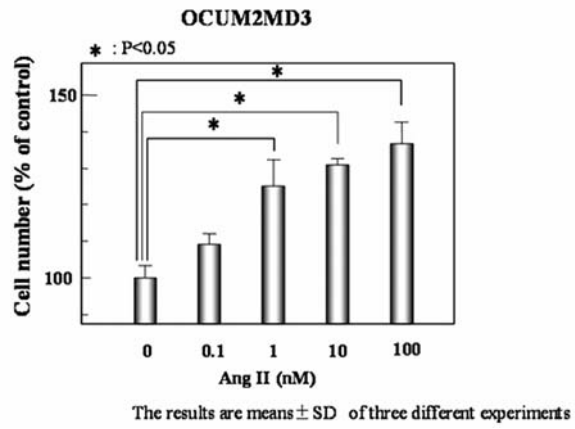

Figure 4. The effect of angiotensin II upon the proliferation of human cultured gastric cancer cells was quantified by MTT assay. Cells were treated in the absence or presence of different concentrations (0.1 to 100 nM) of angiotensin II. Angiotensin II induced proliferative activity in a dose-dependent manner. Results are mean ± SD of three different experiments.

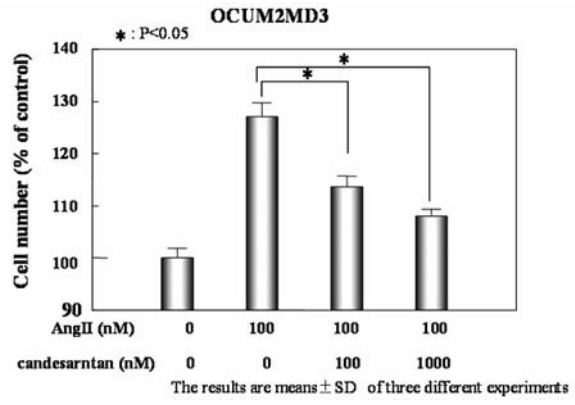

Figure 5. The effect of candesartan upon angiotensin II-induced proliferative response. Cells were pre-incubated with candesartan (100 and 1000 nM) before stimulation with 100 nM angiotensin II. Angiotensin II-induced cell proliferation was significantly reduced by pre-treatment of candesartan in a dose-dependent manner. Results are mean ± SD of three experiments.

antagonist, candesartan, using OCUM2MD3 cells. Pretreatment with candesartan completely inhibited angiotensin II-induced proliferative response at a concentration of 1 μM (Fig. 5). Because candesartan (0.1-10 μM) did not have cytotoxic effect on OCUM2MD3 cells by MTT assay (data not shown), these data demonstrate that the suppressive effect of candesartan on cell growth through the inhibition of angiotensin II as a specific AT1 receptor antagonist.

Angiotensin II increase the expression of phosphor-ERK in human gastric cancer cells. Next, we investigated the signaling molecules in pathway downstream of AT1 receptor *in vitro* using a gastric cancer cell line, OCUM2MD3. ERK1/2 is a key regulatory pathway that controls the cellular response to proliferation, differentiation and apoptosis. The phosphorylation status of ERK1/2 in OCUM2MD3 was determined after OCUM2MD3 cells were treated with 100 nM angiotensin II for the indicated period time up to 30 min, using antibody that specifically recognize phosphorylated forms. In a time course experiment, we found that 100 nM angiotensin II stimulated maximally at 5 min the phosphorylation of ERK1/2, which returns to basal level after 10 min (Fig. 6). This result indicates that angiotensin II activated the pERK1/2 signaling pathway in OCUM2MD3.

NF-κB activity. To investigate whether angiotensin II induces the activation of NF-κB, electrophoretic mobility shift assays were performed. Moderate NF-κB DNA binding activity was shown in nuclear extracts from untreated OCUM2MD3 cells. This basal constitutive NF-κB expression in gastric cancer cells has been described previously (27). However, the amount

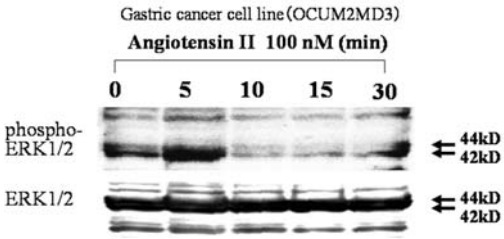

Figure 6. We found by Western blot analysis that angiotensin II stimulated phosphorylation of ERK1/2 in a time course experiment. OCUM2MD3 cells were serum-deprived for 24 h then treated with 100 nM angiotensin II. Cells were harvested at the indicated time after angiotensin II treatment and lysates were prepared for Western immunoblotting. The maximal phosphorylation of ERK1/2 was observed at 5 min.

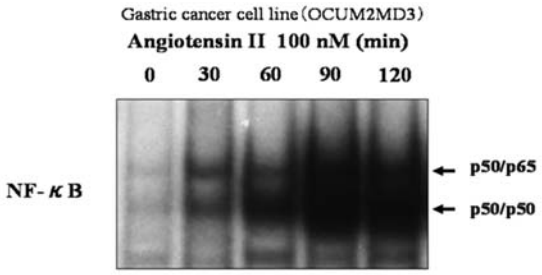

Figure 7. The effect of angiotensin II on NK-κB binding acitivity. OCUM2MD3 cells were serum-deprived for 24 h then treated with 100 nM angiotensin II. Cells were harvested at the indicated time after angiotensin II treatment and nuclear extrants from the cells were analyzed by EMSA. Strong NF-κB binding was present after 60 min of culture with 100 nM angiotensin II in OCUM2MD3.

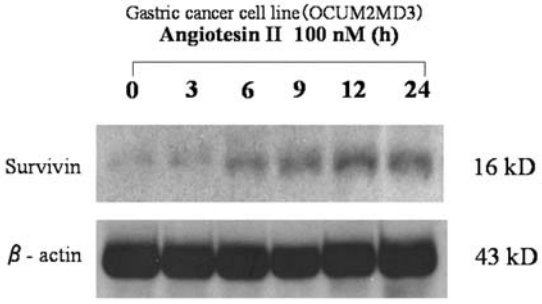

Figure 8. Angiotensin II induced up-regulation of surviving expression in OCUM2MD3. Cells were serum-deprived for 24 h then treated with 100 nM angiotensin II for the indicated time and lysates were subjected to Western immunobloting. The maximal level was reached 24 h after the cells were exposed to angiotensin II.

of NF-κB binding activity increased as early as 30 min and was sustained for over 120 min in the OCUM2MD3 cells treated with 100 nM angiotensin II (Fig. 7). Supershift analysis identified the detected DNA-protein complexes contained both p50/p65 heterodimeres and p50/p50 homodimers (data not shown).

Survivin expression. A unique feature of survivin, which is a member of the IAP family of antiapoptotic genes, is its differential expression in tumor vs. normal tissues (28) and overexpression of survivin has been found in many cancers, including gastric cancer (29). We investigated whether angiotensin II correlates with survivin up-regulation in OCUM2MD3 cell lines. Surviving protein expression was up-regulated by angiotensin II treatment (Fig. 8). The maximal level was reached 24 h after the cells were exposed to angiotensin II.

Treatment of gastric cancer with angiotensin II receptor blocker in vivo using a mouse model of peritoneal carcinomatosis. To determine whether AT1 receptor blocker could inhibit gastric cancer progression *in vivo*, we used OCUM2MD3 (i.p.) nude mice as a model of peritoneal carcinomatosis and evaluated the effect of candesartan on survival of mice. We performed an autopsy to determine the cause of death as soon as they were dead. All mice both in the control group and in the candesartan administered group developed abdominal swelling with bloody ascites and many metastatic nodules in mesentery and retroperitoneum (Fig. 9). Twenty-three days after initiation of the experiment, mice administered vehicle alone started to die and all animals in this group died after 34 days (median survival, 26 days). In mice administered candesartan, the survival time was longer (median survival 41 days) than that in control group (Fig. 10). A significant difference in median survival was observed between candesartan and control groups.

Discussion

Previous studies showed that local RAS exists in various malignant tumor tissues and suggested that the main effector peptide angiotensin II could act a key factor for tumor growth via AT1 receptor. Therefore, considerable attention has been focused on the development of RAS blockade therapy as a new strategy for cancer treatment (8-16,30).

We previously demonstrated that tissue angiotensin II concentrations within pancreatic ductal cancers were significantly higher than those of normal pancreas and colon cancers. This study also showed AT1 receptor protein was overexpressed immnohistochemically in pancreatic ductal cancer cells, whereas the colon cancer cells had extremely weak or negative immunoreactivity for AT1 receptor (7). In gastric caner, it has been also reported that AT1 receptor is overexpressed in cancer tissues (18-21), little information exists on the local synthesis of angiotensin II. Classically, the physiological activity of angiotensin II has long been recognized to be dependent on conversion of its precursor angiotensin I by angiotensin-converting enzyme (ACE). However, a number of *in vitro* studies have recently demonstrated angiotensin II forming enzymes other than ACE in the local tissues, such as kallikrein-type serine protease (trypsin, kallikrein, tonin) and chymase-type serine protease (chymase, cathepsin G) (31-36).

In gastric cancer, several studies have reported over-expression of these angiotensin II forming enzymes. Nagahara *et al* recently reported kallikrein 6 protein was overexpressed in gastric cancer tissues and high kallilkrein 6 expression levels were associated with lymphatic invasion and poor prognosis (37). It has been also reported that human gastric carcinoma cell lines secrete trypsin (trypsinogen) protein at high levels (38,39). In addition, Kondo *et al* have

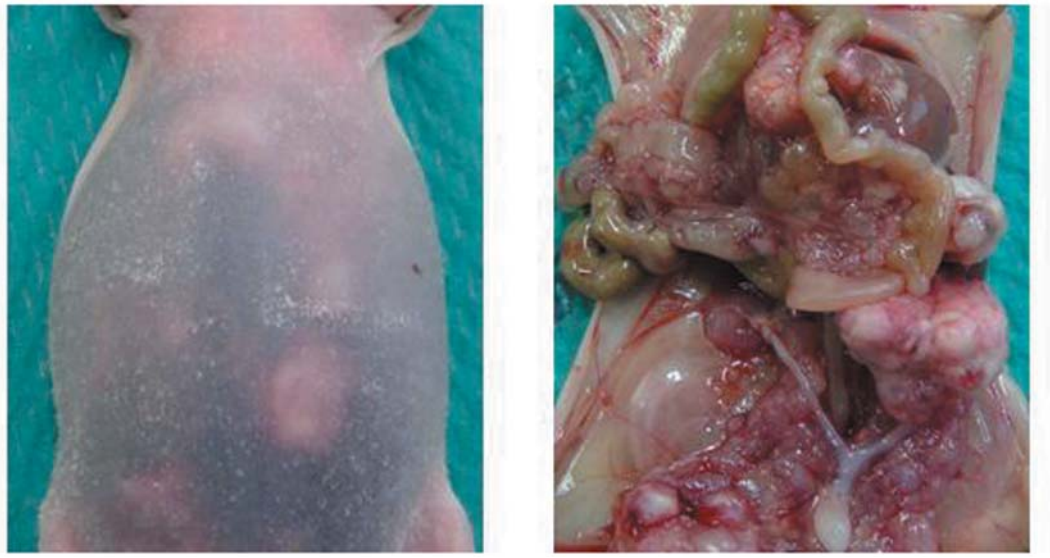

Figure 9. A mouse model of peritoneal carcinomatosis. OCUM2MD3 cells were injected intra-peritoneally into nude mice on day 0. We performed an autopsy to determine the cause of death as soon as they were dead. All mice that received i.p. injections of OCUM2MD3 developed abdominal swelling with bloody ascites. Many metastatic nodules were found in mesentery and retroperitoneum.

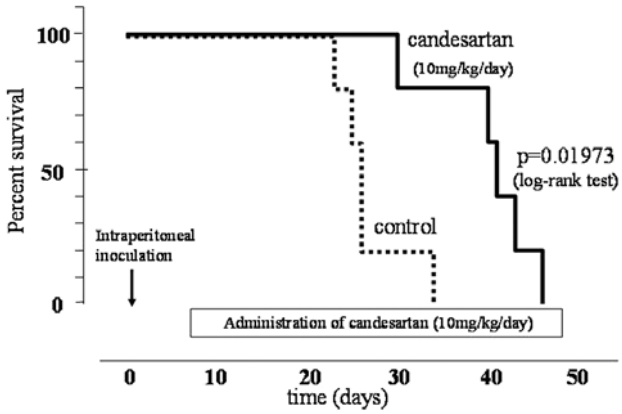

Figure 10. The effect of candesartan on survival of the mice given peritoneal inoculation of OCUM2MD3 cells. Dotted line, control mice (n=5). Solid line, mice treated with candesartan (n=5). The mice were housed under identical conditions and the date of death of each mouse was recorded. The difference in the survival times between the two groups of mice was stastically singnificant (P=0.01973, log-rank test).

demonstrated chymase-positive cell counts in the gastric cancer region that were significantly higher than in the normal region and the number of chymase-positive cell counts correlated significantly with tumor angiogenesis (40).

Because these results indicated the local synthesis of angiotensin II and the cancer development via AT1 receptor, we focused on the concentration of angiotensin II to confirm the local angiotensin II generating system. In the current study, we clearly showed a sharp contrast between human gastric cancer tissues and the corresponding normal counterpart with respect to the concentration of angiotensin II. This is, to our knowledge, the first demonstration of a local angiotensin II generating system in human gastric cancer. In addition, we showed that angiotensin II stimulates cell proliferation through AT1 receptor activation *in vitro*. This angiotensin II-induced cell proliferation was reduced by pre-treatment of the cells for AT1 receptor antagonist. The result suggests angiotensin II, which is locally generated in gastric cancer tissues might be involved in the progression of the gastric cancer as local paracine and autocrine systems.

Then, we focused on the mechanism of angiotensin II-induced transduction on cell proliferation and survival, which are closely related to the development and sensitivity for chemotherapy of gastric cancer.

AT1 receptor belongs to the superfamily of heterotrimeric G protein-coupled receptors (GPCR). GPCR - epidermal growth factor receptor (EGFR) cross-talk pathways are widely established and it has been reported that stimulation of angiotensin II involves transactivation of the EGFR, resulting in the activation of the MAPK pathway in various cell types (16,41-45).

ERK1/2, which is the best characterized of the MAPK, has the effect of transduction the signal to the nucleus and of increasing the level of expression of genes that are related to cellular proliferation (46). We have shown that angiotensin II transduced mitogenic signals leading to cell proliferation of gastric cancer cells through ERK1/2 activation.

The transcription factor NF-κB regulates the expression of numerous genes, including survival factors and cell growth regulatory molecules. NF-κB suppresses apoptosis by inducing expression of a number of genes whose products inhibit apoptosis, including IAPs and Bcl-2 families (47). In the current study, we confirmed that angiotensin II increased the DNA-binding activities of NF-κB in gastric cancer cells by EMSA. These data indicate that angiotensin II-induced NF-κB activation may be one of the important mechanisms causing cell survival in gastric cancer. To investigate the effects of angiotensin II on survival signal through NF-κB pathway more precisely, we next focused on the expression of survivin, which is a newly identified member of the

inhibitor of apoptosis protein (IAP) family. This anti-apoptotic protein is expressed in embryonic and fetal organs, but it has not been reported in differential normal tissues. In contrast, survivin is overexpressed in most human tumor types (28), including gastric cancer (29) and high expression of survivin was shown to be associated with resistance to chemotherapy and poor prognosis of carcinomas of the lung, breast, colon, esophagus and stomach (48-53). Thus, survivin is at present validated as a cancer therapeutic target. In the present study, we demonstrated the expression of representative molecules, survivin was upregulated by 100 nM angiotensin II. Our results might support that AT1 receptor blockade therapy provides clinical benefits by directly inducing cancer cell apoptosis and conferring sensitivity to conventional chemotherapy agents. Although there was no data on the relation between NF-κB activation and survivin expression, precise potential downstream of NF-κB activation that promote cell survival in response to angiotensin II are currently under investigation in our laboratory.

We finally investigated the potential role of AT1 receptor blockade therapy *in vivo* using a murine model of peritoneal carcinomatosis of gastric cancer. To date, several additional studies have shown that AT1 receptor blocker inhibited tumor growth and metastasis in experimental models (9,12-14,19,54,55). However, there has been no study demonstrating the anti-tumor effect by evaluating the survival time. In the present study, we demonstrated that oral administration of candesartan at dose of 10 mg/kg/day significantly extended survival of mice. The data suggest that angiotensin II also plays a critical role in tumor progression *in vivo* and further validate *in vitro* results.

Furthermore, we hypothesized that the AT1 receptor blockade therapy has a clinical potential for peritoneal carcinomatosis of gastric cancer. Human gastric carcinoma develops peritoneal metastasis frequently with extensive stromal fibrosis, resulting in various serious complications such as ileus, hydronephrosis and obstructive jaundice. Therefore, to prevent intraperitoneal fibrosis is considered as an important strategy to improve the treatment for peritoneal carcinomatosis of gastric cancer. Meanwhile, recent studies have shown that RAS plays a pivotal role in liver fibrosis (56-58) and demonstrated that the clinically used AT1 receptor blockers significantly attenuated experimental liver fibrosis (59,60). In the present study, we established a peritoneal carcinomatosis model by using human scirrhous gastric cancer cell line OCUM2MD3. It was previously reported that these cells, intraperitoneally inoculaed, had developed peritoneal metastasis accompanied by stromal fibrosis of the peritoneum in nude mice (22,23). The result of our *in vivo* study supports the assumption that AT1 receptor blockade therapy provides a new strategy for peritoneal carcinomatosis through suppression of both tumor growth and stromal fibrosis.

In summary, our results demonstrate that the high concentration of angiotensin II and AT1 receptor expression in gastric cancer tissues, which unequivocally provides *in vivo* evidence for a local angiotensin II-generating system in human gastric cancer. We further uncovered novel signaling molecules, ERK and NF-κB, responsible for angiotensin II-induced cell proliferation and survival and showed up-regulation of the broad spectrum apoptosis inhibitor survivin. We also confirmed the anti-tumor effects of angiotensin II in a murine model of peritoneal carinomatosis of gastric cancer.

Because AT1 receptor blocker has been widely used clinically as an antihypertensive agent without serious effects, it may also be available as an anticancer agent and could be effective for targeted therapy of not only chemoresistance of advanced gastric cancer, but also peritoneal carcinomatosis.

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