

Angiotensin II type 1 receptor expression in human pancreatic cancer and growth inhibition by angiotensin II type 1 receptor antagonist

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Abstract We investigated the expression of angiotensin II type 1 receptor (AT1) in pancreatic cancer. Both AT1 mRNA and protein were expressed in human pancreatic cancer tissues and cell lines. Binding assays showed that pancreatic cancer cells have specific binding sites for angiotensin II and that binding could be eliminated by treatment with a selective AT1 antagonist in a dose-dependent fashion. Surprisingly, the growth of cancer cells was significantly suppressed by treatment with antagonist, also in a dose-dependent manner. These observations suggest AT1 plays an important role in pancreatic cancer growth. Furthermore, ligand-induced inhibition of AT1 may be a useful therapeutic strategy. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Angiotensin II; Pancreatic cancer; Angiotensin II type 1 receptor; Angiotensin II type 1 receptor antagonist

1. Introduction

Pancreatic cancer is the fifth leading cause of cancer deaths in Japan, and its overall 5-year survival rate is only 9.3% [1]. In many patients suffering from pancreatic cancer, invasions to surrounding organs and hematogenous metastasis are already present at the time of diagnosis. Because there is no useful therapy in pancreatic cancer except for curative operation, late diagnosis and the aggressive nature of pancreatic cancer result in a poor survival rate. To improve the prognosis of pancreatic cancer, it is extremely important to resolve its growth mechanism.

Angiotensin II (Ang-II) is the main effector peptide of the renin-angiotensin system and it exerts a variety of actions on the cardiovascular and renal systems. Recently, two main subtypes of Ang-II receptors have been found using selective antagonists and named type 1 receptor (AT1) and type 2 receptor (AT2) [2,3]. Growth-modulating effects of these receptors were reported in coronary endothelial cells and vascular smooth muscle cells [4,5], where AT1 caused cell proliferation, whereas AT2 acted in an anti-proliferative manner. Expression profiles of these receptors in the cardiovascular

system demonstrated that AT1 was dominantly expressed in physiological conditions, but AT2 was expressed in some morbid conditions, such as cardiac hypertrophy and myocardial infarction [6,7].

Despite the fact that the role of Ang-II and its receptors has been well discussed in the cardiovascular field, few reports can be found about the expression of AT1 in cancer cells [8–11], and no reports were found about the relation between AT1 and the growth of cancer cells. In the present study, we examined the expression of AT1 in human pancreatic cancer, and are the first to demonstrate that AT1 regulates the growth of pancreatic cancer.

2. Materials and methods

2.1. Cell culture and tissue specimens

The pancreatic cancer cell lines used in this study were PANC-1, MIA PaCa-2, and Capan-2, and they were purchased from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, USA) supplemented with 10% fetal bovine serum (FBS; Iwaki, Japan), 10 U/ml penicillin–streptomycin (Gibco-BRL, USA), and incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. A selective antagonist of AT1, L-158,809 (kindly supplied by Merck and Co., USA), was dissolved in dimethyl sulfoxide and applied to cells at a concentration of 0.1% of the medium volume. Fresh pancreatic cancer tissues and normal pancreatic tissues were surgically obtained after obtaining informed consent from patients. All pancreatic cancers were of ductal origin. For RNA purification, specimens were immediately frozen in liquid nitrogen, and stored at –80°C until extraction.

2.2. RNA purification and RT-PCR

For RNA purification, frozen tissues were homogenized using a microhomogenizer (Cosmo Biol. Co., Ltd., Japan), and total RNA from these tissues and cell lines was prepared using the Fast RNA Kit-Green[®] (Bio-101, USA). The primer sequences of AT1 and G3PDH were as follows: 5'-AGATGATTGTCCCAAGCTG-3' (forward), and 5'-GCTTCTTGGTGGATGAGCTT-3' (reverse), for human AT1; and 5'-TCCACCACCCTGTTGCTGTA-3' (forward), and 5'-ACCACAGTCCATGCCATCAC-3' (reverse), for G3PDH. The expected sizes of the PCR products for AT1 and G3PDH are 1010 bp and 450 bp, respectively. A 200 ng quantity of total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Toyobo Co., Ltd., Japan) at 42°C for 30 min in a 20 µl mixture in the presence of 25 pmol of oligo(dT)₂₀ primer. A 20 µl amount of the reverse transcribed mixture was subjected to PCR in a 100 µl mixture in the presence of 1 U of KOD Dash[®] (Toyobo Co., Ltd., Japan) and 20 pmol of each primer. PCR was performed for 35 cycles (denaturation at 98°C for 15 s, annealing at 60°C for 3 s and extension at 74°C for 45 s). PCR products (10 µl) were separated in 1.5% agarose gels, stained with SYBR-green (Eugene, USA), and visualized under ultraviolet light.

2.3. Western blot analysis

Lysates were prepared by treating cells with a lysis buffer consisting

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Abbreviations: Ang-II, angiotensin II; AT1, angiotensin II type 1 receptor; AT2, angiotensin II type 2 receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline

of 125 mM Tris-buffered saline (pH 6.8), 2% sodium dodecyl sulfate (SDS), 5% glycerol, 0.003% BPB and 1% β -mercaptoethanol. SDS-PAGE was performed on 10% polyacrylamide gels, and the resolved proteins were transferred to a nitrocellulose membrane. The membrane was blocked with horse serum and incubated with an anti-AT1 rabbit polyclonal antibody (2 μ g/ml; sc-1173, Santa Cruz Biotechnology, USA). After incubating with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, USA), the membrane was developed by Western Blot Chemiluminescence Reagent Plus (NEN[®] Life Science Products, USA), and exposed to autoradiography film (NEN[®] Life Science Products, USA). For neutralization of AT1 antibody, we reacted antibody with a 10-fold excess of peptide antigen (sc-1173P, Santa Cruz Biotechnology, USA).

2.4. Immunocytochemistry and immunohistochemistry

For immunocytochemistry, pancreatic cancer cells were grown on glass coverslips and the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. The cells were washed and incubated for 1 h with the primary antibody at a 1:250 dilution. After washing with PBS, they were incubated for 1 h with FITC-labeled anti-rabbit IgG. The coverslips were washed with PBS, mounted on glass slides and viewed with a confocal laser scanning microscope (TCS-NT, Leica-laser-technik GmbH, Germany). Immunostaining of surgically resected tissues was performed on formalin-fixed, paraffin-embedded tissues using a standard avidin-biotin-peroxidase technique. After treatment by microwaves, endogenous peroxidase activity was suppressed with 0.3% hydrogen peroxide and methanol. The sections were sequentially treated with 5% normal goat serum, primary antibodies (1:250 dilution), biotinylated anti-rabbit IgG, and an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine was used as a chromogen in the presence of hydrogen peroxide. For non-immune staining, we used PBS instead of the primary antibody. Hematoxylin was used as a counterstain. Study specimens were evaluated independently by two investigators (Y.F. and T.S.) and reviewed by a pathologist.

2.5. Receptor binding assay

Binding assays were performed on 96-well plates containing 1×10^4 cells per well. Before the binding assay, the medium was changed to DMEM containing 0.1% FBS for 24 h. Binding was carried out for 1 h at 4°C in 30 μ l of binding medium (Gibco-BRL, USA) containing 0.5% bovine serum albumin with [¹²⁵I]Ang-II (Amersham Pharmacia Biotech Japan, Tokyo, Japan), in the presence of unlabeled Ang-II (Sigma-Aldrich Co., USA) or L-158,809. After incubation, cells were rinsed with binding medium three times at 4°C. The cell layer was then lysed in NaOH (0.1 N), and radioactivity was counted in a γ -counter (Aloka, USA). Each treatment was performed in triplicate.

2.6. Cell proliferation assay

Cells were plated on 96-well plates in DMEM containing 10% FBS for 12 h. The medium was then changed to DMEM containing 0.1% FBS for 24 h. In proliferation assays, the cells were exposed to different concentrations of L-158,809 or vehicle for 24 h. All proliferation assays were examined using a cell counting kit (Dojindo, Japan), and cell proliferation was quantified using a plate analyzer (Toyo Sokki, Japan). Each treatment was performed in triplicate.

2.7. Statistical analysis

Statistical analysis was performed using Scheffé's *F* test. Results are presented as the mean \pm S.E.M. of triplicate samples from at least two independent experiments. A probability level of $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. AT1 expression in pancreatic cancer tissues

We first examined the expression of AT1 in human pancreatic tissues. RT-PCR demonstrated that AT1 mRNA was expressed in six of eight pancreatic cancer tissues (Fig. 1A). In contrast, it was expressed in only one of four normal pancreatic tissues (Fig. 1B). Moreover, immunohistochemical staining revealed that AT1 protein was also expressed in 27 out of 50 cases of pancreatic cancer (Fig. 1C). On the other hand,

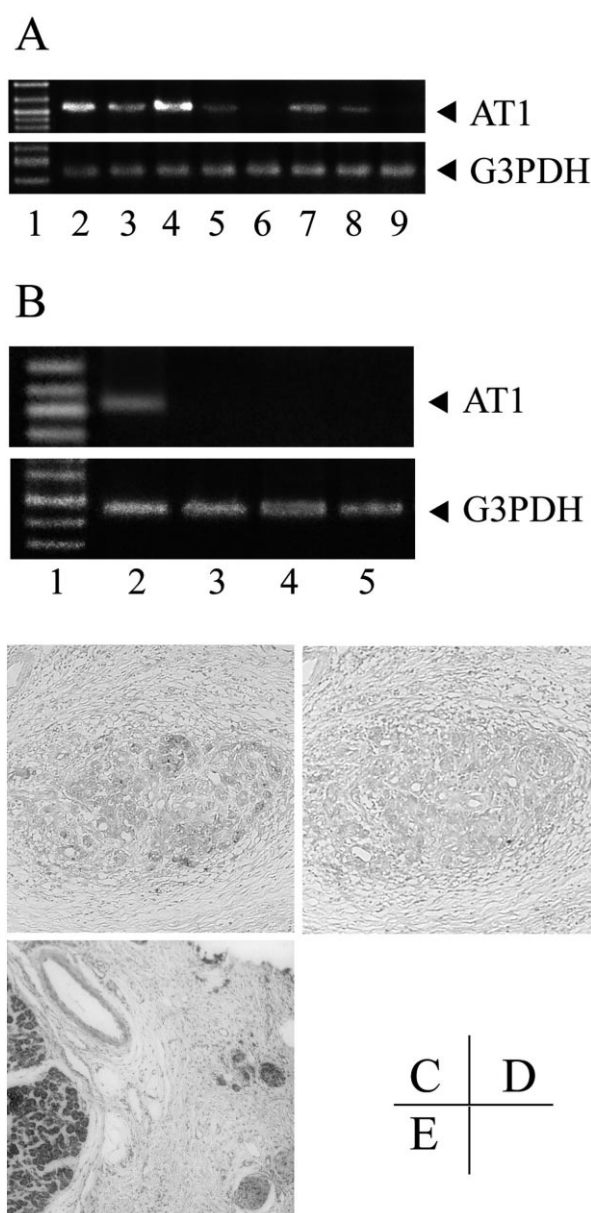


Fig. 1. Expression of AT1 mRNA and protein in pancreatic tissues. A: Total RNA from pancreatic cancer tissues was extracted and amplified by RT-PCR. The arrowhead at the 1010-bp band indicates the position of the AT1 mRNA. The arrowhead at the 450-bp band indicates the position of G3PDH. Lanes are as follows: lane 1, molecular marker; lanes 2–9, pancreatic carcinoma tissues. B: Total RNA from normal pancreatic tissues was extracted and amplified by RT-PCR. Lanes are as follows: lane 1, molecular marker; lanes 2–5, normal pancreatic tissue. C: Expression of AT1. D: Its non-immune staining in human pancreatic carcinoma tissue by immunohistochemistry. E: Expression of AT1 in normal pancreas tissue by immunohistochemistry.

AT1 protein was negligibly expressed in normal pancreatic tissues, and only observed in islands of Langerhans in some cases (Fig. 1E). These findings suggested that AT1 was over-expressed in pancreatic cancer.

3.2. AT1 expression in pancreatic cancer cells

We next investigated the role of AT1 in pancreatic cancer cell lines. As shown in Fig. 2A, the 1010-bp PCR products corresponding to AT1 mRNA were observed in all cell lines.

To elucidate the post-transcriptional regulation of AT1 we next performed Western blot analysis using a specific anti-body. The results demonstrated that 44-kDa proteins were found in all three cell lines, and that those bands disappeared upon treatment with the AT1 blocking peptide (Fig. 2B). We then examined the localization of AT1 protein in these cells using immunofluorescence staining. Immunoreactive green signals were detected in the cell membrane and cytoplasm of these cells (Fig. 2C). No nuclear staining was observed. It is reported that the AT1s are internalized within the cell and recycled to the cell surface [12]. The cytoplasmic localization we observed may contribute to the receptor internalization. In addition, these cellular distributions may depend on the extent of receptor occupancy.

3.3. Receptor binding assay

We next examined the binding function between AT1 in cancer cells and Ang-II. Because the most common type of pancreatic cancer is well-differentiated, we chose to use Capan-2 cells in this binding assay. Exogenous iodinated Ang-II was able to saturate Ang-II receptors on Capan-2 cells (Fig.

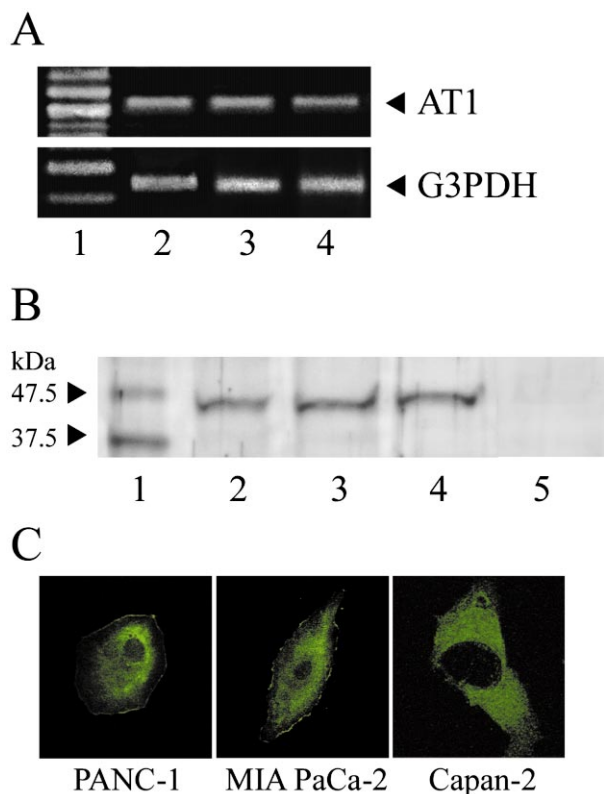


Fig. 2. Expression of AT1 mRNA and protein in three pancreatic carcinoma cell lines. A: Total RNA from pancreatic carcinoma cells was extracted and amplified by RT-PCR. The arrowhead at the 1010-bp band indicates the position of the AT1 mRNA. The arrowhead at the 450-bp band indicates the position of G3PDH. B: Total cell lysates were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and incubated with an AT1-specific antibody. In A and B, lanes are as follows: lane 1, molecular marker; lane 2, PANC-1; lane 3, MIA PaCa-2; lane 4, Capan-2; lane 5, PANC-1 + blocking peptide. C: The expression of the AT1 receptor was demonstrated by immunocytochemistry. Cells were grown on coverslips, fixed and stained with a FITC-labeled anti-AT1 receptor antibody.

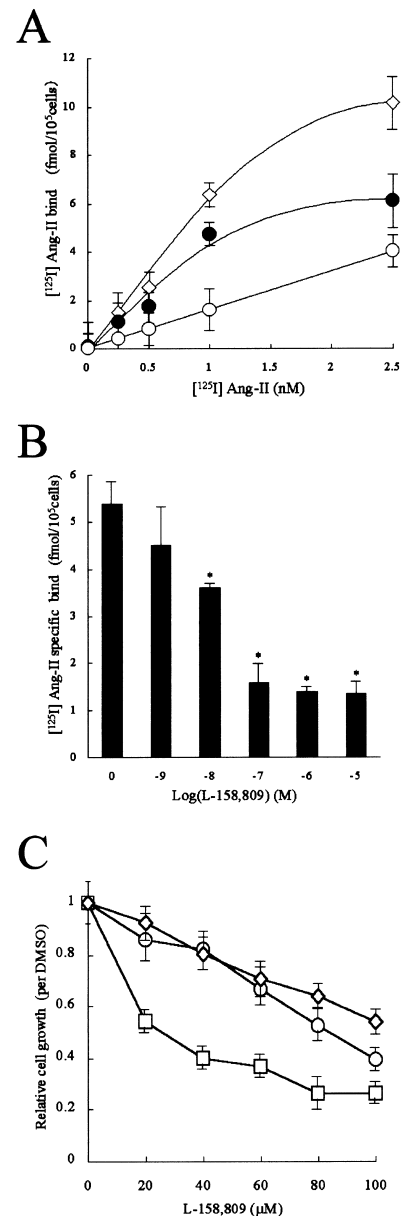


Fig. 3. Binding assay and L-158,809-induced growth suppression on pancreatic carcinoma cell lines. A: The graph shows saturation binding of [¹²⁵I]Ang-II to Capan-2 cells. Specific binding (●) was calculated by subtracting the non-specific binding (○) from the total binding (◇). B: A displacement experiment against the binding of [¹²⁵I]Ang-II on cultured Capan-2 cells was performed. Binding was carried out for 1 h at 4°C in binding medium with [¹²⁵I]Ang-II and L-158,809. The specific binding of [¹²⁵I]Ang-II was dose-dependently competed with L-158,809. *P* values are a comparison between the untreated group and the treated group (**P* < 0.05). C: Cells were exposed to different concentrations of L-158,809. Each point represents a relative growth ratio to control. L-158,809 showed growth inhibition in a dose-dependent manner. PANC-1 (○), MIA PaCa-2 (◇), Capan-2 (□).

3A). The specific binding was calculated by subtracting non-specific binding from total binding. Scatchard plot analysis demonstrated that Capan-2 cell have a specific binding site for Ang-II with an equilibrium binding constant (*K_d*) value of 6.1 nM and a maximum binding capacity (*B_{max}*) of 9 fmol/10⁶ cells (data not shown). Displacement experiments were performed on cultured Capan-2 cells to classify the kinds of

receptor. The specific binding of [125 I]Ang-II was dose-dependently competed with L-158,809 (Fig. 3B). Ang-II binding to cells was suppressed by 74.1% when the dose of inhibitor used was up to 10^{-7} M. These data show that most of the Ang-II binding sites on Capan-2 cells were AT1s.

3.4. Growth inhibition by AT1-selective antagonist

Since pancreatic cancer cells expressed AT1, which was able to bind natural Ang-II, we next examined the effects of AT1 on the growth of pancreatic cancer cells. According to the result of the binding assay, we selected the concentrations of L-158,809 in the range from 10^{-9} to 10^{-6} M. But cell growth was not affected in these ranges. In the range of 0–100 μ M, L-158,809 inhibited growth in a dose-dependent manner (Fig. 3C). The specific IC_{50} values for the reduction in cell proliferation were calculated using each dose–response curve and were determined to be approximately 85 μ M in PANC-1 cells, 30 μ M in MIA PaCa-2 cells, and 120 μ M in Capan-2 cells. These results suggested that the effect of L-158,809 on cell growth was dependent on cellular differentiation. And still more, there is a discrepancy in concentration between binding and growth assays. One of the possible reasons for this discrepancy is that the pancreatic cancer cells used in this study (Capan-2) may have not enough AT1 receptors for L-158,809 to inhibit its growth. Judging from this possibility, it may be reasonable that the higher dose of L-158,809 is necessary to inhibit the growth of Capan-2 cells. In this point of view, further studies will be needed to understand the signal pathways for cell proliferation in Capan-2.

To confirm that the growth inhibition did not interfere with cell viability, the trypan blue exclusion assay and light microscopic observation were performed. The results demonstrated that the proportion of viable cells and cell morphology were not influenced, suggesting that the anti-mitotic activity of L-158,809 is not due to the cytotoxicity of this drug.

It has been suggested that there are some important factors that promote the growth of pancreatic cancer. Gastrin, secretin, cholecystokinin, and epidermal growth factor have been reported to promote the proliferation of pancreatic cancer cells [13,14]. Recently, we have also reported that phospholipase A_2 stimulates the proliferation of pancreatic cancer cells [15]. To the best of our knowledge, there have been no reports investigating the relationship between the angiotensin receptor and cancer growth. In the present study, we have demonstrated that a selective antagonist of AT1 suppresses the growth of human pancreatic cancer. A previous study reported that angiotensin-converting enzyme (ACE) inhibitors exhibit antimitotic activity in pancreatic cancer cells [16]. Furthermore, a clinical retrospective study of the effects of ACE inhibitors on cancer has been reported [17]. Studies by Lever et al. indicated that the incidence of cancer was reduced by continuous intake of an ACE inhibitor, although the anti-tumorigenic mechanisms were not determined sufficiently. Judging from these reports and the results presented here, it is suggested that Ang-II and AT1 play an important role in maintaining the growth of human pancreatic cancer.

In other neoplastic cells and tissues, it was reported that two subtypes of Ang-II receptors are present in various proportions. For instance, AT1s were detected in normal and diseased human breast tissues [10] but most of the Ang-II receptors detected in colorectal cancer cells were AT2 receptors [11]. In the present study we did not examine the other type of receptors for Ang-II. The results of binding assays showed that the specific antagonist of AT1 was not able to completely displace Ang-II from all binding sites. Therefore, further studies will be needed to define the other type of receptor on pancreatic cancer cells.

In conclusion, this is the first study to investigate the expression of AT1 in human pancreatic cancer and to explore the effects of an AT1 antagonist on this cancer. Our data suggest that AT1 is a target for the prevention of pancreatic cancer. Further studies to determine the mechanisms associated with growth inhibition by the AT1 antagonist are needed to understand this process.

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