

Angiotensin II Type 1 Receptor Antagonist Candesartan as an Angiogenic Inhibitor in a Xenograft Model of Bladder Cancer

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Abstract Purpose: There have been several studies on the antitumor activity of angiotensin II type 1 receptor (AT1R) antagonists. In this study, we evaluated the efficacy of the AT1R antagonist candesartan in bladder cancer.

Experimental Design: For the study *in vitro*, human bladder cancer cells (KU-19-19) were cultured with or without angiotensin II and candesartan. Various cytokines and cell viability were analyzed. For the study *in vivo*, a tumor xenograft model was prepared in nude mice using KU-19-19 cells. Mice were given candesartan daily by oral gavage. Microvessel density, expression of vascular endothelial growth factor (VEGF), and apoptosis were assessed.

Results: Candesartan did not induce direct toxicity in KU-19-19 cells, but VEGF and interleukin-8 were significantly lower in candesartan-treated cells (2.55 ± 0.25 and 6.58 ± 0.48 pg/ 10^3 cells) than in the angiotensin II-treated control cells (3.16 ± 0.42 and 7.91 ± 0.69 pg/ 10^3 cells). In mice, candesartan both at doses of 2 and 10 mg/kg/d significantly suppressed tumor growth in mice (35.4% and 33.5% reduction in tumor volume). Microvessel density was significantly decreased by candesartan (9.8 ± 2.8 per field) compared with the control group (17.6 ± 6.0 per field), and VEGF expression was significantly suppressed by this AT1R antagonist. However, candesartan did not induce apoptosis of cancer cells in the tumor.

Conclusions: Specific blockade of AT1R prevented bladder tumor growth by inhibiting angiogenesis. However, its antitumor effect was not due to direct toxicity. Because AT1R antagonists are widely used to treat hypertension, and a 2 mg/kg/d dose level of candesartan is clinically achievable, this AT1R antagonist could also be used to treat bladder cancer.

Bladder cancer is one of the most aggressive epithelial tumors characterized by a high rate of early systemic dissemination; it is the fifth most common cancer in the United States (1). Of the 57,000 new cases of bladder cancer seen annually in the United States, ~30% are diagnosed at their initial presentation as muscle invasive, and in 50% of them, bladder cancer will progress to metastatic disease. Muscle-invasive bladder cancer is therefore a significant cause of morbidity and mortality. Further evidence for its aggressive nature is that only 50% to 60% of such patients survive for up to 5 years after definitive radiation or radical surgical excision (2). Despite the continuous search for and development of even more effective chemotherapies for advanced urothelial carcinoma, only a proportion of patients with advanced disease will respond to therapy. It is thus necessary to identify new targets to treat a greater range of patients.

Local angiogenesis is recognized as necessary for tumor growth, invasion, and metastasis. In addition, microvessel density of the tumor has been proposed as a prognostic factor for several tumors, including bladder cancer (3). Philp et al. reported that the microvessel density of a bladder tumor was a significant predictor of death and also showed a significant correlation with tumor stage (4). Thus, angiogenesis is an attractive new target for novel drugs against bladder cancer.

Concern regarding the potential role of angiotensin II in angiogenesis and promotion of tumor growth has been growing in the past few decades. Angiotensin II is known as a key biological peptide in the renin-angiotensin system, which regulates blood pressure and renal hemodynamics, and angiotensin II type 1 receptor (AT1R) antagonists are widely used as antihypertensive drugs (5). Some tumor cells, such as melanoma, pancreatic cancer, renal cancer, breast cancer, and glioblastoma, have been reported to express AT1R (6–10), and there have been several studies on the potential antitumor effect of AT1R antagonists mediated by its inhibition of angiogenesis in tumors expressing AT1R (6–8, 11). Lever et al. reported the first clinical evidence that long-term angiotensin II blockade might have a protective effect against carcinogenesis (12), and Koh et al. reported genetic polymorphisms of the angiotensin I-converting enzyme gene and the AT1R gene related to risk of breast cancer among Chinese women (9). For these reasons, we tested the effects of the AT1R antagonist candesartan on tumor angiogenesis in bladder cancer. We also examined AT1R expression in several bladder cancer cell lines

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and clinical samples of human bladder cancer, to see whether targeting AT1R for tumor angiogenesis was clinically feasible.

Previous reports have not fully elucidated the mechanism of the antitumor effects of AT1R antagonists. Vascular endothelial growth factor (VEGF) has been reported to be related to angiogenesis promoted by angiotensin II (7, 8), but little is known about the effects of AT1R antagonists on the secretion of the other angiogenic factors by cancer cells. In the present study, we used the bladder cancer cell line KU-19-19, which produces multiple cytokines, including granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor, interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor- β , and β -hCG (13, 14). Therefore, we investigated the effect of the AT1R antagonist candesartan on the secretion of cytokines by KU-19-19 cells, mainly of angiogenic factors, such as basic fibroblast growth factor, IL-6, IL-8, G-CSF, granulocyte macrophage colony-stimulating factor, and VEGF. Furthermore, we sought to determine whether the AT1R antagonist itself induced cytotoxicity and apoptosis in bladder cancer cells.

Materials and Methods

Cell lines and agents. KU-19-19, a cytokine-producing, aggressive, human bladder cancer cell line (13), was mainly used, and bladder cancer cell lines T24, BC47, and MBT-2, which were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), were used to

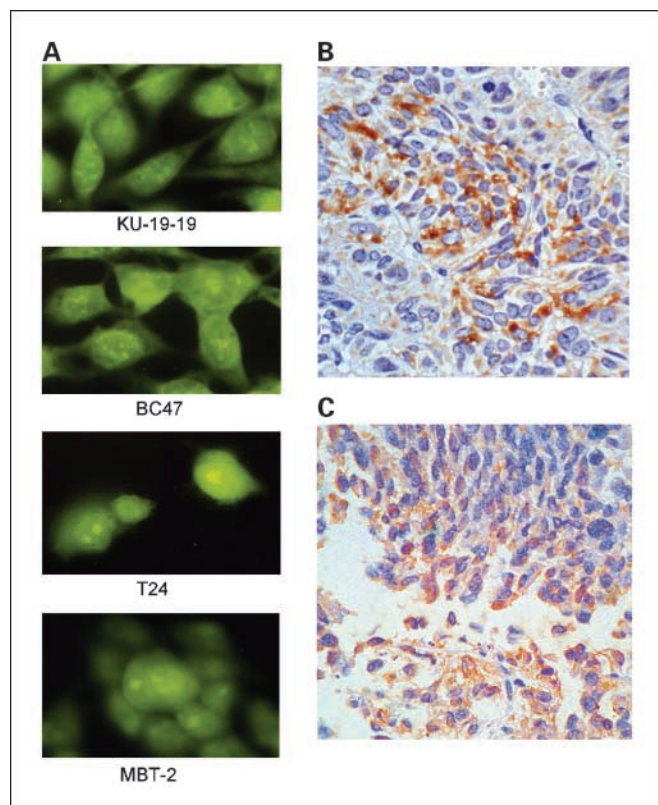


Fig. 1. AT1R expression in bladder cancer. *A*, flow cytometric detection. Typical positive staining of KU-19-19, BC47, T24, and MBT-2 cells. Magnification, $\times 800$. *B*, immunohistochemical analysis of KU-19-19 mice xenograft tumor. Magnification, $\times 400$. *C*, immunohistochemical analysis of human urothelial carcinoma (grade 3, $\geq pT_2$). Magnification, $\times 400$.

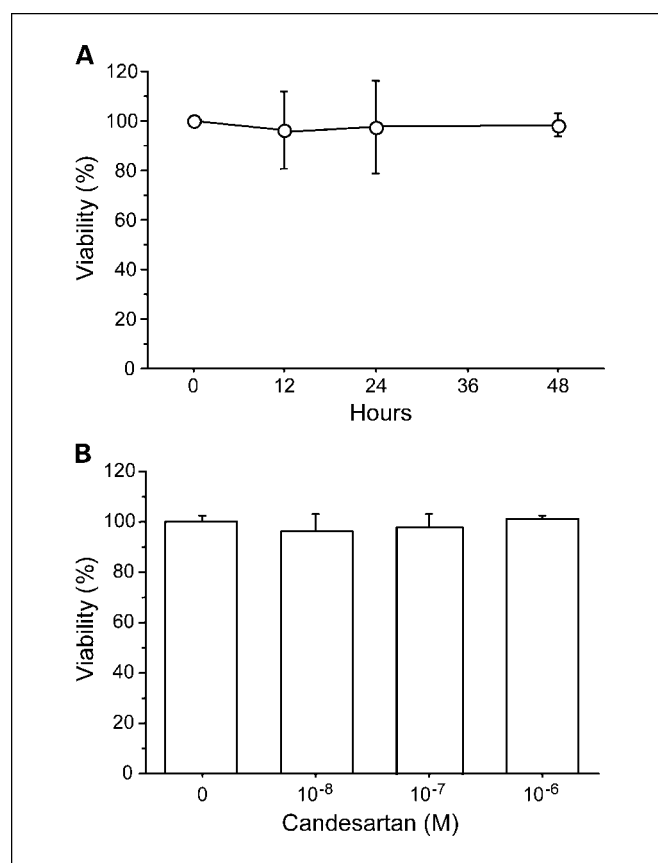


Fig. 2. Cytotoxic effects of candesartan on KU-19-19. Cells were treated at 10^{-7} mol/L of candesartan for various periods of time (*A*) and with various concentrations of candesartan for 48 hours (*B*). Cell viability was assessed by Alamar Blue assay. Points/columns, mean from at least three individual experiments; bars, SE.

examine AT1R expression. These cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Dainippon Pharmaceutical, Tokyo, Japan). The AT1R antagonist candesartan was generously supplied by Takeda Chemical Industries (Osaka, Japan).

Cell growth assay. KU-19-19 cells were seeded at a cell density of 2×10^4 per well in 96-well plates and allowed to grow overnight. Then the cells were treated with various concentrations of candesartan for various periods of time. Cell viability was determined by the Alamar Blue assay to examine the cytotoxicity and antiproliferative effect of candesartan (15). The absorbance value of each well was determined in a microplate reader (Funakoshi, Tokyo, Japan).

Flow cytometric detection for AT1R. After harvesting, cells were fixed with 70% ethanol for 1 hour at 4°C. The primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was applied to the cells for 1 hour at room temperature. Subsequently, cells were incubated with the FITC-labeled secondary antibody for 30 minutes and then subjected to fluorescence microscopy.

Cytokines assays in conditioned medium. KU-19-19 cells were seeded in 60-mm culture dishes at a cell density of 10^5 per dish in 5 mL of culture medium and allowed to attach for 24 hours. Then the medium was replaced with 5 mL of fetal bovine serum-free medium, and angiotensin II and/or candesartan were added at various concentrations to the medium. After culture for various periods of time, all the supernatant in each dish was collected, centrifuged, and stored at -80°C until the assay. The concentration of basic fibroblast growth factor, G-CSF, granulocyte macrophage colony-stimulating factor, IL-6, IL-8, and VEGF in the cultured medium was determined by a double-ligand ELISA assay (Quantikine, R&D Systems, Wiesbaden, Germany).

Murine xenograft model of bladder cancer. Nude athymic BALB/c mice, 6 weeks of age with an average body weight of 20 g, were obtained from Sankyo Lab Service Co. (Tokyo, Japan). KU-19-19 cells (2×10^6 cells), suspended in 50 μ L of Matrigel (Becton Dickinson Labware, Lincoln Park, NJ), were implanted s.c. in the flank of each nude mouse. The mice were assigned to three groups, each consisting of ~20 animals. The mice were administered 2 or 10 mg/kg candesartan daily by gavage, starting on the day of the cell implantation. These dose levels were chosen because they had proved effective in our previous pulmonary metastasis model of murine renal cancer (8). The animals were carefully monitored, and the tumor was measured twice a week. The tumor volume (V) was calculated according to the formula $V = AB^2 / 2$, where A is the greatest diameter, and B is the diameter perpendicular to A . On the 28th day, the animals were killed, and the s.c. tumors were harvested. All animal procedures were carried out according to the government guidelines for animal experiments, and a protocol was reviewed and approved by the animal care committee of our institution.

Immunostaining of the tumors for AT1R, CD34, and VEGF. Formalin-fixed, paraffin-embedded tissue sections (4 μ m) were deparaffinized, rehydrated, and washed in PBS. Endogenous peroxidase was quenched. A blocking step was included using 1% bovine serum albumin together with avidin and biotin blocking solutions. The tissues were incubated with any of the following primary antibodies: anti-CD34 monoclonal antibody (Nichirei, Tokyo, Japan), anti-VEGF polyclonal antibody (Neomarkers, Fremont, CA), and anti-AT1R polyclonal antibody (Santa Cruz Biotechnology), at 4°C overnight. A biotinylated secondary antibody was applied, and then the tissues were incubated with the avidin-biotin peroxidase complex, and color was developed with diaminobenzidine. After washing, the slides were counterstained with 10% hematoxylin for 1 to 2 minutes.

Microvessels in the tumor were counted after immunostaining with anti-CD34 monoclonal antibody. Five microscopic fields at the highest magnification from three different specimens were examined for the presence of microvessels, and microvessel density was determined as the average number of microvessels per $\times 200$ field.

The intensity of VEGF staining in tumor tissues was graded on a scale of 0 to +3, with 0 indicating no detectable staining and +3 indicating the strongest staining. Immunopositivity of tumor tissues for VEGF was assessed at a high power ($\times 400$) by two independent investigators in a blinded fashion (8).

Apoptosis was measured by terminal deoxynucleotidyl transferase-mediated nick-end labeling assay using the commercially available Apoptosis *in situ* detection kit (Wako, Osaka, Japan). The average number of stained cells was counted, and apoptosis index was calculated as the average number of five areas in a $\times 400$ field.

Clinical samples of bladder cancer. Archival paraffin-embedded sections were obtained from 52 patients who had undergone transurethral resection of bladder cancer between January 1994 and December 2001. There were 45 men and 7 women, with a mean age of 66.6 years, and histologic type was urothelial carcinoma in all of them.

Statistical analysis. Data are expressed as the mean value \pm SE. One-way ANOVA combined with Bonferroni's t test was used to compare each variable. $P < 0.05$ was considered significant. χ^2 test was also used to analyze differences in the score used for semiquantitative evaluation.

Results

AT1R expression in bladder cancer cell lines and clinical samples. We confirmed the expression of AT1R in bladder cancer cells, KU-19-19 xenograft tumors, and human samples by flow cytometry (Fig. 1A) and immunohistochemistry (Fig. 1B). AT1R was clearly detected by flow cytometry in KU-19-19, BC47, T24, and MBT-2 cells (Fig. 1A); in KU-19-19 xenograft tumors, AT1R was expressed mainly in the cytoplasm of the cells and epithelial cells of microvessels (Fig. 1B). We also observed that 17 (32.7%) of 52 tumor specimens from patients with bladder cancer expressed AT1R (Fig. 1C).

Cytotoxic effect of candesartan on KU-19-19 cells. As shown in Fig. 2A and B, candesartan did not affect cell viability or proliferation *in vitro*, regardless of the duration (12-48 hours) of treatment at the concentrations (10^{-8} to 10^{-6} mol/L) employed in this study.

Effect of candesartan and angiotensin II on cytokines secretion by KU-19-19 cells. At the 12-hour time point, KU-19-19 cells were found to have spontaneously secreted IL-8, G-CSF, VEGF, granulocyte macrophage colony-stimulating factor, IL-6, and basic fibroblast growth factor (Table 1). Under angiotensin II stimulation, the secretion of IL-8, G-CSF, and VEGF increased in relation to the concentration of angiotensin II (Fig. 3A). VEGF and IL-8 in the cultured medium of cells treated with 10^{-7} mol/L candesartan (2.55 ± 0.25 and 6.58 ± 0.48 pg/ 10^3 cells) were significantly lower than those in the control group (3.16 ± 0.42 and 7.91 ± 0.69 pg/ 10^3 cells) under exposure to 10^{-7} mol/L angiotensin II (Fig. 3B), but secretion of the other cytokines was not significantly affected (Table 1).

Effects of candesartan on KU-19-19 tumor growth in nude mice. As shown in Fig. 4A and B, the growth of engrafted

Table 1. Effects of angiotensin II and the AT1R antagonist candesartan on the secretion of cytokines by KU-19-19 cells (mean \pm SE, pg/ 10^3 cells)

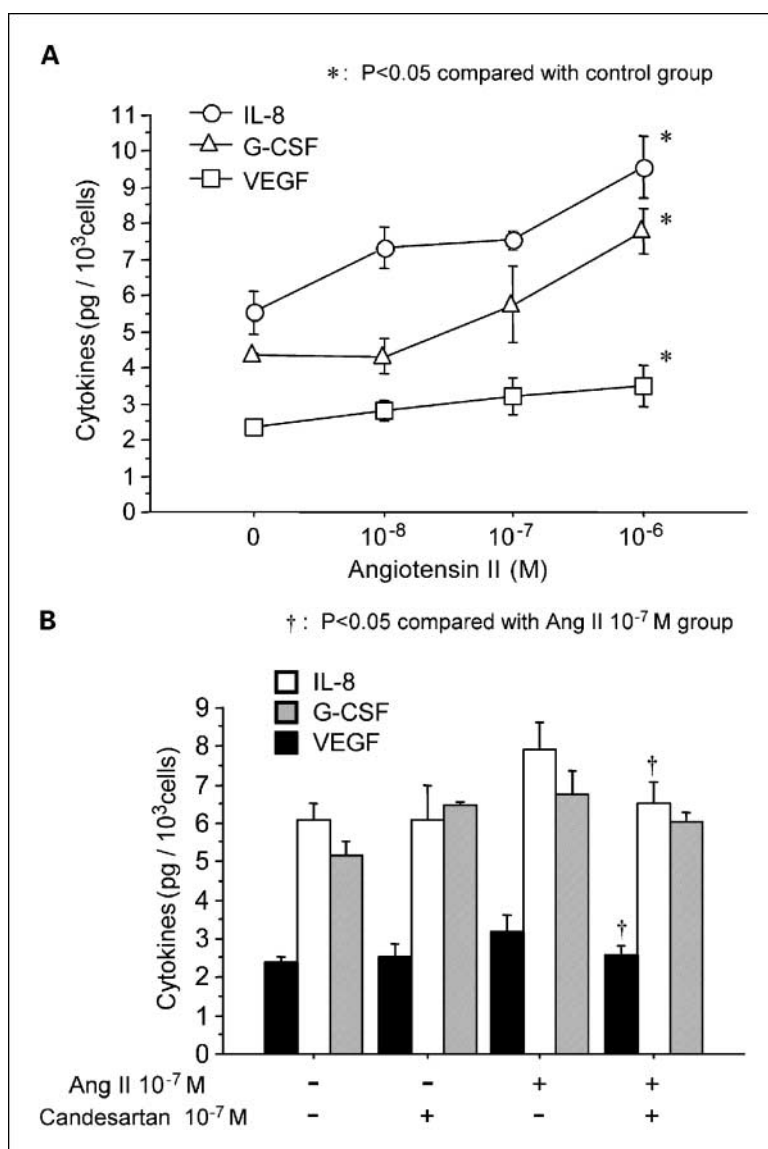
| | Control | Candesartan | Angiotensin II | Angiotensin II + candesartan |
|--------------------------------|-----------------|-----------------|------------------|------------------------------|
| Angiotensin II 10^{-7} mol/L | – | – | + | + |
| Candesartan 10^{-7} mol/L | – | + | – | + |
| IL-8 | 6.28 \pm 0.60 | 6.08 \pm 1.44 | 7.56 \pm 0.62* | 6.58 \pm 0.63 [†] |
| G-CSF | 4.83 \pm 0.22 | 6.47 \pm 0.10 | 6.47 \pm 0.22* | 6.06 \pm 0.10 |
| VEGF | 2.38 \pm 0.21 | 2.44 \pm 0.30 | 3.10 \pm 0.42* | 2.55 \pm 0.32 [†] |
| GM-CSF | 0.04 \pm 0.02 | 0.08 \pm 0.01 | 0.11 \pm 0.02 | 0.15 \pm 0.03 |
| IL-6 | 0.04 \pm 0.14 | 0.04 \pm 0.01 | 0.03 \pm 0.01 | 0.05 \pm 0.02 |
| bFGF | 0.02 \pm 0.00 | 0.01 \pm 0.00 | 0.02 \pm 0.00 | 0.01 \pm 0.00 |

Abbreviations: GM-CSF, granulocyte macrophage colony-stimulating factor; bFGF, basic fibroblast growth factor.

* $P < 0.05$ compared with the control group.

[†] $P < 0.05$ compared with the angiotensin II 10^{-7} mol/L group.

Fig. 3. Effects of angiotensin II and candesartan on IL-8, G-CSF, and VEGF secretion by KU-19-19 cells. **A.** stimulation of cytokine secretion with various concentrations of angiotensin II and candesartan (10^{-8} to 10^{-6} mol/L, for 12 hours). Points, mean; bars, SE. **B.** suppression of cytokine secretion with 10^{-7} mol/L candesartan under exposure to 10^{-7} mol/L angiotensin II (*Ang II*) for 12 hours. Columns, mean; bars, SE.



tumors was significantly inhibited in the candesartan groups (35.4% and 33.5% reduction in tumor volume at 2 and 10 mg, respectively; $P < 0.0001$) compared with the control group at the 28th day of treatment. Significant differences in tumor volume were observed between the control and candesartan treatment groups as early as on the 13th day of treatment; however, the reduction rates did not differ significantly between the two candesartan groups throughout the treatment period.

Effects of candesartan on tumor angiogenesis, VEGF secretion, and apoptosis. Blood vessels in the tumors from the control mice showed well-developed vascular networks. In contrast, the vessels in the tumors of candesartan-treated mice formed poorly developed networks (Fig. 5A, *a* and *b*). In the candesartan groups, microvessel density was significantly lower than that in the control group (2 mg: 11.6 ± 0.4 , $P < 0.01$, $n = 5$; 10 mg: 9.79 ± 0.6 , $P < 0.01$, $n = 5$ versus 17.6 ± 1.0 in the control, $n = 5$; Table 2). VEGF expression in the candesartan group was also lower than in the control group (1.8 ± 0.83 versus 2.4 ± 0.54 ; Fig. 5B, *a* and *b*; Table 2). However, the apoptotic index of the

tumor in the 10^{-7} mol/L candesartan group (31.2 ± 7.3) did not differ significantly from that in the control group (30.8 ± 8.5 ; Fig. 5C, *a* and *b*; Table 2).

Discussion

The results of our study showed that specific blockade of AT1R prevented the angiogenesis and growth of xenograft tumors developed by human bladder cancer cells. Angiogenesis is a key process involved in pathologic conditions, such as tumor growth and metastasis of bladder cancer (3, 4). Hence, understanding the mechanism implied in the angiogenic reaction is important for the development of new therapies. Although angiotensin II has been known to promote tumor growth and angiogenesis, the blockade of angiotensin II has been considered a noteworthy anticancer and antiangiogenesis therapeutic option in recent years (16). However, the role of angiotensin II in angiogenesis is still controversial.

First, we confirmed AT1R expression in some bladder cancer cell lines (KU-19-19, BC47, T24, and MBT-2) and clinical tissue

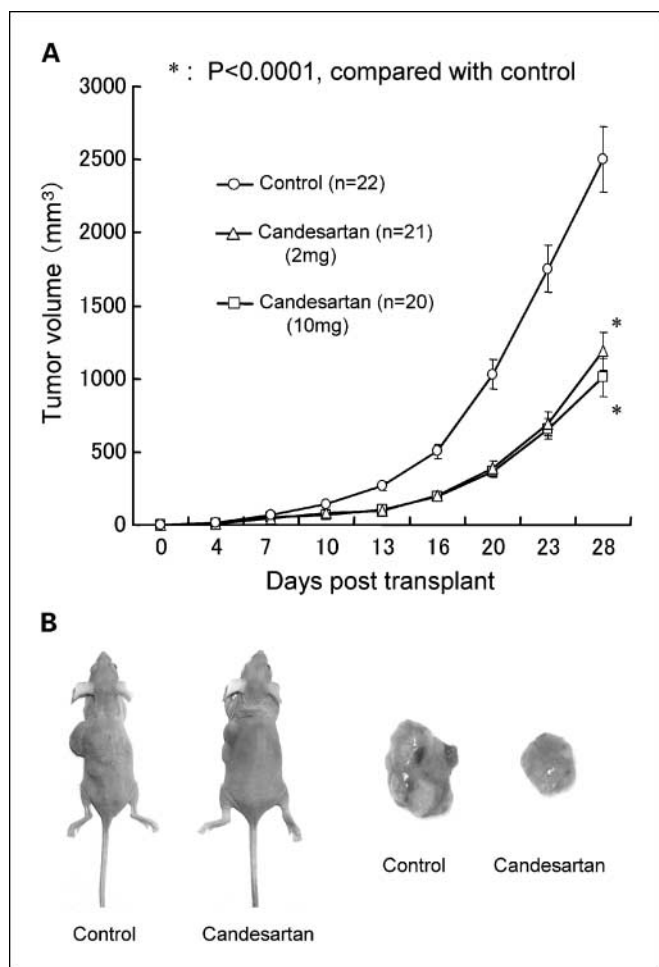


Fig. 4. Efficacy of candesartan on tumor growth in a mouse xenograft model of bladder cancer. KU-19-19 cells (2×10^6) were implanted in the flank of nude mice. *A*, from the day of implantation and for 28 days, 2 or 10 mg/kg candesartan was given once daily by gavage ($n = 22$, control group; $n = 21$, 2 mg/kg candesartan group; $n = 20$, 10 mg/kg candesartan group). Points, mean tumor volume (mm^3); bars, SE. *B*, gross findings of the tumor from a control mouse (*left*) and a 10 mg/kg candesartan-treated mouse (*right*) harvested on the 28th day.

specimens, as well as in some other cancers (6–11). The results encouraged the validity of an AT1R antagonist therapy for many types of bladder cancer. Immunostaining of KU-19-19 xenograft tumors revealed AT1R expression in the cytoplasm of tumor cells and epithelial cells of microvessels. We did not detect any differences in AT1R intensity in the tumor between control and candesartan-treated mice; thus, we suggest that the AT1R antagonist did not affect AT1R expression in our study. On the other hand, Fujita et al. reported AT1R antagonist-induced down-regulation of AT1R in the endothelial cells of microvessels in pancreatic cancer. And they suggested that this AT1R down-regulation might weaken the angiogenic and tumor-proliferative effect of angiotensin II (11). Because the cellular characteristics and metabolic requirements of bladder cancer differ from those of other tumors, and because these cancers arise from different sources, the difference in AT1R expression observed in each type of cancer should not be a surprise.

Angiogenesis is a complex process, and a large number of factors are involved in tumor angiogenesis. The present study was undertaken to investigate the effects of the AT1R antagonist

candesartan on the secretion of cytokines, especially of angiogenic factors. We have already shown that candesartan prevents pulmonary metastasis of murine renal cancer by inhibiting tumor angiogenesis through the suppression of VEGF (8). Indeed, in this study, we observed that candesartan suppressed VEGF in human bladder cancer *in vivo* and in KU-19-19 cells *in vitro*. We also observed that candesartan suppressed VEGF secretion under exposure to angiotensin II, although angiotensin II increased cellular VEGF secretion in bladder cancer cells. Interestingly, we found that IL-8 secretion was increased by angiotensin II and was suppressed by candesartan the same as with VEGF. Ito et al. investigated the relationship between IL-8 and angiotensin II and showed that candesartan suppressed IL-8 secretion in human vascular smooth muscle cells (17). In the oncologic field, this was the first report of AT1R antagonist-induced suppression of IL-8 secretion in a bladder cancer cell line. VEGF and IL-8 are two of the most important cytokines that regulate angiogenesis (18) and are recognized as important prognostic factors of bladder cancer and some other cancers (3, 19, 20). In this study, G-CSF

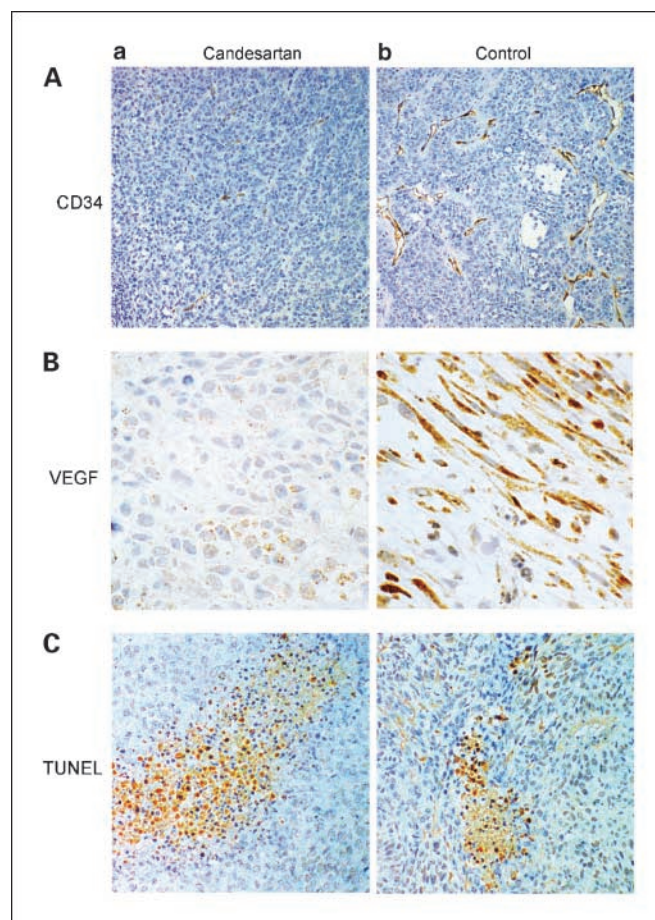


Fig. 5. Immunohistochemical study of KU-19-19 xenograft tumors from mice treated with candesartan. *A*, vascular staining of KU-19-19 xenograft tumors. Magnification, $\times 200$. *a*, CD34 expression in the 2 mg/kg candesartan-treated group. *b*, CD34 expression in the control group. *B*, VEGF expression in KU-19-19 xenograft tumors. Magnification, $\times 400$. Representative areas in tissue sections from tumors of the 2 mg/kg candesartan-treated group (*a*) and the control group (*b*). *C*, apoptosis was investigated using the terminal deoxynucleotidyl transferase-mediated nick-end labeling assay. Magnification, $\times 200$. *a*, representative section from the 2 mg/kg candesartan-treated group. *b*, representative section from the control group.

Table 2. Tumor volume, microvessel density, VEGF intensity, and apoptotic index in mice xenograft tumors from mice treated with candesartan harvested after 28 days of treatment (mean \pm SE)

| Treatment | Tumor volume (mm ³) | Microvessel density (per field) | VEGF intensity | Apoptosis index |
|------------------------|---------------------------------|---------------------------------|----------------------------|-----------------|
| Control | 2,762 \pm 802 | 17.6 \pm 1.0 | 2.4 \pm 0.5 | 30.8 \pm 8.5 |
| Candesartan (2 mg/kg) | 1,181 \pm 171* | 11.6 \pm 0.4 [†] | 1.8 \pm 0.8 [†] | 30.0 \pm 4.6 |
| Candesartan (10 mg/kg) | 1,022 \pm 176* | 9.8 \pm 0.6 [†] | 0.7 \pm 0.6 [†] | 31.2 \pm 7.3 |

*P < 0.0001 compared with control.
[†]P < 0.01 compared with control.

secretion was obviously stimulated by angiotensin II in KU-19-19 cells *in vitro*. However, we did not detect a significant suppression of G-CSF secretion by the AT1R antagonist. Obermueller et al. showed that constitutive expression of G-CSF induced the transformation of a benign tumor into a malignant and strongly angiogenic tumor, and that G-CSF enhanced persistent angiogenesis (17). Natori et al. showed that G-CSF markedly promoted the growth of colon cancer associated with enhancement of neovascularization *in vivo*, whereas G-CSF had no effect on cancer cells proliferation *in vitro* (21). Granulocyte macrophage colony-stimulating factor, IL-6, and basic fibroblast growth factor were not affected by angiotensin II stimulation or candesartan treatment in our study. Taken together, interruption of these cytokines may inhibit angiogenesis and hence tumor growth and metastasis (22). Therefore, AT1R antagonists may exert an antitumor effect through the suppression of several angiogenic cytokines, such as VEGF and IL-8, and its consequent inhibition of multistep angiogenesis. Further investigation is necessary to prove this hypothesis.

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