Angiotensin II receptor blocker shows antiproliferative activity in prostate cancer cells: A possibility of tyrosine kinase inhibitor of growth factor

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Abstract

Angiotensin II (A-II) receptor (AT_1 receptor) blockers (ARB) are a class of antihypertensive agent. It is known that they suppress signal transduction pathways mediated by growth factors [*e.g.*, epidermal growth factor (EGF)] through the AT_1 receptor in vascular endothelial cells. In the present study, we demonstrated that A-II activates the cell proliferation of prostate cancer as well as EGF. In addition, we showed that A-II induces the phosphorylations of mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 3 (STAT3) in prostate cancer cells. In contrast, ARB was shown to inhibit the proliferation of prostate cancer cells stimulated with EGF or A-II, the mechanism of which is through the suppression of MAPK or STAT3 phosphorylation by ARB. Oral administration of ARB to nude mice inhibited the growth of prostate cancer xenografts in both androgen-dependent and androgen-independent cells in a dose-dependent manner. Microvessel density was reduced in xenografts treated with ARB, which means ARB inhibits the vascularization of xenografts. Expression of AT_1 receptor mRNA was examined by reverse transcription-PCR using 10 pairs of human prostate cancer and normal prostate tissues. AT_1 receptor expression in human prostate cancer tissue was higher (9 of 10 cases) than that in normal prostate tissue. These results suggest the possibility that ARB is a novel therapeutic class of agents for prostate cancer, especially hormone-independent tumors. (Mol Cancer Ther. 2003;2:1139–1147)

Introduction

Hormone therapy for prostate cancer is based on the theory of inducing cancer cell death (apoptosis) by decreasing serum androgen levels and blocking the androgen receptor (AR), resulting in the slowing of disease progression. Initial hormone therapy generally provides good efficacy against prostate cancer with a high response rate of 80–90%. However, most cases receiving hormone therapy develop resistance to the treatment within several years. To date, although various kinds of therapies for patients with hormone-refractory cancer have been studied, no effective therapy has been reported.

Refractory prostate cancer has been attributed to factors that include amplification or point mutations of AR (1) in addition to the existence of AR cofactors (2). Other possible factors include the secretion of various growth factors in an autocrine or paracrine loop, specifically epidermal growth factor (EGF) (3), keratinocyte growth factor (4), fibrosis growth factor (5), and insulin-like growth factor (6). After binding to these growth factors, their cognate receptors transmit signals through the tyrosine kinase domain and initiate signal transduction cascades critical for cell growth or differentiation. In this respect, tyrosine kinase inhibitors (7) or antagonists of growth factor receptors (8) as molecular targeted drugs have recently been developed and given to patients with refractory prostate cancer.

Angiotensin II (A-II) is well known to be associated with hypertension as a main effector peptide of the renin-angiotensin system, and the molecular mechanisms have recently been elucidated. For example, the A-II directly activates not only the mitogen-activated protein kinase (MAPK) but also the Janus tyrosine kinase-signal transducer and activator of transcription (STAT) pathway through AT_1 receptor in smooth muscle cells and cardiac myocytes (9, 10). Furthermore, A-II activates the collagen I gene through the MAPK or extracellular signal-regulated protein kinase pathway (11) and mediates angiogenesis and the transcription of growth-related factors through the AT_1 receptor (12) as a result. These effects are mostly inhibited by AT_1 receptor blockers (ARB), which have been given as antihypertensive agents.

The objective of the present study was to gain an insight into the cellular events after A-II binding to its receptors and leading to prostate cancer cell proliferation. To this end, we examined in prostate cancer cells the involvement of the signal transduction pathways, MAPK and STAT3, which are thought to be among the major mediators of the mitogenic action of A-II and other growth factors related to cancer cell proliferation. In addition, we investigated whether these effects occurred in prostate cancer cells *in vitro* or *in vivo* using an ARB.

Materials and Methods

Tissue Samples and Cell Lines

Paired tissue samples of human prostate cancer and normal prostate tissue were obtained by total prostatectomy for prostate cancer at Yokohama City University Hospital

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(13). Briefly, after removal, tissue samples were immediately frozen and stored at −80°C until the experiments. Informed consent was obtained from the patients for the samples to be used in this study. Human prostate cancer cell lines (LNCaP, PC3, and DU145 cells) were obtained from the American Type Culture Collection (Rockville, MD). LNCaP and PC3 cells were cultured in F-12 medium and DU145 cells were cultured in MEM medium supplemented with 10% FCS under 3.5% CO_2. In the experiments of this study, these cells were cultured in phenol red-free RPMI + 0.1% bovine serum albumin (BSA) and stimulated with reagents.

Reagents

A-II, PD123319, and EGF were purchased from Sigma (Atlanta, USA), and candesartan (CV11974 and TCV116) was provided by Takeda Pharmaceutical Co. (Osaka, Japan). CV11974 is the active metabolite of TCV116 and used for *in vitro* experiment. TCV116 is the prodrug of CV11974 and used for *in vivo* experiment. Recombinant interleukin-6 (IL-6) was purchased from R&D Systems, Inc. (Minneapolis, MN). Antiphospho-MAPK antibody, anti-MAPK antibody, antiphospho-STAT3 antibody, and anti-STAT3 were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Cell Growth Analysis

Cell growth was estimated by counting the cell number using a microcellcounter (Toha Co., Tokyo). Briefly, LNCaP and DU145 cells were seeded onto 12-well plates at a density of 10^4 to 10^5 cells/well. Cells were cultured in phenol red-free RPMI + 0.1% BSA for 18–24 h before experiments and then treated with A-II or EGF for 5 days. Simultaneously, the cells were pretreated by CV11974 for 4 h and cultured in phenol red-free RPMI + 0.1% BSA in the presence of A-II or EGF for 5 days. After incubation in 3.5% CO_2 at 37°C, cells were harvested with trypsin and cell numbers were determined by a cell counter on day 5.

RNA and Reverse Transcription-PCR

LNCaP, DU145, and PC3 cells were cultured in their respective media and harvested for reverse transcription-PCR. Total RNA of cells or prostate tissues was extracted using Isogen (Nippon Gene, Tokyo) and then converted into cDNA by Moloney murine leukemia virus reverse transcriptase. PCR of the AT_1 receptor and β-actin was performed under the following conditions: denaturing for 30 s at 95°C, annealing for 30 s at 55°C for AT_1 or 60°C for β-actin, and elongation for 30 s at 72°C, with a total of 30 cycles for AT_1 and 27 cycles for β-actin. The sequences of oligonucleotides as forward or reverse primers of AT_1 and β-actin were as follows:

AT_1: forward 5′-GTAGCCAAAGTCACCTGCATC-3′ and reverse 5′-CAGTCACGTATGATGCCTAGT-3′ and β-actin: forward 5′-TAATACGACTCACTATAGG-GAGAGCGGGAAATCGTGCGTGACATT-3′ and reverse 5′-GATGGAGTTGAAGGTAGTTTCGTG-3′.

PCR products (10 μl) were loaded on 1.5% agarose gel containing ethidium bromide. Semiquantitation of AT_1 receptor mRNA was performed in pairs of tumor and normal tissues. In brief, each band of AT_1 receptor mRNA on the agarose gel, which was normalized by β-actin, was analyzed using NIH imaging software.

Western Blot Analysis

LNCaP and DU145 cells were cultured in phenol red-free RPMI + 0.1% BSA for 2 days before experiments. Then, cells were harvested after A-II, EGF, or IL-6 treatment as indicated in the figures. Cells were pretreated with CV11974 for 4 h, stimulated with the reagents, and harvested at indicated times in the figures. Cells under the appropriate conditions were washed twice with ice-cold PBS, lysed in ice-cold buffer consisting of 20-mM Tris (pH 8.0), 137-mM NaCl, 10% glycerol, 0.1% SDS, 0.5% NP40, 100-mM sodium fluoride, 200-mM sodium orthovandate, 1-mM EGTA, 2-mM phenylmethylsulfonyl fluoride, 1-mg/ml leupeptin, and 3-mg/ml aprotinin and centrifuged (30 min, 4°C, 14,500×*g*). Following quantitation, 30 μg of each cell lysate were added to SDS gel-loading buffer (containing a reducing agent) and boiled for 5 min. The samples were subjected to SDS-PAGE on 12% gel and electrotransferred to Immobilon-P purchased from Millipore (Bedford, MA). After blocking the membrane with 5% albumin, Western blotting was performed using the antibody of interest and the product was detected with an enhanced chemiluminescence detection system (Amersham Bioscience Co., Piscataway, NJ).

Antitumor Activity of CV11974 in Nude Mice

The antitumor activity of TCV116 (an AT_1 receptor antagonist) was determined in athymic nude mice bearing DU145 tumors. DU145 cells (5×10^6) were injected into the flank region of athymic nude mice (4–6 weeks old), and treatment was started on day 10 when the tumor measured 5 mm in diameter. Each mouse received one of two different doses of TCV116 (2.5 or 5.0 mg/kg/day). Each group consisted of 10 animals. The control group received only the diluent. LNCaP cells (10^7) mixed with Matrigel (Becton Dickinson, Franklin Lakes, NJ) were injected into the flank region of athymic nude mice. Treatment was started on day 9 after the inoculation of tumor. Each mouse received one of two different doses of TCV116 (5.0 or 10 mg/kg/day). Each group consisted of five animals. The control group received only the diluent. Tumors were measured with a caliper every 7 days. The volume of the tumor was calculated using the formula: tumor volume (mm^3) = length × $(\text{width})^2$ × 0.5. Each tumor volume on the first day when each mouse received treatment was expressed as a relative tumor volume of 1.0.

Microvessel Density

To assess the density of blood vessels, immunohistochemical staining for mouse CD31 was performed according to a previous report (14) with minor modifications. Briefly, frozen nude mouse xenografts embedded in OCT compound (Sakura Finetechnol. Co. Ltd., Tokyo) were cut with a cryostat. Sections were fixed with ice-cold acetone for 5 min. After drying, the sections were immersed in 0.3% hydrogen peroxide-containing methanol to inactivate intrinsic peroxidase followed by treatment with 10%

normal goat serum. Then, the sections were treated with rat antimouse CD31 antibody (PharMingen, San Diego, CA, diluted to 1:100) at 4°C overnight. The labeled antigen was visualized by streptavidin-biotin complex method followed by diaminobenzidine reaction. Two investigators counted the microvessels independently in a blinded fashion. The tissues were examined at high power (400×), and the four fields with the highest microvessel density (MVD) were identified for vessel count. The mean number of CD31-positive vessels in the four selected fields (high-power field, 40× objective and 10× ocular, 0.185 mm^2/field) was used to express the vascular density (15).

Statistics

Values are given as means ± SD. For the results of cell number and xenograft volume, group data were compared by unpaired Student's *t* test. AT_1 expression in prostate tissue was compared between cancer and normal tissues using Mann-Whitney's *U* test. A *P* value of <0.05 was considered statistically significant.

Results

ARB Inhibited Prostate Cancer Cell Growth

To determine whether the AT_1 receptor is expressed in human prostate cancer cells, we analyzed AT_1 receptor mRNA level in LNCaP, PC3, and DU145 cells. As shown in Fig. 1A, the AT_1 receptor was most strongly expressed in LNCaP cells and was moderately expressed in DU145 cells. We therefore used LNCaP and DU145 cells in the present study.

To investigate the effect of A-II on human prostate cancer cells, we applied it with its cognate receptor blocker, CV11974, a selective blocker for the AT_1 receptor. As shown in Fig. 1, B and C, A-II treatment increased the number of prostate cancer cells, LNCaP (androgen-dependent cell) and DU145 (androgen-independent cell), in a dose-dependent manner. Furthermore, CV11974 significantly suppressed the cell growth induced by A-II treatment in both cell lines.

Next, we investigated the effect of CV11974 on human prostate cancer cells when these cells were treated with EGF, an important factor for the growth and development of cancer. EGF treatment increased the cell number of both prostate cancer cell lines (Fig. 2, A and B). Interestingly, when CV11974 was added to cells treated with EGF, growth was suppressed in both cell lines as shown in Fig. 2. In LNCaP cells, cell number was decreased by 20% for 5 days by ARB treatment. Similarly, DU145 cell proliferation induced by EGF was suppressed by 26% with CV11974 treatment as shown in Fig. 2B. When EGF and A-II were simultaneously added in the media of LNCaP cells, the cell growth was synergistically increased in comparison with EGF or A-II treatment alone (data not shown).

We investigated whether another ARB, AT_2 receptor blocker, could suppress cell growth induced by A-II or EGF treatment. PD123319, a specific blocker of AT_2 receptor, was added to LNCaP cells with EGF or A-II treatment. Unlike the action of an ARB, the cell proliferation was not inhibited and was conversely activated by PD123319 treatment as shown in Fig. 2C.

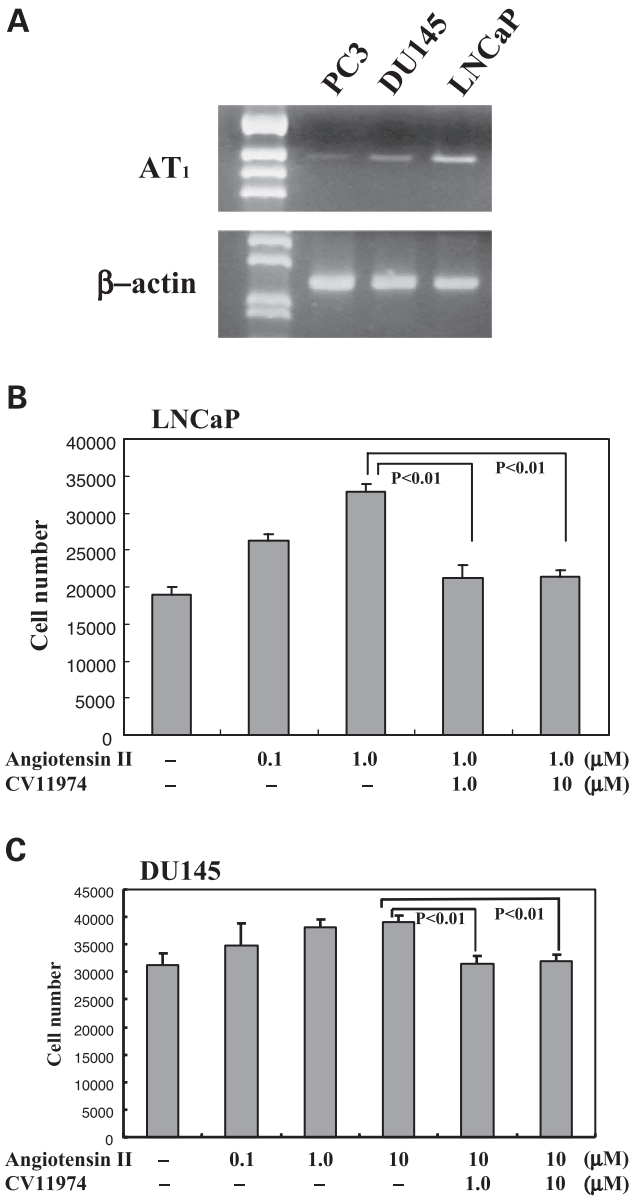

Figure 1. AT_1 receptor expression in prostate cancer cells and inhibition of cell proliferation by ARB in LNCaP and DU145 cells. **A,** total RNA from prostate cancer cell lines (LNCaP, DU145, and PC-3) was extracted and AT_1 receptors were detected by reverse transcription-PCR. **B,** LNCaP cells were cultured in phenol red-free RPMI + 0.1% BSA in the presence of A-II (0, 0.1, and 1.0 μM) for 5 days. Simultaneously, LNCaP cells were pretreated with 1.0 or 10 μM CV11974 for 4 h and cultured in phenol red-free RPMI + 0.1% BSA in the presence of 10-μM A-II for 5 days. **C,** DU145 cells were cultured in phenol red-free RPMI + 0.1% BSA in the presence of A-II (0, 0.1, 1.0, and 10 μM) for 5 days. Simultaneously, DU145 cells were pretreated with 1.0 or 10 μM CV11974 for 4 h and cultured in phenol red-free RPMI + 0.1% BSA in the presence of 10-μM A-II for 5 days. After 5 days of stimulation, all cell numbers were counted with a hemocytometer. $P < 0.01$, $n = 4$.

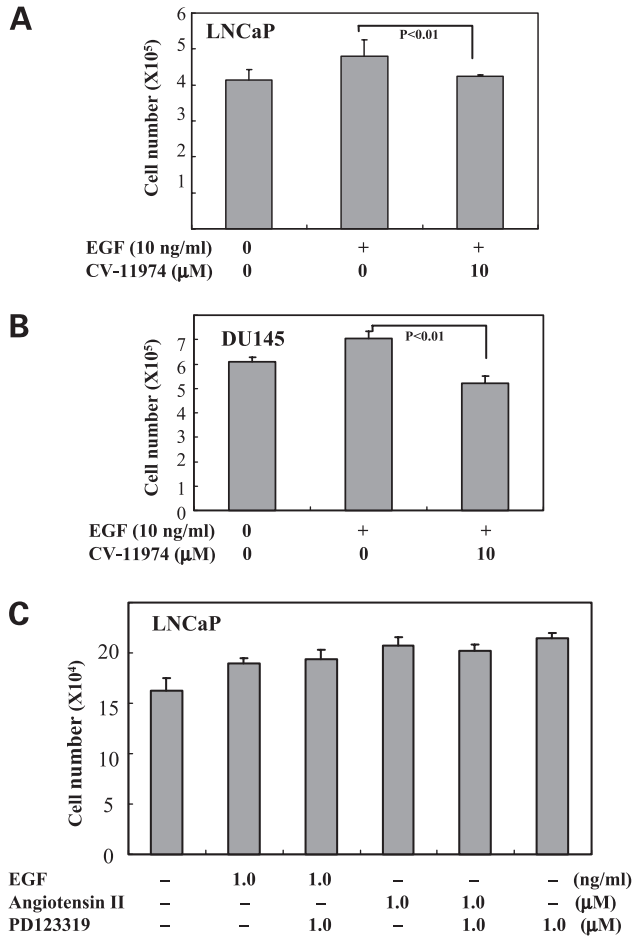

Figure 2. Cell proliferation of DU145 and LNCaP stimulated with EGF and ARB. **A** and **B,** cells were cultured in phenol red-free RPMI + 0.1% BSA in the presence of 10-ng/ml EGF for 5 days. Simultaneously, cells were pretreated with 10-μM CV11974 for 4 h and cultured in phenol red-free RPMI + 0.1% BSA in the presence of EGF for 5 days. **C,** LNCaP cells were cultured in phenol red-free RPMI + 0.1% BSA in the presence of A-II (0 and 1.0 μM) or EGF (0 and 1.0 ng/ml) for 5 days. Simultaneously, these cells were pretreated with 1.0-μM PD123319 for 4 h and cultured in phenol red-free RPMI + 0.1% BSA in the presence of 1.0-μM A-II or 1.0-ng/ml EGF for 5 days. After 5 days of stimulation, viable cells were counted. *Columns,* mean of four experiments; *bars,* SD. $P < 0.01$.

Therefore, only AT_1 selective blocker has the potential to suppress cell proliferation treated with A-II or EGF.

AT_1 Receptor Antagonist Blocked A-II-Stimulated or EGF-Stimulated Signaling in Prostate Cancer Cells

We investigated whether treatment with A-II induced the activation of signal transduction pathways in LNCaP cells. Western blot analysis showed that A-II stimulation increased tyrosine phosphorylation levels of proteins (at least four proteins; data not shown) following stimulation with EGF, which is well known to enhance the cell proliferation of prostate cancer cells through various signal transduction systems. As the next step, we investigated whether A-II could induce the activation of MAPK and STAT3 in LNCaP cells, which have important roles in signaling to mediate cell proliferation induced by EGF stimulation. MAPK was activated immediately after stimulation with A-II as shown in Fig. 3A. Similarly, A-II induced much stronger phosphorylation of STAT3 than that induced by EGF (Fig. 3A). Additionally, CV11974 suppressed in a dose-dependent manner the activation of MAPK and STAT3 induced by A-II stimulation (Fig. 3B).

To investigate whether CV11974 can suppress the activation of signal transduction pathways by EGF, we carried out Western blotting of phosphorylated MAPK or STAT3. Fig. 3C shows that CV11974 suppressed their phosphorylation. More interestingly, this phosphorylation induced by IL-6, which is known to be associated with cell proliferation of androgen-independent prostate cancer, was suppressed by CV11974 (Fig. 3D).

Based on the above observations in LNCaP cells, we then performed Western blot analyses to investigate

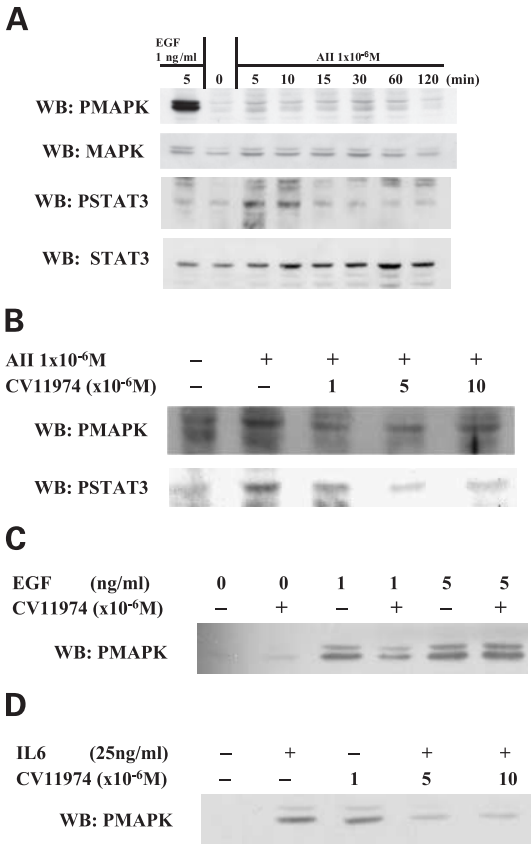

Figure 3. Activation of signal transduction pathways by A-II and suppression by ARB in LNCaP cells. Cells were cultured with phenol red-free RPMI + 0.1% BSA for 2 days before experiments. **A,** cells were harvested at the indicated times after 1.0-μM A-II or 1-ng/ml EGF exposure. The cells were lysed, and detergent extracts were immunoblotted with phospho-MAPK or phospho-STAT3 antibodies. **B,** cells were pretreated with 1.0, 5.0, and 10 μM CV11974 for 4 h and harvested at 15 min after 1.0-μM A-II exposure. The cells were lysed, and detergent extracts were immunoblotted with phospho-MAPK or phospho-STAT3 antibodies. **C,** cells were pretreated with 1.0-μM CV11974 for 4 h and harvested at 15 min after 1.0 or 5.0 ng/ml EGF. **D,** cells were pretreated with 1.0, 5.0, and 10 μM CV11974 for 4 h and harvested at 15 min after 25-ng/ml IL-6 exposure. The cells were lysed, and detergent extracts were immunoblotted with phospho-MAPK antibodies.

whether these phenomena also occur in DU145 cells. As shown in Fig. 4A, A-II treatment showed clear induction of phosphorylated MAPK, which peaked after 10 min of treatment. To determine whether CV11974 can block the A-II-induced phosphorylation of MAPK in DU145 cells, further Western blot was performed. In cells pretreated with CV11974 for 4 h, phosphorylation of MAPK by 10-μM A-II treatment was inhibited by CV11974 in a dose-dependent manner as shown in Fig. 4B. We next examined whether MAPK phosphorylation induced by EGF treatment is suppressed by CV11974 in DU145 cells in a similar way to that in LNCaP cells. As expected, when DU145 cells were pretreated with CV11974, MAPK phosphorylation induced by EGF treatment was reduced (Fig. 4C).

We conclude from these data that CV11974 can down-regulate the A-II/EGF signaling pathways in both androgen-dependent and androgen-independent prostate cancer cells.

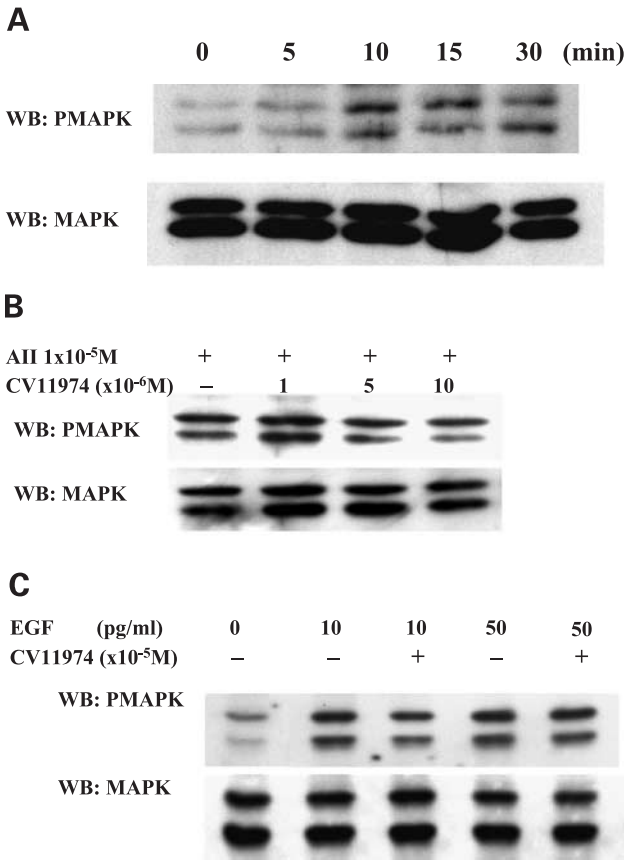

Figure 4. Immunoblots showing the induction of MAPK phosphorylation by A-II in DU145 cells. Cells were cultured with phenol red-free RPMI + 0.1% BSA for 2 days before experiments. **A,** cells were harvested at 0, 5, 10, 15, and 30 min after 10-μM A-II exposure. The cells were lysed, and detergent extracts were immunoblotted with the indicated antibodies. **B,** cells were pretreated by 1.0, 5.0, and 10 μM CV11974 for 4 h and harvested after 15 min of 10-μM A-II exposures. The cells were lysed, and detergent extracts were immunoblotted with the indicated antibodies. **C,** cells were pretreated with 10-μM CV11974 for 4 h and harvested at 15 min after 10 and 50 pg/ml EGF exposure. The cells were lysed, and detergent extracts were immunoblotted with phospho-MAPK antibody.

Antitumor Activity of ARB

To determine whether the *in vitro* antiproliferative activity of an ARB could be translated to antitumor activity *in vivo*, TCV116, an ARB, was given in athymic nude mice with tumor xenografts of DU145 cells. TCV116 is the prodrug of CV11974 and was used only for *in vivo* experiments. When the tumors reached about 5 mm in diameter, the animals were given TCV116 at 2.5 or 5.0 mg/kg/day. The control group received water containing sodium hypochlorite (10 ppm). As shown in Fig. 5A, at 4 weeks, control animals had developed large tumors of 27 ± 12.3 relative volume compared with those at 0 week. Mice treated with TCV116 at 2.5 or 5.0 mg/kg/day showed inhibition of tumor relative volume at 4 weeks by 13.1 ± 3.5 or 7.1 ± 5.9, respectively. There were statistically significant differences in tumor relative volume between control and TCV116-treated mice (2.5 mg/kg/day: $P < 0.05$ and 5.0 mg/kg/day: $P < 0.01$, respectively).

To investigate whether these observations *in vivo* are also shown in androgen-dependent cells, LNCaP cells were established as xenografts in nude mice. Mice were treated from day 9 with TCV116 of 5.0 or 10 mg/kg/day. As shown in Fig. 5B, there was a significant difference in tumor growth between control and 10-mg/kg/day group ($P < 0.05$) as early as day 16. Furthermore, there were significant differences in tumor growth between control and TCV116-treated mice (5 and 10 mg/kg/day; $P < 0.05$) on day 23. Thus, TCV116 could suppress tumor growth of not only androgen-independent DU145 cells but also androgen-dependent LNCaP cells.

Early reports have demonstrated that A-II induced angiogenesis in the rabbit cornea (16), embryonic chorioallantoic membrane (17), and rat cremaster muscle (18). In the present study, we confirmed the antitumor effect of an AT_1 receptor antagonist; hence, we measured MVD of xenografts in mice treated with TCV116. Immunohistochemical staining for mouse CD31 revealed a marked difference in microvessel numbers of xenografts between control and TCV116-treated mice as shown in Fig. 5C. MVD was quantitated in three xenografts each in the control and treatment groups. As shown in Fig. 5D, the TCV116 treatment group had a reduced mean MVD of 37.5 ± 12.4 compared with a mean value of 72.5 ± 9.7 in the control group ($P < 0.01$).

AT_1 Receptor Expression in Human Prostate Adenocarcinoma

To determine whether the AT_1 receptor is expressed in human prostate tissue, we analyzed AT_1 mRNA levels in prostatic adenocarcinomas and adjacent normal prostate tissue obtained from 10 patients who underwent radical prostatectomy. As shown in Fig. 6A, the AT_1 receptor was expressed in both normal and malignant tissues obtained from the same patients. Next, we performed semiquantitated reverse transcription-PCR analysis, which indicated that AT_1 mRNA level was significantly higher ($P < 0.01$) in tumors (9 of 10 tumors or 90%) than in normal tissue (Fig. 6B). We then analyzed AT_1 receptor levels in metastatic lymph nodes and bones of

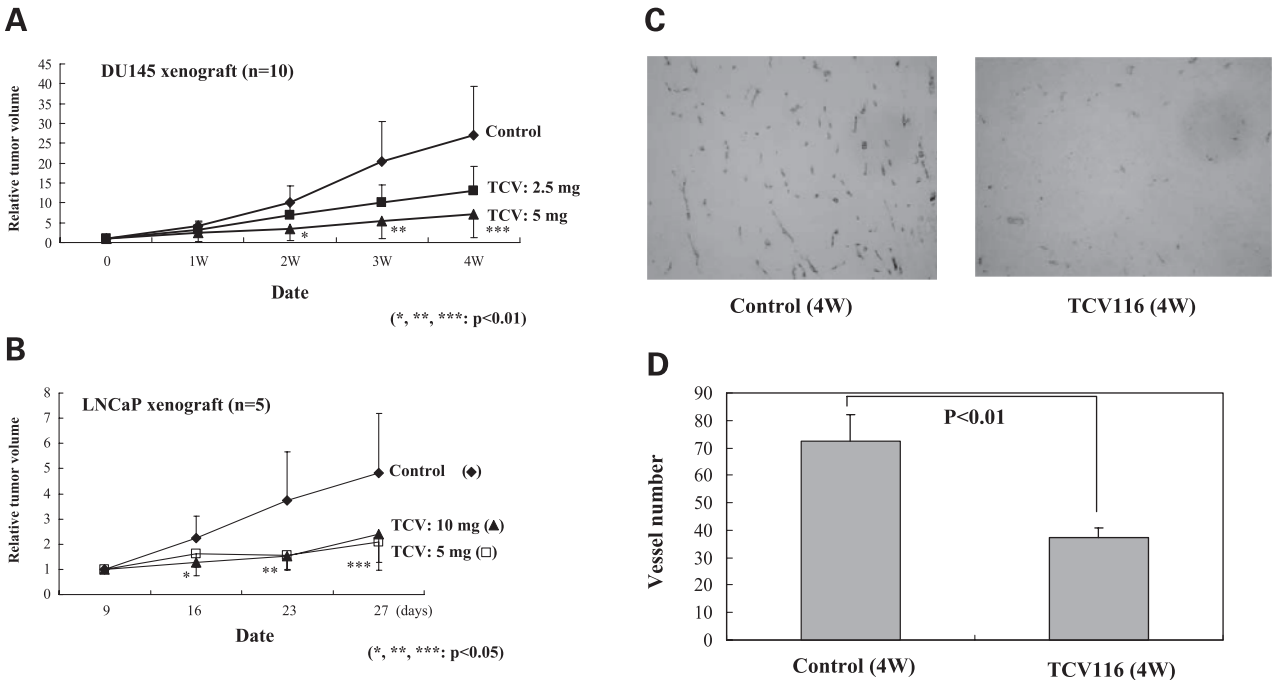

Figure 5. Antitumor activity and inhibition of tumor angiogenesis by ARB (TCV116). **A,** tumor growth of DU145 xenografts was measured weekly. Mice were given TCV116 [■ 2.5 mg/kg/day p.o., ▲ 5.0 mg/kg/day p.o., ◆ no treatment (control)]. The number of nude mice in each group was 10. *, **, *** $P < 0.01$, relative tumor volume of the 5.0-mg/kg/day TCV116 group was significantly different from that of the control group. **B,** tumor growth of LNCaP xenografts was measured at indicated times. Mice were given TCV116 [□ 5.0 mg/kg/day p.o., ▲ 10 mg/kg/day p.o., ◆ no treatment (control)]. The number of nude mice in each group was 5. *, **, *** $P < 0.05$, relative tumor volume of the 10-mg/kg/day TCV116 group was significantly different from that of the control group. **C,** immunohistochemical staining for mouse CD31 showed a difference in MVD between tumors of control (4 weeks) and 5.0-mg/kg/day TCV116-treated mice (4 weeks). **D,** mean values of tumor MVD were blotted from three tumors each in control (4 weeks) and 5.0-mg/kg/day TCV116-treated mice (4 weeks). $P < 0.01$.

hormone-refractory cases. Their AT_1 mRNA levels were higher than those of tumor tissues obtained at operation from patients who had not been treated preoperatively (data not shown).

Discussion

Although there is an apparent low cancer prevalence in hypertensive patients receiving angiotensin-converting enzyme inhibitors (19), the molecular mechanisms have never been elucidated. We here present strong evidence of the effects of A-II and ARB on prostate cancer cells and the tumor growth of prostate cancer. Our present data clearly indicate that A-II enhanced the proliferation of prostate cancer cells through AT_1 receptor-mediated activation of MAPK and STAT3 phosphorylation. Cell proliferation was induced by A-II in androgen-dependent and androgen-independent cancer cells (LNCaP and DU145), and the kinetics were similar to those of another growth factor (EGF) previously observed. This study provides evidence that the mitogenic action of A-II in prostate cancer cells is meditated by the MAPK or STAT3 cascade. Furthermore, evaluation of mRNA expression of the AT_1 receptor by reverse transcription-PCR confirmed A-II-induced activation of mitogenic signal transduction pathways in prostate cancer.

These *in vitro* and *in vivo* data, which indicate that an ARB could inhibit the cancer cell proliferation and angiogenesis, encouraged us to examine the effects of this drug on patients with hormone-refractory prostate cancer (HRPC). Indeed, about half of the patients experienced a sustained prostate-specific antigen (PSA) decrease or stabilization by the treatment. In one patient, candesartan administration resulted in a marked size reduction of lung metastatic lesions, with a marked fall in PSA (data not shown).

A-II is known to have a variety of effects mainly on the cardiovascular system (20, 21). A-II activates not only the MAPK but also the Janus tyrosine kinase-STAT pathway directly through the AT_1 receptor in smooth muscle cells and cardiac myocytes (9, 10). Fujiyama *et al.* (12) reported that A-II transactivated the EGF receptor (EGFR) via the AT_1 receptor and induced angiogenesis by enhancement of the angiogenic activity of vascular endothelial growth factor (VEGF). Furthermore, an *in vivo* corneal assay demonstrated the inhibition of angiogenesis by an ARB.

In a progressive renal injury model, AT_1 receptor antagonist treatment improved renal dysfunction, with reduced gene expression of transforming growth factor-β1 (TGF-β1; 22). Besides its action on the cardiovascular system, ARB could limit the progression of hepatic fibrosis associated with a decrease in the expression of TGF-β (23). Intriguingly, TGF-β1 is known to be one of specific cytokines

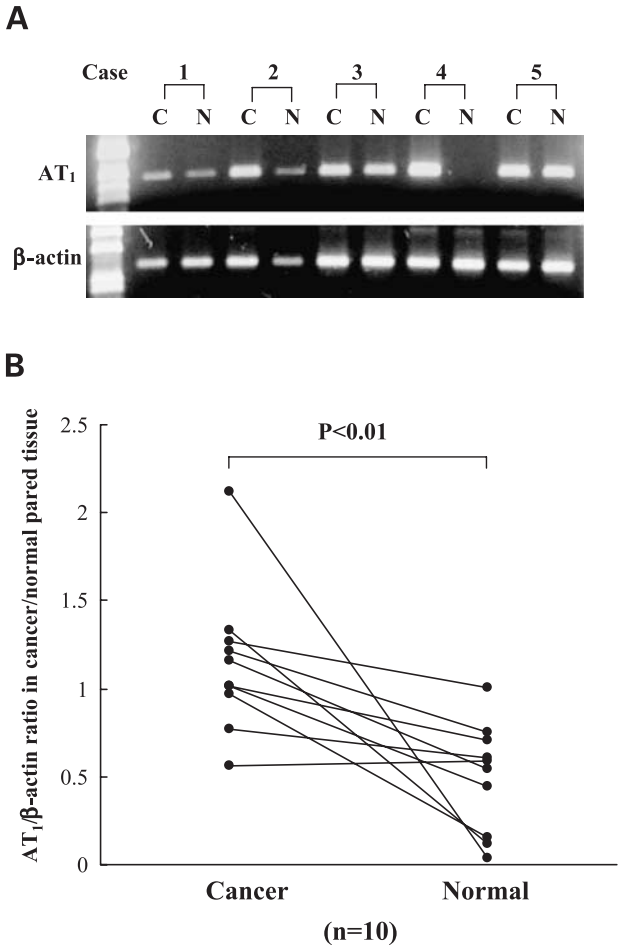

Figure 6. Detection of AT_1 receptors in prostate cancer tissue. **A,** total RNA was extracted from the prostate of untreated patients and AT_1 receptors were detected in pairs of tumor and normal tissues by reverse transcription-PCR. **B,** semiquantitation of AT_1 receptor expression in pairs of tumor and normal tissues by reverse transcription-PCR analysis using NIH imaging software. All expression levels were corrected by β-actin expression and showed differences between prostate cancer and normal tissues. AT_1 expression was compared between cancer and normal tissues using Mann-Whitney's *U* test. $P < 0.01$.

associated with the growth of prostate cancer. In particular, elevated plasma TGF-β1 has been reported in patients with invasive or metastatic prostate cancer (24, 25). Furthermore, TGF-β1 has been demonstrated by immunohistochemical staining to accumulate in primary and metastatic prostate tissues (26–28). An ARB, therefore, has the potential to suppress the development of prostate cancer by the inhibition of TGF-β1 expression.

In various neoplastic cells and tissues, two subtypes of A-II receptors, AT_1 and AT_2, have been detected. AT_1 receptors were detected in pancreatic cancer and breast cancer tissues as well as in both normal tissues (29, 30). In addition, in colorectal cancer cells, AT_2 receptors were mostly present (31). Recently, two reports concerning tumor growth inhibition by an ARB in cancer cells were published. One report showed that pancreatic cancer cells expressed the AT_1 receptor, and cell growth was significantly suppressed by treatment with an ARB in a dose-dependent manner (29). The other report demonstrated the presence of AT_1 receptors on C6 glioma cells and the effect of an ARB on the growth and angiogenesis of C6 rat glioma (32). Recently, Egami *et al.* demonstrated that the host A-II-AT_1 receptor pathway in mice was strongly associated with angiogenesis of tumor growth induced by VEGF from tumor-associated macrophages (33). As the next step, a clinical trial using an ARB as adjuvant treatment might be planned for malignant tumors with AT_1 receptor expression.

We suggest several factors through which ARB could inhibit the tumor growth of metastatic lesions in HRPC. (1) Prostate cancer cells and tissues have AT_1 receptors, especially abundant in refractory prostate cancer including metastatic lesions, from the results of reverse transcription-PCR analyses. (2) Because HRPC cells secrete various growth factors and cytokines as paracrine or autocrine factors (*e.g.*, EGF, IL-6, and tumor necrosis factor-α), ARB could suppress the signal transduction by these growth factors or cytokines. (3) A-II was shown to augment angiogenesis (17, 18) and cause up-regulation of heparin binding-EGF expression (34). Thus, ARB treatment could suppress tumor angiogenesis as demonstrated in the present study. (4) In prostate cancer, TGF-β promotes tumor progression by stimulating angiogenesis and metastasis (35). Interestingly, it was reported that an ARB could suppress the expression of TGF-β (36). Hence, ARB probably exhibit efficacy against the angiogenic properties of TGF-β, particularly in AT_1 receptor-rich lesions.

Several recent studies indicated that prostate stromal cells contained the AT_1 receptor, and the cell number was increased by A-II treatment (37). It is well known that prostatic stromal cells, especially fibroblasts, are involved in the development of HRPC accompanied by secretion of several growth factors (38–41). We confirmed that A-II facilitated the secretion of several growth factors and cytokines from prostatic stromal cells.[1] In contrast, ARB could suppress the secretion of cytokines from these cells. Therefore, A-II may be involved in the development of HRPC; furthermore, the local renin-angiotensin system may play a role in the mechanism controlling the development of HRPC.

We indeed observed prolonged stabilization or a decrease of PSA values over a long period by candesartan treatment. If candesartan directly affected PSA synthesis in tumor cells, the PSA changes would occur immediately after the start of treatment. To confirm whether this drug interacts with PSA gene transcription or secretion by human prostate cancer LNCaP cells, we investigated the changes in PSA secretion. As a result, we observed no suppression of PSA level by treatment with androgen and

[1] H. Uemura, H. Ishiguro, and Y. Kubota, unpublished data.

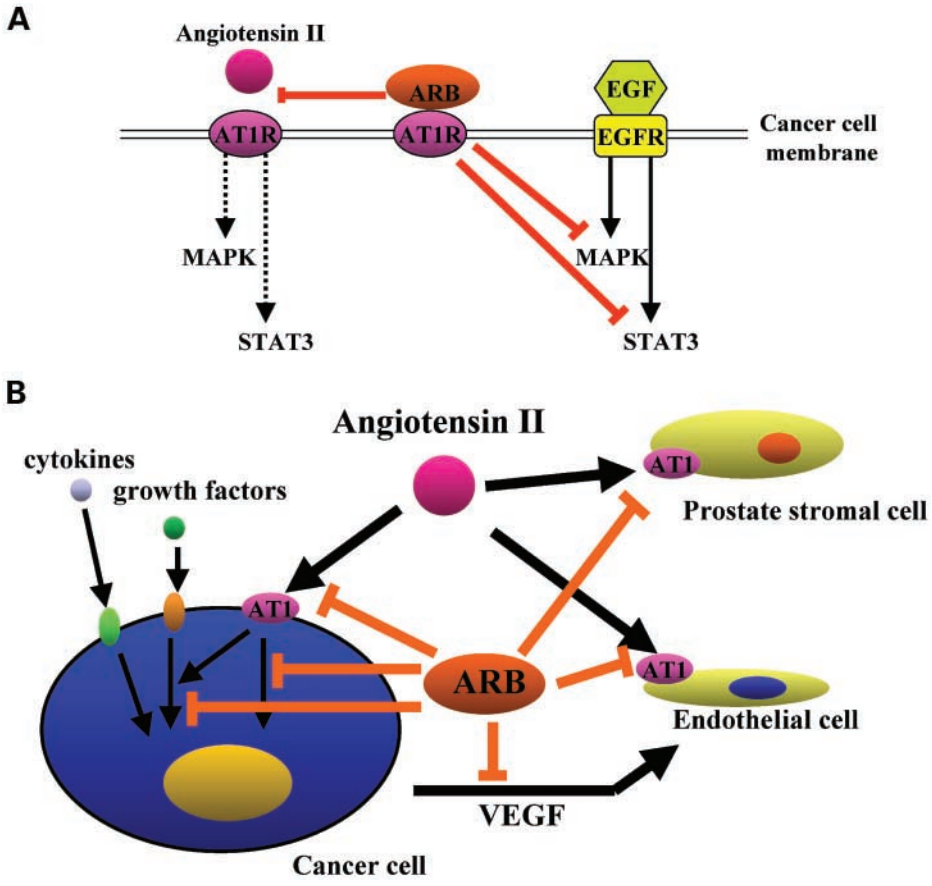

Figure 7. Putative mechanisms of ARB at multiple sites in prostate cancer tissue. **A,** ARB suppresses cell proliferation of prostate cancer by interaction with signal transduction via EGFR, which means ARB suppresses the phosphorylation of MAPK or STAT3, or by blocking A-II binding to AT_1 receptor. **B,** A-II activates cell proliferation of vascular endothelial, prostate stromal, and cancer cells. ARB inhibits cell proliferation induced by A-II and suppresses MAPK and STAT3 phosphorylation activated by growth factors (EGF, etc.) or cytokines (IL-6, etc.). In addition, ARB probably inhibits vascularization by inhibition of VEGF production in prostate cancer cells. From these results, ARB is suggested to affect multiple sites in prostate cancer tissue, resulting in modulation of tumor growth.

an ARB. In addition, we speculate that the reason it required a long time to observe the effects of this drug is based on its pharmacological effects. Candesartan has unique properties in that it binds selectively to the AT_1 receptor and disassociates very slowly (42). Additionally, if an ARB suppresses angiogenesis by inhibiting VEGF expression in metastatic tumors, in practice, it would take a long time to observe a decrease in serum PSA.

Of importance in this study is the fact that the mechanisms of ARB action are relatively well understood in vascular cells, and we need to know whether these phenomena also occur in prostate cancer cells. As depicted in Fig. 7A, ARB could indeed inhibit the cell proliferation of prostate cancer by interaction with signal transduction via the EGFR or by blocking A-II binding to the AT_1 receptor. In addition, we speculate that A-II is involved in several pathways in the development of prostate cancer and that ARB interacts with these pathways as depicted in Fig. 7B. Briefly, treatment with an ARB might reduce the secretion of growth factors or cytokines (*e.g.*, TGF-β and IL-6) by prostate stromal cells as a paracrine loop. Furthermore, ARBs might influence vascular endothelial cells, leading to suppression of tumor angiogenesis.

Although we do not think the dose of candesartan used in this study was enough to control the tumor growth of prostate cancer completely, we believe that this agent has a novel ability to suppress it. Further studies are needed to investigate the molecular mechanisms of AT_1 receptor antagonists in hormone-independent prostate cancer cells and extend their application to a clinical trial of treatment for the advanced stages of prostate cancer.

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