INTRODUCTION

Prostate cancer has become the most common malignancy in men in western countries such as Europe and the United States while its incidence is low in Asian countries. It has been estimated that there will be approximately 240,890 new cases of prostate cancer and 33,720 deaths from prostate cancer in the United States in 2011 [1], and the prevalence of prostate cancer has also been increasing in Japan [2]. Androgen ablation therapy is widely accepted and carried out for prostate cancers because androgens are essential for the development and growth of normal prostate and prostate cancer cells [3]. However, outgrowth of hormone-independent cancer cells occurs within several years and eventually leads to a fatal outcome in many cases [4].

Accumulating evidence suggests that the renin-angiotensin system (RAS) is involved in maintenance of blood pressure as well as progression of various cancers, such as breast, lung, kidney, stomach, colorectum, ovary, and bladder [5]. Angiotensin II is a main effector molecule of the RAS and is an octapeptide hormone with diverse biological activity through binding to typical G protein-coupled receptors, angiotensin II receptor type 1 (AT1R) and type 2 (AT2R). AT1R is expressed in diverse adult tissues and mediates cell proliferation, migration, angiogenesis, and inflammatory responses via G protein-dependent and independent signaling including the MAPK and STAT signal pathways [6]. AT2R is predominantly expressed at a high level in the fetus, and its expression is low in adult tissues, being detectable in heart, kidney, pancreas, adrenal gland, uterus, ovary, and brain [7]. In contrast to AT1R, AT2R has been shown to exert an antagonistic effect against many AT1R-mediated actions [5,8]. Recent studies have demonstrated that an angiotensin II receptor blocker (ARB) has the potential to decrease serum prostate-specific antigen (PSA) level and improve performance status in some patients with castration-resistant prostate cancer (CRPC) [9,10]. Moreover, expression of AT1R and angiotensinogen in CRPC was significantly higher than that in normal prostate tissue or hormone-naive prostate cancer [11]. These facts prompted us to confirm the direct effects of ARBs on prostate cancer and investigate the mechanisms of their suppression of prostate cancer growth and progression.

We have established a transgenic rat for adenocarcinoma of prostate (TRAP) model bearing a probasin promoter/simian virus 40 (SV40) T antigen construct, which features development of high-grade prostatic intraepithelial neoplasia (PIN) from 4 weeks of age and well-moderately differentiated adenocarcinoma with high incidences by 15 weeks of age [12,13]. These characteristics of TRAP have been shown to be very suitable for evaluation of strategies for chemoprevention and treatment with ARBs in vivo.

Here, we showed that ARBs attenuate prostate cancer development in TRAP rats and androgen receptor (AR)-mediated transcriptional activity in human prostate cancer cells. Also, we showed that an ARB delayed PSA progression in patients with local recurrence after radical prostatectomy clinically.

MATERIALS AND METHODS

Chemicals, Reagents, Plasmids, and Cell Line

Telmisartan was provided by Boehringer Ingelheim (Ingelheim, Germany) and candesartan was from Takeda Pharmaceutical Co. Ltd. (Osaka, Japan). MG132 was purchased from Calbiochem (EMD Biosciences, Inc., San Diego, CA), diarylpropionitrile (DPN) from Tocris Bioscience (Bristol, UK), and biochanin A from Sigma (St. Louis, MO). The PSA promoter reporter construct (pGL3/PSA promoter) was donated by Dr. Chawnshang Chang, University of Rochester Medical Center. An expression vector for human ERβ (pCXN2/ERβ) was provided by Dr. Masami Muramatsu (Saitama Medical University, Japan). To generate an expression vector for FLAG-tagged human AR (pCMVTag/hAR), the human AR open reading frame was amplified by PCR and cloned into the pCMV-Tag2 vector at *Bam*HI/*Xho*I sites. The human prostate cancer cell lines LNCaP (androgen-dependent) and VCaP (androgen-independent), and the non-tumorigenic prostate epithelial cell line RWPE-1 were obtained from the American Type Culture Collection (Manassas, VA). Four androgen-independent (AI) sublines derived from LNCaP were established after seven repeated cycles of incubation using the following culture medium; RPMI1640 containing 10% FBS for 5–7 days or RPMI1640 containing 10% charcoal-stripped FBS for 2–3 weeks. AI sublines were designated AI-1, -5, -5s, and -8, respectively (Fig. S1A,B).

Animals

Male heterozygous TRAP rats established in our laboratory with a Sprague–Dawley genetic background were used in the present study. They were housed at three animals per cage on wood-chip bedding in an air-conditioned animal room at 23 ± 2°C and 50 ± 10% humidity. Food and tap water were available ad libitum.

Experimental Protocol

Experiment 1. A total of 36 heterozygous male TRAP rats of 3 weeks of age were randomly divided into

three groups. Rats in Group 1 as a control received basal diet and tap water. The rats in Groups 2 and 3 continuously received 2 or 10 mg/kg/day telmisartan in drinking water for 12 weeks, respectively.

Experiment 2. A total of 48 heterozygous male TRAP rats of 3 weeks of age were randomly divided into four groups. Rats in Group 1 as a control received basal diet and tap water. The rats in Groups 2–4 continuously received 2 or 10 mg/kg/day candesartan or 10 mg/kg/day telmisartan in drinking water for 12 weeks, respectively.

In both experiments, measurement of blood pressure was performed at weeks 4, 7, and 11, and the experiments were terminated at week 15. The prostate was removed and fixed in formalin. A part of the prostate glands was immediately frozen in liquid nitrogen and stored at −80°C until processed. Testosterone and estrogen levels in serum were analyzed using radioimmunoassay by a commercial laboratory (SRL, Inc., Tokyo, Japan). The present experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of Nagoya City University Graduate School of Medical Sciences.

Assessment of Prostate Neoplastic Lesion Development

Neoplastic lesions in the prostate gland of TRAP rats were evaluated as previously described [14]. Briefly, neoplastic lesions were classified into three types: low-grade PIN (LG-PIN), high-grade PIN (HG-PIN), and adenocarcinoma. The relative numbers of acini with the histological characteristics of each type, that is, LG-PIN, HG-PIN, and adenocarcinoma, were quantified by counting the total acini in each prostatic lobe.

Immunoblot Analysis

Immunoblot analysis was performed as described previously [15]. Briefly, frozen ventral prostate tissues were homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl (pH 8.0), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, and protease inhibitor cocktail [Complete, Roche]). The antibodies used were cyclin D1 (Oncogene Science, Cambridge, MA), caspases 3, 7, and 9, Erk 1/2 and phophs-Erk1/2, p38 MAPK and phospho-p38 MAPK (Cell Signaling Technology, Danvers, MA), AR and SV40 T antigen (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), VEGF (IBL Co. Ltd., Fujioka, Japan) and β-actin (Sigma). The intensity of each band was measured using Image J 1.440 (National Cancer Institute, Bethesda, MD).

Immunohistochemistry

Deparaffinized sections were incubated with diluted antibodies for Ki-67 (Novocastra Laboratories Ltd., Newcastle, UK), SV40 T antigen (Santa Cruz Biotechnologies), phospho-p38 MAPK (Thr180/Tyr182), and cleaved caspase 3 (Asp175: Cell Signaling Technology, Danvers, MA). Apoptotic cells in the prostate were detected using an In Situ Apoptosis Detection Kit (TUNEL method) according to the manufacturer's instructions (Takara Bio Inc., Ohtsu, Japan). Labeling indices for Ki-67, TUNEL, cleaved caspase 3 or phospho-p38 MAPK were generated by counting over 1,000 cells mainly in HG-PIN under microscope at high magnification and they were expressed as numbers of positive cells per 100 cells. To evaluate the effect of telmisartan against angiogenesis, microvessels were detected using anti-factor 8-related antigen (DAKO, Glostrup, Denmark).

Cell Proliferation Assay

Cell proliferation of prostate cancer cell lines was assessed by 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate tetrazolium salt (WST-1) assay (Roche Applied Science, Mannheim, Germany). Briefly, cells were seeded in 96-well plates at 1×10^4 cells/well in 200 μl of culture media. ARBs or ERβ agonists were added 24 hrs after seeding and incubated for 3 days. WST-1 reagent was added to each well with incubation for 60 min at 37°C, and then each well were measured for absorbance at 430 nm.

Ubiquitylation Assay

LNCaP cells were transfected with Flag-tagged human AR (pCMVTag/hAR) using Nucleofector II (Amaxa AG, Koeln, Germany), seeded into a 6-well plate and incubated for 24 hr. Cells were treated with ARBs and/or 1 μM MG132 for 24 hr, and then lysed with RIPA buffer supplemented with COMPLETE protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The extracts were immunoprecipitated with anti-Flag antibody (Sigma) and protein G sepharose (GE Healthcare Bio-sciences AB, Uppsala, Sweden) for 3 hr at 4°C. After washing the agarose with RIPA buffer, Laemmli sample buffer was directly added to the agarose and heated to 85°C for 10 min. Samples were subjected to immunoblot analysis using anti-ubiquitin antibody (Santa Cruz).

Reporter Gene Assay

LNCaP cells were transfected with the pGL3/PSA promoter using Nucleofector II. Twenty-four hours after transfection, 5 nM DHT and/or ARB was added.

Cells were lysed with the buffer supplied in the kit 72 hr after transfection. The luciferase assay was conducted using the dual-luciferase reporter assay system (Promega Corporation, Madison, WI) according to manufacturer's protocol. Data shown represent the mean and standard deviation of four independent data points.

Microarray Analysis

Total RNA was isolated from ventral prostate tissues as en bloc by phenol–chloroform extraction (ISOGEN, Nippon Gene Co. Ltd., Toyama, Japan), and fluorescent cRNA amplification was performed using a Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies, Palo Alto, CA) according to the manufacturer's instruction. Total RNA from ventral prostate of F344 rat was used as a reference RNA. Quality of total and amplified cRNAs was examined with a high-resolution electrophoresis system, Agilent 2100 Bioanalyzer (Agilent Technologies). Gene expression analysis was performed using a Whole Rat Genome oligo DNA microarray (4 × 44k; Agilent Technologies). The slides were hybridized with Cy3- or Cy5-labeled cRNA for 16–18 hr at 60°C, washed in 0.5 × SSC/0.01% SDS buffer for 5 min at room temperature, then 0.06 × SSC buffer for 2 min, and desiccated with a centrifuge. The slides were scanned with a DNA Microarray Scanner (Agilent Technologies) at two wavelengths to detect emission from both Cy3 and Cy5. Genes with significantly different expression levels were revealed by Significance Analysis of Microarray (ver 2.0; delta = 0.34; http://www-stat.stanford.edu/~tibs/SAM/).

Real-Time RT-PCR

Total RNA was isolated from ventral prostate tissues as en bloc using an RNeasy Mini kit (Qiagen, Valencia, CA). Total RNAs were reverse-transcribed with the Thermoscript first-strand synthesis system (Invitrogen Corporation, Carlsbad, CA), and real-time RT-PCR was performed using a LightCycler (Roche Diagnostics GmbH). The oligonucleotides listed in Table S1 as primers.

RNA-Interference

siRNAs were designed and obtained from RNAi Co. Ltd. (Tokyo, Japan), AR, ERβ and control siRNA sequences were 5′-GAGGAGCUUUCCAGAAUCUGU-3′, 5′-GGAAAUGCGUAGAAGGAAUUC-3′ and 5′-GUACCGCACGUCAUUCGUAUC-3′, respectively. LNCaP cells were transfected with siRNAs using Nucleofector II.

Statistical Analysis

Differences in incidences or means between groups were determined by analysis of variance (ANOVA), followed by the Dunn' multiple comparison test or Dunnett's post-hoc test with GraphPad Prism (version 5.0c; GraphPad Software, Inc., La Jolla, CA), respectively.

Intervention Study

After Institutional Review Board approval was obtained, data from 234 patients undergoing radical prostatectomy (RP) at Yokohama City University Hospital and Center Hospital from March 1999 to July 2007 were entered into our database. Biochemical failure (BCF) was defined as a single PSA level of >0.2 ng/ml. The PSA-doubling time (DT) was calculated by log-linear regression and analyzed, respectively, as from the nadir PSA level after RP up to 0.2 ng/ml, and from 0.2 ng/ml to about 1.0 ng/ml. Briefly, PSA-DT was calculated starting with the nadir PSA value after RP, and included all PSA values up to 0.2 ng/ml used in the PSA-DT calculations, and also required patients to have a minimum of two values separated by at least 3 months, which was designated as ePSA-DT. In cases with a PSA value over 0.2 ng/ml, PSA-DT was calculated using PSA values after BCF (i.e., >0.2 ng/ml), as opposed to ePSA-DT, which was computed using all values after BCF up to about 1.0 ng/ml, at which point secondary therapy, for example, radiotherapy or hormonal therapy, was started, which was designated as aPSA-DT.

Eighteen patients were enrolled in this study. All patients underwent RP, and the specimens showed adenocarcinoma pathologically, with tumor stage T2 or T3, without lymph node metastasis (pathological stage: T2 or T3 N0M0). At BCF of PSA values, they received olmesartan (Dai-ichi Sankyo Co., Tokyo, Japan), an ARB, at 10–20 mg once daily till they received secondary therapy when their PSA values were over 1.0 ng/ml. ePSA-DT was calculated using all PSA values from the PSA nadir after RP until the start of olmesartan. Then, aPSA-DT was calculated using all PSA values after the start of olmesartan to the start of secondary therapy, at which point their PSA values were over 1.0 ng/ml. As for the control, 9 patients were not treated after RP until PSA level was over 1.0 ng/ml. ePSA-DT was calculated starting with the nadir PSA value after RP and included all PSA values up to 0.2 ng/ml, and aPSA-DT was calculated using all PSA values from BCF (>0.2 ng/ml) to over 1.0 ng/ml.

We compared ePSA-DT and aPSA-DT of olmesartan-treated patients and control patients. Also, the ratio of aPSA-DT/ePSA-DT was compared between

olmesartan-treated patients and control patients by Wilcoxon signed-rank test. Furthermore, the time to PSA progression over 1.0 ng/ml after BCF and from the nadir PSA value ($TTPP_{1.0}$) was estimated using Kaplan–Meier method and analyzed using log rank test.

RESULTS

Animal Experiment UsingTRAP Rats

Experiment 1. One rat in the control group was omitted from the effective animals because of suffering from cachexia due to the spontaneous development of leukemia. Blood pressure of TRAP rats treated with telmisartan was significantly lowered in a dose-dependent manner (Table S2). Telmisartan decreased mean body weight and increased kidney weight but did not influence the ventral prostate and liver weights (Table S2, Fig. S2A). Histologically, stromal edema was demonstrated in the kidneys of TRAP rats given telmisartan but tubular or glomerular damage was not evident (Fig. S2C). Serum levels of testosterone and estradiol were not affected by telmisartan (Table S2). In the lateral prostate, a significant decrease in the incidence of adenocarcinoma was observed (Table I). In the ventral prostate, there was a marked or partial pathologic response to telmisartan treatment, as demonstrated by a significant reduction in the amount of prostatic neoplastic lesions in TRAP rats; however, small foci of adenocarcinoma still remained, so there was no significant difference in the incidence of PIN or adenocarcinoma in the prostate of TRAP rats (Table I). Quantitative evaluation of the proportion of preneoplastic and neoplastic lesions in the prostate gland showed significant suppression of progression from LG-PIN to HG-PIN or adenocarcinoma in rats treated with telmisartan (Table II). There was a significant increase in the apoptotic index in the prostate of TRAP rats given telmisartan (Fig. 1A), although Ki-67 index was not different among the groups (Fig. 1B). In the ventral prostate, immunoblot analyses showed activation of caspases 3 and 7 and inactivation of p38 MAPK in rats treated with telmisartan, while expression of cyclin D1 was not altered in all groups (Fig. 1E). Both caspase 3 activation and decreased expression of phospho-p38 MAPK were confirmed by immunohistochemistry (Fig. 1G,I). There was no difference in the expression of AT1R in the ventral prostate between telmisartan-treated and control rats (Fig. 1K). ARB exerted suppressive effects on the growth of prostate cancer via the inhibition of angiogenesis in a tumor xenograft model [16]. The possibility that telmisartan augmented apoptosis in prostate cancer through suppression of tumor angiogenesis in TRAP rats was examined. Unexpectedly, VEGF protein expression in the ventral prostate was not altered by telmisartan treatment (Fig. S3), and there was no difference in microvessel density among the groups (Table S3).

Experiment 2. To confirm the reproducibility of the suppressive effect of telmisartan on prostate carcinogenesis, we performed an experiment with a similar design to that of experiment 1. Candesartan, a selective AT1R blocker with no PPARγ agonistic activity at usual doses, was applied for comparison with the effects of telmisartan. Body weight gain and organ weights were not affected by candesartan (Table S4, Fig. S2B). Serum levels of testosterone and estradiol were not significantly different between telmisartan and candesartan treatment (Table S4). Prostate adenocarcinomas were found only in the ventral and lateral lobes, and a significant decrease in its incidence was observed in the lateral prostate and suppression of the progression of prostatic lesions from LG-PIN to

TABLE I. Incidences of Adenocarcinoma of the Prostate Glands of TRAP Rats

Treatment	No. of rats	Ventral AC (%)	Lateral AC (%)	Dorsal AC (%)
Experiment 1				
Control	11	11 (100)	9 (82)	0
TS 2 mg/kg/day	12	11 (92)	8 (67)	0
TS 10 mg/kg/day	12	9(75)	4 (33)*	0
Experiment 2				
Control	12	12(100)	12 (100)	0
CS 2 mg/kg/day	12	12(100)	10 (83)	0
CS 10 mg/kg/day	12	11 (92)	5 (42)**	0
TS 10 mg/kg/day	12	12(100)	4 (33)**	0

TS, telmisartan; CS, candesartan; AC, adenocarcinoma.
*$P < 0.05$ versus control.
**$P < 0.01$ versus control.

TABLE II. Quantitative Evaluation of Neoplastic Lesions in Ventral Prostate of TRAP Rats Treated With ARBs

Treatment	No. of rats	LG-PIN	HG-PIN	AC
Experiment 1				
Control	11	7.2 ± 2.3	90.1 ± 2.2	2.7 ± 1.2
TS 2 mg/kg/day	12	11.9 ± 4.2*	86.7 ± 4.2	1.4 ± 0.4**
TS 10 mg/kg/day	12	12.5 ± 5.0**	85.9 ± 4.8*	1.6 ± 0.6**
Experiment 2				
Control	12	10.6 ± 4.5	83.1 ± 3.4	6.3 ± 2.8
CS 2 mg/kg/day	12	13.5 ± 5.9	82.6 ± 4.9	3.9 ± 2.1*
CS 10 mg/kg/day	12	18.4 ± 4.9**	78.9 ± 4.5	2.7 ± 1.2***
TS 10 mg/kg/day	12	22.5 ± 8.6***	75.0 ± 7.8**	2.5 ± 1.3***

Values (mean% ± SD) are the relative number of acini with histological characteristics against whole number of acini.
LG-PIN, low-grade prostatic intraepithelial neoplasia; HG, high grade; AC, adenocarcinoma; TS, telmisartan; CS, candesartan.
*$P < 0.05$ versus control.
**$P < 0.01$ versus control.
***$P < 0.001$ versus control.

HG-PIN or adenocarcinoma were found in rats given telmisartan and high-dose candesartan (Tables I and II). The numbers of apoptotic cells in both the ventral and lateral prostate of rats treated with both telmisartan and candesartan were significantly increased as compared with the controls, whereas there was no obvious difference in Ki-67 labeling index (Fig. 1C,D). Immunoblot and immunohistochemical analyses clearly demonstrated activation of caspases 3 and 7 and a tendency for inactivation of p38 MAPK in the ventral prostate of rats treated with both candesartan and telmisartan, as likewise shown in experiment 1 (Fig. 1F,H,J). There was no significant difference in AT1R expression among the groups (Fig. 1L).

Suppressive Effects of ARBs on the Expression and Transcriptional Activity of Androgen Receptor (AR)

We examined the effects of ARBs on AR expression because previous clinical studies demonstrated that ARBs have potential to decrease serum PSA level in prostate cancer patients. AR protein expression was down-regulated in the ventral prostate of TRAP rats while SV40 T antigen protein and AR mRNA expression did not differ among the groups (Fig. 2A,B). Immunohistochemical analysis revealed that all prostate epithelial cells, including neoplastic and normal-looking cells, expressed SV40 T antigen at almost similar levels (Fig. S4A). Real-time RT-PCR of the androgen responsive gene, GK11, known as an ortholog of human PSA, demonstrated significant down-regulation by ARB treatment while probasin expression levels showed no clear alteration (Fig. S4B,C). In human prostate cancer and prostate epithelial cells, all cells used in the present study expressed both AT1R and AT2R but its levels were variable (Table S5). In LNCaP cells, ARBs repressed both AR and PSA protein expression although real-time RT-PCR analysis of the AR gene showed no obvious difference among treatments (Fig. 2C,E). ARBs also suppressed both AR and PSA expression in VCaP cells, androgen-independent prostate cancer cells harboring wild type AR (Fig. 2D). The suppressive effect of ARBs on AR protein expression was blocked by the proteasome inhibitor, MG132, suggesting that a proteasome-dependent pathway is involved in ARB-induced AR protein down-regulation (Fig. 2F). Subsequent luciferase reporter assays clearly demonstrated significant inhibition of AR transcriptional activity, this finding being considered to simply reflect down-regulation of AR protein expression by ARBs (Fig. 2G).

In RWPE-1 cells, normal epithelial cells from the peripheral zone of the human prostate immortalized with human papilloma virus 18, candesartan did not affect cell growth while high-dose of telmisartan attenuated cell proliferation (Fig. S5).

Estrogen Receptor β (ERβ) Upregulation by ARBs in Prostate of TRAP Rats and Human Prostate Cancer Cell Lines

To further investigate the downstream molecule(s) of AT1R responsible for suppression of prostate carcinogenesis, we performed microarray analysis using ventral prostate tissue of TRAP rats. According to comprehensive mRNA profiling by DNA microarray, 28 genes were up-regulated and 43 were down-regulated in the telmisartan treatment group over the control (Delta = 0.340; Fig. S6). Table S6 showed significant genes detected microarray analysis. Among these genes, we focused on ERβ as one of the genes up-regulated by ARB treatment because the majority

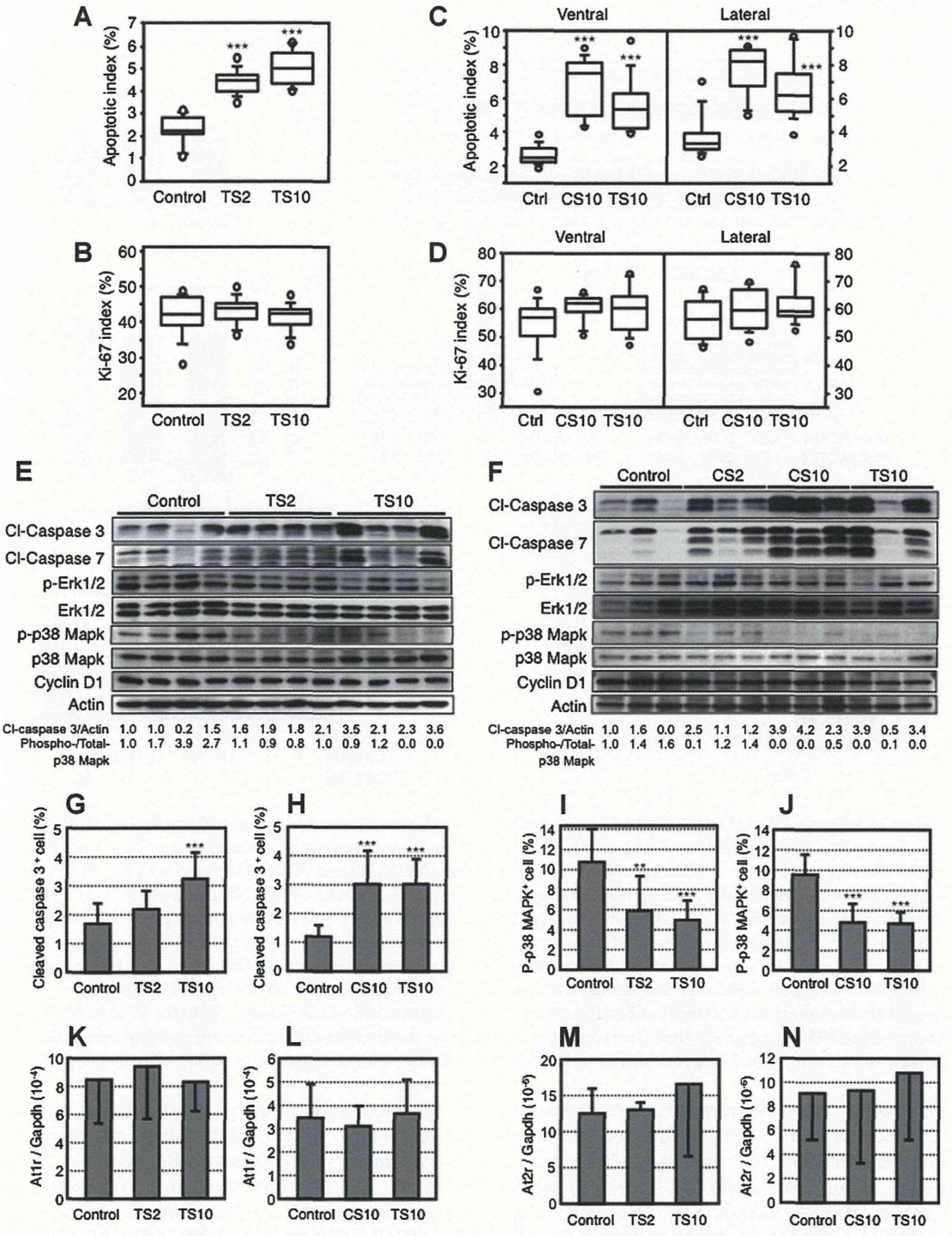

Fig. 1. Labeling indices for apoptosis (TUNEL), Ki-67 positive cells and immunoblot analysis in prostate of TRAP rats treated with ARB. Box plot data for TUNEL (**A**), Ki-67 (**B**) indices in ventral prostate in Experiment 1, and TUNEL (**C**) and Ki-67 (**D**) indices in each prostatic lobe in Experiment 2. Labeling indices were counted in prostate epithelial cells of all rats and more than 1,000 cells were evaluated to give percentage values. $^{***}P < 0.001$ versus control. **E**,**F**: Immunoblots of protein lysates (20 μg) of ventral prostate in Experiment 1 and 2, respectively, were probed with antibodies to cleaved caspases 3 and 7, MAPKs, cyclin D1 and β-actin. Quantitative data for labeling of cleaved caspase 3 (**G**,**H**) or phospho-p38 MAPK (**I**,**J**) in the ventral prostate in Experiments 1 and 2, respectively. Quantitative data for angiotensin II receptor type 1 (**K**,**L**) and type II (**M**,**N**) expression in the ventral prostate in Experiments 1 and 2, respectively. Data except immunoblotting were analyzed using all animal samples. **, $^{***}P < 0.01$ and 0.001 versus control. TS2 and TS10, telmisartan 2 and 10 mg/kg/day, respectively; CS2 and CS10, candesartan 2 and 10 mg/kg/day, respectively; Ctrl, control.

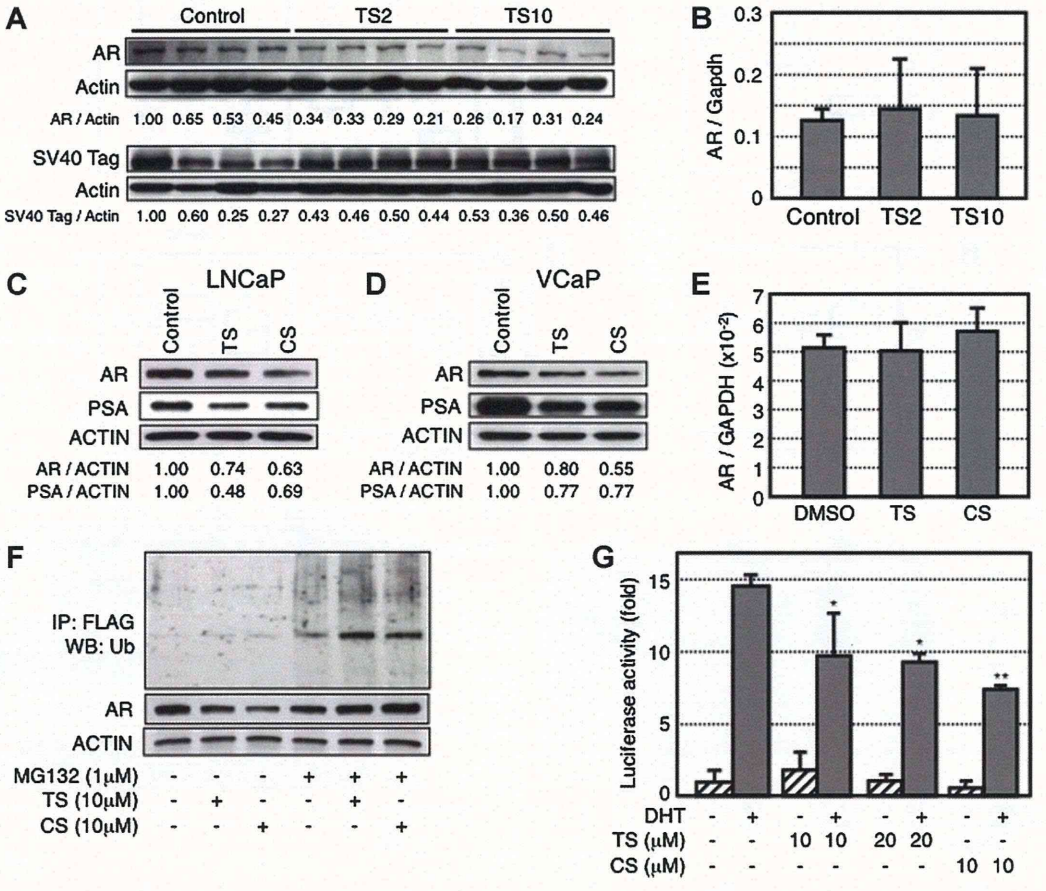

Fig. 2. Effect of ARBs on AR expression in TRAP rats or human prostate cancer cells. Immunoblot (**A**) or Real-time-RT-PCR (**B**) analysis for AR in the ventral prostate of TRAP rats. The intensity of each band was measured and normalized to actin. Data were analyzed using all animal samples and represent mean ± SD. Immunoblot analysis for AR in LNCaP (**C**) and VCaP (**D**) treated with 10 μM TS or CS for 3 days. The intensity of each band was measured and normalized to actin. **E**: Real-time RT-PCR for AR in LNCaP exposed 10 μM TS or CS for 3 days. **F**: Ubiquitylation assay for AR in LNCaP cells transfected with FLAG-tagged human AR, treated with 10 μM TS, CS and/or 1 μM MG132 for 24 hr. Protein extracts prepared from treated or untreated cells were subjected to immunoprecipitation using anti-FLAG antibody. The ubiquitylation status of AR was analyzed by immunoblotting using anti-ubiquitin antibody. **G**: Inhibition of PSA promoter luciferase activity by ARBs in LNCaP cells. Cells were transfected with both a PSA promoter reporter construct (pGL3/PSA promoter) and a control phRL-TK Renilla luciferase vector. Cells were incubated in the absence or presence of 5 nM DHT and/or ARBs for 48 hr in RPMI1640 containing 10% charcoal-stripped FBS without phenol red. Data represent the mean and standard deviation of four independent data points. $^{*},^{**}P < 0.05$ and 0.01 versus no treatment control, respectively. TS2 and TS10, telmisartan 2 and 10 mg/kg/day, respectively; CS, candesartan.

of the genes other than ERβ were involved in the regulation of blood pressure. First, we needed to confirm ERβ expression in TRAP rat prostate in both in vivo experiments, as we have done. As expected, significant elevation of ERβ mRNA was observed in the ventral prostate of TRAP rats by quantitative RT-PCR (Fig. 3A,B), and a similar phenomenon was found in LNCaP cells exposed to ARBs (Fig. 3C).

Up-Regulation of ERβ Induce Suppression of AR Transcriptional Activity and Prostate Cancer Cell Growth

Luciferase reporter assay demonstrated that forced expression of ERβ in LNCaP cells clearly inhibited AR-mediated transcriptional activity in both ligand-dependent and -independent manners (Fig. 3D). Treatment with selective ERβ agonists, diarylpropionitrile (DPN) and biochanin A, suppressed both growth and AR-mediated transcriptional activity of LNCaP cells as did ARBs (Fig. 3E,F). Immunoblot analysis revealed that selective ERβ agonists down-regulated PSA expression but AR expression was increased in LNCaP cells (Fig. 3G). Selective ERβ agonist-induced cell growth suppression was blocked by siRNA-mediated knock-down of AR expression, suggesting that the suppressive action of ERβ was via the AR signaling pathway (Fig. 3H). Knock-down of ERβ expression by siRNA did not affect on cell

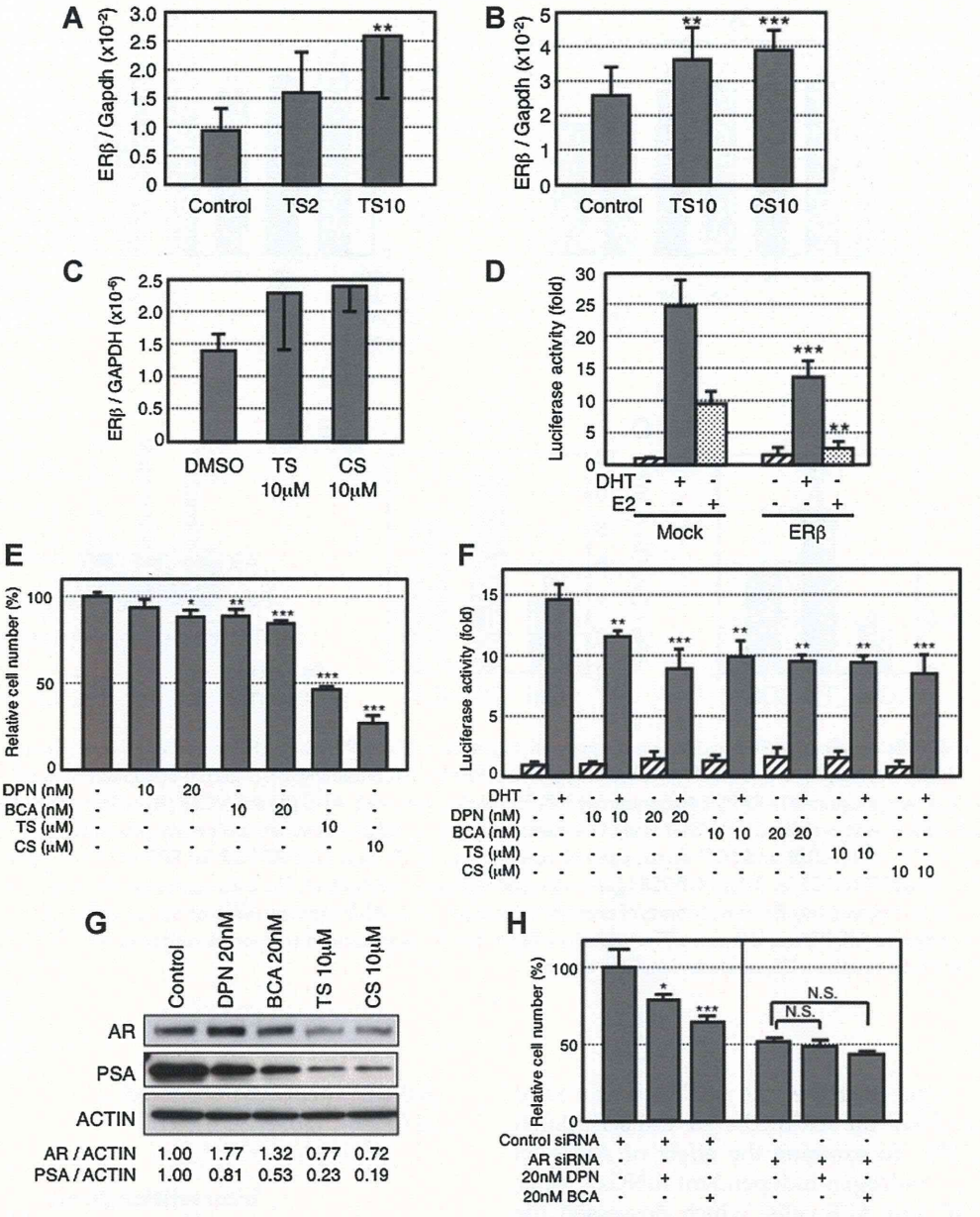

Fig. 3. Effects of ARBs on ERβ mRNA expression in TRAP rats or LNCaP cells. Real-time-RT-PCR for ERβ in ventral prostate of TRAP rats from Experiment 1 (**A**) and from Experiment 2 (**B**) Data in (A) and (B) were analyzed using all animal samples. **C**: Real-time RT-PCR for ERβ using total RNA of LNCaP cells treated with ARBs for 3 days. **D**: Inhibition of PSA promoter luciferase activity by ERβ in LNCaP cells. Cells were transfected with pGL3/PSA promoter, phRL-TK Renilla luciferase vector and expression plasmid encoding human ERβ (pCXN2/ERβ) or mock vector (pCXN2). Cells were incubated in the absence or presence of 5 nM DHT or 100 nM 17β-estradiol (E2) for 48 hr. Data represent the mean and standard deviation of four independent data points. **E**: Cell growth after chemical exposure of ARBs or selective ERβ agonist in LNCaP. Cells were exposed to chemicals for 72 hr, and cell growth was evaluated by WST-1 assay. **F**: Inhibition of PSA promoter luciferase activity by ARBs or selective ERβ agonist in LNCaP cells. Cells were transfected with the same as (D). Cells were treated in the absence or presence of 5 nM DHT and/or ARBs or a selective ERβ agonist as indicated for 48 hr in RPMI1640 containing 10% charcoal-stripped FBS without phenol red. Data represent the mean and standard deviation of four independent data points. **G**: Immunoblots of protein lysates of LNCaP exposed ARBs or selective ERβ agonist for 3 days. The intensity of each band was measured and normalized to actin. **H**: LNCaP cells were transfected with either control siRNA or AR siRNA and then were exposed to selective ERβ agonists 1 day after transfection. Cells were incubated with RPMI1640 containing 5% FBS without phenol red for 3 days. Cell growth was evaluated by WST-1 assay. N.S., not significant. TS2 and TS10, telmisartan 2 and 10 mg/kg/day, respectively; CS10, candesartan 10 mg/kg/day; DPN, diarylpropionitrile; BCA, biochanin A. *,***$P < 0.05$ and 0.001 versus control, respectively.

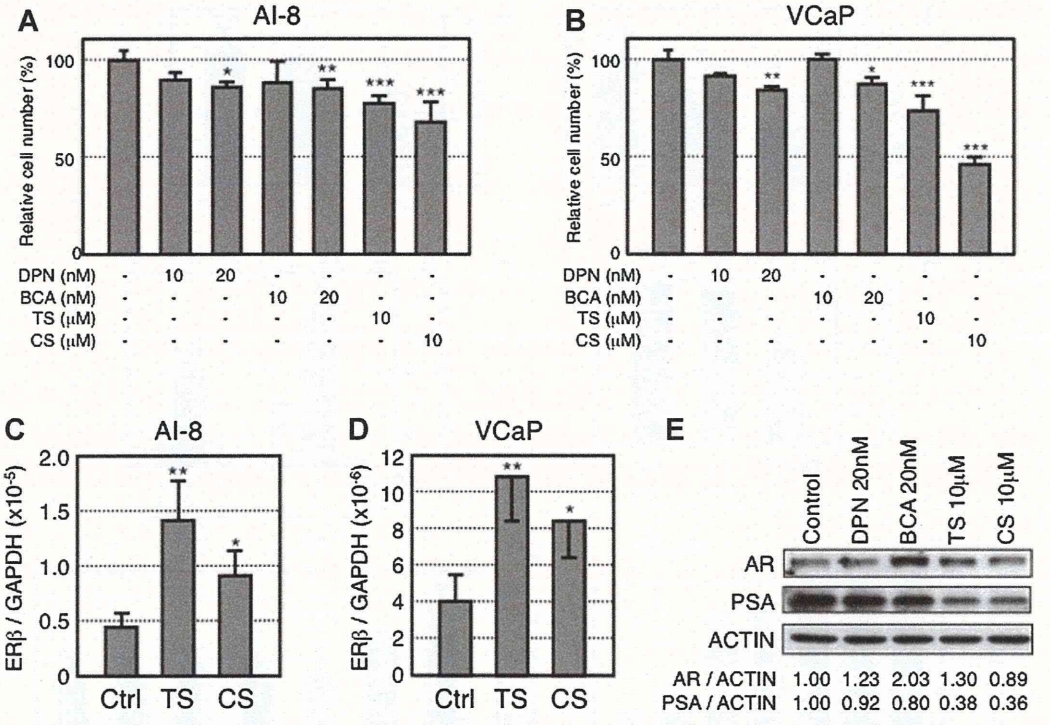

Fig. 4. Effects of ARBs or selective ERβ agonist on the growth, expression of AR, PSA, and ERβ in androgen-independent human prostate cancer cell lines, LNCaP subline, AI-8, and VCaP. AI-8 was incubated in RPMI1640 containing 10% charcoal-stripped FBS and 5 nM DHT without phenol red and VCaP was incubated in RPMI1640 containing 10% FBS with phenol red. AI-8 (**A**) and VCaP (**B**) cells were exposed to chemicals for 72 hr, and cell growth was evaluated by WST-1 assay. The mean values of six independent data points are presented and SD values are indicated by bars. *,**,***$P < 0.05$, 0.01, and 0.001 versus control, respectively. (**C**,**D**) Real-time RT-PCR for ERβ using total RNA of AI-8 (C) and VCaP (D) exposed 10 μM TS or CS for 3 days. GAPDH gene was used as an internal control. The data represent the mean ± SD. *,**$P < 0.05$ and 0.01 versus control, respectively. **E**: Immunoblots of protein lysates (20 μg) of AI-8 exposed ARBs or selective ERβ agonist for 3 days were probed with antibodies to AR, PSA, and β-actin. The intensity of each band was measured and normalized to actin. DPN, diarylpropionitrile; BCA, biochanin A; TS, telmisartan; CS, candesartan; Ctrl, control.

proliferation, suggesting the role of ERβ was a mild additional modifier on AR-mediated transcriptional activity (Fig. S7). To examine the effect of ARBs on the growth of androgen-independent LNCaP cells, we used VCaP and AI-8 cells, which possessed the highest PSA expression among the four sublines (Fig. S1C). Selective ERβ agonists as well as ARBs inhibited cell proliferation of both AI-8 and VCaP cells (Fig. 4A,B). Immunoblot analysis revealed that AR expression was increased while PSA was decreased by treatment with selective ERβ agonists in AI-8 cells (Fig. 4E). On the other hand, ARBs clearly repressed PSA expression although the AR protein level was slight increased with telmisartan (TS) and decreased with candesartan (CS) treatment compared with the no-treatment control. These results are in marked contrast to the findings seen in androgen-dependent parental LNCaP cells as shown in Fig. 3G. Up-regulation of ERβ mRNA expression by ARB treatment was observed in both AI-8 and VCaP cell (Fig. 4C,D).

Intervention Study

Background data of the patients are shown in Table S7. aPSA-DT was significantly longer than ePSA-DT in both the olmesartan-treated patients (see Materials and Methods Section), median 436.3 versus 128.7 days ($P = 0.004$; Fig. 5A). However, the ratio of aPSA-DT/ePSA-DT was significantly longer in olmesartan-treated patients (3.36 times) than in control patients (1.80 times) ($P = 0.0156$), indicating that olmesartan treatment could delay PSA progression (Fig. 5B). Fig. 5C shows that $TTPP_{1.0}$ from biochemical failure (0.2 ng/ml) to over 1.0 ng/ml in olmesartan-treated patients was significantly longer than that in control patients (no treatment; $P = 0.026$). Similarly, the time for PSA progression from the PSA nadir to over

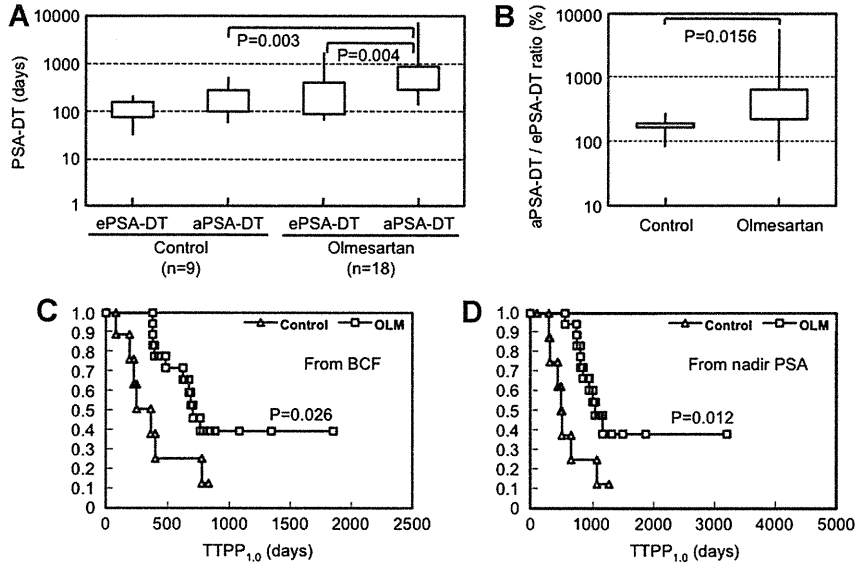

Fig. 5. PSA-DT in control and olmesartan-treated patients. **A**: ePSA-DT and aPSA-DT in control and olmesartan-treated patients. aPSA-DT was significantly longer than ePSA-DTs in olmesartan-treated patients ($P = 0.004$) or aPSA-DT in control patients ($P = 0.003$). **B**: The ratio of aPSA-DT/ePSA-DT was significantly higher in olmesartan-treated patients (3.36 times) than in control patients (1.80 times) ($P = 0.0156$). Kaplan–Meier plots illustrating time to PSA progression in control and olmesartan (OLM)-treated patients. **C**: Time to PSA progression from biochemical failure (0.2 ng/ml) to over 1.0 ng/ml in olmesartan treated patients was significantly longer than that in control patients ($P = 0.026$). **D**: Time to PSA progression from PSA nadir to over 1.0 ng/ml in olmesartan-treated patients was significantly longer than that in control patients ($P = 0.012$). BCF, biochemical failure.

1.0 ng/ml in olmesartan-treated patients was significantly longer than that in control patients ($P = 0.012$) as shown in Fig. 5D.

DISCUSSION

It is widely accepted that there are multiple processes in the development of cancer; that is, initiation, promotion and progression. In 1976, Sporn proposed "cancer chemoprevention" as a strategy for prevention of cancer to delay the development of clinically evident disease by suppression of progression from precancerous lesions to invasive cancer by giving natural or synthetic compounds [17]. Prostate cancer is known to be strongly associated with aging, that is, about three-quarters of cases worldwide occur in men aged 65 years or more [18]. Therefore, prostate cancer is an attractive target for cancer chemoprevention because of the high population incidence and long latent period, and several dietary factors as well as genetic background have been linked to risk and progression of prostate cancer [19,20]. In fact, a large number of observational or intervention studies have been conducted using vitamins, phytochemicals, and minerals [21,22]. Moreover, three large scale randomized clinical trials, SELECT (Selenium and Vitamin E Cancer Prevention Trial) [23], PCPT (Prostate Cancer Prevention Trial) [24], and REDUCE (Reduction by Dutasteride of Prostate Cancer Events) [25], have been completed, and the latter two trials using 5α-reductase inhibitors showed a reduction of prostate cancer risk, although adverse effects including sexual dysfunction were observed. From the viewpoint of sexual dysfunction, ARB could restore rather than induce these side effects [26–28].

The present study demonstrated suppressive effects of ARBs on prostate tumor progression in an in vivo animal model, with decrease in the development of both HG-PINs and adenocarcinoma and consequent increase in LG-PINs in the ventral prostate. We have validated the chemopreventive effects of various chemicals including anti-androgens using our TRAP rats [14,15,29,30]. This transgenic rat is characterized by the sequential development of prostatic lesions, that is, LG-PINs, HG-PINs and adenocarcinomas in almost all acini in the entire ventral and lateral lobes. The SV40 T antigen used in the transgene of the TRAP rat acts as a potent oncoprotein to strongly stimulate cell growth and the development of adenocarcinomas in almost all acini in the entire prostatic lobes through inhibiting both the pRB and p53 tumor suppressor pathways. Since expression of SV40 T antigen is regulated by the androgen-dependent probasin promoter, it is speculated that cancer