Research Article

Cancer Prevention Research

Naftopidil, a Selective α_1-Adrenoceptor Antagonist, Suppresses Human Prostate Tumor Growth by Altering Interactions between Tumor Cells and Stroma

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Abstract

In prostate cancer, tumor–stroma interactions play a critical role in the promotion of tumorigenesis, and thus the prevention of those interactions is a promising target to suppress tumor growth. Several studies demonstrated that alpha$_1$-adrenoceptor (α_1-AR) antagonists, therapeutic drugs for benign prostatic hyperplasia, have growth inhibitory effects on human prostate cancer (PCa) cells through induction of apoptosis or G_1 cell-cycle arrest. However, their direct actions on stromal cells surrounding cancer cells have not yet been elucidated. In this study, we investigated the effects of subtype-selective α_1-AR antagonists (naftopidil, tamsulosin, and silodosin) on prostate tumor growth with a focus on the role of stroma, using commercially available fibroblast cells (PrSC). Tumorigenic studies *in vivo* showed significant reductions in tumor growth when E9 cells (an androgen low-sensitive LNCaP subline) grafted with PrSC were treated with naftopidil. In *in vitro* analyses, naftopidil and silodosin showed antiproliferative effects on PCa cells regardless of androgen sensitivity and α_1-AR subtype expression. In PrSC, a strong growth inhibitory effect was observed with naftopidil but not silodosin. Flow cytometric analysis revealed that naftopidil, but not silodosin, induced G_1 cell-cycle arrest in both PCa cells and PrSC. In naftopidil-treated PrSC, total interleukin-6 protein was significantly reduced with increased suppression of cell proliferation. Silodosin induced weak early apoptosis only in PCa cells. These findings demonstrated that naftopidil strongly suppressed cell proliferation of stromal cells, resulting in decreased tumorigenic soluble factor, suggesting that naftopidil might be effective in preventing stromal support of tumor cells.
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Introduction

Recently, several studies demonstrated that alpha$_1$-adrenoceptor (α_1-AR) antagonists, which are generally prescribed for benign prostatic hyperplasia (BPH) patients, have antiproliferative effects on prostate cancer (PCa). Epidemiologically, 2 observational cohort studies have shown an apparent low prevalence of PCa in BPH patients receiving α_1-AR antagonists. Harris et al. reported that the administration of quinazoline-based α_1-AR antagonists (subtype nonselective: doxazosin or terazosin) significantly decreased the incidence of PCa (1). Murtola et al. reported that the administration of α_1-AR antagonists (subtype nonselective: alfuzosin or subtype-selective: tamsulosin) decreased the incidence of high-grade PCa; the decreasing trend correlated with the cumulative duration of α_1-AR antagonists use (2).

BPH is a common prostatic disease in elderly men. In regard to treatment for BPH, α_1-AR antagonists are the most widely used therapeutic agents. Although the incidence of BPH increases with age, this increase occurs faster than that of PCa (3). In addition, most PCa arises in the prostate concomitant with BPH elsewhere (4). This means that, in general, the administration of α_1-AR antagonists for BPH treatment often comes before diagnosis of PCa. Accordingly, it is very interesting to examine the possibility of α_1-AR antagonists suppress prostate tumor growth. Previous studies have documented the induction of apoptosis in PCa cells by subtype nonselective α_1-AR antagonists (doxazosin, terazosin, and prazosin; refs. 5, 6). In addition, the G_1 cell-cycle arrest was induced in PCa cells by subtype-selective α_1-AR antagonist naftopidil, which is only marketed in Japan (7). The underlying molecular mechanism by which α_1-AR antagonists inhibit PCa cells is gradually being elucidated.

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In general, cancer cells exist in a microenvironment composed of extracellular matrix (ECM), tumorigenic soluble factors, and stromal cells including fibroblasts, smooth muscle cells, endothelial cells, macrophages, and other cells (8). In this tumor microenvironment, various cytokines or growth factors act as messengers in reciprocal tumor–stroma interactions (9). Fibroblasts are likely sources of tumorigenic soluble factors that affect the growth of cancer cells. Thus, they have a crucial influence on the growth and progression of tumors (10). The normal prostate has an abundance of stromal components, mainly well-differentiated smooth muscle cells. In prostate tumors, however, fibroblasts surrounding cancer cells are associated not only with the initiation of cancer cells but also with tumor growth and progression to androgen independence (11–13). Olumi et al. demonstrated stroma-induced malignant transformation, indicating that fibroblasts surrounding epithelia play an important role in PCa development (14). Other groups reported that coinoculation of PCa cells with fibroblasts increased tumorigenicity and implied the promotion of angiogenesis (11, 15, 16). Therefore, the inhibition of tumor–stroma interactions with addition of the suppression of cancer cell growth might lead to synergistic effects on tumor control.

In the present study, we investigated the effects of subtype-selective α_1-AR antagonists (naftopidil, tamsulosin, and silodosin) on prostate tumor growth with a focus on the role of stroma, especially commercially available fibroblast cells (PrSC). Although we have already reported that the α_{1D}-selective antagonist naftopidil suppresses the growth of androgen receptor-positive human PCa line LNCaP and the androgen receptor-negative human PCa line PC-3 by induction of G_1 cell-cycle arrest (7), little is known about the relationship between the growth inhibitory effects and the androgen receptor signal or the α_1-AR signal in PCa cells. Thus, we also investigated the biochemical mechanisms of subtype-selective α_1-AR antagonists in PCa cells with a focus on the androgen sensitivity of the androgen receptor-positive PCa cells and the α_1-androgen receptor selectivity of each antagonist.

Materials and Methods

Materials

Normal human prostate total Poly A+ RNA (636124) was purchased from Clontech Laboratories, Inc. The 3 clinically used subtype-selective α_1-AR antagonists were kindly provided by the following pharmaceutical companies: naftopidil from Schering-Plough Co; tamsulosin hydrochloride from Astellas Japan, Inc; and silodosin from Daiichi Sankyo Co., Ltd. Selective α_{1A} antagonist RS100329 and selective α_{1D} antagonist BMY7378 were purchased from Tocris Bioscience and Sigma-Aldrich, Inc, respectively. Mouse monoclonal anti-human Ki-67 antigen (clone MIB-1) antibody was purchased from Dako Cytomation, Inc. Rat monoclonal anti-mouse CD31/PECAM-1 (MEC13.3) and mouse monoclonal anti-human p27kip1 (57) antibodies were purchased from BD Bioscience. Mouse monoclonal anti-p21waf1 (HZ52) antibody was purchased from Millipore Co. Mouse monoclonal anti-actin (AC-15) antibody was purchased from Sigma-Aldrich, Inc.

Cell cultures

The androgen-sensitive, androgen receptor-positive human PCa cell line LNCaP was obtained from the American Type Culture Collection. Androgen low-sensitive, androgen receptor-positive E9 cells were derived from a parental LNCaP cell population by a limiting dilution method in regular culture conditions (17). Androgen-insensitive, androgen receptor-positive AIDL cells were established from parental LNCaP cells by continuous passaging in hormone-depleted conditions (18). LNCaP and E9 cells were cultured in phenol red (+) RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution. AIDL cells were cultured in phenol red–free RPMI-1640 supplemented with 10% charcoal-stripped (CS) FBS and 1% antibiotic/antimycotic solution. The parental LNCaP, E9, and AIDL lines expressed similar levels of androgen receptor in culture, but prostate-specific antigen (PSA) protein was detected only in parental LNCaP cells (19). The androgen sensitivity of parental LNCaP, E9, and AIDL was confirmed by the change of PSA mRNA expression in cell culture with synthetic androgen R1881 (19). Human prostate stromal cell PrSC was purchased from Lonza Group Ltd. PrSC was maintained using SCGM Bullet Kit (Lonza Group Ltd) as recommended.

In vivo tumor growth

Subconfluent cultures of PCa cells (parental LNCaP, E9, and AIDL lines) and PrSC were trypsinized and counted. Xenografts without PrSC contained 4×10^5 PCa cells. Mixtures with PrSC were prepared for each PCa cell type by mixing 1×10^5 cancer cells and 3×10^5 PrSC in suspension. Pelleted cells were resuspended in 50 μL of neutralized type I rat tail collagen gels, and then grafted beneath the renal capsule of male athymic mice (7 to 8 weeks old).

Subsequently, we evaluated the effects of administration of subtype-selective α_1-AR antagonists on growth of tumors from an E9 + PrSC combination. Cellular combinations were prepared by mixing 1×10^5 E9 cells and 3×10^5 PrSC in suspension. Xenografts were transplanted into male athymic mice as described above. Treatments were started 7 days after transplantation. Each subtype-selective antagonist was administered orally (10 mg/kg/d) as a suspension in 0.5% of carboxymethylcellulose through a 22-gauge gavage needle for 28 days. The control group received only the diluent. All animals were maintained in a specific pathogen-free environment. The Mie University's Committee on Animal Investigation approved the experimental protocol.

Processing of tumors and immunohistochemistry

Mice were sacrificed 35 days after transplantation. Grafts were harvested and their weights were measured.

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Table 1. Sequences of primers used for reverse transcriptase PCR

Gene name	Sequence	Annealing, °C
Vimentin		
Sense	5′-TGAGTACCGGAGACAGGTGCAG-3′	55
Antisense	5′-CTTGAAACGGCAACTTCGACGAT-3′	
Collagen I		
Sense	5′-CCCGGGTTTCAGAGACAACTTC-3′	55
Antisense	5′-CTAACGACCTTATTTCGTACACCT-3′	
TN-C		
Sense	5′-GAGAGCAATCCAGCGACCATC-3′	55
Antisense	5′-CTTCTGTGGCAACCGGTTTAAA-3′	
αSMA		
Sense	5′-CAATGTCCCTGCCATGTACGTC-3′	55
Antisense	5′-AGATACTTCCGATACGGGACGG-3′	
Myosin		
Sense	5′-TTCTGGAGGCTTTCGGCAAC-3′	55
Antisense	5′-AGTGCCCAATGTAGCACCCT-3′	
Desmin		
Sense	5′-AATACCGACACCAGATCCAGTCCTA-3′	55
Antisense	5′-CTCCTGGCTAAACGGTCACTC-3′	
α_{1A}- adrenoceptor		
Sense	5′-TTAGTCATGCCCATTGGGTCTT-3′	60
Antisense	5′-TATGGGTACGAGGTCGGTTC-3′	
α_{1B}- adrenoceptor		
Sense	5′-GCTCTACCGCTTGGCTCCTT-3′	60
Antisense	5′-GGCACAAGTTCCACCACAAGA-3′	
α_{1D}- adrenoceptor		
Sense	5′-CCGCTCGGCTCCTTGTT-3′	60
Antisense	5′-GCGGAAGGAGGCAGAGGA-3′	
GAPDH		
Sense	5′-GCACCGTCAAGGCTGAGAAC-3′	60
Antisense	5′-AGGTGACCGCAGAAGTGGT-3′	

Tissues were fixed in 10% neutral-buffered formalin for regular H&E (hematoxylin and eosin) staining and human-specific Ki-67 staining or in IHC zinc fixative (BD Biosciences Pharmingen) for mouse-specific CD31 staining. Then, they were dehydrated and embedded in paraffin.

Every harvested tumor was analyzed for cell proliferation (Ki-67 labeling index) and microvessel density (MVD). Immunohistochemical (IHC) staining was performed with a Vectastain ABC elite kit (Vector Laboratories Inc) following our previously reported protocols (19). The extent of Ki-67 labeling was expressed as the percentage of positive nuclear cells. A "microvessel" was defined as mouse-specific CD31-positive endothelial cells that formed a vascular lumen. The numbers were determined by 2 investigators independently in a blinded fashion.

RNA extraction, cDNA preparation, and reverse transcriptase (RT)-PCR

Total RNA was extracted from each cell line using the Qiagen mini RNA Easy kit (Qiagen Inc). cDNA was reverse transcribed from 2 μg of total RNA using High Capacity RNA-to-cDNA Kit (Life Technologies Co) in a total volume of 20 μL. RT-PCR were performed using 2 μL of cDNA with Takara Ex Taq (Takara Bio Inc) with specific primers (Table 1). After PCR, the amplified RT-PCR products were electrophoretically analyzed through 1.6% of ethidium bromide–stained agarose gel and photographed under UV light.

Cell viability assay

LNCaP (and derived) cell lines were plated onto 12-well plates (Nunc; 1.0×10^4 parental LNCaP cells per well; 0.75×10^4 E9 cells per well; 2.0×10^4 AIDL cells per well). Two days after seeding, cells were treated with each α_1-AR antagonist (10 μmol/L) dissolved in dimethylsulfoxide (DMSO) or vehicle (0.1% DMSO) and incubated for 4 days. Then, cells were detached by trypsinization and counted using a hemocytometer. Cell viability was measured by trypan blue exclusion. The PrSC line was seeded into 96-well culture plates at a density of 5×10^2 cells per well. After 24 hours, cells were treated with various concentrations (0, 0.1, 1, and 10 μmol/L) of each α_1-AR antagonist

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and incubated for 3 days. Viable cells were measured using a spectrophotometric cell counting kit (Dojindo Lab).

Cell-cycle analysis

E9 (1×10^5 cells) and PrSC (2×10^5 cells) were seeded onto 6-well plates (Nunc) and 90-mm culture dishes (Sumitomo Bakelit Co. Ltd). Two days after seeding, cells were treated with either 50 μmol/L (E9) or 25 μmol/L (PrSC) of each α_1-AR antagonist or vehicle (0.1% DMSO) for 48 hours. After treatment, cells were isolated and the nuclei stained using the Cycle TEST PLUS DNA Reagent kit (BD Immunocytometry Systems). To determine cell-cycle distribution, the DNA content of stained cells was analyzed on a BD FACS Cant II flow cytometer (Becton Dickinson).

Detection of apoptosis

E9 cells were seeded onto 6-well plates at a density of 1×10^5 cells per well. Two days after seeding, E9 cells were treated with each α_1-AR antagonist (50 μmol/L) for 48 hours. Cells were trypsinized and collected, and then cells were stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) simultaneously using the Annexin V-FITC Apoptosis Detection kit (BioVison). The cell suspensions were analyzed with a BD FACS Canto II flow cytometer (Becton Dickinson) to determine the percentage of apoptotic (FITC-stained cells) and necrotic cells (PI-stained cells). A minimum of 20,000 cells were collected for all the samples.

Western blot analysis

Extracted proteins were separated by gel electrophoresis and transferred to Immobilon polyvinylidene difluoride (PVDF) membranes following our previously reported protocol (7). The specific protein bands were detected with the LAS-4000 mini (Fuji Photo Film) using a Super Signal West Pico reagent kit (Pierce Biotechnology). Anti-p21waf1, p27kip1, and actin were used at dilutions of 1:2,500, 1:2,500, and 1:5,000, respectively.

Detection of interleukin 6 secretion from PrSC

PrSC was seeded onto 6-well plates at a density of 1×10^4 cells per well. Twenty-four hours after seeding, cells were treated with each α_1-AR antagonist (10 μmol/L) for 3 days. Cells were harvested and counted, and culture supernatants were collected on day 4. For the quantitative determination of interleukin (IL)-6, the collected aliquots of medium were subjected to ELISA assays using a Quantikine human IL-6 immunoassay kit (R&D Systems).

Statistical analysis

In vivo data obtained from vehicle and α_1-AR antagonist administration groups were analyzed using Student's t test with Dunnet multiple comparison. Other data from *in vitro* experiments were analyzed using Student's t test. Values were expressed as the means ± SD. Values of $P < 0.05$ were considered statistically significant.

Results

Effects of combining PrSC with LNCaP sublines on tumorigenesis *in vivo*

We used the commercially available human PrSC cell line as a source of stromal cells to simulate the tumor microenvironment. We first confirmed the characteristics of PrSC using RT-PCR analysis. PrSC expressed mRNAs of fibroblastic/myofibroblastic differentiation markers *vimentin, tenascin (TN)-C*, and α *smooth muscle actin (SMA)*, but not smooth muscle differentiation markers *myosin* or *desmin* (Fig. 1A). PrSC expressed mRNA of tumorigenic growth factors, *fibroblast growth factor (FGF)-2* and *FGF-7*, whereas the mRNA expression of *insulin-like growth factor (IGF)-I* was quite low (data not shown). In PrSC, the expression of androgen receptor protein was not detected, and no changes in cell proliferation were observed in culture with additional dihydrotestosterone (DHT; data not shown).

Parental LNCaP, E9, and AIDL lines were grafted alone or in combination with PrSC into athymic mice. As shown in Figure 1B, no significant difference of tumor weights was observed when LNCaP or its sublines were grafted without stroma. On the other hand, PCa tumors grown by combining E9 with PrSC resulted in significantly larger tumors (87.8 ± 21.9 mg) compared with tumors from LNCaP combined with PrSC (60.4 ± 35.0 mg; $P < 0.05$). There was no significant difference between LNCaP + PrSC tumors and AIDL + PrSC tumors. IHC analysis showed that cell proliferation (Ki-67 labeling index) in PCa + PrSC tumors was significantly higher than those of tumors without PrSC for all PCa cells ($P < 0.01$; Fig. 1C). As for the MVD in tumors, there was a statistically significant increase between grafting E9 or AIDL alone versus adding PrSC ($P < 0.01$; Fig. 1D).

Effect of subtype-selective α_1-AR antagonists on tumors formed by combining E9 and PrSC

Of the 3 cell lines, we found that E9 cells showed the highest responsiveness and most stable tumorigenesis when coinoculated with PrSC. Therefore, we chose the E9 + PrSC model to evaluate the effects of subtype-selective α_1-AR antagonists (naftopidil, tamsulosin, and silodosin) on PCa tumorigenesis *in vivo*.

We grafted E9 + PrSC cell mixtures into athymic mice and gave the subtype-selective α_1-AR antagonist (10 mg/kg/d) to the grafted mice daily by oral administration for 28 days. Figure 2A reveals the characteristic gross appearance and Ki-67 staining of E9 + PrSC tumors treated with vehicle or subtype-selective antagonists (naftopidil, tamsulosin, or silodosin). E9 + PrSC xenografts formed spheroidal reddish tumors filled with blood. The average relative rate of E9 + PrSC tumor weights divided by body weight in control animals, naftopidil-, tamsulosin-, and silodosin-treated animal were 0.369%, 0.192%, 0.267%, and 0.281%, respectively (Fig. 2B). A statistically significant reduction of relative rate was observed only in naftopidil-treated mice ($P < 0.01$). The Ki-67 index in E9 + PrSC

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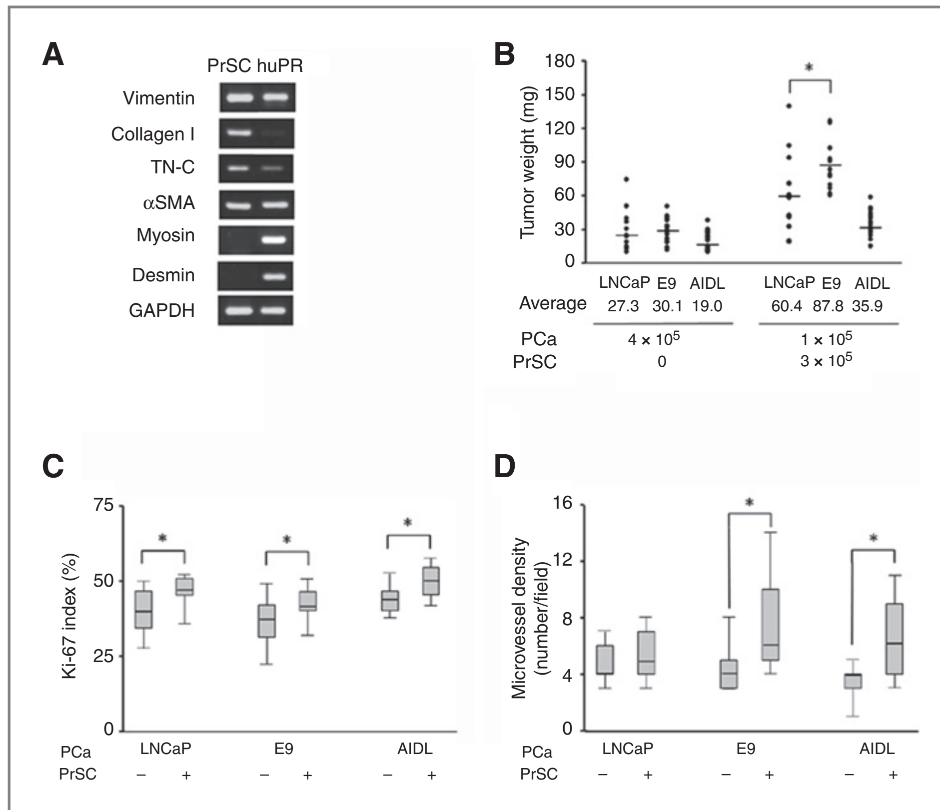

Figure 1. Effects of mixing tumor lines with PrSC on their tumorigenesis *in vivo*. A, gene expression of differentiation markers in PrSC. To confirm gene expression, we performed RT-PCR analysis. cDNA made from normal human prostate (huPR) poly A+ RNA was used as a positive control. The products were resolved on 1.6% agarose gels and visualized with ethidium bromide. B, comparison of tumor weights obtained by grafting animals with tumor lines alone or mixed with stroma. The tumor weights were measured 35 days after transplanting. *, $P < 0.05$ versus LNCaP + PrSC tumor. C, comparison of cell proliferation (Ki-67 labeling index) obtained by grafting animals with tumor lines alone or mixed with stroma. The Ki-67 positive cells were counted in 10 different areas at 400× magnification. *, $P < 0.01$ versus PCa + PrSC tumor. D, comparison of MVDs of tumors obtained by grafting animals with tumor lines alone or mixed with stroma. The mouse-specific CD31-positive vessels with lumens were counted in 10 different areas at 200× magnification. *, $P < 0.01$ versus PCa + PrSC tumor.

tumors was decreased by all antagonists ($P < 0.01$; Fig. 2C). MVD in E9 + PrSC tumors was decreased by treatment of naftopidil and silodosin ($P < 0.01$; Fig. 2D).

Gene expression of α_1-AR subtypes in PCa cells and PrSC

There were differences in the mRNA expression level of the α_1-*AR* subtypes among the 3 PCa cell lines. α_{1a}-*AR* subtype mRNA was detected in LNCaP and AIDL, whereas its mRNA expression in E9 was quite low (Fig. 3A). Similar levels of α_{1d}-*AR* subtype mRNA were detected in LNCaP and E9 but not in AIDL. α_{1b}-*AR* subtype mRNA was not detected in any of the PCa cells. Although PrSC expressed all α_1-*AR* subtype mRNAs, expression of α_{1a}-subtype mRNA was comparatively low.

Effects of subtype-selective α_1-AR antagonists on cell proliferation *in vitro*

Naftopidil (α_{1D}-selective), silodosin (α_{1A}-selective), and RS100329 (α_{1A}-selective) showed approximately equivalent growth inhibitory effects on LNCaP, E9, and AIDL (Fig. 3B). Tamsulosin (α_{1A}- and α_{1D}-selective) and BMY 7378 (α_{1D}-selective) showed comparatively weak inhibitory effects on AIDL. With regard to PrSC, similar to PCa cells, growth inhibitory effects were observed by treatment with naftopidil, silodosin, and RS100329 in dose-dependent manners (Fig. 3C). The growth inhibitory effect of naftopidil was greater than that of silodosin.

Analyses of antiproliferative mechanisms of subtype-selective α_1-AR antagonists

To investigate whether subtype-selective α_1-AR antagonists affect the cell cycle, we carried out flow cytometric analysis and Western blot analysis of cell-cycle regulatory proteins. Compared with vehicle, naftopidil but neither tamsulosin nor silodosin increased the population of cells in the G_0/G_1 phase, which was accompanied by a decrease in the S/G_2 phase in E9 cells (Fig. 4A). Similar results were observed in PrSC with lower concentrations than that required to induce G_1 cell-cycle arrest in E9 cells

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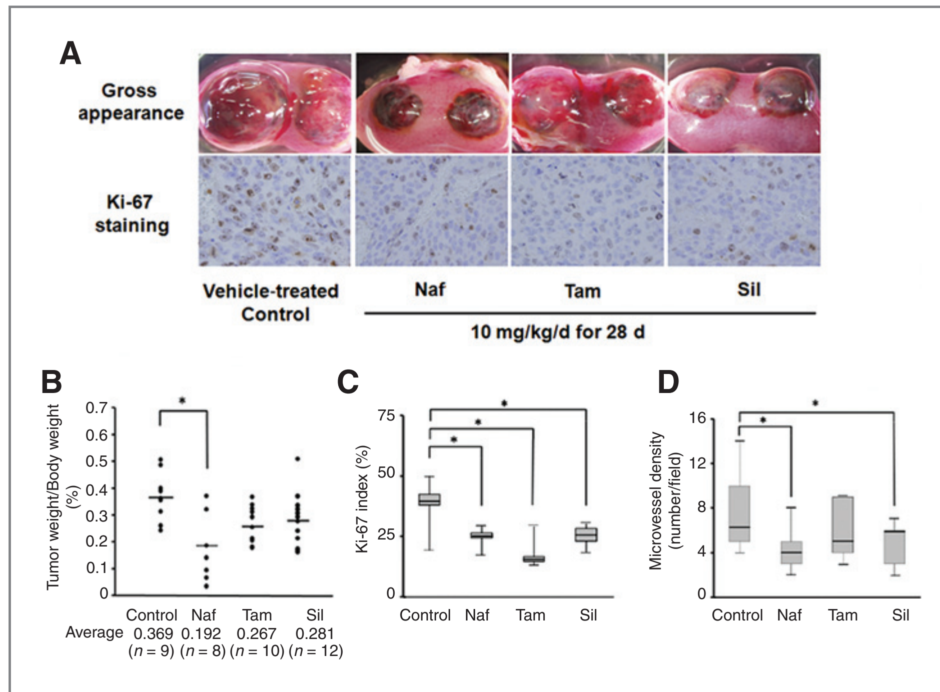

Figure 2. Effect of subtype-selective α_1-AR antagonists on tumorigenesis of E9 + PrSC. E9 cells and PrSC were combined in collagen gels and then grafted in athymic mice. Grafted mice were treated orally with an α_1-AR antagonist (10 mg/kg/d) beginning 7 days after transplant and continued for 28 days. Tumor weights were measured 35 days after transplanting. A, characteristic gross appearances of E9 + PrSC tumors and representative images of Ki-67 stain of E9 + PrSC tumors. B, relative rate of E9 + PrSC tumor weights divided by body weight in mice treated with each subtype-selective α_1-AR antagonist. *, $P < 0.01$ versus vehicle-treated control. C, the Ki-67 index in E9 + PrSC tumors. *, $P < 0.01$ versus vehicle-treated control. D, MVD in E9 + PrSC tumors. CD31-positive vessels with lumens were counted in 10 different areas at 200× magnification. *, $P < 0.01$ versus vehicle-treated control. Naf, naftopidil; Tam, tamsulosin; Sil, silodosin.

(Fig. 4B). As shown in Figure 4C, naftopidil-treated E9 cells increased the level of cell-cycle regulatory protein p27, whereas no increase of p27 protein was observed in naftopidil-treated PrSC.

To confirm the apoptotic effect of subtype-selective α_1-AR antagonists, we used flow cytometry to characterize early apoptosis in annexin V–stained cells. Silodosin-treated E9 cells had a greater proportion of cells in the early phase of apoptosis (Table 2). Although statistical analysis indicated a significant difference ($P < 0.05$), the increased ratio was modest. Naftopidil and tamsulosin were not involved in apoptosis induction. Increased caspase-3 activity was observed only in silodosin-treated E9 cells.

To evaluate the effect of subtype-selective α_1-AR antagonists on secretion of IL-6 derived from PrSC, we performed ELISA. The total amount of IL-6 protein secreted from PrSC was decreased by naftopidil treatment (Fig. 5B). Real-time PCR analysis showed that the expression of *FGF-2* and *FGF-7* mRNAs by PrSC was not changed by treatment with any subtype-selective α_1-AR antagonist (data not shown).

Discussion

We have demonstrated that subtype-selective α_1-AR antagonists have antiproliferative effects on PCa growth in both *in vitro* and *in vivo* systems. The major finding in this study was that oral administration of subtype-selective α_1-AR antagonist, naftopidil, suppressed E9 + PrSC tumor growth *in vivo*. The antiproliferative effect of naftopidil was due to inhibition of cell-cycle progression in both PCa cells and PrSC. Naftopidil was the only α_1-AR antagonist that showed strong antiproliferative effects on stromal cells among the 5 subtype-selective α_1-AR antagonists which included tamsulosin, silodosin, RS100329, and BMY7378. In addition, we showed that the antiproliferative effect on stromal cells resulted in the suppression of tumorigenic soluble factor derived from stromal cells. These results suggest that the marked decline of tumor weight caused by naftopidil treatment might be due to its antiproliferative effects on both PCa cells and stromal cells. It is very attractive that naftopidil exhibits this unique antiproliferative action on stromal cells.

Previous studies have reported that tumor–stroma interactions could play a role in the promotion of prostate carcinogenesis (16). Kawada et al. reported that PrSC-conditioned medium increased PCa cell proliferation *in vitro*, suggesting the significance of prostate stroma secreted factors in tumor–stroma interactions (20). FGF-2 and FGF-7 play an important role as mediators involved in tumor–stroma interactions (10). In PrSC, mRNA expressions of *FGF-2* and *FGF-7* were detected (data not shown). Previous report showed that responsiveness of E9 cells to FGF-2 and

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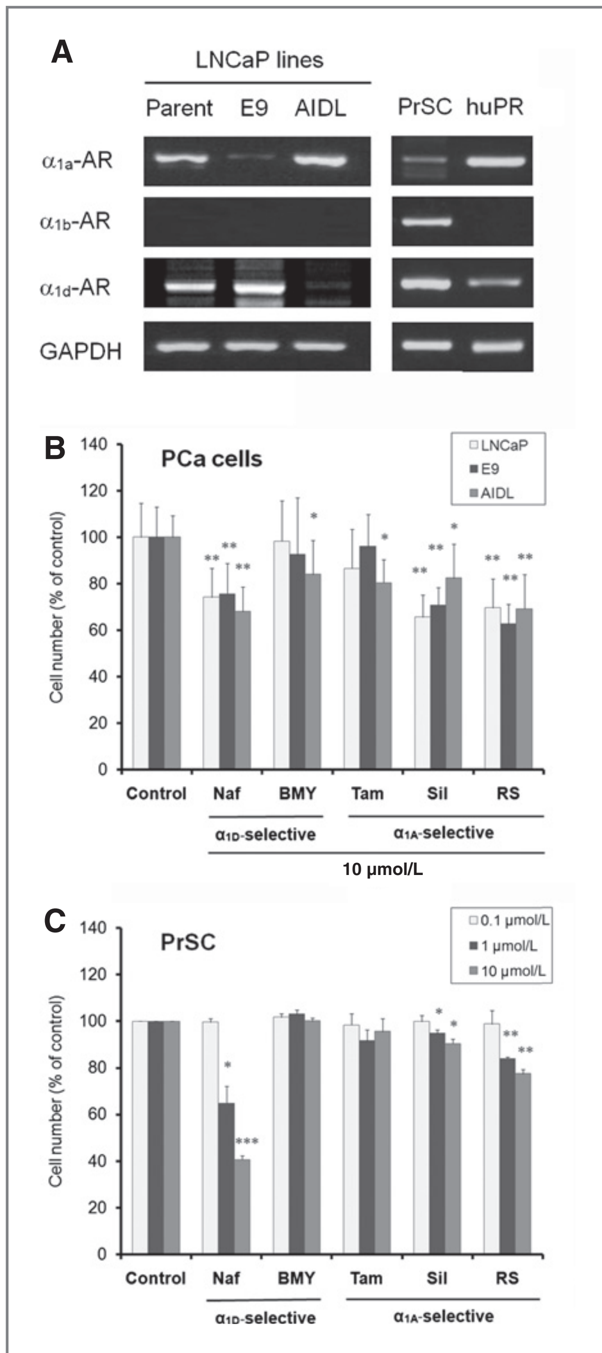

Figure 3. Effects of subtype-selective α_1-AR antagonists on cell proliferation *in vitro.* A, gene expression of α_1-AR subtypes in PCa cells and PrSC. cDNA made from normal human prostate (huPR) poly A + RNA was used as a positive control. The products were resolved on 1.6% agarose gels and visualized with ethidium bromide. B, effects of subtype-selective α_1-AR antagonists on PCa cell proliferation *in vitro*. PCa cells were exposed to 10 μmol/L of each α_1-AR antagonist for 4 days. C, PrSC was exposed to various concentrations (0.1, 1, and 10 μmol/L) of each α_1-AR antagonist for 3 days. Values represent the means ± SD percentage of viable cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus vehicle-treated control. Naf, naftopidil; BMY, BMY7378; Tam, tamsulosin; Sil, silodosin; RS, RS100329.

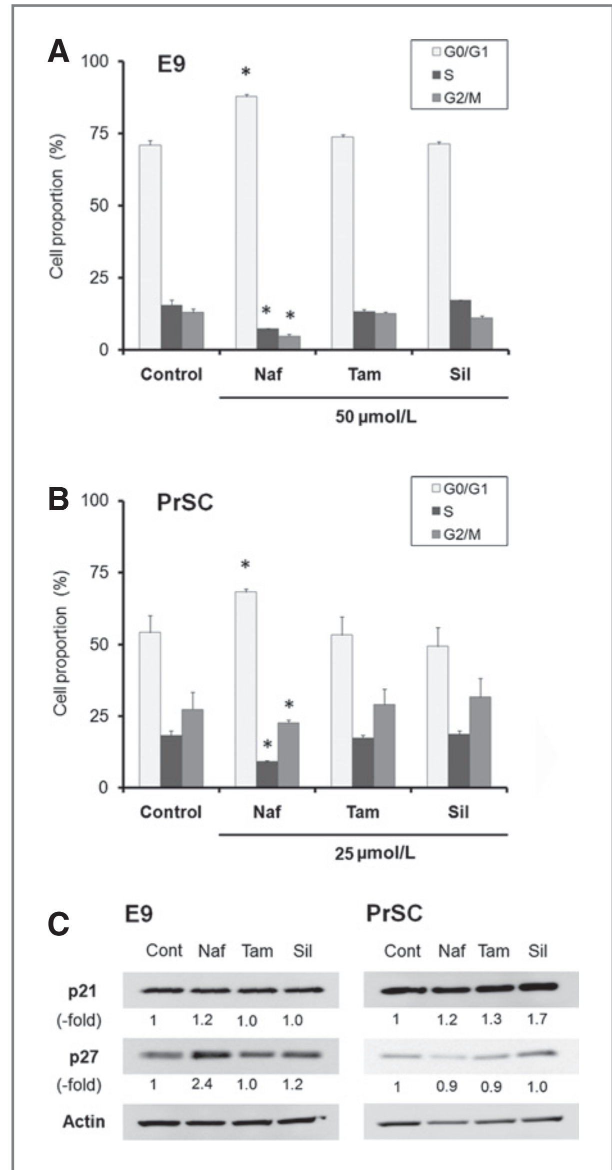

Figure 4. Effects of subtype-selective α_1-AR antagonists on cell cycles of E9 cells and PrSC. A and B, cell-cycle analyses of cells treated with 50 μmol/L (E9 cells, A) or 25 μmol/L (PrSC, B) of each subtype-selective α_1-AR antagonist for 48 hours. Distribution of cell-cycle phases is shown for E9 cells and PrSC. Each value represents the means ± SD percentage. *, $P < 0.05$ versus vehicle-treated control. C, expression of cell-cycle regulatory proteins (p21 and p27) was determined by Western blotting analysis. Cell lysates (30 μg) from E9 cells or PrSC were separated by electrophoresis with 12.5% SDS-polyacrylamide gel. Blots were probed with indicated antibody after transferring to a PVDF membrane. Cont, control; Naf, naftopidil; Tam, tamsulosin; Sil. silodosin.

FGF-7 stimulation were higher than that of parental LNCaP cells (19), suggesting that E9 cells made larger tumor in combination with PrSC compared with LNCaP cells *in vivo*. Uemura et al. showed that the administration of angiotensin II receptor blockers (ARB), that are widely used as antihypertensive drugs, suppressed PC-3 + PrSC tumor growth *in vivo* by inhibition of PrSC cell proliferation

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Table 2. Effects of subtype-selective α_1-AR antagonists on apoptosis in E9 cells *in vitro*

	Control	Naf	Tam	Sil
Flow cytometric analysis				
Viable cells	91.0 ± 2.2	91.4 ± 0.5	91.9 ± 0.2	89.7 ± 2.2
Early apoptosis	2.0 ± 0.4	1.7 ± 0.3	1.9 ± 0.2	3.8 ± 0.2[a]
Luminescence detection				
Caspase-3	100 ± 6.5	105.8 ± 6.1	106.7 ± 15.5	124.0 ± 7.2[a]
Caspase-9	100 ± 6.4	99.8 ± 5.8	106.1 ± 8.9	108.8 ± 9.0

NOTE: E9 cells were treated with each subtype-selective α_1-AR antagonist (50 μmol/L) for 48 hours, and then stained simultaneously with annexin V and PI. The samples were subjected to flow cytometry to detect early apoptosis. Values represent the means ± SD percentage of cells. In addition, caspase-3/-9 activity was measured by luminescence detection. Results are represented as the percentage of the activity compared with control.
Abbreviations: Naf, naftopidil; Tam, tamsulosin; and Sil, silodosin.
[a]$P < 0.05$ versus vehicle-treated control.

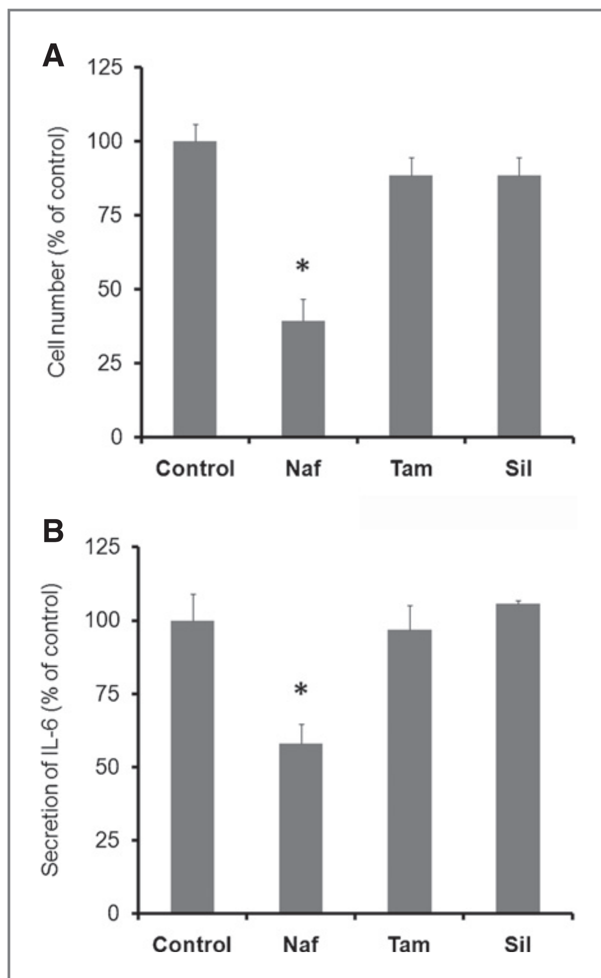

Figure 5. Effect of subtype-selective α_1-AR antagonists on secretion of IL-6 from PrSC. A, effects of subtype-selective α_1-AR antagonists on PrSC proliferation *in vitro*. PrSC was exposed to 10 μmol/L of each α_1-AR antagonist for 3 days. B, secretion of IL-6 from PrSC was measured by ELISA. Results are presented as the percentage compared with control. *, $P < 0.05$ versus vehicle-treated control. Naf, naftopidil; Tam, tamsulosin; Sil, silodosin.

and IL-6 secretion from PrSC (15). It is highly important that naftopidil shows antiproliferative effects not only on cancer cells but also on stromal cells, and that stromal cell growth was inhibited by lower concentrations than that required to inhibit cancer cells. In our *in vivo* tumorigenic study, although difference of a given concentration from clinically relevant therapeutic dose range was the smallest in naftopidil, the highest decline of tumor weight was observed with naftopidil treatment. From these findings, we suggest that subtype-selective α_1-AR antagonists such as naftopidil, which inhibit stromal cell growth and suppress the secretion of tumorigenic soluble factor, might prevent tumor–stroma interactions in PCa progression. For instance, naftopidil showed antitumor effects through suppression of IL-6 protein secreted from stromal PrSC. IL-6 plays an important role in tumorigenesis and is considered a positive growth factor for most PCa cells and an important messenger linking stromal and epithelial prostate cells (21). IL-6 is also involved in the regulation of PCa cell growth (22). The inhibition of IL-6 secreted from stromal cells likely contributes to the suppression of prostate tumor.

We found strong evidence for the antiproliferative effects of highly α_{1A}-selective RS100329 on both PCa cells and PrSC (Fig. 3). Naftopidil and RS100329 both possess a phenylpiperazine-based structure and arrest cells in G_1. Interestingly, the Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) inhibitor KN-62 has a phenylpiperazine structure and induces G_1 cell-cycle arrest in small cell lung carcinoma (SCLC) cells (23, 24). In this study, the phenylpiperazine-derived, α_{1D}-selective antagonist BMY7378 did not induce G_1 cell-cycle arrest at low concentration (10 μmol/L; Fig. 3), whereas 5 times concentration of BMY7378 induced very weak G_1 cell-cycle arrest in E9 cells (data not shown). Taken together, it is possible that the α_1-AR antagonists with a phenylpiperazine-based structure might suppress PCa cell proliferation through G_1 cell-cycle arrest, whereas modification of the substituent group could change the properties of the compound, resulting in other effects on cells. The quinazoline-derived,

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subtype nonselective α_1-AR antagonist doxazosin is a well-investigated α_1-AR antagonist with antitumor effects (25–29). Kyprianou et al. focused attention on the quinazoline-based structure of doxazosin and indicated doxazosin-induced apoptosis might be independent of the α_1-AR signal and the biological characteristics of the cells (5, 30). Garrison and his colleagues demonstrated that novel lead quinazoline-derived compound (DZ-50) has demonstrated to reduce endothelial cell viability via a nonapoptotic mechanism, even though it has quinazoline structure (31). The characteristic of the compound depends strongly on substituent group. Thus, it is possible that BMY7378, which has phenylpiperazine structure, did not show any significant cell-cycle arrest at low concentration. Our studies suggest strongly that differences in antiproliferative mechanisms among α_1-AR antagonists are mainly attributable to their chemical structures.

Tamsulosin and silodosin showed the similar tendency *in vivo*, that is, a small decline of E9 + PrSC tumor growth. Suppression of E9 + PrSC tumor growth during silodosin treatment might have induced some early apoptosis in PCa cells. Silodosin showed antiproliferative effects on E9 cells through apoptosis induction, whereas the apoptosis-inducing action was modest in comparison with that of quinazoline-derived α_1-AR antagonists (5). On the other hand, tamsulosin did not show antiproliferative effect on either PCa cells or PrSC *in vitro*, similar to previous reports (5, 6). Tamsulosin induced neither induction of G_1 cell-cycle arrest, nor apoptosis, nor suppression of tumorigenic soluble factors derived from PrSC. It is interesting that these antagonists have shown different antiproliferative mechanisms for androgen low-sensitive E9 cells expressing a_{1d}-*AR* subtype but not α_{1a}-*AR* subtype.

In regard to treatment for BPH, α_1-AR antagonists are the most widely used therapeutic agents. α_1-AR antagonists are divided into 2 groups on the basis of differential selectivity to α_1-AR; subtype nonselective agents and subtype-selective agents. In Japan, using subtype-selective antagonist is mainstream treatment for BPH because such agents have fewer side effects on the cardiovascular system than subtype nonselective antagonists (32). In this study, we used 3 typical, clinically used, subtype-selective α_1-AR antagonists which were developed in Japan. Tamsulosin, an α_{1A}-AR and α_{1D}-AR selective antagonist, is commonly used around the world. Silodosin, a highly selective α_{1A}-AR antagonist, has been recently approved for clinical use in Japan, Europe, and the United States. Naftopidil, which is only marketed in Japan, has a comparatively higher selectivity for α_{1D}-AR than the other 2 antagonists. α_{1D}-selective naftopidil and highly α_{1A}-selective silodosin showed antiproliferative effects on PCa cells regardless of the androgen sensitivity and the α_1-AR subtype expression in PCa cells *in vitro* cell culture. These results suggest that neither the androgen receptor nor the α_1-AR signal in PCa cells may be involved in their antiproliferative effects. In clinical aspects, naftopidil has high tolerability with few adverse effects (33, 34). Thus, certain clinical benefit obtained from long-term internal use of naftopidil might be prospective. Naftopidil may be considered for long-term prevention, that is, it is possible that naftopidil could suppress progression to clinical PCa in patients with latent PCa which is concomitant with BPH. In future, further investigation for a prospective study would be necessary to confirm the antitumor effects from oral administration of naftopidil on patient with PCa.

In conclusion, our findings advanced understanding of the effects of subtype-selective α_1-AR antagonists on both PCa and stromal cells. This study provides strong evidence that oral administration of α_{1D}-AR antagonist naftopidil decreases PCa tumor growth by altering tumor–stroma interactions. Furthermore, the antiproliferative effect of naftopidil is not related to androgen sensitivity of the cells or the α_1-AR subtype expression in PCa cells, suggesting that naftopidil might induce G_1 cell-cycle arrest in various types of cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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