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Effects of Curcumin Combined With the 5-alpha Reductase Inhibitor Dutasteride on LNCaP Prostate Cancer Cells

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Abstract. *Background/Aim: Curcumin is a natural compound of turmeric, which inhibits prostate cancer cell proliferation. This study examined whether treatment of LNCaP prostate cancer cells with the combination of curcumin and dutasteride, a 5-alpha reductase inhibitor, affect proliferation and the amount of testosterone and dihydrotestosterone. Materials and Methods: LNCaP Cells were incubated with curcumin or the combination of curcumin and dutasteride and cell proliferation was measured at 72 h. LC-MS/MS was used to determine testosterone and dihydrotestosterone concentrations in prostate cancer cells. Results: Curcumin combined with dutasteride suppressed proliferation and affected apoptosis of LNCaP cells. The combination of curcumin and dutasteride also reduced the amount of testosterone and dihydrotestosterone in LNCaP cells. The secretion of prostate-specific antigen was inhibited by the combination treatment in a dose-dependent manner. Conclusion: Treatment with the combination of curcumin and dutasteride may interfere with the intra-tumoral androgen activity.*

Androgen signaling plays a key role in the propagation and differentiation of prostate gland cells and also in prostate carcinogenesis (1). The 5-alpha reductase inhibitor dutasteride inhibits conversion of testosterone to dihydrotestosterone. Dutasteride is used to treat benign prostatic hyperplasia (BPH) as it shrinks the prostate and reduces the levels of prostate-specific antigen (PSA) by at least 40% (2). Relative to testosterone, dihydrotestosterone has considerably more potent biological activities in various tissues (3). The Reduction by Dutasteride of Prostate Cancer Events (REDUCE) trial is a randomized controlled trial, which tests dutasteride for prostate cancer chemoprevention over a 4-year period in patients between 50 to 75 years of age who had prostate-specific antigen (PSA) levels of 2.5 to 10.0 ng per milliliter (4). Risk of prostate cancer was reduced by approximately 25% in randomized trials using dutasteride (4, 5); therefore, inhibiting the conversion of testosterone into dihydrotestosterone might reduce the risk of prostate cancer.

Curcumin, a spice often used as an ingredient in curry and other dishes, is an active ingredient comprising about 5% of turmeric. Previously, we have reported that curcumin can affect steroidogenesis in prostate cancer. The study indicated that curcumin significantly reduced testosterone levels in LNCaP cells through AKR1C2 expression, and thereby, suppressed the proliferation of LNCaP and 22Rv1 prostate cancer cells, which expressed the androgen receptor (6). In addition, the suppression of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) has validated that curcumin has powerful anti-oxidative and anti-inflammatory functions (7) and triggers apoptosis through DNA damage, thus impeding the progression of various cancers (8).

As both dutasteride and curcumin have suppressive effects on intratumoral androgen production, the current study examined whether dutasteride and curcumin had an additive inhibitory effect on cell proliferation and PSA production. The experimental results showed that curcumin and dutasteride have an inhibitory effect on the malignant progression of prostate cancer, supporting the clinical potential of this combination for the prevention and therapy of prostate cancer.

Materials and Methods

Human prostate cancer cell line and cell culture. The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in 10-cm diameter dishes with RPMI 1640 Medium (Gibco, Gran Island, NY, USA) containing 10% (v/v) fetal bovine

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Key Words: Curcumin, dutasteride, testosterone, prostate cancer.

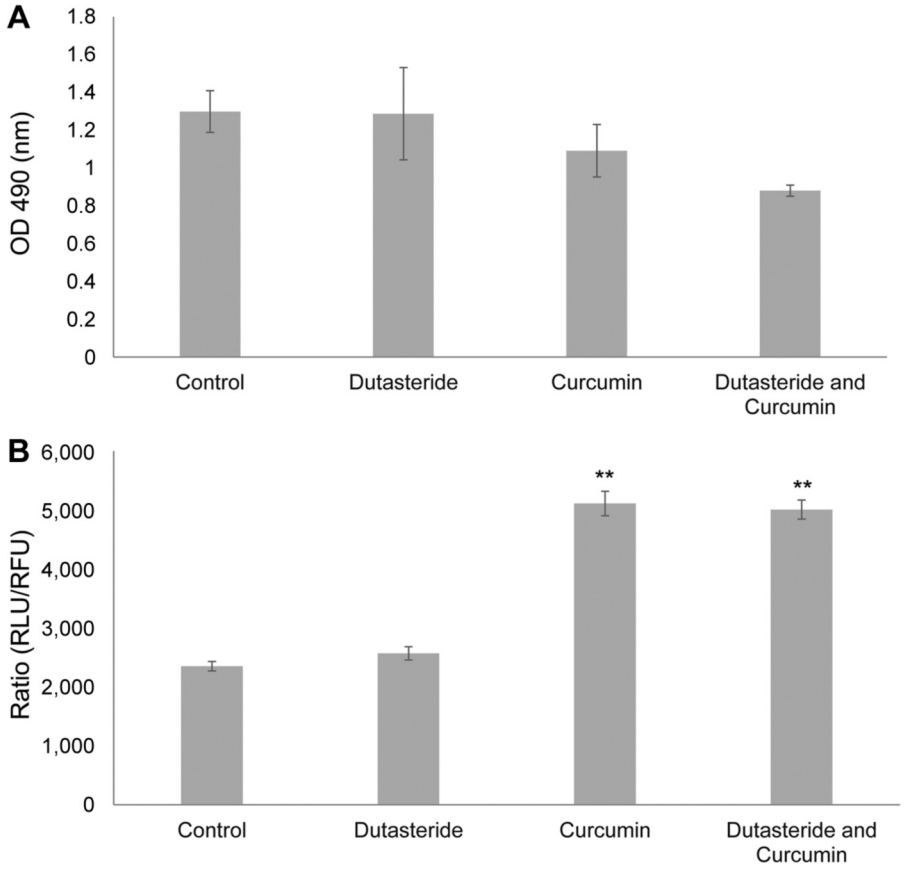

Figure 1. *Effects of curcumin and dutasteride on LNCaP cell growth. A) Cells were treated with 10 μM curcumin or/and 10 μM dutasteride, and MTS proliferation assays were performed. Each point represents the mean±SEM of six wells. The combination of curcumin and dutasteride has an inhibitory effect on the viability of LNCaP cells, although no statistical significance was observed (p=0.087) compared with the control (0 μM). B) Induction and quantification of apoptosis were examined following treatment of curcumin and dutasteride. LNCaP cells were incubated with 25 μM curcumin alone and with 10 μM dutasteride for 6 h. The error bar represents SEM from 6 wells. The vertical axis shows the ratio of caspase-3/7 activity [relative luminescence units (RLU)] to viable cells [relative fluorescence units (RFU)]. A statistical significance (**p<0.01) is indicated compared with the control (0 μM).*

serum (Gibco) and 1% penicillin-streptomycin (Gibco) at 37˚C under 5% CO_2, and passaged with TrypLE Select (Gibco) every three days to maintain a cell monolayer. To determine testosterone and dihydrotestosterone levels in LNCaP cells, charcoal stripped-FBS (Gibco) was used in the medium instead of FBS for 24 h before curcumin treatment. LNCaP cells were characterized for androgen receptor expression.

An effective preparation of curcumin (THERACURMIN) (9), a nano-particle colloidal dispersion with improved oral bioavailability (THERACURMIN, Theravalues Corp., Tokyo, Japan), was obtained. Curcumin was dissolved in DMSO at a concentration of 10 mM and stored at –20˚C until use. 10 μM dutasteride (Cayman Chemical Company, Ann Arbor, MI, USA) was used in this study.

Cell proliferation assay. LNCaP cells were incubated with 10 μM curcumin with or without 10 μM dutasteride. Cell proliferation was then measured by an MTS assay (Promega Corp., Madison, WI, USA) at 72 h. Quintuplet assay repeats were conducted for all treatments, and the results are presented as the mean of three separate experiments.

Apoptosis assay of viability and caspase activation. To determine apoptosis induced by curcumin with or without dutasteride, we measured caspase-3/7 activity using the ApoLive-Glo Multiplex assay (Promega, Corp.). LNCaP cells were plated in a 96-well plate at a density of 1×10^5 cells/ml and incubated at 37˚C overnight. The cells were then incubated with 25 μM curcumin with or without 10 μM dutasteride for 6 h. In accordance with the manufacturer's protocol, after incubation, 20 μl of viability reagent were added to each well and the cells were incubated again for 30 min at 37˚C. Fluorescence (RFU: relative fluorescence units) was measured at a $400_{EX}/505_{EM}$ wavelength for viability employing a fluorescence spectrophotometer F-7000 (Hitachi, Tokyo, Japan). Finally, we added 100 μl of Caspase-Glo 3/7 Reagent to each well and incubated the cells for 1 h at room temperature. Luminescence (RLU: relative luminescence units) was measured using the luminometer AB-2350 (Atto, Tokyo, Japan) to detect apoptosis.

Immunoblotting analysis. Subconfluent LNCaP cells were treated with different concentrations of curcumin with or without 10 μM

dutasteride for 24 h. The cells were washed twice with cold PBS and then lysed in RIPA buffer on ice for 10 min. Supernatants were collected following centrifugation of cell lysates at 15,000 g for 10 min at 4˚C. Protein concentrations were measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). Proteins were separated by SDS-PAGE (5-20%, SuperSep Ace, Wako, Tokyo, Japan) and transferred to a PVDF membrane (Millipore Corporation, Billerica, MA, USA). The membrane was blocked in 1% BSA (bovine serum albumin) in TBS (Tris-buffered saline) (Blocker: Thermo Fisher Scientific, Inc.) for 1 h at room temperature and then incubated with i) p-ATM (Abcam, Cambridge, UK, clone#EP1890Y; 1:1,000 dilution) rabbit monoclonal (phospho S1981), ii) gamma-H2AX (Cell Signaling, Beverly, MA, USA; 1:1,000 dilution) rabbit polyclonal antibody, iii) cleaved PARP (Cell Signaling; 1:1,000 dilution) rabbit polyclonal antibody, iv) androgen receptor (Santacruz, Dallas, TX, USA, 1:1,000 dilution) rabbit polyclonal antibody, v) AKR1C2 (ABGENT, WuXi, China; 1:1,000 dilution) rabbit polyclonal antibody, vi) Cytochrome P450(17α) (CYP17A1) (Santa Cruz, clone#D-12; 1:1,000 dilution) rabbit monoclonal antibody, vii) steroid 5α-reductase type 1 (SRD5A1) (Proteintec, WuHan, China; 1:1,000 dilution), and viii) steroid 5α-reductase type 2 (SRD5A2) (Abcam, clone#EPR6181(B); 1:1,000 dilution) rabbit monoclonal antibody overnight at 4˚C. β-actin antibody (Sigma-Andrich Co., St. Louis, MO, USA. Clone#AC-15; 1:10,000 dilution) was used as an internal loading control. Blots were washed three times (15 min each) with TBST (Tris-buffered saline containing 0.1% Tween-20). The blots were then incubated with HRP-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA, USA) at a dilution of 1:3,000 for 1 h at room temperature and washed three times (15 min each) with TBST. Proteins were visualized with ECL Western Blotting Detection Reagents (GE Healthcare, Little Chalfont Buckinghamshire, UK) and LAS-3000 mini detection system (Fujifilm Medical Systems, Tokyo, Japan). An image processing program (ImageJ, NIH, Bethesda, MD, USA) was used to quantify the band density.

Mass-spectrometric assay of testosterone and dihydrotestosterone. A brief description of modified procedures is presented here; detailed description can be found in (10, 11). Cultured LNCaP cells were suspended in 1 ml of distilled water and homogenized by Ultra-Turrax homogenizer to extract steroids. The concentration of testosterone and dihydrotestosterone was determined using an LC-MS/MS system consisting of an Agilent 1290 Infinity LC systems (Agilent technologies, Santa Clara, CA, USA) and an API-5000 triple stage quadrupole mass spectrometer (Applied Biosystems, Chicago, IL, USA). MS analysis was conducted with electro spray ionization (ESI) in the positive-ion mode. The LC-MS/MS system was operated in the multiple reaction monitoring mode to monitor the m/z transition, from 394 to 253 for Testosterone-picolinoyl, and from 396 to 203 for dihydrotestosterone-picolinoyl (10). The limits of quantification were 1 pg for testosterone and dihydrotestosterone.

PSA measurement in conditioned medium. LNCaP cells were cultured in growth medium for 24 h and then treated with curcumin, dutasteride, or their combination. After a 72-h treatment, the conditioned media were collected, centrifuged to remove residual cells, and stored at –20˚C. The quantitative analysis of the amount of PSA secreted by LNCaP cells was conducted by an immunoassay procedure using a Human (free PSA) ELISA kit (Abcam). An MTS assay was performed at the same time, and results were used for normalization of PSA levels.

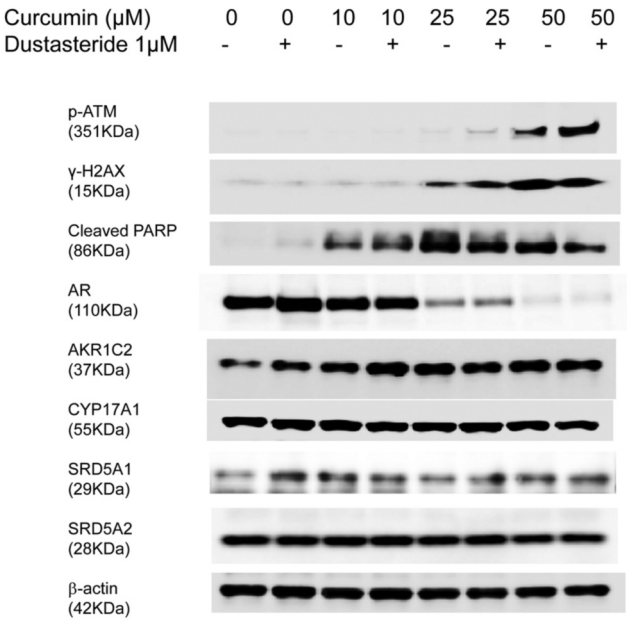

Figure 2. *Activation of DNA damage response and the expression of enzymes involved in testosterone metabolism following treatment with curcumin, dutasteride or their combination. Immunoblotting conducted after a 48-hour treatment resulted in the detection of protein expression. The protein bands from top to bottom show phosphorylation of ataxia-telangiectasia-mutated kinase (ATM), phosphorylation of H2AX (g-H2AX), poly (ADP-ribose) polymerase (PARP) cleavage androgen receptor (AR), Aldo-Keto reductase 1C2 (AKR1C2), Cytochrome P450(17α) (CYP17A1), steroid 5α-reductase type 1 (SRD5A1), steroid 5α-reductase type 2 (SRD5A2) and β-actin. The internal control used in this study was a mouse anti-human β-actin antibody.*

Statistical analysis. Data are expressed as mean±SEM. For statistical analysis, one-way ANOVA tests were used (SPSS). *$p<0.05$ and **$p<0.01$ were considered statistically significant.

Results

Curcumin and dutasteride inhibited the growth of LNCaP cells. The effects of curcumin, dutasteride and their combination on cell proliferation were determined by performing an MTS assay on LNCaP cells which express the androgen receptor at various concentrations. The combination of curcumin and dutasteride had an inhibitory effect on the growth of the cells. Viability of LNCaP cells decreased by 16%, as shown in Figure 1A, when 10 μM of curcumin was added to the culture medium. LNCaP cell growth was not suppressed by the use of dutasteride alone. Although not statistically significant, cell growth was inhibited by 33% ($p=0.087$) with the combination treatment of 10 μM curcumin and 10 μM dutasteride.

Curcumin and dutasteride treatment induced apoptosis. Caspase-3/7 activity was assayed to determine apoptosis and the ApoLive-Glo Multiplex Assay was used for quantifying

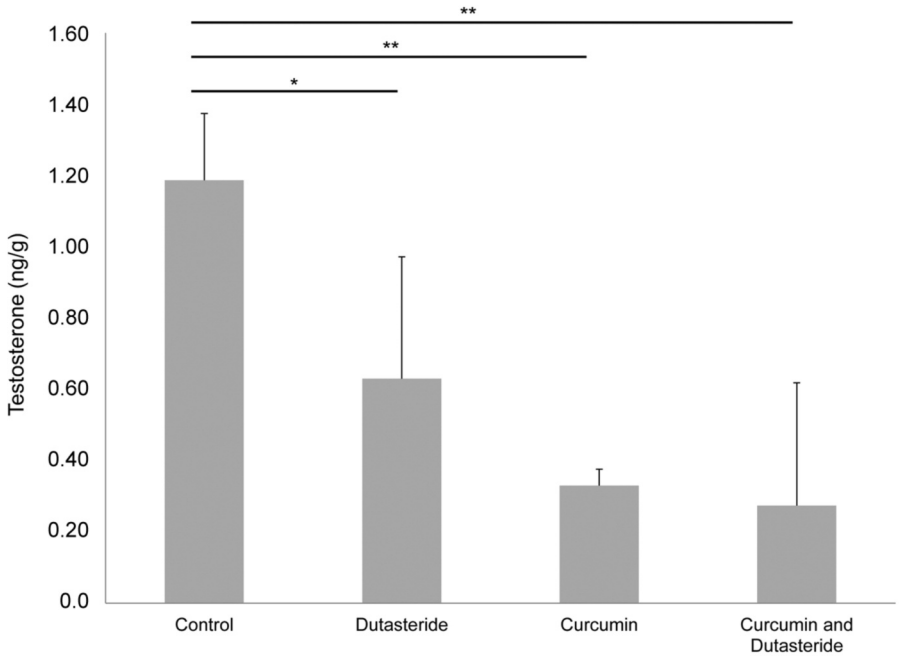

Figure 3. *Testosterone levels in LNCaP cells after curcumin and dutasteride treatment. A statistically significant effect is shown following treatment with curcumin (10, 50 μM) and curcumin with dutasteride (10 μM) in comparison with the control (*p<0.05, **p<0.01).*

apoptosis and the ration of caspase-3/7 activity (RLU) to viable cells was determined as described in Materials and Methods. Treatment with 25 μM curcumin resulted in a significant increase in the ratio of caspase-3/7 activity (RLU) to viable cells (RLU), as shown in Figure 1B, demonstrating that curcumin-induced apoptosis ($p<0.01$). Dutasteride alone did not induce apoptosis of LNCaP cells. It is also shown that the combination of 25 μM curcumin and 10 μM dutasteride induced apoptosis compared to the control ($p<0.01$), but no additional effect was observed.

Curcumin and dutasteride triggered DNA damage response and PARP cleavage. To determine how curcumin and dutasteride suppress cell proliferation and induce apoptosis, we examined the phosphorylation of ATM and histone H2AX as well as the occurrence of poly (ADP-ribose) polymerase (PARP) cleavage, a marker of apoptosis, by immunoblotting. In addition, to evaluate the effect of curcumin with and without dutasteride on steroidogenesis-related proteins in LNCaP prostate cancer cells (Figure 2), we examined the expression of androgen receptor, AKR1C2, CYP17A1, SRD5A1 and SRD5A2 using western immunoblotting. LNCaP cells were treated with 10 to 50 μM curcumin, 1 μM dutasteride, and their combination, for 24 h each.

At the concentrations of 25 μM and 50 μM, curcumin inhibited the expression of androgen receptors and induced phosphorylation of ATM and histone H2AX in LNCaP cells. Moreover, a combination of curcumin and dutasteride, compared to curcumin alone, induced higher levels of ATM and histone H2AX phosphorylation.

The expression of AKR1C2, CYP17A1, SRD5A1 and SRD5A2, which are involved in dihydrotestosterone production, was examined to determine changes in testosterone metabolism in prostate cancer cells treated with curcumin and/or dutasteride. Expression of these proteins was not affected by treatment with curcumin and/or dutasteride, with the exception of AKR1C2. The results showed a dose-dependent increase in AKR1C2 expression in LNCaP cells treated with curcumin (10 μM to 50 μM) and 1 μM dutasteride (Figure 2). The combination of curcumin and dutasteride induced higher levels of AKR1C2 expression compared to treatment with curcumin alone. We obtained similar results using 22Rv1 cells, another androgen receptor-positive prostate cancer cell line.

Treatment with curcumin and dutasteride decreased the levels of testosterone and dihydrotestosterone in LNCaP cells. The levels of testosterone and dihydrotestosterone in LNCaP decreased after 24 h treatment with 10 μM and 50 μM curcumin, respectively. Testosterone levels were reduced from 1.19 ng/g (control without curcumin) to 0.63 ng/g (10 μM dutasteride), 0.33 ng/g (50 μM curcumin) and 0.27 ng/g (50 μM curcumin and 10 μM dutasteride) (Figure 3). A considerable decrease was also observed in dihydrotestosterone levels, from 0.34 ng/g (control) to 0.04 ng/g (10 μM

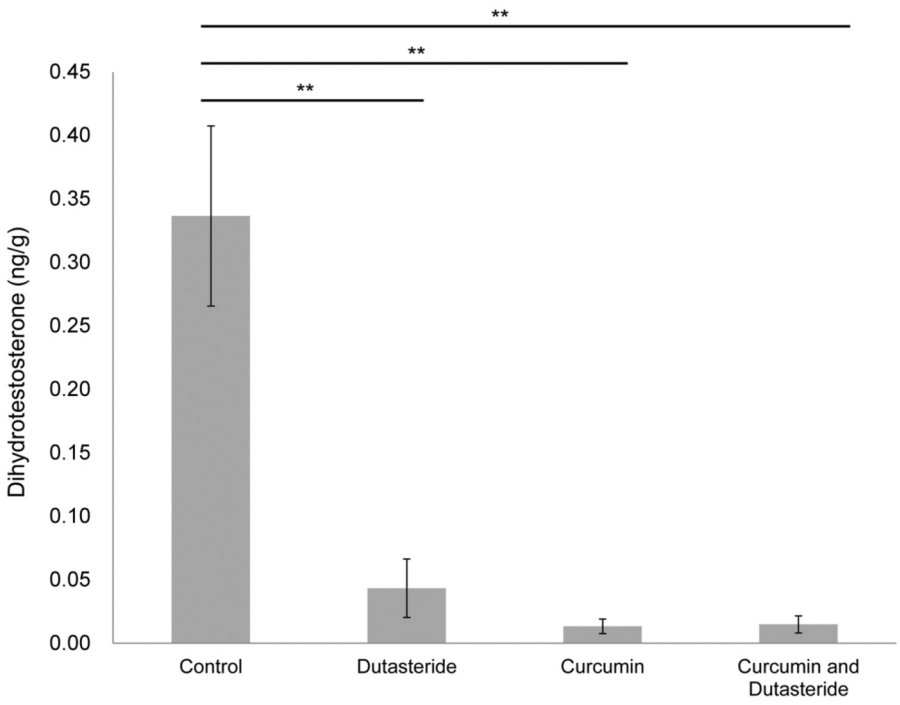

Figure 4. *Dihydrotestosterone levels in LNCaP cells after curcumin and dutasteride treatment. A statistically significant effect is shown following treatment with curcumin (10, 50 μM) and curcumin with dutasteride (10 μM) compared with the control (**p<0.01).*

dutasteride), 0.01 ng/g (50 μM curcumin), and 0.01 ng/g (50 μM curcumin and 10 μM dutasteride) (Figure 4).

The combination of curcumin and dutasteride inhibited PSA production. The effects of curcumin, dutasteride and their combination on PSA production were determined by performing an immunoassay on LNCaP cells. Treatment of the cells with 5 μM curcumin alone resulted in 58% inhibition of PSA secretion to the supernatant compared to the control. Treatment of cells with 5 μM dutasteride resulted in 48% inhibition. However, treatment with the combination of 5 μM curcumin and 5 μM dutasteride resulted in 70% inhibition of PSA production in LNCaP cells (Figure 5). These results indicated that LNCaP cells treated with curcumin and/or dutasteride produce significantly lower amounts of PSA. Cells that were treated with the combination of 5 μM curcumin and 5 μM dutasteride showed an even greater decrease in PSA levels (Figure 5).

Discussion

Testosterone and dihydrotestosterone are critical for the development, maturation, and maintenance of the prostate as well as for carcinogenesis. Blocking testosterone production is a common strategy used to treat metastatic prostate cancer, and successfully reduces the size of local prostate cancer and metastases. High testosterone levels in prostate tissue are linked to a high Gleason score, an advanced clinical stage, and a high percentage of positive biopsy cores in patients with prostate cancer (12). In addition, many studies have shown the involvement of intratumoral steroidogenesis of testosterone and dihydrotestosterone in prostate cancer cells (13). In particular, an increase in *de novo* testosterone synthesis was shown by an upregulation of enzymes that synthesize testosterone and dihydrotestosterone in castration-resistant prostate cancers which responded well to hormone therapy (13). Although, association between androgen concentrations and prostate cancer occurrence has been challenging to validate in epidemiologic studies (14), there is evidence that intervention in the intraprostatic hormonal environment may reduce the prevalence of some prostate cancers (15). Human aldo-keto reductases (AKRs) are able to convert potent sex hormones into their cognate inactive metabolites and *vice versa*. In prostate cells, AKR1C2 (3α-HSD) acts as a 3-ketosteroid reductase to lower the dihydrotestosterone levels and prevent androgen receptor activation. Curcumin has been shown to induce the expression AKR1C2 in prostate cancer by promoting dihydrotestosterone metabolism (16). On the other hand, dutasteride, a 5-alpha-reductase inhibitor, has been shown to inhibit the conversion of testosterone to dihydrotestosterone (2). In this study, the combination of curcumin and

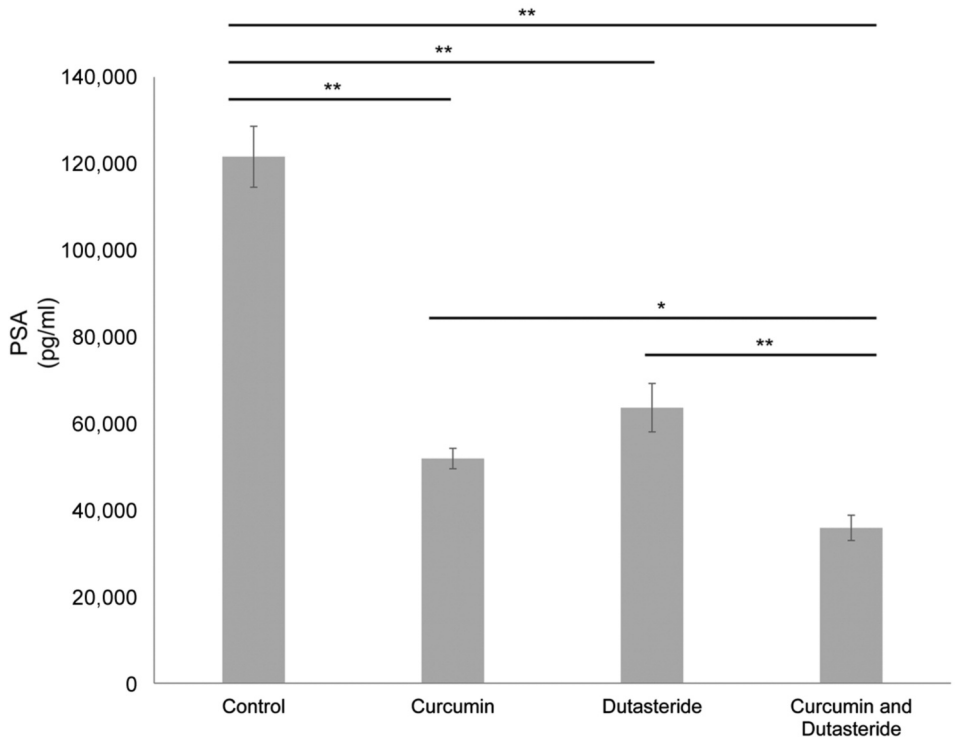

Figure 5. *PSA secretion in LNCaP cells after treatment with 5 μM curcumin and/or 5 μM dutasteride (*p<0.05, **p<0.01).*

dutasteride decreased the intratumoral levels of testosterone and dihydrotestosterone, apoptosis, and PSA production. Although no agreement has yet been reached on the efficacy of suppressing androgen concentrations in prostate cancer tissues for the prevention of prostate cancer, we must consider the impact that androgens and the manipulation of androgen metabolism, have on prostate growth and the intraprostatic hormone environment in men with different stages and pathological grades of the disease.

PSA is an extremely useful tumor marker for prostate cancer screening and treatment decisions, but its remarkable sensitivity introduces the possibility of over-treatment in some cases (17, 18). Previously, we administered either supplements containing curcumin as the primary component or a placebo daily for 6 months to 89 patients who had experienced a negative prostate biopsy result. PSA levels were measured at 0, 3, and 6 months. As a result, the experimental group, comprised of subjects with PSA levels measuring 10 ng/ml or greater, experienced a statistically significant decrease in PSA levels in comparison with to the placebo group ($p<0.001$) (19). In this study, we demonstrated that the combined effect of curcumin and dutasteride on PSA production reduced intracellular androgen levels. Evidence suggests that the serine protease activity of PSA may be involved in the invasion and metastasis of prostate cancer (20). Mammary serine protease inhibitor (maspin) has been characterized as a tumor suppressor gene and is down-regulated in prostate cancer. Curcumin-mediated maspin induction has been suggested to have therapeutic potential as it has been shown to inhibit the invasion of cancer cells (21). If combined, dutasteride and polyphenols, such as curcumin, could extend the PSA elevation period during postoperative PSA failure, and might present a useful treatment option in terms of patient quality of life (22). A prospective randomised phase III trial is on-going to investigate the effect of curcumin on the recurrence-free survival prostate cancer patients after radical prostatectomy. These various experimental results suggest that, through a variety of action mechanisms, curcumin, dutasteride and their combination have an inhibitory effect on the malignant progression of prostate cancer. Our research team will conduct a clinical investigation to examine whether curcumin and dutasteride have an additional inhibitory effect on PSA and whether they suppress oncogenesis. We will examine whether administration of curcumin and dutasteride could inhibit postoperative rising of PSA levels in subjects who had already received a radical prostatectomy as treatment for localized prostate cancer. Further basic and clinical investigation into the effects of curcumin and dutasteride on androgen signal suppression may produce clinically important results and open new avenues in cancer prevention strategies.

Disclosure Statement

The Authors have no conflict of interest.

Conflicts of Interest

The Authors declare no potential conflicts of interest in relation to this study.

Authors' Contributions

Hisamitsu Ide: contributed substantially to the study's conception and design, performing experiments, data acquisition and analysis, and writing of the article. Hiroaki Nakayama: contributed substantially to the study's conception and design, performing experiments, data acquisition and analysis, and writing of the manuscript. Yan Lu and Ayano Takei: contributed substantially to performing experiments and data acquisition and analysis. Akiyoshi Osaka, Gaku Arai and Kazunori Fukuda: contributed substantially to performing experiments and data acquisition and analysis. Hiroshi Okada and Kazutaka Saito: contributed substantially to data interpretation and revision of the article. Shigeo Horie: contributed substantially to the study's conception and design, data interpretation, and revision of the article.

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