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Vitamin C supplementation prevents testosterone-induced hyperplasia of rat prostate by down-regulating HIF-1α☆

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Abstract

Benign prostatic hyperplasia (BPH) is a disease that impairs the well-being of many aged men. To alleviate BPH symptoms or to find a cure for this disease, key molecules should be identified that control prostate cell proliferation. Recently, HIF-1α has attracted attention in this context, because it is highly expressed in hyperplasic prostates and prevents prostate cell death. Thus, given that vitamin C inhibits HIF-1α expression in several malignant tumors, we examined its therapeutic potential in BPH. HIF-1α was noticeably induced by testosterone in prostate cells, and this HIF-1α induction was abolished by vitamin C. Vascular endothelial growth factor (VEGF) promoter activity reporter assays and semi-quantitative RT-PCR revealed that vitamin C inhibited HIF-1-dependent VEGF expression. Furthermore, HIF-1α suppression by vitamin C was rescued by knocking down HIF-prolyl hydroxylase-2, suggesting that vitamin C destabilizes HIF-1α via prolyl hydroxylation. Moreover, vitamin C treatment abolished cell proliferation induced by testosterone treatment to the control level. These results suggest that vitamin C inhibits testosterone-induced HIF-1α expression and by so doing effectively prevents prostate hyperplasia. In male rats, testosterone treatment for 4 weeks induced prostate hyperplasia. Furthermore, HIF-1α and VEGF levels were significantly elevated in hyperplasic prostates. In vitamin C-treated rats, however, most prostate hyperplasia parameters and prostrate HIF-1α/VEGF levels were markedly reduced. Accordingly, our findings indicate that vitamin C could be further developed clinically for use as an anti-BPH agent.

Keywords: Benign prostate hyperplasia; HIF-1α; testosterone; Vitamin C; Cell death

1. Introduction

Benign prostatic hyperplasia (BPH) and prostate cancer are accompanied by uncontrolled tissue growth, and during this process, the tissue microenvironment becomes hypoxic, which in turn stimulates angiogenesis. Today, angiogenesis is viewed as a compelling means of preventing excessive tissue growth, and, in fact, several angiogenesis inhibitors have already been developed for the treatment of hypervascular tumors [1]. During tumor angiogenesis, vascular endothelial growth factor (VEGF) functions as a potent angiogenic factor [2] and its expression is known to be regulated by HIF-1 (a transcription factor).

HIF-1 is composed of HIF-1α and HIF-1β, and induces many of the proteins essential for cell survival in hypoxia. Of these two subunits, HIF-1β is constitutively expressed, but HIF-1α is tightly regulated by oxygen tension in terms of its protein stability and activity. Under normoxic conditions, two proline residues (aa. 402 and 564) of HIF-1α are hydroxylated by HIF-1-prolyl hydroxylases (PHDs) [3–5], which causes pVHL-E3 ubiquitin–ligase complex to recruit HIF-1α for ubiquitination [6]. However, when oxygen supply is limited, HIF-1α degradation ceases and HIF-1α associates with HIF-1β in the nucleus to form functional HIF-1.

HIF-1α activity is controlled by several independent steps. As mentioned above, PHDs are enzymes that hydroxylate HIF-1α [7]. They use oxygen as a substrate for this purpose and require Fe^{2+} and 2-oxoglutarate as co-factors. In particular, vitamin C also participates in the HIF-1α hydroxylation process. The oxygen-dependent regulation of HIF-1α activity is another step in this process. Factor inhibiting HIF-1α (FIH) is an enzyme which hydroxylates an asparagine residue (aa. 803) of HIF-1α, and when so hydroxylated, HIF-1α cannot recruit p300/CBP coactivator and thus loses its transcriptional ability [8]. Like PHDs, FIH also requires Fe^{2+}, 2-oxoglutarate and vitamin C for its full activation. Therefore, if any of these co-factors is limited, HIF-1α becomes stabilized and activated regardless of oxygen level. In addition, to this hydroxylation process, HIF-1α is known to be

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induced by some growth factors which accelerate the *de novo* synthesis of HIF-1α protein [9,10], and, similarly, testosterone has also been recently reported to stimulate HIF-1α synthesis even under normoxic conditions [11,12].

A growing body of evidence supports the notion that vitamin C is negatively involved in the pathogenesis of BPH. For example, case-control studies have demonstrated that men who consume fruits and vegetables rich in vitamin C have a lower incidence of BPH [13,14]. Furthermore, vitamin C plasma concentrations were found to be significantly lower in BPH patients than in normal healthy controls [15]. Accordingly, clinical studies suggest that vitamin C lowers the risk of BPH development, and because vitamin C generally acts to reduce oxidative stress, it might be expected that it influences the development of BPH. However, questions about the nature of the mechanism underlying the anti-BPH action of vitamin C remain open.

Given that prostate growth requires angiogenesis and that HIF-1 promotes angiogenesis via VEGF, we hypothesized that HIF-1 inhibition prevents prostate overgrowth via VEGF down-regulation. In the present study, we chose to examine the effect of vitamin C, because it is a known HIF-1 inhibitor with proven safety. Accordingly, we examined whether vitamin C inhibits HIF-1 and prevents BPH in testosterone-treated rats.

2. Materials and methods

2.1. Materials and reagents

Testosterone, vitamin C and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The FIH inhibitor, dimethyloxalylglycine (DMOG), was obtained from Frontier Scientific (Logan, UT, USA) and [α-^{32}P]CTP (18.5TBq/mmol) from NEN Scientific (Boston, MA, USA). Culture media, fetal bovine serum and Lipofectamine 2000 reagent were purchased from Invitrogen (Carlsbad, CA, USA). Anti-HIF-1α antiserum was generated in rabbits against a bacterially expressed fragment encompassing amino acids 418 to 698 of human HIF-1α, as previously described [16].

2.2. Cell culture

LNCaP (a human prostate cancer cell) and RWPE-1 (a nonneoplastic human prostate epithelial cell) were obtained from ECACC (London, UK) and ATCC (Manassas, VA, USA), respectively. LNCaP cells were cultured in RPMI-1640 medium supplemented with 10% heat-activated fetal bovine serum, L-glutamine (2 mM), HEPES (10 mM), penicillin and streptomycin, and RWPE-1 cells were maintained in keratinocyte serum-free medium supplemented with 5 ng/ml human recombinant EGF and 0.05 mg/ml bovine pituitary extract (Invitrogen) in a humidified condition with 5% CO_2 at 37°C.

2.3. Preparation of siRNA and transfection

A PHD2-targeting siRNA was synthesized by Invitrogen and its sequence corresponds to the coding region 885–905 of human PHD2 (NM_022051). This siRNA was transfected into cells using Lipofectamine RNAiMAX (Invitrogen). Cells were used for experiments 24 h after transfection.

2.4. Reporter assay

Luciferase reporter plasmid containing HRE of the rat *VEGF* gene was a gift from Dr. Eric Huang (University of Utah, Utah). Cells were transfected with 1 μg of reporter plasmid using Lipofectamine (Invitrogen) and lysed to determine luciferase and β-gal activities. Luciferase activities were measured using a Lumat LB9507 luminometer (Berthold), and initially measured activities were divided by β-gal activity to normalize transfection efficiencies.

2.5. Semiquantitative RT-PCR

To quantify VEGF, HIF-1α and β-actin mRNA levels, we utilized semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) method and autoradiography, as previously described [16]. Total RNAs were isolated using Trizol (Invitrogen).

2.6. Immunoblotting

Total or nuclear extracted proteins were separated on 8% or 15% SDS/polyacrylamide gels and transferred to Immobilon-P membranes (Millipore), which were then blocked with 5% nonfat milk in TTBS for 1 h and then incubated overnight at 4°C with anti-HIF-1α (diluted 1:2000), anti-ARNT (diluted 1:3000, Santa Cruz Biotechnology), antitubulin (diluted 1:3000, Santa Cruz) or anti-VEGF (diluted 1:3000, Santa Cruz) antibodies in blocking solution. Horseradish peroxidase-conjugated antirabbit and antimouse sera (GE Healthcare) were used as secondary antibodies after dilution at 1:5000 in blocking solution for 1 h. Antigen–antibody complexes were visualized using an Enhanced Chemiluminescence Plus Kit (GE Healthcare).

2.7. The BPH animal model

To examine the effect of vitamin C on testosterone-stimulated prostate growth, 3-month-old male Sprague-Dawley rats (300–350 g) were obtained from The Orient Company (Gyeong-Gi Do, Korea). Rats were subcutaneously injected with corn oil in the control group (n=5), with corn oil mixed with testosterone (10 mg/kg per day) in the BPH group (n=5), or with testosterone plus vitamin C (intraperitoneally, 100 mg/kg per day) in the VC group (n=5) for 4 weeks. At 1 day after the final injection, prostates were excised, weighed and used for the following experiments. To monitor BPH progress, rat blood was sampled from tail veins and prepared for plasma prostate specific antigen (PSA) level determination by ELISA (USCN Life Science and Technology, Missouri, TX, USA), according to the manufacturer's instructions. All animal procedures were performed in accord with the *Seoul National University Laboratory Animal Maintenance Manual*.

2.8. Immunohistochemistry

Excised prostates were fixed with formalin and embedded in paraffin. Serial tissue sections (6 μm thick) were cut from each paraffin block. The sections were deparaffinized, rehydrated through a graded alcohol series and heated in 10 mM sodium citrate (pH 6.0) for 5 min in a microwave to retrieve the antigens. Nonspecific sites were blocked with a solution containing 2.5% bovine serum albumin (Sigma-Aldrich) and 2% normal rabbit serum in PBS (pH 7.4) for 1 h, and the sections were then incubated overnight at 4°C with anti-HIF-1α (Santa Cruz) or anti-PCNA (Santa Cruz) antibody diluted 1:100 in blocking solution [17]. As negative controls, sections were incubated in blocking solution without primary antibody. Immune complexes were detected by sequentially incubating slides with horseradish peroxidase-conjugated secondary antibodies (diluted 1:200), an ABC kit (Vector, Burlingame, CA, USA) and a DAB kit (Dako Cytomation, Glostrup, Denmark).

2.9. Analysis of apoptosis

ApopTag *in situ* apoptosis detection kits (Oncor, Gaithersburg, MD, USA) were used to evaluate apoptotic cell death. Prostate sections were incubated with TdT enzyme solution at 37°C for 2 h, and reactions were terminated by incubation in a stop/wash buffer. Sections were then incubated with antidigoxigenin peroxidase followed by diaminobenzidine and 0.01% H_2O_2 for 5 min, counterstained with hematoxylin and examined under an optical microscope.

2.10. Statistics

All data were analyzed using Microsoft Excel 2006 software. The Mann–Whitney U test was used to see statistical differences, and all comparisons were two sided. Results are expressed as means and standard deviations, and differences were considered significant when P values were <.05.

3. Results

3.1. Vitamin C inhibited testosterone-induced HIF-1α expression in prostate cells

In testosterone-sensitive LNCaP cells, HIF-1α was induced by testosterone under normoxic conditions, while HIF-1β (ARNT) and β-tubulin levels were unaffected (Fig. 1A). However, this testosterone effect in LNCaP was abolished by vitamin C treatment in a dose-dependent manner (Fig. 1B). Likewise, vitamin C also noticeably inhibited the testosterone-dependent induction of HIF-1α in a nonneoplastic prostate epithelial cell line, RWPE-1 (Fig. 1C). Since RWPE-1, vs. LNCaP, expressed a lower level of HIF-1α, we analyzed the nuclear fractions in which HIF-1α is enriched and measured the LaminB level as an internal standard. Moreover, the VEGF promoter activity and VEGF mRNA expression were found to be induced by testosterone and decreased to the control level by vitamin C treatment (Fig. 1D and E). These results suggest that vitamin C attenuates VEGF induction in response to testosterone by inhibiting HIF-1α expression and transcriptional activity.

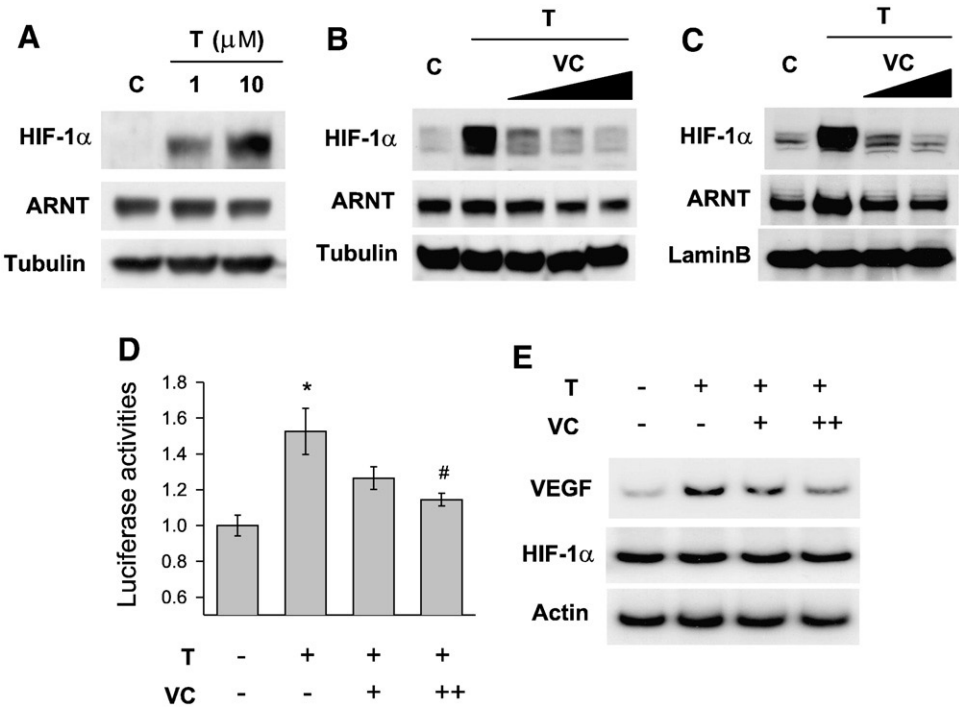

Fig. 1. Vitamin C attenuated HIF-1α expression induced by testosterone. (A) Testosterone-induced HIF-1α expression. LNCaP cells were incubated in serum-free medium for 24 h and then treated with 1 or 10 μM testosterone for 24 h. HIF-1α and ARNT proteins in total cell lysates were analyzed by Western blotting, and β-tubulin was used as a loading control. (B) HIF-1α suppression by vitamin C in the LNCaP cells. After 24 h of incubation with 2 μM testosterone, cells were further incubated with PBS or vitamin C (final concentration of 10, 50 or 100 μM) for 8 h. Proteins were analyzed by Western blotting. (C) HIF-1α suppression by vitamin C in the RWPE-1 cells. After 48 h of incubation with 5 μM testosterone, cells were further incubated with PBS or vitamin C (final concentration of 25 or 100 μM) for 8 h. Nuclear proteins were analyzed by Western blotting. (D) VEGF promoter activity. LNCaP cells were cotransfected with VEGF promoter-luciferase plasmid and with β-gal plasmid. After 24 h of incubation in serum-free medium, 2 μM testosterone was treated with 25 or 100 μM vitamin C (+, ++) for 24 h. Luciferase activity was normalized vs. β-gal activity. Data are relative values vs. untreated controls. Bars represent the means±S.D. of nine experiments. *P<.05 vs. the untreated control; #P<.05 vs. testosterone treatment only. (E) mRNA expressions. After incubating for 24 h in serum-free medium, LNCaP cells were treated with 2 μM of testosterone alone or with 25 or 100 μM vitamin C (+, ++) for 24 h. Total RNAs were isolated and the mRNA levels of VEGF, HIF-1α and actin were analyzed by semiquantitative RT-PCR and autoradiography.

3.2. Vitamin C inhibited prostate cell proliferation induced by testosterone

To evaluate the effect of vitamin C on prostate cell growth, LNCaP cells were treated with testosterone and/or vitamin C for 5 days. The culture dishes containing testosterone showed a higher cell density than the control dish. However, vitamin C cotreatment reduced the cell density to the control level without any distinct differences in cell morphology (Fig. 2A). When counting the numbers of viable cells, it was found that testosterone increased the cell number by 31% vs. the control and that vitamin C cotreatment reduced the cell number by 44% vs. the testosterone-treated group (Fig. 2B). These results suggest that vitamin C has an antiproliferative action in testosterone-stimulated prostate cells.

3.3. HIF-1α suppression by vitamin C depended on PHD2

Since PHD2 is known to mainly contribute to HIF-1α degradation in normoxia, we examined the involvement of PHD2 in the vitamin C action on HIF-1α degradation. In both LNCaP and RWPE-1 cells, vitamin C suppressed the testosterone-induced HIF-1α expression, which was antagonized by PHD2 knockdown. The PHD2-silencing effect of siRNA was verified by measuring PHD2 protein levels (Fig. 3A). Moreover, we inhibited the enzymatic activity of PHD2 using DMOG and found that DMOG noticeably recovered the HIF-1α expression suppressed by vitamin C in both cell lines (Fig. 3B). These results suggest that vitamin C destabilizes HIF-1α by stimulating PHD2.

3.4. Vitamin C prevented prostate growth induced by testosterone

After 4 weeks of testosterone treatment, serum PSA levels (mean±S.D., n=5 each) of control, testosterone-treated and testosterone plus vitamin C-treated rats were 257±77.7, 843.8±307.8 and 474.4±192.6 pg/ml, respectively. Individual PSA values and the results of statistical analyses are plotted in Fig. 4A. Serum PSA levels significantly increased after testosterone injections and this was effectively prevented by vitamin C treatment. Prostates were excised and weighed at this time, and results are plotted in Fig. 4B. Mean prostate weights (mean±S.D., n=5) were 470±110 mg in the control, 904±138 mg in the testosterone-treated and 804±127 mg in the testosterone plus vitamin C-treated group. As was expected, the prostates of testosterone-treated rats were larger than those of controls, and those of vitamin C-treated rats were smaller than those of testosterone-treated rats. Despite a significant PSA reduction by vitamin C, mean PSA values in the testosterone-treated and testosterone plus vitamin C-treated groups were not statistically different. To further evaluate the effect of vitamin C on prostate growth, we analyzed other BPH parameters in tissue sections. Structurally, prostates were composed of an epithelial layer, a stroma and a fluid-filled lumen. To evaluate prostate growth, we measured epithelial cell layer area. As demonstrated by Fig. 4C, which shows microscopic views of rat prostates, epithelial cell layers and stromal spaces of the prostates of testosterone-treated rats were larger than those of controls. Interestingly, epithelial cell layers were found to be noticeably different in the testosterone-treated and vitamin C-treated groups, whereas few differences were observed between stromal

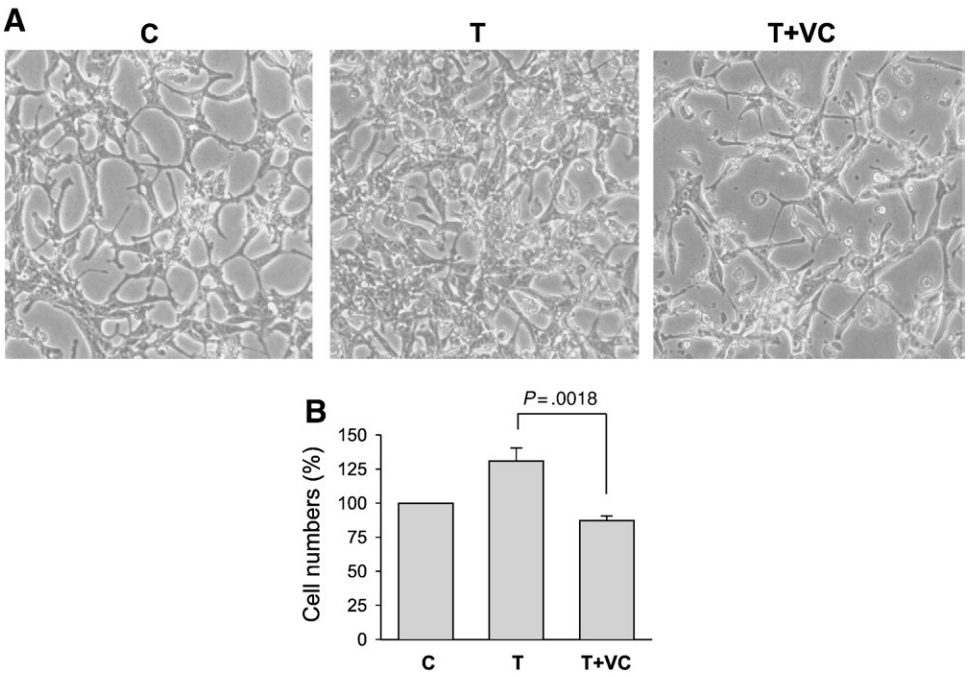

Fig. 2. Vitamin C inhibited prostate cell proliferation induced by testosterone. To show the inhibitory effect of vitamin C on the prostate epithelial cell proliferation, LNCaP cells were treated with testosterone only or cotreated with vitamin C. Testosterone at 5 μM was treated once, and vitamin C at 500 μM was treated twice a day for 5 days. (A) Photography of cells after incubation for 5 days. (B) Cell proliferation was analyzed by counting viable cells in the trypan blue exclusion assay. C, Untreated control; T, testosterone alone; T+VC, cotreatment with testosterone and vitamin C.

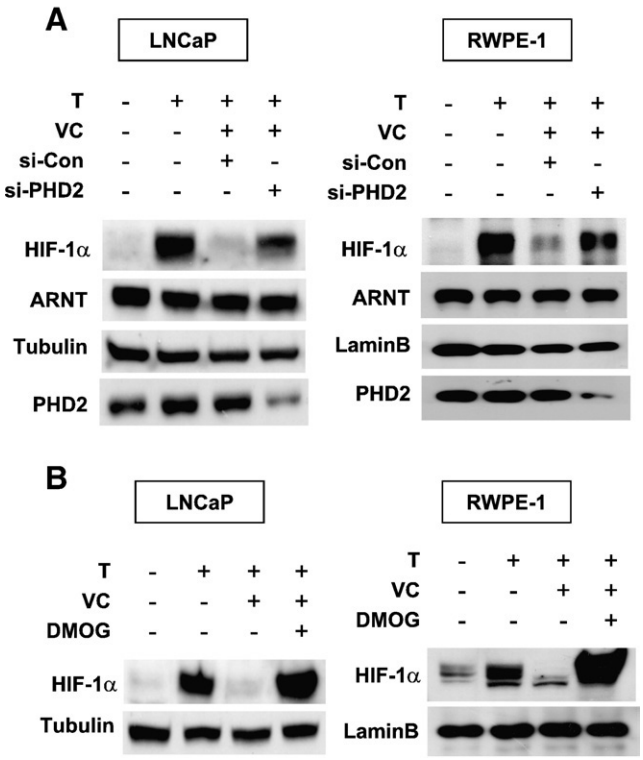

Fig. 3. Vitamin C suppressed HIF-1α in a PHD2-dependent manner. (A) The effect of PHD2 silencing on HIF-1α expression. LNCaP and RWPE-1 cells were transfected with 50 nM control or PHD2 siRNA using Lipofectamine RNAiMAX. After 24 h of treatment with 2 μM testosterone, cells were treated with 100 μM of vitamin C for 8 h. Proteins were obtained from total cell lysates of LNCaP or from nuclear extracts of RWPE-1 and analyzed by Western blotting. (B) The effect of PHD inhibition on HIF-1α expression. LNCaP and RWPE-1 cells were incubated with 2 μM testosterone for 24 h, pretreated with 1 mM of DMOG (a PHD inhibitor) for 1 h and then incubated with 100 μM vitamin C for 8 h.

spaces. Individual data and statistical results are plotted in Fig. 4D. Unlike prostate weight, epithelial cell proliferation was significantly reduced by vitamin C. These results indicate that vitamin C has an inhibitory effect on prostate epithelial cell proliferation, but is less effective at inhibiting stromal enlargement.

3.5. Vitamin C reduced HIF-1α and VEGF expression induced by testosterone in rat prostate

To examine the effect of vitamin C on HIF-1α expression, we analyzed its expression immunohistochemically. Compared to control prostates, testosterone-treated group prostates showed higher numbers of HIF-1α-positive cells, and this was significantly lower in the vitamin C group (Fig. 5A). HIF-1α-positive cell counts were as follows: control (mean±S.D.), 8.3±1.2 cells/100 cells; testosterone-treated group, 19.1±5.4 cells/100 cells; and the testosterone plus vitamin C-treated group, 11.9±3.1 cells/100 cells. We also analyzed VEGF levels in prostate homogenates by Western blotting. VEGF expression levels were calculated as ratios of VEGF band intensities to β-tubulin intensities (Fig. 5B). VEGF levels in prostate tissues increased in the testosterone-treated group but not in the testosterone plus vitamin C-treated group. As was observed in cultured cells, vitamin C was found to significantly reduce HIF-1α and VEGF expressions *in vivo*.

3.6. Vitamin C inhibited prostate hyperplasia in testosterone-treated rats

As was expected, PCNA, a cell proliferation marker, was highly expressed in testosterone-treated prostates (6.0±1.1 cells/100 cells) vs. the control (1.6±0.7 cells/100 cells), and this was significantly higher than in vitamin C-treated prostates (2.9±0.6 cells/100 cells) (Fig. 6A). TUNEL staining (a marker of apoptosis) showed that testosterone prevented epithelial cell death (0.4±0.1 TUNEL-positive cells per 100 cells vs. 4.9±3.1 for the control) and that vitamin C cotreatment increased TUNEL-positive cell numbers (6.9±1.0 cells/100 cells) to a level higher than that of the control level (Fig. 6B).

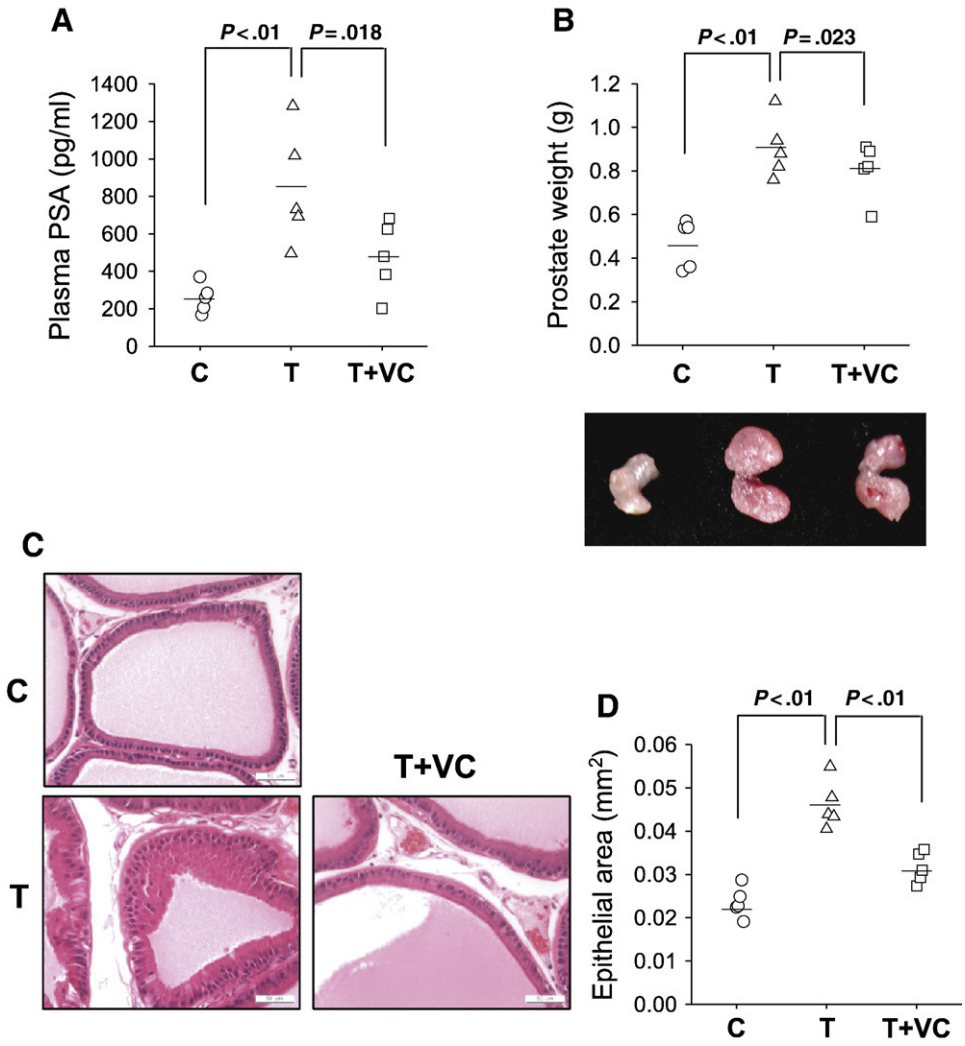

Fig. 4. Vitamin C retarded prostate epithelial growth induced by testosterone. (A) Plasma level of PSA. After treating rats with testosterone only or with 100 mg/kg (intraperitoneally, once a day) of vitamin C for 4 weeks, plasma PSA levels were analyzed using ELISA kits (USCN Life Science and Technology). Points represent individual results and short horizontal lines represent mean values. C, Untreated control (n=5); T, testosterone (subcutaneous injection) alone (n=5); T+VC, cotreatment with testosterone (subcutaneous) and vitamin C (intraperitoneally) (n=5). (B) After 4 weeks of treatment, prostates were excised and weighed. The lower panel represents prostrate gross morphologies. (C) Prostate histology. Prostates were fixed, embedded in paraffin and sectioned at 6 μm. Sections were stained with hematoxylin and eosin (H&E) and visualized at 100×. (D) Epithelial areas. Areas of epithelial cell layers in H&E-stained slides were analyzed using the ImageJ 1.36 b image analysis software (NIH, USA).

These results suggest that vitamin C has antiproliferative and proapoptotic activities in prostate epithelial cells.

4. Discussion

Given that prostate growth depends on angiogenesis and that vitamin C inhibits HIF-1, we tested the possibility that vitamin C prevents prostate hyperplasia by targeting HIF-1. In cultured prostate cells, testosterone stimulated HIF-1α and VEGF expression, and this was attenuated by vitamin C cotreatment. Also, vitamin C inhibited the prostate cell proliferation induced by testosterone. Mechanistically, vitamin C is likely to repress HIF-1α by stimulating the enzymatic action of PHD2. In our testosterone-induced rat BPH model, vitamin C reduced various parameters of prostate hyperplasia and inhibited HIF-1α and VEGF expressions in prostate tissue. These results encourage us to suggest that HIF-1 is a potential target for preventing/treating BPH and that vitamin C should be viewed as a HIF-1-targeting anti-BPH agent.

Vitamin C is widely used as an antioxidant in health foods. Furthermore, a growing body of evidence suggests that the biological effects of vitamin C are due to its specific targeting of cell signaling and gene regulation systems. Indeed, vitamin C is known to repress various transcription factors, such as IRF and NF-κB, and to suppress their downstream genes [18–20]. In addition, HIF-1α inhibition by vitamin C has been reevaluated because vitamin C initiates HIF-1α degradation by activating PHDs. In particular, a tumor xenograft study demonstrated that vitamin C has *in vivo* anticancer activity by targeting HIF-1α [21], and other studies have also shown that vitamin C has HIF-1α inhibitory effects in various tumor cells [22–24].

These findings indicate that vitamin C has the potential to prevent BPH, which resembles cancer in some aspects, and this possibility is supported by the findings of the present study.

Benign prostatic hyperplasia and prostatic carcinoma share a phenomenon in the sense of accompanying with angiogenesis-dependent tissue growth. Hypoxia is generally developed within rapidly growing tumors and is a robust stimulus for the production of angiogenic factors and consequent vessel formation. Thus, angiogenesis inhibitors have been viewed for some time as promising anticancer agents, and, in fact, several antiangiogenic drugs are currently available and others are undergoing clinical trials [1]. In this

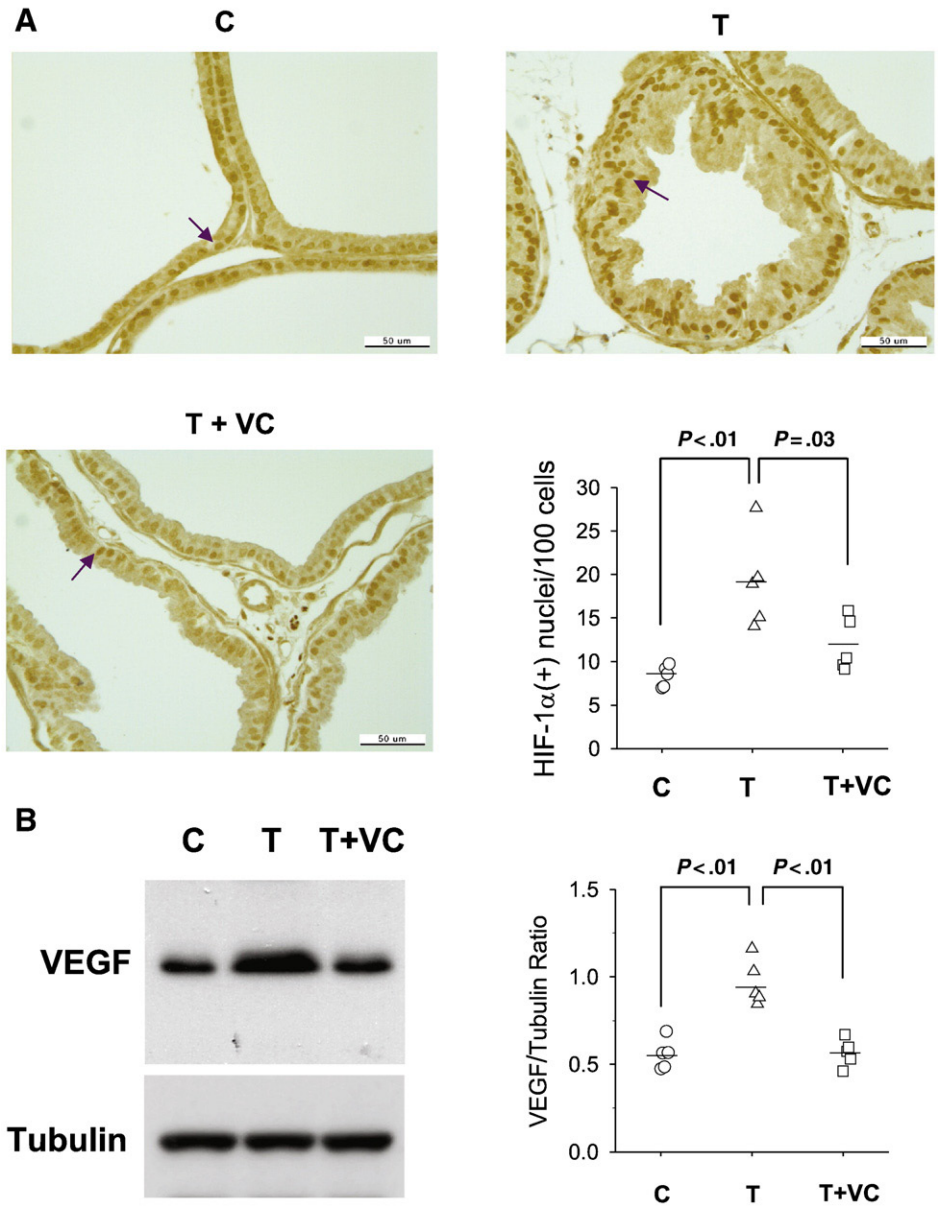

Fig. 5. Vitamin C suppressed HIF-1α and VEGF expressions induced by testosterone. (A) HIF-1α expression was analyzed immunohistochemically in the prostate tissues after treatment for 4 weeks. When the density of nuclear HIF-1α over background density staining exceeded twofold (determined using Adobe Photoshop Elements 2.0), cells were regarded as being HIF-1α positive. C, T and T+VC represent control, testosterone alone and testosterone plus vitamin C, respectively. Data in the lower right panel represent HIF-1α-positive cell (arrows) numbers per 100 cells and short horizontal lines represent mean values. (B) VEGF expression. Prostate tissues were homogenized and 50-μg aliquots of protein were subjected to Western blotting (left panel). Protein band densities of VEGF and tubulin were analyzed using ImageJ 1.36b (NIH). Ratios of VEGF to tubulin are plotted in the right panel.

respect, monoclonal antibodies and chemical inhibitors against VEGF receptor are being developed as anticancer agents [25,26]. In the present study, vitamin C was found to inhibit HIF-1-dependent VEGF expression in both neoplastic and nonneoplastic prostate epithelial cells and in the prostates of BPH rats.

Despite significant reductions in most BPH parameters, prostate weights were found to be only marginally reduced by vitamin C. This disappointing finding was also mentioned in an article published recently concerning the testing of a phosphodiesterase 5 inhibitor [27]. This finding might be explained by our histological data (Fig. 4C), which demonstrates that vitamin C reduced epithelial cell layers but did not affect stromal and lumen sizes. The prostate is composed of several epithelial cell layers, stroma layers and lumen space, and, histologically, the stroma and lumen occupy more space than the epithelial cell layer. Therefore, even a large reduction in epithelial cell proliferation is unlikely to cause a large reduction in prostate weight. Thus, we speculate that the observed marginal effect of vitamin C on prostate weight is attributable to the prostrate structure. Nevertheless, epithelial growth is the prime event in BPH development, and, thus, long-term vitamin C treatment may eventually reduce the prostate size. In the present study, we treated rats with vitamin C only for 4 weeks, which is a relatively short time as compared with that required for BPH development in man.

Because rodents can synthesize vitamin C, the biological effects of exogenous vitamin C may have been underestimated in the present study. Nevertheless, 4 weeks of supplementation was found to have a significant effect on rat prostate growth. On the other hand, man is unable to synthesize vitamin C, and insufficient vitamin C in diet can

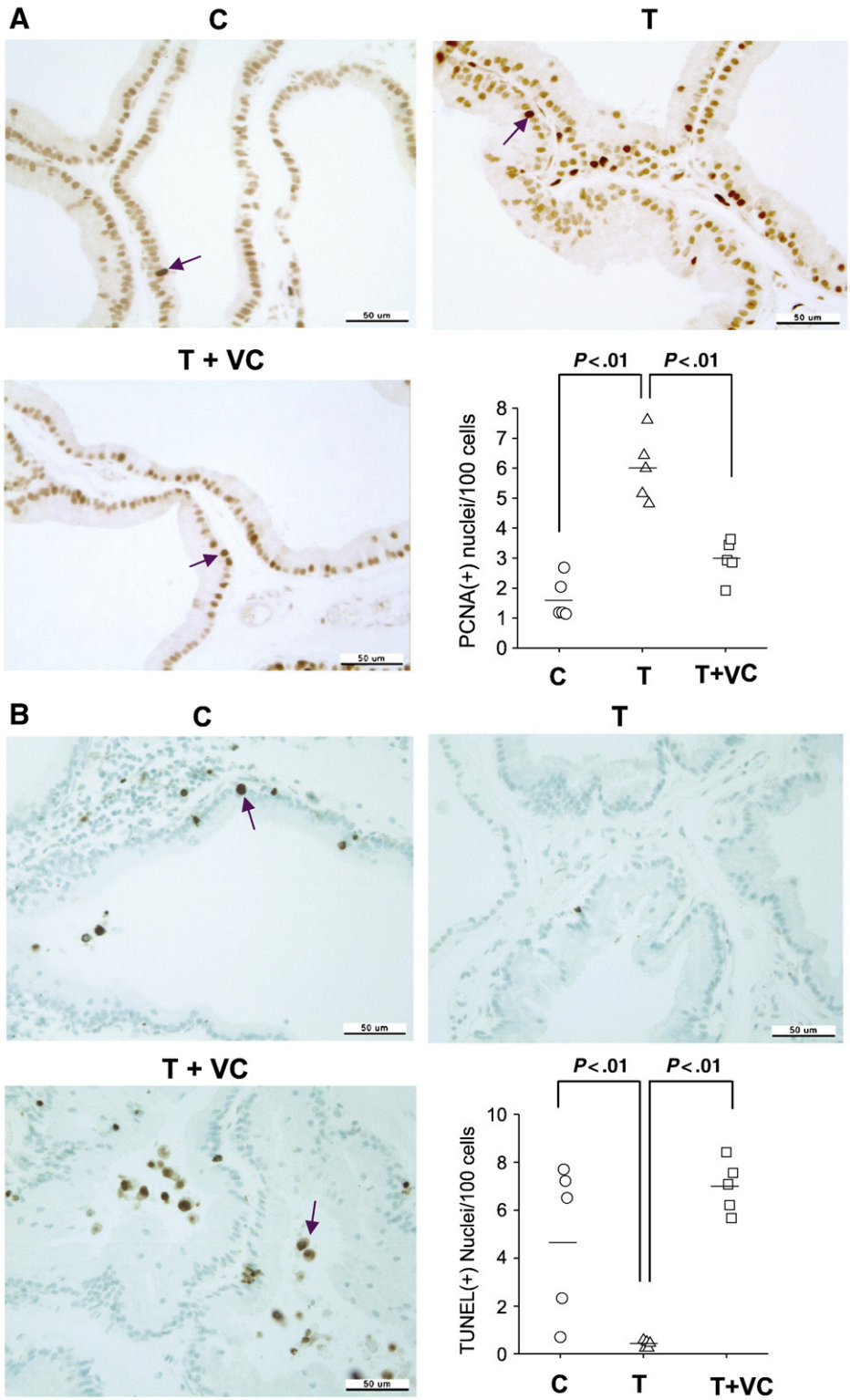

Fig. 6. Vitamin C inhibited prostate epithelial cell proliferation and survival. (A) Cell proliferation. PCNA expression was analyzed immunohistochemically. A PCNA density vs. background density ratio of >5 was regarded as indicating PCNA positivity (also determined using Adobe Photoshop Elements 2.0). Results are presented as PCNA-positive cell (arrows) numbers per 100 cells and are plotted in the lower right panel. C, T and T+VC represent control, testosterone alone testosterone plus vitamin C, respectively. (B) Apoptosis. Apoptotic cell death was analyzed by TUNEL staining. Results are presented as numbers of TUNEL-positive nuclei (arrows) per 100 cells and are plotted in the lower right panel.

cause various physiopathologies. Furthermore, conditions like cancer and inflammation are known to induce vitamin C deficiency, and, therefore, we consider that the anti-HIF and anti-BPH effects of vitamin C are expected to be greater in man.

Normal plasma concentrations of vitamin C are 0.6 to 20 mg/dl, and some tissues (the eye lens and adrenal and pituitary glands) contain at least twice this amount [28]. Since Cameron et al. [29] reviewed vitamin C treatment in cancer patients in 1979, it has been

used since as a supplement during cancer therapy. In particular, intravenous supplementation of vitamin C is believed to help achieve cancer control [30]. In several clinical studies, increasing vitamin C doses have been administered to 100 g/day to maintain a plasma concentration of several hundred milligrams per deciliter [31,32]. On the contrary, Gaziano et al. [33] recently reported epidemiological results regarding the effects of vitamins E and C on cancer therapy: neither vitamin has any beneficial effects in various cancers including prostate cancer. However, it should be noted that there are big differences in the vitamin C doses among these studies. The volunteers in the research of Gaziano et al. [33] took 0.5 g of vitamin C daily, whereas cancer patients in previous studies were treated with 20-fold or higher doses of vitamin C. Therefore, higher doses of vitamin C may be required to support cancer therapy. If nothing else, these clinical reports support the safety of vitamin C in man. In the present study, we injected 100 mg/kg of vitamin C daily into rats, which equates to a daily dose of 7 g/70 kg of body weight, which is within the vitamin C dose range tolerable to human. Accordingly, it may be that vitamin C at such a tolerable dosage is effective at preventing BPH in man. This notion is certainly worth considering in the context of retarding prostate growth in BPH patients. Furthermore, well-designed clinical studies are required to explore the possibilities.

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