

## Vitamin E succinate inhibits human prostate cancer cell growth via modulating cell cycle regulatory machinery<sup>☆</sup>

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### Abstract

Several epidemiological studies have demonstrated that vitamin E is a chemopreventative agent for prostate cancer.  $\alpha$ -Tocopheryl succinate (VES), a derivative of vitamin E, effectively modulates prostate cancer cell growth. However, little is known about the mechanisms regarding this action. Here we show that VES causes human prostate cancer cell LNCaP arrest at G1 phase. This effect is accomplished through VES significantly decreasing expression of the cell cycle regulatory proteins cyclin D1, D3, and E, cdk2 and 4, but not cdk6. Furthermore, VES reduces cdk4 kinase activity, Rb phosphorylation, and cyclin E mRNA expression. Recently there is increasing interest in the protective effect of the VES and selenium combination on prostate cancer. Here we show that VES and selenium work through different mechanisms to exert their inhibitory effects on prostate cancer cells. Taken together, our studies suggest that VES-mediated prostate cancer cell G1/S arrest is a consequence of the regulation of multiple molecules of the cell cycle regulatory machinery.

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Vitamin E succinate (VES), a derivative of vitamin E, is a potent antiproliferative agent for many tumor cells [1]. Although Vitamin E exerts its physiological function mainly through its antioxidant character, VES does not possess antioxidant ability unless its succinate functional group is hydrolyzed [2]. In vitro and in vivo studies have shown that anti-tumor activities of VES involve several mechanisms, including DNA synthesis arrest, induction of apoptosis and differentiation, and regulation of the transforming growth factor- $\beta$  (TGF $\beta$ ), the protein kinase C (PKC), Fas, and the mitogen-activated protein kinase (MAPK) pathways [3–9]. Recently, our group demonstrated that VES inhibits prostate cancer cell

growth in part through the suppression of the androgen receptor/prostate specific antigen (AR/PSA) pathway [10].

Prostate cancer is the second leading lethal cancer in American men [11]. Prostate cancer usually progresses from an androgen-dependent to an androgen-independent stage after an initial positive response to hormonal therapy. Presently there is no effective treatment for androgen-independent prostate cancer [12]. Several epidemiological studies suggest that vitamin E is a chemopreventative agent for prostate cancer [13,14]. Cell culture studies further indicate that VES is a promising anti-tumor agent that can selectively inhibit growth in human prostate cancer cells but not normal prostate cells [10,15]. However, little is known about how VES affects the cell cycle in human prostate cancer cells.

Many reports indicate that cell cycle arrest leads to cell growth inhibition and/or apoptosis. The cell cycle is coordinately controlled by cyclin-dependent kinases (cdks) and their cyclin partners, whose levels fluctuate

<sup>☆</sup> **Abbreviations:** VES, vitamin E succinate,  $\alpha$ -tocopheryl succinate; SeMet, seleno-L-methionine; Rb, retinoblastoma; cdk, cyclin-dependent kinase; CKI, cdk inhibitor; PCNA, proliferating cell nuclear antigen; LI, labeling index; PBS, phosphate-buffered saline; AR, androgen receptor; PSA, prostate specific antigen.

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throughout the cell cycle [16]. The Rb–E2F pathway plays a central role in cell cycle regulation [17]. Disruption of the Rb–E2F pathway has been associated with various tumors [18]. In this study, we found that VES-mediated inhibition of prostate cancer cells is partly due to cell cycle arrest at the G1/S phase. VES suppressed the expression of cell cycle regulatory proteins cyclin D1, D3, and E, and cdk2, 4 but not 6 in LNCaP cells. In addition, VES reduced cdk4 kinase activity, Rb phosphorylation, and consequently inhibited mRNA expression level of cyclin E, an E2F target gene. We also compared the effects of VES with the effects of selenium, another agent that is under investigation to test its protective effect on prostate cancer either alone or in combination with vitamin E in the SELECT project [19]. Although both agents induced cell cycle arrest in the G1 phase [20], our data suggested that the molecular mechanisms might be different. This finding may have clinical significance and future applications.

## Materials and methods

**Chemicals, reagents, and antibodies.** + $\alpha$ -Tocopheryl succinate (VES) and seleno-L-methionine (SeMet) were purchased from Sigma. Antibodies specific for cdk2, cdk4, cdk6, cyclin E, cyclin D1, cyclin D3, and actin were purchased from Santa Cruz Biotechnology. Antibodies specific for phosphorylated retinoblastoma protein (Rb, 14001A) and retinoblastoma protein (Rb, 14061) were from BD Pharmingen. Ki-67 and PCNA antibodies were from DAKO.

**Cell culture conditions.** The LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA). LNCaP cells were maintained in phenol red-free RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100  $\mu$ g/mL penicillin–streptomycin in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. Experiments were performed when cells were at approximately 60% confluence, with RPMI 1640 media supplemented with 5% FBS.

**MTT assay.** LNCaP cells were seeded into 12-well dishes ( $5 \times 10^4$  cells/well) for 48 h and then treated with either VES or SeMet in different doses as indicated in figures for another 48 h. Two hundred  $\mu$ L MTT (5 mg/mL) was added into each well. After 3 h incubation at 37 °C, 2 mL of 0.04 M HCl in isopropyl alcohol was added into each well. The absorbency was read at a wavelength of 595 nm.

**Cell block and immunocytochemistry testing.** Cultured cells were harvested, pelleted, and resuspended in 35  $\mu$ L HistoGel (Richard-Allan Scientific, Kalamazoo, MI). The mixture was fixed in 10% formalin and embedded in paraffin to make a cell block. Paraffin embedded cell blocks were sectioned and mounted on chemically charged slides. Sections were deparaffinized according to established procedures. Immunocytochemical stains were performed using antibodies specific for Ki-67 (Clone MIB-1, DAKO, CA) and PCNA (Clone PC-10, DAKO, CA), followed by DAKO EnVision+ System (DAKO, CA) according to manufacturer's instructions and counterstained in Mayer's hematoxylin blue in 0.3% ammonia water. Slides were mounted using an aqueous media and observed with a light microscope. For negative controls, sections were stained in the same manner except that primary antibodies were omitted.

**Cell cycle analysis (flow cytometry assay).** LNCaP cells grown in 100 mm dishes were treated with either 20  $\mu$ M VES or 150  $\mu$ M SeMet for the indicated time. At the end of each time period of treatment, the cells were digested by trypsin–EDTA. As many as  $1 \times 10^6$  cells were

harvested and the cell pellets were fixed in 70% ethanol at 4 °C. After 12 h, cells were centrifuged (1000g, 7 min, 4 °C), resuspended in PBS containing 0.05 mg/mL RNase A (Sigma), then incubated at room temperature for 30 min. After washing, the cells were stained with 10  $\mu$ g/mL propidium iodide, filtered through a 60  $\mu$ m mesh, and analyzed by flow cytometry (FACSCalibur, BD Company). Ten thousand cells were analyzed with MODFIT software (Verity Software House, Inc).

**Western blot analysis.** Fifty  $\mu$ g protein from total cell lysates was resolved by SDS–PAGE gel and transferred to nitrocellulose membranes. After blotting with blocking buffer (PBS containing 0.1% Tween 20 and 10% serum) for 1 h, the membrane was incubated with primary antibody for 1 h at room temperature. The membrane was then incubated with AP-conjugated second antibodies for another 1 h at room temperature. The proteins were detected by alkaline phosphatase reagents (Bio-Rad).

**Northern blot analysis.** Total cellular RNA was extracted and purified using Trizol (Gibco), according to manufacturer's instructions. Fifteen  $\mu$ g of total RNA was electrophoresed on a 1% agarose formaldehyde gel and transferred to Hybond-N+ membrane (Amersham Pharmacia Biotech). Cyclin E cDNA was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP. The membranes were prehybridized and hybridized using Rapid-Hyb system (Amersham Pharmacia Biotech). Expression was detected by PhosphorImage analysis. Cyclin E plasmids were provided by Dr. Yan Geng (Harvard Medical School, MA).

**Immunoprecipitation and kinase assay.** Five hundred  $\mu$ g cell lysate protein was incubated with 1.5  $\mu$ g cdk4 polyclonal antibody c22 (Santa Cruz) and 500  $\mu$ L IP buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, and 1 mM DTT] for 2 h at 4 °C with constant rocking. Twenty-five  $\mu$ L protein A/G beads were then added into each tube and incubated for another 2 h with constant rocking. After four washes with IP buffer, the beads were resuspended in 30  $\mu$ L kinase buffer [50 mM Hepes (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM DTT, 2.5 mM EGTA, 10 mM  $\beta$ -glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM NaF, 20  $\mu$ M ATP, and 100  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP], with 0.2  $\mu$ g purified GST-Rb C-terminus (773–928 aa) and incubated at 30 °C for 30 min. The reaction was stopped by the addition of loading buffer. After boiling for 5 min, the samples were separated by gel electrophoresis and visualized by autoradiography [21,22].

## Results

### *VES inhibits prostate cancer cell growth and proliferation and arrests cell cycle at G1 phase*

To assess the growth inhibitory effect of VES in LNCaP cells, the MTT growth assay was performed. We found that VES inhibited cell growth in a dose-dependent manner (Fig. 1A). Twenty  $\mu$ M VES inhibited cell growth by 45% after 48 h treatment. Such a dose is close to the physiological concentration (19–29  $\mu$ M) of  $\alpha$ -vitamin E in human serum [23]. Moreover, there is no toxicity or side effects in mice after injection of 50  $\mu$ L of 100 mM VES, which increases VES concentration in several tissues to as high as 80  $\mu$ M [9]. Therefore, we used VES at 20  $\mu$ M in the following study.

VES also inhibited the expression of the proliferating cell nuclear antigen (PCNA) and Ki-67 in the LNCaP cell line (Fig. 1B). PCNA and Ki-67 are nuclear proteins that are tightly associated with cell proliferation [24]. Here we used a cell block technique and immunocytochemistry to evaluate the effect of VES on LNCaP cell

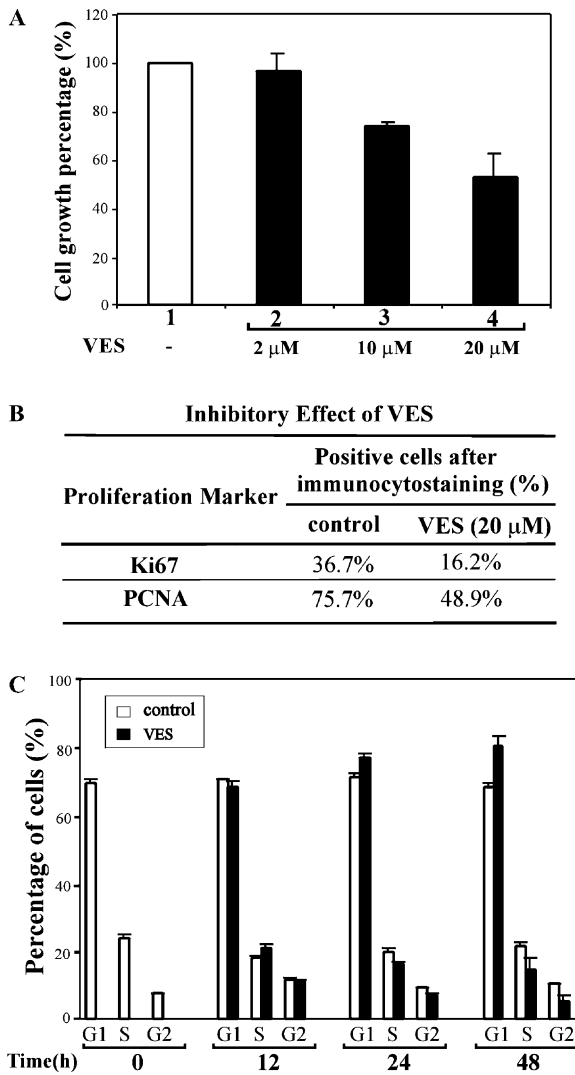


Fig. 1. VES inhibits LNCaP cell growth and induces cell arrest in G1/S phase. (A) VES inhibits cell growth of LNCaP cells. LNCaP cells were treated with different concentrations of VES (2, 10, and 20 μM) and harvested 48 h after treatment. Effects of VES on LNCaP were measured by MTT assay. The control group was 0.1% ethanol treatment and set as 100%. The absorbance was read by spectrometer at 595 nm. Data bar represents mean values and standard deviations of triplicate samples. (B) VES inhibits cell proliferation marker PCNA and Ki-67 of LNCaP cells. Cells treated with or without 20 μM VES were harvested and embedded into paraffin. Immunocyto staining using antibodies specific for Ki-67 or PCNA was performed on formalin-fixed, paraffin-embedded cell block sections. The percentages of positive cells after immunocyto staining were counted on 5 random areas under microscope. (C) VES induces cell arrest in G1/S phase. LNCaP cells were treated with 20 μM VES for 12, 24, and 48 h, while 0.1% (v/v) of ethanol treatment served as control. Cells were harvested and prepared for propidium iodide staining and flow cytometric analysis. As many as 1 × 10<sup>6</sup> cells were stained with propidium iodide and prepared for FACS analysis. Ten thousand cells were analyzed with MODFIT software. The percentages of the total cells were calculated on the results of mean values and standard deviations of three independent samples.

proliferation. VES treatment decreased the labeling index (LI) of Ki-67 from 36.7% to 16.2% and decreased LI of PCNA from 75.7% to 48.9%.

Next we examined the cell cycle distribution after VES treatment. As shown in Fig. 1C, LNCaP cells responded to VES treatment and accumulated in G1 phase of the cell cycle starting from 24 h after treatment, with significant accumulation in G1 phase after 48 h treatment. After 24 h treatment, LNCaP cells in the G1 phase increased from 71.3% to 76.9% of the total cell population, 7.9% above control. After 48 h VES treatment, 80.27% cells accumulated in G1 phase, 18% more than the control cells. Concomitantly, cells in S phase were reduced by 32% and cells in G2 phase were reduced by 50.5%.

Together, these data showed that VES inhibited prostate cancer cell growth and proliferation as evidenced by the reduction of Ki-67 and PCNA expression and the arrest of cell cycle at G1 phase.

*VES regulates expression levels of the cell cycle regulatory proteins*

To determine the molecular mechanism involved in the G1/S cell cycle arrest caused by VES, the expression levels of the cell cycle regulatory proteins that control G1/S progression were examined (Fig. 2). Treatment with 20 μM VES resulted in reduction in the protein expression of cyclin D1, cyclin D3, cdk2, cdk4, and cyclin E in LNCaP cells. However, little effect was observed on cdk6 expression, indicating that such effects were specific. Our data found that cdk4 and cyclin D1 levels were reduced significantly within 12 h of VES treatment and cyclin D3, cyclin E, and cdk2 were

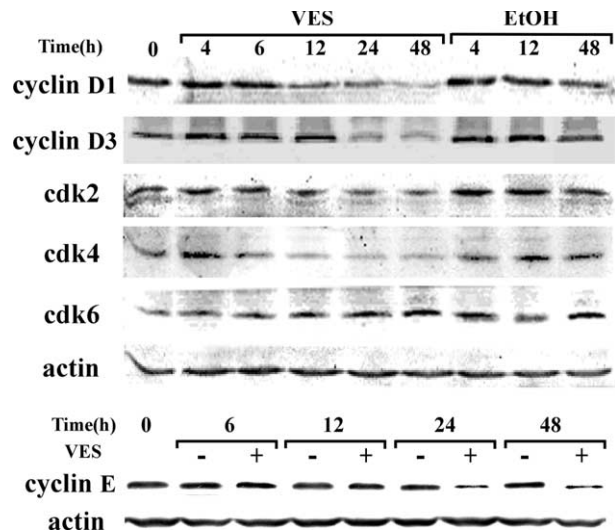


Fig. 2. VES regulates some checkpoint proteins that control G1/S phase. VES regulates protein expression of the cell cycle from G1 to S phase. LNCaP cells were treated with or without 20 μM VES for the indicated time periods. Western blot was used to detect the protein levels of cyclin D1, cyclin D3, cdk2, cdk4, cdk6, and cyclin E with actin expression for equal-loading. The results represent at least three independent experiments.

reduced after 24 h. The expression level of cyclin D2 was too low to be detected and was not used to examine VES effect in LNCaP cells (data not shown). Furthermore, we found that VES did not alter the expression of some cell cycle related proteins: p53, nuclear transcription factor- $\kappa$ B (NF- $\kappa$ B), Bcl2, or caspase 3 (data not shown). Therefore, it appears that VES prevented the cells from entering the S phase by selectively reducing the expression of several cell cycle regulatory proteins.

#### VES inhibits Rb–E2F pathway

G1/S transition is controlled by the Rb–E2F pathway. The protein levels of cyclin D and cdk4 decreased after VES treatment, strongly suggesting that VES may affect the cdk4 complex activity. We used immunoprecipitation followed by *in vitro* GST-Rb C-terminus kinase assay to compare cdk4 complex activity. As shown in Fig. 3A, the activation of cdk4 complex activity and

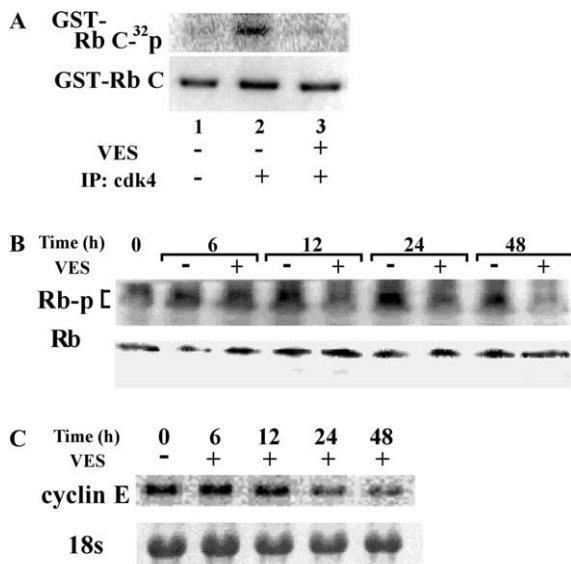


Fig. 3. VES downregulates Rb–E2F pathway. (A) VES suppresses the cdk4-associated kinase activity. The cells treated with (lane 3) or without VES (lane 2) for 48 h were harvested. Five hundred  $\mu$ g cell lysate was immunoprecipitated with cdk4 polyclonal antibody, with IgG as the control (lane 1). The complex was assayed for GST-Rb C-terminal (722–928 aa) kinase activity. The GST-Rbc protein amount was stained with Coomassie blue to check for equal loading. The results are representative of three independent experiments. (B) VES inhibits phosphorylation of the Rb protein. Western blot was used to detect phosphorylation levels of Rb and Rb protein level extract from cells treated with VES and control (0.1% ethanol) at indicated times. For the phosphorylation of Rb, the lysate was separated on 7% SDS-PAGE gel whereas for detecting total Rb protein, the lysate was separated on a 12% SDS-PAGE gel. The results are representative of at least three independent experiments. (C) VES inhibits the expression of cyclin E, a E2F target gene, at mRNA levels. LNCaP cells were treated with VES (20  $\mu$ M) for the indicated time periods. Northern blot was used to detect cyclin E mRNA levels with 18s rRNA as an equal-loading control. The results are representative of at least three independent experiments.

the phosphorylation level of Rb were dramatically inhibited by 48 h VES treatment (lane 2 vs. lane 3). We next examined whether endogenous phosphorylation levels of Rb protein changed in response to VES treatment. As shown in Fig. 3B, Rb phosphorylation was reduced after 12 h, while the Rb protein level showed little change even after 48 h of VES treatment.

After hyperphosphorylation, Rb dissociates from the transcriptional factor E2F, allowing E2F to activate genes which are critical for the progression of the cell cycle to S phase [25]. Because cyclin E is one of E2F's target genes, we next examined its expression as downstream effect of reduced Rb phosphorylation in prostate cancer cells. As shown in Fig. 3C, cyclin E mRNA levels decreased significantly after 24 h treatment, which is consistent with the data that showed cyclin E protein levels were significantly decreased after 24 h of VES treatment (Fig. 2). Taken together, these studies suggested that VES might first target the cyclin D–cdk4/6 complex, inhibit Rb phosphorylation, retain Rb–E2F complex, and concurrently repress the expression of E2F target genes, cyclin E, at the mRNA and protein levels.

#### VES and SeMet regulate cell cycle through different mechanisms

Recently both VES and SeMet have attracted much attention due to their potential preventative effects, individually or in combination, on prostate cancer [19]. Therefore, we compared the effects of VES versus selenium on cell cycle regulatory proteins in LNCaP cells. The physiological blood concentration of selenium is between 82 and 620  $\mu$ g/L (about 1–10  $\mu$ M) [26]. However, SeMet at 10  $\mu$ M did not induce growth inhibition (data not shown). A concentration of 150  $\mu$ M has been used by other researchers to achieve the inhibition of prostate cancer cell growth. It was thought that local tissue concentrations of selenium might reach that level [20,27]. Therefore, 150  $\mu$ M SeMet was applied in this study. As shown in Fig. 4A, 20  $\mu$ M VES inhibited LNCaP cell growth by 47%, while 150  $\mu$ M SeMet inhibited cell growth by 37%. Moreover, a combination of VES and SeMet significantly inhibited cell growth by 78%.

Flow cytometry studies showed that VES and SeMet arrested cells at G1 phase to a similar extent (Fig. 4B). Both VES and SeMet blocked cells at G1 phase, about 18% more when compared to control cells. However, the decreased percentages at S phase and G2 phase were different. After VES treatment, the percentage of cells in both S phase and G2 phase was reduced significantly by 31.6% and 50.5%, respectively. After SeMet treatment, only the percentage of cells in S phase was reduced significantly.

To further study the mechanisms of the inhibitory effects for these two compounds, we compared the

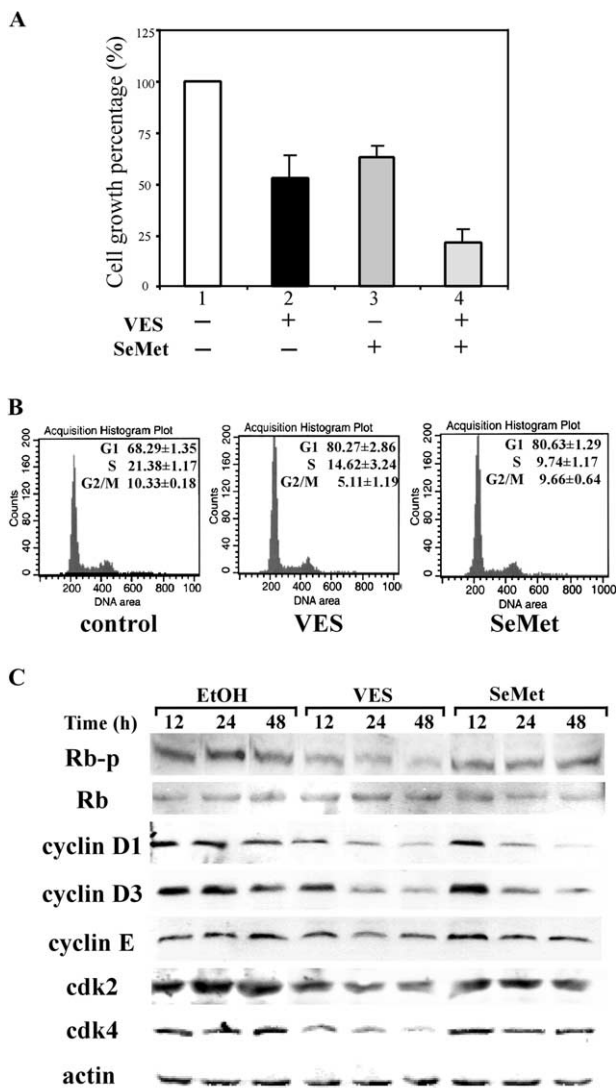


Fig. 4. Comparison of the effect of VES versus the effect of SeMet. (A) Both VES and SeMet can inhibit cell growth. Cells treated with SeMet (150  $\mu$ M), VES (20  $\mu$ M), or VES plus SeMet were harvested at 48 h and measured by MTT assay. The control group was 0.1% ethanol treatment and set as 100%. The absorbance was read by spectrometer at 595 nm. Data bar represents the mean value and standard deviations of triplicate samples. (B) Both VES and SeMet can induce cell cycle at G1 phase. LNCaP cells treated with 20  $\mu$ M VES, or 150  $\mu$ M SeMet, or 0.1% ethanol (control) for 48 h. The cells were trypsinized. As many as  $1 \times 10^6$  cells were stained with propidium iodide and prepared for FACS analysis. Ten thousand cells were analyzed with MODFIT software. The results were representative of three independent experiments. (C) VES and SeMet have different effects on some cell cycle checkpoint proteins. LNCaP cells treated with VES (20  $\mu$ M), or SeMet (150  $\mu$ M), or 0.1% ethanol (control) were harvested at the indicated time intervals. Proteins were detected by immunoblotting with the indicated antibodies.

effects of VES and SeMet on some cell cycle regulatory proteins. Rb phosphorylation levels were reduced with VES treatment. However, as shown in Fig. 4C, 48 h of SeMet treatment had little effect on Rb phosphorylation level. Both VES and SeMet decreased cyclin D1 and

cyclin D3 protein expression levels. The protein levels of cyclin E, cdk2, and cdk4 were reduced after VES treatment within 12 h or 24 h, but not after SeMet treatment. Taken together, our data suggest that VES and SeMet may prevent prostate cancer through various pathways and target various cellular proteins.

### Discussion

The studies reported here provide a new mechanism for VES inhibition of prostate cancer cell growth and proliferation. We demonstrated that VES-induced prostate cancer cell growth arrest occurs partly because VES modulates the cell cycle machinery, resulting in cell cycle blockage at the G1/S phase. Previously, we, along with other groups, reported three mechanisms including AR/PSA pathway [10], Fas–apoptosis pathway [15], and DNA synthesis arrest [3], for the VES-mediated prostate cancer cell growth inhibition. VES-induced cell cycle arrest has been reported in cells other than prostate cancer cells. VES induced G1 arrest in breast cancer cells [28], but caused G2 arrest in lymphoid cells [29]. Therefore, it is likely that VES regulation of the cell cycle varies with cell line. Our findings further demonstrated that such a VES effect is specific on cell cycle regulatory proteins, but not on apoptosis related proteins, such as p53, NF- $\kappa$ B, or caspase 3. Under our conditions, VES is able to cause cell cycle arrest without triggering apoptosis (TUNEL assay and DNA ladder assay, data not shown). There are several possible reasons why apoptosis was not observed under our conditions. First, different doses of an agent may exert different mechanisms in the same cells. For example, cdk inhibitor CGP74514 induces cell cycle arrest at a low concentration (1  $\mu$ M); while at a high concentration (5  $\mu$ M) it can induce apoptosis [30]. The concentration of VES required to induce apoptosis by other groups was 20  $\mu$ g/mL (about 40  $\mu$ M) or higher. In our study we used VES at 20  $\mu$ M. Second, some components in the serum can prevent cell apoptosis. We used a higher serum concentration in the cell culture than other groups, 5% compared to 2% or lower. The combination of higher serum concentration and physiological dose of VES might make it difficult to induce apoptosis in LNCaP cells. One more possible reason that apoptosis was not observed in our system may be due to the duration of VES treatment. When the cells were treated with VES for 96 h under our conditions, more cells were floating and apoptosis marker caspase 3 expression level decreased significantly (data not shown). Together, under our conditions, we observed that VES induced cell cycle arrest without triggering apoptosis.

Both VES and selenium have potential protective effects on prostate cancer and go through different pathways to influence the cell cycle. Currently, SELECT

(the Selenium and Vitamin E Cancer Prevention Trial), the largest-ever prostate cancer prevention trial is ongoing. It is of interest to compare VES to selenium in their functional mechanisms, which will provide some basic understandings for the clinical study.

During the preparing and submission process of this manuscript, one report studying the vitamin E effect on prostate cancer cells cycle was published by Venkateswaran et al. [31]. While the study supported our conclusion that VES/vitamin E arrests prostate cancer cell at the G1 phase, significant differences arise among studies. They focused on cyclin E–cdk2 complex and examined only three cell cycle regulatory proteins, including cyclin E, cdk2, and p27 expression levels after VES treatment. In our study, we studied a wider range of cell cycle regulatory proteins and obtained a more comprehensive conclusion. We found that cyclin D and cdk4 are more important proteins for early VES regulation and VES also influences the Rb–E2F pathway, resulting in inhibiting E2F's target gene, cyclin E. Our data suggested that VES has an effect on prostate cancer cells in a time-dependent manner. Taken together, our study provides comprehensive information about the effects of VES on prostate cancer cell cycle. This study provides some of the molecular basis for using VES clinically as a possible anti-prostate cancer agent.

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