Synergy between Selenium and Vitamin E in Apoptosis Induction Is Associated with Activation of Distinctive Initiator Caspases in Human Prostate Cancer Cells

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ABSTRACT

The ongoing Selenium and Vitamin E Chemoprevention Trial is designed to evaluate the efficacy of these two agents, either individually or in combination, in reducing the incidence of prostate cancer in healthy men over 55 years of age. Little information, however, is available on the potential synergy between vitamin E and selenium in chemoprevention. The present study was aimed at addressing this gap of knowledge with the use of the androgen-unresponsive, p53-null, PC-3 human prostate cancer cell line. The growth-inhibitory activity of vitamin E appeared to be dependent on the chemical form. In our hands, d-tocopherol and d-tocopheryl succinate (VES) was much more potent than either dl-tocopherol or dl-tocopheryl acetate. Combining VES with methylseleninic acid (MSA), a selenium metabolite, produced a synergistic effect on cell growth suppression. The synergy was accounted for primarily by an augmented apoptotic response. Poly(ADP-ribose) polymerase cleavage and activation of specific caspases were confirmed by Western blot analysis. The caspases that were commonly modulated by either VES or MSA included initiator caspases-8 and -10, as well as executioner caspases-3, -6, and -7. In contrast, caspase-9 was activated only by VES, whereas caspases-1 and -12 were activated only by MSA. Based on the above information, it is proposed that the mitochondrial pathway and the endoplasmic reticulum stress/cytokine signaling pathway might be involved in apoptosis induction by VES and MSA, respectively. These two pathways may act in cooperation to switch on the full force of the apoptotic machinery when cells are treated with both VES and MSA.

INTRODUCTION

Prostate cancer is the second most common cancer, as well as the second leading cause of cancer death in men in the United States. The estimated lifetime risk of developing prostate cancer is 16.6% for Caucasian and 18.1% for African-American men; their lifetime risk of dying from the disease is 3.5% and 4.3%, respectively (1). The public health impact from prostate cancer has spawned a growing interest in chemoprevention. One such initiative is the Selenium and Vitamin E Chemoprevention Trial, which was launched in 2001. This is a Phase III, double-blind, placebo-controlled, 12-year trial designed to evaluate the efficacy of these two agents, either individually or in combination, in reducing the incidence of prostate cancer in healthy men over 55 years of age (2).

Selenium is considered one of the most promising candidates for preventing prostate cancer. The justification is based on the findings of a previous large-scale cancer prevention trial, in which prostate cancer was evaluated as a secondary end point. In the study by Clark et al. (3) involving 1312 subjects (974 of them were men), the goal was to determine whether a nutritional dose of selenium was able to prevent basal cell or squamous cell skin carcinoma. Although the results on skin cancer were negative, the trial produced a striking observation that selenium supplementation was associated with a 63% reduction of prostate cancer incidence. This milestone finding has prompted an intensive effort aimed at investigating the biological effects of selenium in human prostate cancer cells. Selenium has been reported to cause cell cycle block, DNA synthesis suppression, and apoptosis induction (4, 5). Additionally, selenium has also been found to down-regulate the angiogenic switch in endothelial cells and prostate cells, an effect that may contribute to its anticancer activity (6, 7).

The supportive evidence on vitamin E and prostate cancer prevention was provided by the α-Tocopherol, β-Carotene Cancer Prevention Study, a controlled trial conducted with 29,133 male smokers in Finland (8). Although the trial was originally designed to examine the role of these nutrients in lung cancer prevention, the analysis uncovered a 40% decrease in prostate cancer mortality in the α-tocopherol group. Numerous studies have demonstrated that vitamin E compounds or their derivatives have anticancer effects in a variety of malignant cell lines (9–13). In prostate cancer cells, vitamin E compounds were found to inhibit cell growth by modulating the cell cycle machinery (14), suppressing DNA synthesis (15), and inducing apoptosis (16, 17). These responses are similar to that of selenium. It has also been reported that vitamin E can attenuate the expression of androgen receptor at both the transcriptional and posttranscriptional levels (18). In the same study, the authors showed that these effects are accompanied by reduced levels of prostate-specific antigen, a marker used to monitor the progression of prostate cancer.

Despite the increasing interest in selenium or vitamin E chemoprevention of prostate cancer, little information is available on the potential synergy between these two agents. The present study was designed to address this gap of knowledge and to gain further insight into the molecular mechanisms that may underlie the interaction between selenium and vitamin E. The research was performed with the use of a human prostate cancer cell line. In general, prostate cells respond poorly to a selenoamino acid such as selenomethionine, and only when it is present at nonphysiological levels in the culture medium. A plausible explanation is that prostate cells have a low capacity in metabolizing selenomethionine, and conversion of selenomethionine to a monomethylated metabolite, as exemplified by methylselenol, is required for the chemopreventive activity of a selenium compound (19–21). For this reason, MSA3 (CH3SeO2H) was developed by Ip et al. (22) specifically for in vitro studies with epithelial cells that lack the ability to produce methylselenol readily. MSA circumvents this problem because it is easily reduced by glutathione and NADPH to methylselenol in a nonenzymatic reaction once taken up by cells. A recent review by Ip et al. (23) contains a detailed elaboration of why MSA is best suited for in vitro experiments. The equation governing the relationship between structure and activity also extends to vitamin E in that not all vitamin E compounds are alike biologically (24–27). In the present study, we started by evaluating several vitamin E compounds in our cell model...
before deciding on the most active compound for additional investigation.

**MATERIALS AND METHODS**

**Chemicals.** MSA was synthesized as described previously (22). Because vitamin E is a generic name for the various tocopherols, the alcohol form is abbreviated here to VE. VE, VEA, and pan-caspase inhibitor z-VAD-fmk (Cl) were purchased from Sigma (St. Louis, MO).

**Cell Culture and Treatments.** The PC-3 human prostate cancer cells were obtained from American Type Culture Collection (Manassas, VA). These cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine and maintained in an atmosphere of 5% CO2 in a 37°C humidified incubator.

**MTT Cell Growth Assay.** This assay is based on the conversion of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. It provides a quantitative determination of cell viability. Cells treated with vitamin E. Vehicle control cultures contained 0.1% ethanol final concentration of ethanol in the culture medium was kept at 0.1% (v/v) in

**Western Blot Analysis.** Western blot analysis was performed using the Trizol-isolated protein. Briefly, ~50 μg of protein were resolved over 10–15% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The blot was blocked in blocking buffer [5% nonfat dry milk, 10 mM Tris (pH 7.5), 10 mM NaCl, and 0.1% Tween 20] at 37°C for 1 h and incubated with the primary antibody overnight at 4°C, followed by incubation with an antimouse or antirabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA) at room temperature for 30 min. Individual proteins were visualized by an enhanced chemiluminescence kit obtained from Amersham Pharma Biotech (Piscataway, NJ). Immunoreactive bands were quantitated by volume densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA), and normalized to actin or GAPDH.

**RESULTS**

**Sensitivity of PC-3 Cells to Different Vitamin E Compounds.** The effects of VE and its two ester derivatives, VEA and VES, on growth of the PC-3 cells were assessed by the MTT assay (Fig. 1). VES treatment at a concentration of 20 μM inhibited the accumulation of PC-3 cells in a time-dependent manner. In contrast, neither VE nor VEA was able to produce a decrease in cell number, even at a concentration as high as 200 μM. The results indicated that VES is much more potent than either VE or VEA in suppressing PC-3 cell growth. Thus VES was the agent of choice in all of the follow-up experiments.

**Synergy between VES and MSA in Cell Growth Inhibition.** The effects of VES and MSA, either alone or in combination, on the accumulation of PC-3 cells were assessed by the MTT assay (Fig. 2). MSA at a concentration of 2.5 μM produced only marginal changes over a course of 72 h (Fig. 2A). Increasing the concentration to 5 μM resulted in a more robust inhibition of ~20% and ~60% at 48 and 72 h, respectively (Fig. 2B). In this experiment, the response to 20 μM VES was not as marked as that seen previously (Fig. 1); nonetheless, the decrease in cell growth was still statistically significant at multiple time points of analysis. A combination of 2.5 μM MSA and 20 μM VES was more effective than either agent alone in growth inhibition only after 72 h of treatment (Fig. 2A), suggesting that the events leading to a synergy required time to take hold. The term “synergy”
is used to describe an outcome in which the response to the combination treatment is statistically greater than the sum of the response to the two single-agent treatments. As shown in Fig. 2B, the synergy was apparently accelerated by using a dose of 5 μM MSA in the combination because the effect was clearly manifested as early as 24 h in culture. The magnitude of the response was also amplified with time.

Cell Cycle Analysis of PC-3 Cells with VES and MSA Treatments. To determine whether the synergy between VES and MSA in PC-3 cell growth inhibition was associated with an enhanced effect on cell cycle arrest, we analyzed cell cycle distribution in the various treatment groups by flow cytometry. Synchronized PC-3 cells were exposed to 20 μM VES, 5 μM MSA, or the combination for 24 h. There was no overt perturbation in cell cycle distribution among the different groups (Fig. 3). BrdUrd labeling analysis showed that MSA treatment inhibited DNA synthesis in a dose-dependent manner at 12 h, whereas the effect of VES was minimal (Fig. 4). Combining VES with either 2.5 μM or 5 μM MSA failed to produce statistically fewer BrdUrd-labeled cells when compared with MSA alone (Fig. 4). The data suggest that MSA probably slowed down the pace of cell cycle progression evenly through G1, S, and G2-M, thereby providing an explanation of why a change in the proportion of cells in different phases of the cell cycle could not be detected. Thus, cell cycle retardation by MSA was likely to contribute to its inhibitory effect on cell growth. On the other hand, because cell cycle block was not enhanced by the combination, we conclude that the synergy between VES and MSA in cell growth inhibition was not due to the two agents working in tandem to cause further delay in cell cycle transit.

Synergy between VES and MSA in Apoptosis Induction. To investigate whether apoptosis augmentation might account for the synergy of growth inhibition, we quantified apoptosis changes by flow cytometry. Staining cells with annexin V and PI allows the resolution of early apoptotic cells (annexin V positive and PI negative) and late apoptotic and necrotic cells (double positive). According to our analysis, early apoptotic cells were present in much lower abundance than...
late apoptotic/necrotic cells across all time points (data not shown), suggesting that the presence of early apoptotic cells was transient. Because the baseline level of early apoptotic cells hovered around a near background value of 0.2%, it was difficult to detect changes in this population. Therefore, we used the percentage of all annexin V-positive cells (i.e., both annexin V single positive and annexin V/PI double positive) as an indicator of cell death. As shown in Fig. 5A, 20 μM VES induced cell death at 72 h, but not at earlier times, whereas the effect of 2.5 μM MSA on cell death was minimal. A trend suggestive of an augmentation in cell death induction was detected with the combination of 2.5 μM MSA and VES after 72 h. The role of MSA became clearer when the concentration of MSA was increased to 5 μM (Fig. 5B). Meaningful increases in cell death were observed with 5 μM MSA or the combination of 5 μM MSA + VES at all three time points. Despite the empirical data showing that the combination effect at 72 h was numerically greater than the sum of the VES effect and the MSA effect, an unambiguous claim of synergy could not be validated statistically due to variability of the data.

In view of the fact that the annexin V/PI double staining method did not yield conclusive evidence of synergy between VES and MSA in apoptosis induction, we turned our attention to the TUNEL assay to see if it might offer more convincing proof. As shown in Fig. 6, VES caused a slight but statistically significant increase in apoptosis at 24 h, and the effect was sustained at longer exposure times. Likewise,
MSA induced apoptosis in a time- and dose-dependent manner. A 72-h exposure was needed to see an increase in apoptosis with 2.5 μM MSA (Fig. 6A). By increasing the concentration of MSA to 5 μM, apoptosis induction was detected as early as 24 h; furthermore, there were signs of an escalation as a function of time of treatment (Fig. 6B). A synergistic effect on apoptosis induction was successfully attained by combining VES with either 2.5 or 5 μM MSA, and the synergy was statistically significant at the 72 h time point (Fig. 6). The definition of synergy for this assay followed the same criteria as that described for the cell growth inhibition assay.

In summary, the results of the two assays were confirmatory in principle and provided strongly suggestive evidence of a synergy between VES and MSA with respect to their ability to induce apoptosis. Although the TUNEL assay proved to be more sensitive than the annexin V/PI double staining assay in the context of our experimental design and cell model, the concordance of the findings was reassuring. Given the above information, we felt ready to examine the molecular basis underlying the augmented apoptosis response elicited by the VES and MSA combination.

PARP Cleavage in PC-3 Cells with VES and MSA Treatments. PARP cleavage, which is accepted as a sensitive marker of caspase-mediated apoptosis, was evaluated in cells treated with either VES or MSA. The results presented in Fig. 7A show that PARP cleavage increased in a dose- and time-dependent manner for VES, less so for MSA. Cotreatment of PC-3 cells with z-VAD-fmk (CI), an irreversible pan-caspase inhibitor, blocked PARP cleavage induced by 40 μM VES (Fig. 7B). The effect was much stronger at 24 h than at 48 h, suggesting that there might be some recovery by VES even in the presence of the caspase inhibitor. Likewise, the presence of z-VAD-fmk at a concentration of 50 μM completely blocked PARP cleavage in cells treated with 10 μM MSA at 24 h (Fig. 7C). Interestingly, the reversal was sustained for at least 48 h. These results indicated that caspases mediated the induction of apoptosis by both VES and MSA. More importantly, the combination of 20 μM VES and 5 μM MSA produced a larger amount of the cleaved PARP, compared with that produced by the single agent (Fig. 7D). The above findings therefore substantiated the phenomenon of augmented apoptosis at the molecular level when cells were treated with both VES and MSA.
Caspase Activation in PC-3 Cells with VES and MSA Treatments. To identify which caspases are involved in the effects of VES and MSA, we examined the activation of executioner and initiator caspases by Western blot analysis. All three executioner caspases, caspases-3, -6, and -7, were cleaved in cells treated with either VES or MSA alone (Fig. 8A). These results were consistent with the corresponding data from PARP cleavage. In contrast, the activation of certain initiator caspases appeared to be agent dependent. As shown in Fig. 8B, caspase-9 was activated only by VES, whereas caspases-1 and -12 were activated only by MSA. It is interesting to note that the selectivity was restricted to just a few initiator caspases. Our data also showed that caspases-8 and -10 were commonly modulated by either VES or MSA. The signature initiator caspase activation profiles suggest that distinct signaling pathways are likely to be involved in apoptosis induction by VES or MSA.

DISCUSSION

This study is the first to demonstrate a mechanism-based synergy between selenium and vitamin E in prostate cancer prevention. The advantage of combining selenium and vitamin E in achieving a greater magnitude of cell growth inhibition is due primarily to an augmented response in apoptosis. We also have suggestive evidence showing that distinctive signaling pathways of caspase activation may be responsible for the mechanism of apoptosis induction triggered by selenium or vitamin E. It is informative to note that the uniquely modulated caspases are confined to the initiator caspases. For example, caspases-1 and -12 are activated only by MSA, whereas caspase-9 is activated only by VES. Aside from these agent-specific caspases, caspases-8 and -10 are activated by either MSA or VES. By targeting the entire battery of initiator caspases, selenium and vitamin E in combination may act in a cooperative fashion to switch on the full force of the apoptotic machinery.

Caspase-1, also known as interleukin 1β-converting enzyme, is an initiator caspase, and its overexpression can result in apoptosis induction (28, 29). Recent study by Winter et al. (30) suggested that deregulation of caspase-1 may play a role in the loss of apoptotic control during prostate carcinogenesis. There is evidence indicating that caspase-1 activation is required during TGF-β1-mediated apo-

Fig. 8. Western blot analysis of cleaved caspases in PC-3 cells treated with different concentrations of VES or MSA. A, executioner caspases-3, -6, and -7 with β-actin as the loading control. B, initiator caspases-9, -1, -12, -8, and -10 with GAPDH as the loading control. The results shown here are representative of those from two independent experiments.
ptosis in human prostate cancer cells (31). Our study has provided the first tantalizing hint to link caspase-1 activation in human prostate cancer cells with selenium-induced apoptosis, thereby suggesting that members of the TGF-β pathway might also be likely targets for the action of selenium.

The ER is the site of protein synthesis, folding, and trafficking. It is also intimately involved in cellular response to stress and intracellular Ca$^{2+}$ levels. Stress signals in the ER, including alterations in calcium homeostasis and the accumulation of unfolded proteins, are known to lead to programmed cell death. Studies in several laboratories have demonstrated that the activation of caspase-12, an ER resident caspase, is essential for ER stress-induced apoptosis (32–37).

A number of factors have been reported to play a role in this response, including calpains, plasma membrane Na/Ca exchanger, tumor necrosis factor receptor-associated factor 2, ER stress sensors IRE1s, and caspase-7. Little information is available on ER stress-induced apoptosis in prostate cancer cells, especially in relation to selenium treatment. Our discovery of caspase-12 activation during MSA-induced apoptosis in PC-3 cells is novel and suggests that a disruption of the ER microenvironment may be integral to the molecular mechanism of the chemopreventive activity of selenium.

Previous studies by Jiang et al. (5) have shown that caspases are essential executioners of MSA-induced apoptosis in DU-145 human prostate cancer cells. These authors examined the effects of MSA on the activation of multiple caspases (caspases-3, -7, -8, and -9), the release of cytochrome c from mitochondria, PARP cleavage, and DNA fragmentation. Their results indicated that MSA-induced apoptosis involved cell detachment as a prerequisite and was principally initiated by caspase-8 activation and subsequently amplified by its cross-talk with other caspases (caspases-9 and -3). In our experience with PC-3 cells treated with MSA, we also observed cell detachment and caspase-8 activation in apoptotic cells. However, the absence of caspase-9 activation by MSA in PC-3 cells suggests that the cross-talk between caspases-8 and -9 is apparently lost. Genotypic differences between the two cell models are likely to account for the discrepancy.

Caspase-9 is a key initiator caspase in the mitochondrial pathway of apoptosis. Damages to the mitochondria result in the release of cytochrome c into the cytosol. Procaspase-9 is recruited and activated by cofactor Apaf-1 through caspase recruitment domain, after cytochrome c binds to Apaf-1 together with dATP. Activated caspase-9 cleaves downstream executioner caspases to initiate the caspase cascade (38–41). Our finding of VES activation of caspase-9 suggests that VES may act through the mitochondrial pathway. In agreement with our study, Weber et al. (42) have observed that Jurkat cells treated with VES resulted in altered mitochondrial structure, generation of free radicals, activation of the sphingomyelin cycle, and relocation of proapoptotic factors such as cytochrome c and Smac/Diablo. They also demonstrated the significance of the protein kinase C/protein phosphatase 2A pathway and bcl-2 as potential mitochondrial targets of VES-induced apoptosis (43). Previous studies by Kline and colleagues (44–50) have established that several signaling pathways, including TGF-β, Fas (CD95), and the mitogen-activated protein kinases extracellular signal-regulated kinase and c-Jun-NH$_2$-terminal kinase, are involved in apoptosis induction by VES in human breast cancer cells. More recently, they reported that Bax, a proapoptotic member of the Bcl-2 protein family, was translocated to the mitochondria in human breast cancer cells treated with VES (51). Taken together, apoptosis induction through the mitochondrial pathway appears to be an important mechanism for the chemopreventive activity of vitamin E.

α-Tocopheryl succinate is the most commonly used form of vitamin E in in vitro studies of cancer research. It is generally assumed that because α-tocopheryl succinate is less hydrophobic than α-tocopherol, it is taken up more efficiently by cells. According to this scenario, the anticancer activity of α-tocopheryl succinate is in fact due to the release of α-tocopherol by intracellular esterases. More than a decade ago, Turley et al. (24) showed that α-tocopheryl succinate at a concentration of 30 µM caused growth arrest in HL-60 cells, but that other forms of vitamin E, including α-tocopherol and α-tocopheryl acetate, did not inhibit cell growth even at a concentration as high as 100 or 200 µM, respectively. Thus the specificity of individual vitamin E compound has been documented for a long time. To take the observation a step further, Fariss et al. (25) compared the cellular effects of α-tocopheryl succinate and α-tocopherylxyloxybutyric acid, a nonhydrolyzable ether form of the succinate analogue. They found that the ester form and the ether form were similar in their ability to inhibit tumor cell proliferation, suggesting that the activity of α-tocopheryl succinate is attributable to the intact compound rather than to α-tocopherol per se. In our study, a concentration of 20 µM α-tocopheryl succinate is sufficient to achieve a 40% growth inhibition in PC-3 cells after 72 h, yet neither α-tocopherol nor α-tocopheryl acetate is able to do so after the same time of exposure and with a dose 10 times higher than that of α-tocopheryl succinate. This result reaffirms the notion that α-tocopheryl succinate is also the active compound for prostate cancer chemoprevention studies in vitro. However, a nonhydrolyzable form of α-tocopheryl succinate (e.g., α-tocopherylxyloxybutyric acid) will have to be used orally to circumvent the hydrolysis of the succinate ester by intestinal esterases. Most of the research on vitamin E chemoprevention was done with cell culture models. Scanty information is available on the anticancer efficacy of the different forms of vitamin E in animal tumor studies. Despite the paucity of knowledge, α-tocopheryl acetate was chosen to be the vitamin E agent in the Selenium and Vitamin E Chemoprevention Trial. The issue concerning the in vivo biological activities of specific vitamin E compounds needs to be clarified so that present and future human intervention trials with vitamin E (used here generically) can be put in the proper perspective.

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