Differential Response of Human Ovarian Cancer Cells to Induction of Apoptosis by Vitamin E Succinate and Vitamin E Analogue, α-TEA

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ABSTRACT

A vitamin E derivative, vitamin E succinate (VES; RRR-α-tocopherol succinate), and a vitamin E analogue, 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-oyloxy acetic acid (α-TEA), induce human breast, prostate, colon, lung, cervical, and endometrial tumor cells in culture to undergo apoptosis but not normal human mammary epithelial cells, immortalized, nontumorigenic breast cells, or normal human prostatic epithelial cells. Human ovarian and cervical cancer cell lines are exceptions, with α-TEA exhibiting greater proapoptotic effects. Although both VES and α-TEA can induce apoptosis within 24 h of treatment, only α-TEA is an effective inducer of apoptosis. VES or α-TEA treatment of cp70 cells with 5, 10, or 20 μg/ml for 3 days induced 5, 6, and 19% versus 9, 36, and 71% apoptosis, respectively. Colony formation data provide additional evidence that cp70 cells are more sensitive to growth inhibition by α-TEA than VES. Differences in stability of the ester-linked succinate moiety of VES versus the ether-linked acetic acid moiety of α-TEA were demonstrated by high-performance liquid chromatography analyses that showed α-TEA to remain intact, whereas VES was hydrolyzed to the free phenol, RRR-α-tocopherol. Pretreatment of cp70 cells with bis-(p-nitrophenoxy) phosphate, an esterase inhibitor, before VES treatment, resulted in increased levels of intact VES and apoptosis. Taken together, these data show α-TEA to be a potent and stable proapoptotic agent for human ovarian tumor cells and suggest that endogenous ovarian esterases can hydrolyze the succinate moiety of VES, yielding RRR-α-tocopherol, an ineffective apoptotic-inducing agent.

INTRODUCTION

Studies in our lab have focused on the antitumor properties of vitamin E compounds. Two compounds in particular, RRR-α-tocopheryl succinate (vitamin E succinate; VES), a vitamin E derivative, and a novel vitamin E analogue, 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-oyloxy) acetic acid, referred to as α-TEA, differ in structure from natural vitamin E (RRR-α-tocopherol) by the addition of a succinic acid group attached at the C6 position of the chroman head structure by an ester linkage (VES) or an acetic acid moiety attached at the C6 position of the chroman head by an ether linkage (α-TEA; Fig. 1; Refs. 1, 2). VES has been shown to be a potent growth inhibitor of a wide variety of cancer cell lines in vitro, as well as an effective tumor growth inhibitor in vivo (1, 3–14). Recently, α-TEA has been shown to reduce tumor burden and lung metastasis in a syngeneic mouse mammary cancer model (2).

Human tumor cells in culture undergo DNA synthesis arrest, differentiation, and apoptosis in response to treatment with VES (reviewed by Ref. 1; Refs. 15–21). Studies on the mechanisms of actions of VES using human breast cancer cells show that MDA-MB-435 cells are nonresponsive to the growth inhibitory properties of transforming growth factor β and Fas/Fas ligand; however, after treatment with VES, responsiveness to both transforming growth factor β and Fas is restored (reviewed by Ref. 1; Refs. 22, 23). Prolonged activation of the c-Jun-NH2-terminal kinase is a key event in VES-induced apoptosis (reviewed by Ref. 1; Ref. 17), and recent studies have shown that c-Jun-NH2-terminal kinase plays a role in Bax translocation to the mitochondria, which results in increased mitochondrial membrane permeability with release of cytochrome c, activation of caspases 9 and 3, and apoptosis (19). Recent studies have demonstrated that α-TEA-induced apoptosis is similar to VES-induced apoptosis in that transforming growth factor β-, Fas-, and c-Jun-NH2-terminal signaling pathways are necessary, at least in part for α-TEA-induced apoptosis (24). Ovarian cancer is the most lethal of the female gynecologic cancers, and there is a great need for novel chemotherapeutic agents to treat this cancer (25). Thus, it was of interest to see if VES or the novel vitamin E analogue α-TEA might be effective agents.

Here, we report that: (a) α-TEA is as effective as VES in inducing apoptosis in human breast, prostate, colon, lung, and endometrial tumor cells in culture and is superior to VES as an inducer of apoptosis in human ovarian and cervical tumor cells; (b) although VES is less effective as an inducer of apoptosis in ovarian cp70 cells, VES and α-TEA are equally effective in inducing DNA synthesis arrest, demonstrating a unique difference between these two compounds signal DNA synthesis arrest and apoptosis; and (c) ovarian cells contain esterases that can hydrolyze the ester linked succinate moiety from VES yielding RRR-α-tocopherol, an ineffective apoptotic-inducing agent. Taken together, these studies demonstrate that α-TEA is a stable compound and a potent inducer of apoptosis in a wide variety of human cancer cells in culture, including human ovarian and cervical cells. In comparison, VES is an effective pro-apoptotic agent for a wide variety of human cancer cells but is less effective than α-TEA in inducing human ovarian and cervical cancer cells to undergo apoptosis.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Sources of cell lines used in these studies are as follows. MDA-MB-435 [provided by Dr. Janet E. Price, Department of Cell Biology, University of Texas M. D. Anderson Cancer Center (Houston, TX)], MDA-MB-231 (American Type Culture Collection, Manassas, VA), and MCF-7 [provided by Dr. Suzanne Fuqua, Baylor College of Medicine (Houston, TX)] are human breast cancer cell lines; MCF-10A (American Type Culture Collection) is an immortalized, nontumorigenic human breast cell line, and human mammary epithelial cells (Cooperative Human Tissue Network, Birmingham, AL) were primary cultures of human mammary cells derived from normal mammaryplast samples. All of the breast-derived cell lines and primary cells were cultured as described previously (23). LNCaP, PC-3, and DU-145 are human prostate carcinoma cell lines [provided by Dr. Claudio Conti, University of Texas M. D. Anderson-Science Park Research Division (Smithville, TX)]. Normal human prostate epithelial cells were pur-
R-α-Tocopherol Succinate (VES), possessing an ester-linked succinate moiety attached to 6C of the chroman head of RRR-α-tocopherol, and vitamin E analogue [2,5,7,8-tetramethyl-2-(4R,8R,12-trimethyltridecyl)chroman-6-yloxy] acetic acid (α-TEA), possessing an ether-linked acetic acid moiety attached to the 6C of the chroman head of RRR-α-tocopherol.

For experiments, human mammary epithelial cells were cultured in mammary epithelial growth medium, both of which are defined media without serum purchased from Clonetics (San Diego, CA). Cell lines were grown as described previously (26). The human colon HT-29 and lung A-549 were purchased from the American Type Culture Collection and cultured according to American Type Culture Collection’s instructions. The human cervical HEK-293 cell line was obtained from Dr. Michael J. Birrer (NIH, Rockville, MD). The human cervical SiHa and CaSkI cell lines were obtained from Dr. Rebecca Richards-Kortum (University of Texas at Austin). The human ovarian carcinoma cell lines OVCA-429, OVCA-433, OVCA 432, HEY, and SK-OV-3 were obtained from Dr. Gordon Mills (University of Texas M. D. Anderson Cancer Center). The ovarian carcinoma cell line HEY was provided by Dr. Gordon Mills (University of Texas at Austin). The human prostate cell line LNCaP was provided by Dr. Joel R. Winer (University of Michigan Medical School, Ann Arbor, MI), and the LNCaP-resistant subclone, LNCaP-RES, was provided by Dr. Michael J. Birrer. The LNCaP-RES cell line was derived through intermittent exposure of LNCaP cells to increasing concentrations of cisplatin (up to 70 μM) in vitro (27). LNCaP and LNCaP-RES cells were grown as monolayers on plastic (Coming Plastic Ware, Coming, NY) and maintained in RPMI 1640 (In Vitro Technologies, Inc., Carlsbad, CA), supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (Sigma Chemical Co., St. Louis, MO). Cultures were routinely examined and shown to be free of Mycoplasma contamination.

**α-TEA and VES Treatments.** For experiments, human mammary epithelial cells were cultured in mammary epithelial growth medium, and prostate epithelial cells were cultured in prostate epithelial growth medium, both of which are defined media without serum purchased from Clonetics (San Diego, CA), PC-3 cells in 1% fetal bovine serum, MDA-MB-435, and LNCaP cells in 2% fetal bovine serum and all other cell types were cultured in 5% fetal bovine serum. Cells were plated at a density of 1 x 10^5 cells/ml in T-75 flasks (20 ml) for high-performance liquid chromatography (HPLC) analyses, T-25 flasks (10 ml) for Western analyses, 12-well plates (1 ml) for apoptosis, or 96-well plates (100 μl) for DNA synthesis arrest and allowed to adhere overnight before treatment initiation. Treatments were conducted at various concentrations of VES (Sigma Chemical Co.) or α-TEA (synthesized by one of the authors, Dr. Jeffrey Atkinson, and described in detail; Ref. 2) in a final concentration of 0.1% ethanol. Vehicle treatments consisted of an equivalent amount of sodium succinate for the highest amount of VES used in the experiment in a final concentration of 0.1% ethanol.

**Esterase Inhibitor Analyses.** Bis-(4-nitrophenyl)phosphate (BNPP), an inhibitor of nonspecific esterases that prevents de-esterification of VES (28), was used to determine whether esterase activity could be responsible for the ability of VES to induce apoptosis in the cp70 cells. CP70 cells at 1 x 10^5 cells/ml in 20 ml/T-25 flask were pretreated with 12.5 or 25 mM BNPP for 30 min before the addition of 5, 10, or 20 μg/ml VES. Cells were cultured for 1, 2, and 3 days, harvested, and analyzed for apoptosis using procedures described below. Cell lysates were analyzed for intact VES versus free RRR-α-tocopherol by HPLC analyses as described below.

**DNA Synthesis Assay.** Detection of DNA synthesis arrest of cp70 cells in response to treatments with VES or α-TEA was assessed by measuring [3H]Thymidine incorporation as described previously (29). Briefly, cp70 cells at 1 x 10^5 cells/ml in 96-well plates (100 μl/well) were cultured with 5, 10, and 20 μg/ml VES or α-TEA for 24 h. During the last 4 h of incubation, cultures were pulsed with 0.5 μCi [3H]Thymidine. Cells were harvested and [3H]Thymidine uptake measured using a Beckman LS5000TD liquid scintillation counter.

**Apoptotic Analyses of 4,6-Dimaminodiphenyl Dihydrochloride-Stained Cells.** Apoptosis was assessed based on nuclear morphology using the fluorescent DNA dye 4,6-diamidino-2-phenylindole, as described previously (23). Cells in which the nucleus contained clearly condensed chromatin or cells exhibiting fragmented nuclei were scored as apoptotic. Apoptotic data are reported as the percentage of apoptosis obtained by determining the number of apoptotic cells versus the total number of cells. For each sample, a minimum of three slides involving >500 cells/slide were scored. Apoptotic data are presented as mean ± SD for three independent experiments.

**Poly(ADP-Ribose) Polymerase (PARP) Cleavage Detection by Western Blot.** Cleavage of PARP by caspase 3 serves as an early indicator of apoptosis (30). CP70 cells were plated at a density of 1.5 x 10^5 cells/ml in T-25 flasks (10 ml/flask) and allowed to adhere overnight. Next, the media were removed and replaced with experimental media containing treatments of VES, α-TEA, vehicle, or VES + BNPP (25 mM), and cells were allowed to incubate for 2 days. Cells were collected by scraping to combine floating and adherent cells and then pelleted by centrifugation at 350 x g. Whole cell lysates were prepared and analyzed by Western blotting performed as previously described with the exception that proteins were separated using SDS-PAGE on a 7.5% gel (23). Proteins were visualized with immune-enhanced chemiluminescence analyses using 1 μg of primary antibody (PARP (H-250), Santa Cruz Biotechnology, Santa Cruz, CA) and peroxidase-conjugated goat anti-rabbit immunoglobulin as the secondary antibody (Jackson Immunoresearch Laboratory, West Grove, PA) at a 1:10,000 dilution.

**Coloncy Assay.** Clonogenic assays were performed on exponentially growing cells using published procedures (31). Briefly, cp70 cells, a total of 1000 to 1 x 10^5, for low to high doses of treatment, respectively, was seeded in 35-10 mm Petri dishes in 2 ml of normal growth media and allowed to adhere overnight. Growth media were removed and replaced with 2 ml of treatment media containing VES (2.5, 5, and 10 μg/ml), α-TEA (2.5, 5, and 10 μg/ml), or untreated. After 9 days of treatment, media were carefully removed, and the cells were stained with 0.1% crystal violet for 30 min. Cells were washed once with PBS, and colonies of >50 cells were counted as survivors. The surviving fraction was calculated for each treatment based on the plating efficiency of the untreated sample, using the formula: surviving fraction = (number of colonies counted at a given level of VES or α-TEA/number of colonies plated) x (control number of cells plated/control number of colonies counted). Each experiment was repeated at least three times.

**Lipid Extraction of Samples.** Lipid extract was performed on whole cell lysates of cp70 samples to determine the amount of cellular uptake of VES and α-TEA. Cells were extracted according to the method of Tirmenstein et al. (32), with slight modifications. Fifty μl of internal standard, RRR-γ-tocopherol (1 mg/ml in ethanol; Sigma Chemical Co.) was added to each sample in disposable 5-ml centrifuge tubes (Sarstedt catalogue no. 58.536; Sarstedt, Inc., Newton, NC) with five to seven Kimble solid glass beads (4 mm). According to the method of Knight et al. (33), cells were resuspended in 0.1 M SDS and homogenized on a mini-beadbeater (model no. 3110B; Crescent Wig-l-bug -Dentsply, Elgin, IL) device for 30 s. Two ml of ethanol were then added to each vial and mixed by vortexing. Homogenates were extracted twice with...
hexane by adding 1 ml of hexane, followed by shaking in the Wig-l-bug for 30 s, centrifugation for 5 min at 1000 × g, and organic layer removed. Hexane extracts of each sample were combined and evaporated under nitrogen and then resuspended in 200 μl of methanol.

**HPLC Analyses.** RRR-α-tocopherol, VES, and α-TEA levels were measured by an internal standard method using reverse-phase HPLC with fluorometric detection as described by Timenstein et al. (32). Forty μl of each sample in methanol were injected into a Waters 717 HPLC equipped with an autosampler. The mobile phase consisted of 96% methanol (HPLC grade; Aldrich, Milwaukee, WI), 4% water, and 0.001% glacial acetic acid. Samples were separated on a Waters spherisorb ODS-2 5μ (250 × 4.6-mm) column (Alltech, Deerfield, IL). Excitation and emission wavelengths of 210 and 300 nm, respectively, were used for all determinations. Quantitation of the separated compounds was performed based on the internal standard method using RRR-α-tocopherol as the internal standard and Millennium-32 chromatography manager software for data analyses (Waters Corp., Milford, MA). α-TEA, VES, and RRR-α-tocopherol peaks were collected, and mass spectrometry with elemental analysis was performed to verify their chemical composition.

**RESULTS**

**Ability of VES and α-TEA to Inhibit Growth of Human Cancer Cells but not Normal or Immortalized Cells.** A comparison of the ability of VES and α-TEA to induce human breast, prostate, colon, lung, ovarian, cervical, and endometrial cancer cells to undergo apoptosis is shown in Table 1. These data show that VES and α-TEA behave in a similar manner in the induction of apoptosis with all tumor cells tested, with the exception of the ovarian and cervical cancer cells. Furthermore, normal human mammary epithelial cells, normal prostate epithelial cells, and immortalized, nontumorigenic breast cells (MCF-10A) were refractive to the apoptotic-inducing properties of both VES and α-TEA. The superior ability of α-TEA, in comparison to VES, to induce apoptosis was initially found in the cp70 and A2780 ovarian cells, with a suggestion that perhaps α-TEA was also more effective in inducing apoptosis in the ME-180 cervical cells. To determine whether this finding was more generalizable, 7 additional ovarian cell lines and 2 additional cervical cell lines were analyzed (Table 1). Data showed α-TEA to be more effective than VES in inducing apoptosis for both ovarian and cervical cell lines (Table 1). The difference in ability of VES and α-TEA to induce apoptosis in cp70 cells formed the basis for additional investigation.

**VES and α-TEA Treatment of Cp70 Cells Induce DNA Synthesis Arrest.** Cp70 cells treated with VES or α-TEA undergo DNA synthesis arrest (Fig. 2, A and B). Treatment of cp70 cells with 5, 10, and 20 μg/ml VES or α-TEA for 1 day induced 31, 47, and 56% and 20, 25, and 68% DNA synthesis arrest (Fig. 2A). Treatment of cp70 cells with 20 μg/ml VES or α-TEA for 2, 3, and 4 days induced 56, 67, and 85% and 68, 77, and 85% DNA synthesis arrest, respectively (Fig. 2B). α-TEA Is Effective in Inducing Cp70 Cells to Undergo Apoptosis; VES Is Less Effective. Treatment of cp70 cells with VES for 2 and 3 days at 5, 10, and 20 μg/ml gave low levels of apoptosis; 3, 17, and 19% for day 2, and 5, 6, and 20% apoptosis for day 3, respectively (Fig. 3, A and B), whereas treatment of cp70 cells in a similar manner with α-TEA gave 3, 26, and 73% apoptosis for day 2 and 9, 36, and 71% apoptosis for day 3 (Fig. 3, A and B).

**Table 1 Comparison of the apoptotic inducing properties of VES and α-TEA, using several different human cancer cell lines**

<table>
<thead>
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<th>Apoptosis Inductiona</th>
<th>VES</th>
<th>α-TEA</th>
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**Fig. 2.** Vitamin E succinate (VES) and α-TEA induce cp70 cells to undergo DNA synthesis arrest. Cp70 cells at 1 × 105 cells/ml in 96-well plates (100 μl/well) were cultured with 5, 10, and 20 μg/ml VES or α-TEA for 1 day (A) or 20 μg/ml of each compound for 1, 2, and 3 days (B). Four h before harvesting, cells were pulsed with 0.5 mCi [3H]thymidine. Cells were harvested and uptake of [3H]thymidine determined. Data are depicted as mean ± SD for three independent experiments.
Additional evidence of apoptosis induced by α-TEA but not with VES was obtained by Western immunoblotting analyses of the caspase 3 substrate PARP (Fig. 3D). Cell lysates from cells treated with 10 μg/ml VES for 1 day showed low levels of PARP cleavage fragment p84, whereas cell lysates from cells treated with 10 μg/ml α-TEA showed the expected M, 84,000 PARP cleavage fragment, providing additional support that α-TEA is an effective inducer of apoptosis in cp70 cells, whereas VES is less effective (Fig. 3D).

α-TEA Is More Effective Than VES in Inhibiting In Vitro Colony Formation by Cp70 Cells. In the colony formation study, the plating efficiency for cp70 cells cultured in media only for 9 days was 46%. The surviving fraction of cp70 cells after treatment for 9 days with 2.5, 5, and 10 μg/ml VES was 1.20 ± 0.14, 0.46 ± 0.34, and 0.06 ± 0.02, respectively, whereas the surviving fraction of cells treated for the same time period with 2.5 and 5 μg/ml α-TEA was 0.62 ± 0.23 and 0.03 ± 0.014, respectively (Fig. 4). No surviving colonies were formed in plates treated with 10 μg/ml α-TEA at any cell concentration tested, including the highest cell number (1 × 10⁶). The colony formation data provide additional evidence that cp70 cells are more sensitive to growth inhibition by α-TEA than VES.

HPLC Analyses of Levels and Integrity of VES and α-TEA in Cp70 Cell Lysates. Levels of VES and α-TEA in cp70 cells increased over 3–6 h after treatment, reaching maximum levels at 12 h following treatment (data not shown). HPLC data depicted in Fig. 5, A and B, are from cells treated for 12 h with α-TEA or VES, respectively. HPLC analyses of cell lysates from α-TEA-treated cp70 cells showed only the α-TEA peak eluting in 11 min, indicating that α-TEA remained structurally intact (Fig. 5A). HPLC analyses of cell lysates from VES-treated cells showed a VES peak eluting at 12 min, as well as a second peak that eluted from the column at 14 min. Mass spectrometry with elemental analysis confirmed the second peak to be RRR-α-tocopherol, providing direct evidence that the ester-linked succinate moiety of VES had been cleaved (Fig. 5B).

Studies designed to determine the effect of RRR-α-tocopherol on VES or α-TEA uptake and apoptosis involved treating cp70 cells with VES or α-TEA singly or in combination with RRR-α-tocopherol, followed by analyses of cell lysates by HPLC or cell populations for

Nuclei of VEH- and VES (20 μg/ml)-treated cp70 cells exhibited uniform dull staining typical of normal intact cells (Fig. 3C, top panels). When cp70 cells were cultured with 20 μg/ml VES + 25 mM esterase inhibitor BNPP, VES induced cp70 cells to undergo apoptosis as depicted by condensed and fragmented nuclei (Fig. 2C, bottom right panel). Nuclei from α-TEA-treated cells showed nuclear condensation and fragmentation (Fig. 3C, bottom left panel). The cp70 parental cells, A2780, also exhibited resistance to VES-induced apoptosis (data not shown).
number of apoptotic cells. HPLC data showed that both VES or α-TEA were taken up by the cells when administered in combination with RRR-α-tocopherol, but VES and α-TEA levels in cotreated cells were lower than singly treated cells (Fig. 5, C and D, respectively). Quantitation of levels of VES or α-TEA uptake/10,000 cells shows that single versus cotreated cells exhibited mean ± SD of 113 ± 34.6 versus 18 ± 12.06 pmol VES and 74 ± 4.24 versus 55 ± 4.95 pmol α-TEA (Fig. 5E). Analyses of the effects of various treatments on induction of apoptosis in cp70 cells showed that RRR-α-tocopherol alone did not induce the cells to undergo apoptosis and that combination treatments of RRR-α-tocopherol with either VES or α-TEA resulted in decreased percentage of apoptotic cells of 28 and 13% in comparison to VES and α-TEA treatments alone (Fig. 5F).

VES Induces Cp70 Cells to Undergo Apoptosis When Cells Are Pretreated with Cellular Esterase Inhibitor BNPP Followed by Treatment with VES. Pretreatment of cp70 cells with 12.5 or 25 mM of the cellular esterase inhibitor BNPP for 30 min did not cause the cells to undergo apoptosis (Fig. 6A). Pretreatment of cells with 25 mM BNPP for 30 min, followed by treatment with VES at 5, 10, and 20 μg/ml for 2 days induced 12, 19, and 37% apoptosis in comparison to cells treated with VES alone which induced 3, 8, and 19%, respectively (Fig. 6A). Pretreatment of cells with BNPP had no effect on α-TEA-induced apoptosis (data not shown).

Additional data showing that treatment with 25 mM BNPP for 30 min enhances VES-induced apoptosis come from Western immunoblotting analyses of cell lysates from 1 × 10⁵ cp70 cells/ml cultured (10-ml volume) in 25-ml flasks with 20 μg/ml α-TEA (A), 20 μg/ml vitamin E succinate (VES, B), or 20 μg/ml VES with (light solid line) or without (darker dotted line) 100 μg/ml RRR-α-tocopherol (C) or 20 μg/ml α-TEA with (light solid line) or without (darker dotted line) 100 μg/ml RRR-α-tocopherol (D) for 12 h. VES and α-TEA levels in cells treated with single or combination treatments with RRR-α-tocopherol are depicted in pmol/10,000 cells as the mean ± SD of three independent experiments (E). Cp70 cells were untreated (UT) or treated for 3 days with 20 μg/ml VES or α-TEA with and without treatment with 100 μg/ml RRR-α-tocopherol or 100 μg/ml RRR-α-tocopherol alone. Cells were collected and apoptosis determined by 4',6-diamidino-2-phenylindole dihydrochloride staining as described previously (F). Apoptotic data are depicted as mean ± SD of three independent experiments. A, B, and C data are representative of multiple experiments.

Fig. 5. High-performance liquid chromatography analyses were conducted, as described in “Materials and Methods,” on cell lysates from 1 × 10⁵ cp70 cells/ml cultured (10-ml volume) in 25-ml flasks with 20 μg/ml α-TEA (A), 20 μg/ml vitamin E succinate (VES, B), or 20 μg/ml VES with (light solid line) or without (darker dotted line) 100 μg/ml RRR-α-tocopherol (C) or 20 μg/ml α-TEA with (light solid line) or without (darker dotted line) 100 μg/ml RRR-α-tocopherol (D) for 12 h. VES and α-TEA levels in cells treated with single or combination treatments with RRR-α-tocopherol are depicted in pmol/10,000 cells as the mean ± SD of three independent experiments (E). Cp70 cells were untreated (UT) or treated for 3 days with 20 μg/ml VES or α-TEA with and without treatment with 100 μg/ml RRR-α-tocopherol or 100 μg/ml RRR-α-tocopherol alone. Cells were collected and apoptosis determined by 4',6-diamidino-2-phenylindole dihydrochloride staining as described previously (F). Apoptotic data are depicted as mean ± SD of three independent experiments. A, B, and C data are representative of multiple experiments.

Fig. 6. Cp70 cells at 1 × 10⁵ cells/ml in 20-ml flasks were either untreated or pretreated with 12.5 or 25 mM esterase inhibitor bis-(p-nitrophenyl) phosphate (BNPP) for 30 min. The untreated and BNPP-treated cells were then cultured with 5, 10, or 20 μg/ml vitamin E succinate (VES) for 3 days, and cells were 4',6-diamidino-2-phenylindole dihydrochloride stained for analyses of apoptosis as described in “Materials and Methods.” Cp70 cells treated with 5, 10, and 20 μg/ml α-TEA served as positive apoptotic control (A). Cell lysates from cp70 cells cultured with 10 μg/ml α-TEA, 10 μg/ml VES, or pretreated with 25 μM for 30 min and then treated with 10 μg/ml VES for 2 days were analyzed by Western immunoblotting for cleavage of caspase 3 substrate poly(ADP-ribose) polymerase (PARP; B). Data depicted in A are mean ± SD of three independent experiments. Data in B are representative of multiple experiments.
DISCUSSION

Despite several treatment options, ovarian carcinoma remains the fifth most common cause of cancer-related mortality in women (34). One major problem in the treatment of ovarian cancers has been the development of multidrug resistance. After treatment with various regimens including cisplatin, [cis-diaminedichloroplatimum(II)], paclitaxel, and doxorubicin (Adriamycin), many patients experience relapse with drug-resistant tumors and die of chemoresistant disease (35, 36). Thus, there is a need for new anticancer drugs effective against ovarian cancers.

VES is a potent growth inhibitor of a wide variety of human cancer cell lines in vitro, as well as an effective tumor growth inhibitor in vivo when administered i.p. (1, 3–13). Human tumor cells in culture undergo DNA synthesis arrest, differentiation, and apoptosis in response to treatment with VES (reviewed by Refs. 1, 15–21). α-TEA is a novel vitamin E analogue, which is currently being characterized for its chemotherapeutic and chemopreventive potential. α-TEA has been shown to decrease tumor burden and reduce lung metastasis in a transplanted syngeneic mouse mammary cancer model (2).

In the studies reported here, we show that α-TEA is as potent a proapoptotic agent as VES for a number of human breast, prostate, colon, lung, and endometrial cells and markedly better than VES as an inducer of apoptosis in human ovarian and cervical cancer cells. These data suggest that ovarian and cervical cancer cells express sufficient esterase activity to render VES less effective than α-TEA by hydrolysis of the ester-linked succinate moiety of VES and raise the possibility that these findings may be extended to other gynecological malignancies. Studies comparing the responsiveness of cp70 cells to VES versus α-TEA show that the cisplatin-resistant cp70 ovarian tumor cells undergo DNA synthesis arrest but exhibit only low levels of apoptosis when cultured with VES versus undergoing both DNA synthesis arrest and apoptosis when cultured with α-TEA. The potential ramifications of this difference in growth inhibition by VES and α-TEA were addressed by conducting clonogenic studies, which showed that α-TEA was superior to VES in preventing cp70 colony formation.

To address the question of why α-TEA is a more effective proapoptotic agent than VES in ovarian tumor cells, cellular uptake and stability of α-TEA versus VES were examined. Previous studies have suggested that the antitumor activity of VES is associated with the intact molecule. We and others have shown that although VES is capable of inducing DNA synthesis arrest, inducing apoptosis, and preventing colony formation in a variety of cancer cells, neither of the breakdown products, namely RRR-α-tocopherol or succinic acid, exhibit this activity (6, 11, 28, 37–41). Furthermore, Fariss et al. (6, 39) have shown that a nonhydrolyzable ether form of VES prevents colony formation of murine leukemia cells and blocks growth of human breast cancer cells, adding additional support that it is the intact molecule that is responsible for the antitumor properties. Thus, the expectation is that cells capable of hydrolyzing VES would not be responsive to VES-mediated antitumor activities. As reported in this article, α-TEA and VES are taken up by cp70 cells, but most of the intracellular VES is hydrolyzed to RRR-α-tocopherol. In contrast, α-TEA remains intact in cp70 cells. The HPLC and apoptosis analyses reported here are the first direct demonstration that when VES is broken down by a cell it loses its capacity to induce apoptosis.

To test the assumption that cellular esterases in the ovarian cp70 cells were responsible for the breakdown of VES, the esterase inhibitor BNPP was used to block esterase activity before VES treatment. Pretreatment of cp70 cells with BNPP resulted in the retention of intact VES and VES proapoptotic effects. Although no references to esterase activity in cp70 cells were not found in the literature, a study by Di Francesco et al. (42) showed that the parental A2780 cells were capable of cleaving phenol-ester derivatives, suggesting that these cells contained esterases. Another interesting observation made in the conduct of these studies is that VES retains the ability to induce DNA synthesis arrest in cp70 cells despite the presence of esterases and hydrolysis. One possible explanation is that VES signals DNA synthesis arrest in a rapid, acute manner, thereby initiating a competent signal before hydrolysis, whereas signaling of apoptosis requires a longer, more chronic presence of intact VES.

Mechanism(s) for how VES or α-TEA induce DNA synthesis arrest in human ovarian cancer cells is not known at present. Studies in human MDA-MB-435 breast cancer cells showed that VES inhibition of DNA synthesis involved a G0 -G1 cell cycle block and marked induction of the expression of the cyclin-dependent kinase inhibitor p21 Waf1/Cip1 at the mRNA and protein level (18). Confirmation of the involvement of p21 in this event was demonstrated by the blockage of VES-induced DNA synthesis arrest by antisense oligomers to p21 (18). Furthermore, studies by Turley et al. (43) reported that VES inhibited the proliferation of the estrogen receptor-negative human breast cancer cell line BT-20 in the G1 phase of the cell cycle, at least in part, by inhibition of the transcriptional activity of E2F through negative regulation via cyclin A binding. Additional studies are needed to understand the mechanism(s) of how VES and α-TEA block DNA synthesis in ovarian cells.

The failure of VES to induce apoptosis in cp70 cells to the same degree as α-TEA most likely is caused by lower intracellular levels of intact VES because of esterase cleavage of the succinate moiety from VES, resulting in RRR-α-tocopherol that does not exhibit apoptotic inducing properties. Another possibility is that intracellular RRR-α-tocopherol resulting from esterase cleavage of the succinate moiety from VES may block the ability of the remaining intact VES to induce apoptosis by some mechanism that we do not understand at this time. Combination treatments of VES or α-TEA + RRR-α-tocopherol reduces the level of intracellular VES and α-TEA and reduces the level of apoptosis induced by these two tocopherol-based compounds. The mechanism(s) whereby RRR-α-tocopherol interferes with intracellular levels of VES and α-TEA and reduces apoptosis are not understood.

In conclusion, these studies demonstrate that the novel vitamin E analogue, α-TEA, is a stable compound and a potent inducer of apoptosis in a wide variety of human cancer cells but not normal cells in culture. It is of significance that α-TEA, a potential chemotherapeutic agent, is structurally stable when administered to human ovarian and cervical cancer cells and acts as a potent proapoptotic agent. Perhaps of more significance, future drug and prodrug design for prevention and treatment of ovarian and cervical cancer need to take into consideration that these cells may express cellular esterases at levels sufficient to cleave critical functional groups.

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Differential Response of Human Ovarian Cancer Cells to Induction of Apoptosis by Vitamin E Succinate and Vitamin E Analogue, α-TEA

Kristen Anderson, Marla Simmons-Menchaca, Karla A. Lawson, et al.

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