Vitamin E inhibits melanoma growth in mice

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Background. *Previous work has demonstrated that vitamin E succinate (VES), an ester analogue of vitamin E, inhibits the growth of melanoma in vitro. However, there is no information about the effect of VES on melanoma in vivo. We investigated the effect of VES on melanoma in vitro and in vivo.*
Methods. *The effect of VES on the proliferation and apoptosis of the B16F10 murine melanoma cell line was determined by a modified Cell Titer 96 AQ assay and a cell death detection enzyme-linked immunosorbent assay, respectively. The in vivo effect of VES on B16F10 melanoma cells allografted in athymic nude mice was investigated. The mechanism of the in vivo antitumor effect of VES was determined by immunohistochemical detection of proliferation and apoptosis.*
Results. *VES decreased cell proliferation (*P = *.0001) and increased cell apoptosis (*P = *.0001) in a dose-dependent manner in vitro. Also, VES significantly inhibited melanoma growth in mice (*P = *.0013). The VES antitumor effect in vivo was associated with a significant increase in the melanoma apoptosis rate (*P = *.0256).*
Conclusions. *This is the first report of the antimelanoma effect of VES in vivo. The mechanism of the antimelanoma effect of VES in vivo involves the promotion of tumor cell apoptosis. These findings support future investigations of VES as a therapeutic micronutrient against melanoma. (Surgery 2002;131:85-91.)*

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VITAMIN E is a generic term that includes all entities that exhibit the biologic activity of natural vitamin E (d-alpha-tocopherol).[1] Natural vitamin E is an essential nutrient that functions as an antioxidant in the human body. Clinical interest in vitamin E is high because optimal intake can help prevent the onset of free radical-related degenerative diseases, such as cancer, atherosclerosis, premature aging, cataract formation, and arthritis.[2-3]

In nature, 8 substances have been found to have vitamin E activity: d-alpha-, d-beta-, d-gamma-, and d-delta-tocopherol, and d-alpha-, d-beta-, d-gamma-, and d-delta-tocotrienol. The acetate and succinate derivatives of the natural tocopherols have vitamin E activity, as do synthetic tocopherols and their derivatives. Of these, d-alpha-tocopherol has the highest biopotency.[4] Since 1982, many studies have demonstrated that vitamin E succinate (VES, RRR-α-tocopherol acid succinate) is the type of vitamin E that is the most potent inhibitor of neoplastic cells in vitro.[5] Reports on the in vivo antitumor activity of VES are scarce. Recently, we demonstrated VES promotion of breast tumor dormancy.[6]

VES has been shown to inhibit the growth and survival of melanoma cells in vitro.[6-20] However, there is no information about the effect of VES on melanoma in vivo. Here, we examine the effects of VES on melanoma cells in vitro and in vivo. We demonstrate that VES profoundly inhibits melanoma in vivo. The in vivo effect of VES was associated with the induction of apoptosis.

MATERIAL AND METHODS

Chemicals. The (+)-α-tocopherol acid succinate (D- form of VES) was purchased from Sigma Chemical Co (St Louis, Mo).

Cell lines. The aggressively growing B16F10 murine melanoma cells and culture conditions were kindly provided by Isaiah Fidler (MD Anderson Cancer Center, Tex). The cells were maintained as monolayer cultures in minimum essential medium supplemented with 5% fetal

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bovine serum, 2 mmol/L of L-glutamine, 100 units/mL of penicillin, and 100 μg/mL of streptomycin, minimum essential medium vitamin supplements, sodium pyruvate, non-essential amino acid (all from Life Technologies, Inc, Grand Island, NY). The cells were incubated at 37°C in a humidified atmosphere with 5% carbon dioxide in the air.

Cell proliferation assay. Cells were grown to 50% to 70% confluence and harvested by trypsinization. The cell number was determined with a hemacytometer and by trypan blue exclusion analysis. The B16F10 (1×10^5 cells/well) was plated in 4 cycles of triplicates in 96-well plates and incubated for 24 hours in media containing 5 to 100 μg/mL of VES, 0.5% ethanol (vehicle), or no additions (control). Cell proliferation activity was determined by the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (MTS, CellTiter 96 Aq_{ueous} non-radioactive cell proliferation assay; Promega Corp, Madison, Wis) according to the manufacturer's instructions.

Cell apoptosis assay. Cells were grown, harvested, and replated as described above. Cell apoptosis was determined by a sandwich enzyme–linked immunosorbent assay (Cell Death Detection ELISA^{plus}; Boehringer Mannheim, Indianapolis, Ind) according to the manufacturer's instructions.

Animals. Female athymic nude mice 6 to 7 weeks of age were purchased from Harlan Sprague Dawley (Madison, Wis) and housed under specific pathogen-free conditions. The mice were allowed to acclimatize for 1 week. The housing, care and use of animals, as well as procedures to minimize discomfort were approved by the Southern Illinois University Laboratory Animal Care and Use Committee accredited by the American Association for Accreditation of Laboratory Animal Care.

Tumor cell inoculation in nude mice. The B16F10 murine melanoma cells were harvested by trypsinization, centrifuged, resuspended in phosphate buffered saline (PBS) solution at a density of 1×10^6 cells/mL, and kept on ice. All mice were inoculated subcutaneously on the right flank with a 0.1 mL mixture (10^6 cells/100 μL PBS) of murine melanoma cells by means of a 27-gauge needle. The VES treatment started 2 days after B16F10 inoculation. The mice received 50 μL of intraperitoneal injections of either VES (60 mg/mL in sesame oil, 150 mg/kg/d, n = 10) or vehicle (sesame oil, n = 10) in 3 cycles of 5 consecutive daily injections followed by 2 days of rest. One week after tumor cells were inoculated, primary tumor volumes were monitored twice a week over a 2-week growth period from caliper measurements according to the formula $T_{vol} = (L + W)/2 \times (L \times W) \times 0.5236$, where L was maximum length of tumor and W was minimum length. Animals were sacrificed 21 days after the inoculation of B16F10 cells, and their tumor volumes were recorded to determine the degree of local growth. Tumors were fixed in 4% paraformaldehyde, pH 7.2, and embedded in paraffin for immunohistochemical detection.

Immunohistochemistry of proliferating cell nuclear antigen. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was performed on formalin-fixed, paraffin-embedded sections (4 μm) of melanoma tumors with the streptavidin-peroxidase technique described previously.[21] Antigen unmasking was performed on the deparaffinized slides by incubation in 10 mmol/L of citrate buffer, pH 6.0, for 5 minutes at 95°C. Sections were incubated with a 1:200 dilution of mouse anti-human PCNA monoclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, Calif). Positive controls consisted of a PCNA antibody with human tonsil tissue. Replacement of the primary antibody with a mouse IgG_{2a} isotype was used as a negative control (Sigma). Sections were then incubated with a biotin-labeled secondary antibody (Goat anti-mouse IgG; KPL, Gaithersburg, Md). Counterstaining with hematoxylin for 2 minutes followed the subsequent reactions with streptavidin-peroxidase conjugate (KPL) and diaminobenzidine (Research Genetics, Huntsville, Ala). Immunostains were quantified by evaluating the proportion of positively stained cells for PCNA from multiple portions of the slides with the microscope computer imaging device software (MCID/M2 software; Imaging Research, St Catherines, Ontario, Canada).

TUNEL assay. Formalin-fixed paraffin-embedded sections (4 μm) of the tumors were subjected to immunostaining for apoptotic cells with the In Situ Cell Death Detection Kit, POD (Boehringer Mannheim). Sections were incubated at 60°C for 30 minutes, deparaffinized, and rehydrated through a graded series of alcohol and water. The sections were pretreated with trypsin (1 mg/mL) for 4 minutes, rinsed with water, and placed in PBS-1% BSA for 5 minutes. The sections were incubated in 0.3% methanol for 30 minutes and rinsed with PBS-1% BSA for 5 minutes to block endogenous peroxidase activity. Cell permeabilization was performed by incubating the sections in 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes on ice and rinsing the sections with PBS-1% BSA twice. Immunostaining was performed according to the manufacturer's instructions by labeling DNA

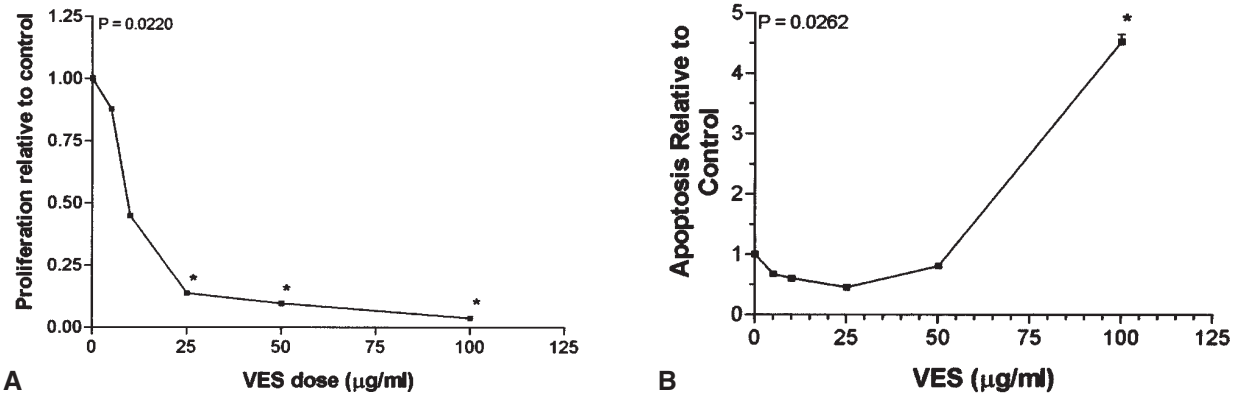

Fig 1. Twenty-four hour dose-dependent effect of VES on B16F10 melanoma cancer cell proliferation (**A**) and apoptosis (**B**). Pearson product moment correlation coefficient indicated that cell proliferation decreased linearly as VES dose increased (**A**, $r = -0.95$, $P = .0001$), and cell apoptosis increased linearly as VES dose increased (**B**, $r = 0.89$, $P = .001$). After 24 hours of incubation, the minimum concentration of VES that significantly inhibited cell proliferation was 25 μg/mL ($P = .0220$), and significantly stimulated cell apoptosis was 100 μg/mL ($P = .0262$)

strand breaks with fluorescein followed by a peroxidase-conjugated anti-fluorescein antibody and was visualized with diaminobenzidine substrate (Research Genetics, Huntsville, Ala). Sections of human tonsil were processed as described above and used as a positive control. Omission of the terminal deoxynucleotidyl transferase enzyme during processing was used as a negative control. Apoptosis was detectable by morphological findings, chromatin condensation, nuclear DNA fragmentation, DNA strand breakage, or apoptotic bodies with a bright-field microscope. Immunostains were quantified by estimating the proportion of positively stained cells for apoptosis from multiple portions of the slides (Imaging Research).

Statistical analysis. Correlation coefficients were computed for VES dose response on cell proliferation and cell apoptosis. In addition, independent *t* tests were used to compare quantified data between groups for proliferation, apoptosis, and immunocytochemistry assays. Split-plot analysis of variance was used to examine changes in tumor volume between the control- and VES-treated groups of mice. Results were considered significant if the *P* value was $< .05$.

RESULTS

VES inhibits growth and promotes apoptosis in melanoma in vitro. We conducted dose-response studies on proliferation and apoptosis of B16F10 murine melanoma cells in vitro to determine the effects of VES on melanoma cancer growth.

The cell proliferation activity was determined by the colorimetric MTS assay. The colorimetric assay determined the number of viable cells proliferating. VES inhibited the proliferation of B16F10 cells in a dose-dependent manner ($r = 0.95$, $P = .0001$). The minimum concentration of VES that significantly inhibited the proliferation of melanoma cancer cells after 24 hours of incubation was 25 μg/mL ($P = .0220$, Fig 1, *A* and *B*).

Cell Death Detection ELISA was performed with B16F10 cells to determine the apoptotic effect of VES on melanoma. The in vitro photometric enzyme-immunoassay determined cytoplasmic histone-associated-DNA-fragments after induced cell death. VES stimulated apoptosis of B16F10 cells in a dose-dependent manner ($r = 0.89$, $P = .0001$). The minimum concentration of VES that significantly induced the apoptosis of B16F10 melanoma cells after 24 hours of incubation was 100 μg/mL ($P = .0262$, Fig 1, *B*).

VES inhibits melanoma tumor growth in nude mice. Because VES inhibited proliferation and stimulated apoptosis in B16F10 cells in vitro, the inhibitory effects of VES on B16F10 tumor growth in vivo was also investigated. Athymic nude mice allografted with B16F10 cells were divided into control and VES treated groups. Tumor growth was monitored 3 times a week. Tumors in the control groups grew rapidly, reaching an average volume of 2350 ± 798 μL by day 15 after the inoculation of B16F10 melanoma cells. In contrast, the inhibition of tumor growth on mice that were administered VES intraperitoneally was profound, with tumor volume remaining at an average of 367 ± 136 μL (Fig 2, *A* and *B*). There were no differences in tumor volumes between the vehicle (sesame oil) and control groups. These results indicate that VES has a significant inhibitory effect on melanoma tumor growth in vivo ($P = .0013$).

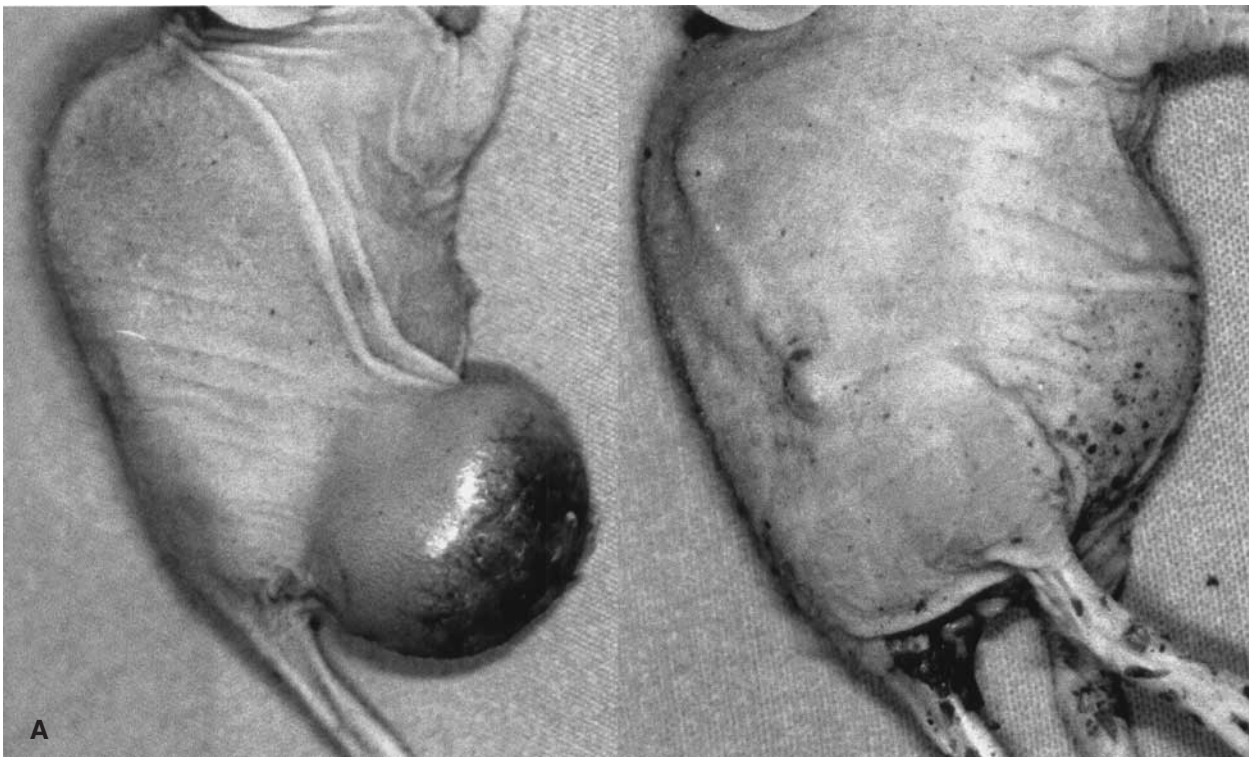

Fig 2. Effect of VES on melanoma tumor growth in vivo. Athymic nude mice were inoculated subcutaneously on the right flank with B16F10 (10^6 cells/100 μL PBS) murine melanoma cells. The mice received either intraperitoneal VES (60 mg/mL in sesame oil, 150 mg/kg/d), vehicle (sesame oil), or no treatment from day 2 after inoculation in 3 cycles of 5 consecutive daily injections. This was followed by 2 days of rest. Mice were sacrificed on day 28. Representative mice (**A**) injected with vehicle (control) or VES (treated). Mice injected with VES intraperitoneally had significantly smaller tumor volumes compared with control mice over time (**B**, $P = .0013$). There were no differences in tumor volumes between vehicle (sesame oil) and control groups. *Sc,* Subcutaneous; *ves,* vitamin E succinate.

VES promotes melanoma apoptosis in vivo. Immunostained sections from the control- and VES-treated groups of animals were analyzed for differences in proliferation and apoptosis to determine whether inhibition of tumor growth in vivo by VES resulted from the inhibition of proliferation or the stimulation of apoptosis. Antiproliferative effects of VES on melanoma tumors were determined by immunolocalization of PCNA from formalin-fixed, paraffin-embedded sections. There was a marginal difference in staining for the proliferation marker, PCNA, in melanoma tumors

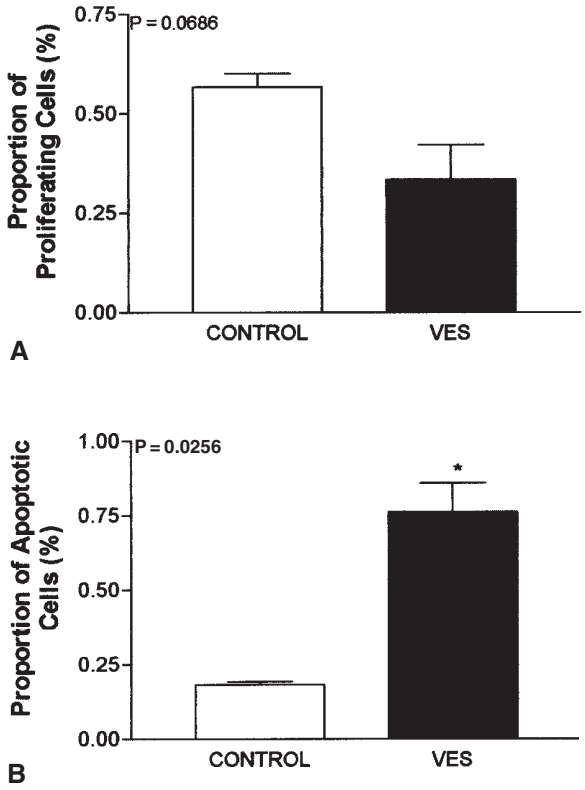

Fig 3. Immunohistochemistry detection of proliferation **(A)**, and apoptosis **(B)** from formalin-fixed, paraffin-embedded section of murine melanoma tumors obtained from athymic nude mice treated with vehicle or VES. There was a marginal difference between control- and VES-treated melanoma cells stained for proliferating cell nuclear antigen (**A**, PCNA, $P = .0686$). A significantly higher number of VES-treated melanoma cells stained positive for apoptosis (**B**, TUNEL, $P = .0256$) compared with the control. *VES*, Vitamin E succinate.

obtained from VES-treated athymic nude mice compared with those from the control mice (33% ± 8% vs 57% ± 3%, $P = .0686$, Fig 3, *A*). Apoptotic effects of VES on melanoma tumors were determined by in situ cell death detection enzyme-linked immunosorbent assay on formalin-fixed, paraffin embedded sections. Apoptosis was detectable by morphologic findings, chromatin condensation, nuclear DNA fragmentation, DNA strand breakage, or apoptotic bodies. Melanoma tumors from VES-treated mice demonstrated a statistically significant increase in apoptosis compared with those from the control (76% ± 9% vs 18% ± 1%, $P = .0256$, Fig 3, *B*, Fig 4). These data suggested that the mechanism of VES in vivo antitumor effects involved the induction of tumor cell apoptosis.

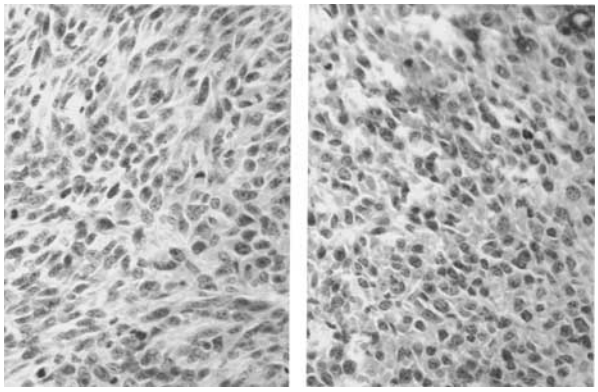

Fig 4. Representative photomicrographs of apoptosis from formalin-fixed, paraffin-embedded sections of murine melanoma tumors obtained from athymic nude mice treated with vehicle or VES. Sections were immunostained by labeling DNA strand breaks with fluorescein followed by a peroxidase-conjugated anti-fluorescein antibody and visualized with diaminobenzidine substrate counterstained with hematoxylin (original magnification, ×100). A significantly high number of VES-treated melanoma cells stained positive for apoptosis ($P = .0256$) compared with the control.

DISCUSSION

In this study, we showed that VES suppressed the growth of B16F10 melanoma cells in nude mice. This is the first report of VES inhibiting melanoma growth in vivo. Reports of VES antitumor effects in vivo are rare possibly because of problems in delivering the intact VES molecule to tumor cells. Our laboratory and others have shown that the inhibitory effect of VES on cell growth is specific for the intact molecule.[6,22] In one study, where tocopherol esters were delivered through the diet, there was intestinal hydrolysis yielding the acid and vitamin E,[23] thus de-esterizing the intact VES molecule. Recently, we observed that the intraperitoneal route of VES administration was effective in inhibiting breast cancer tumor growth.[6] We administered VES to mice xenografted with melanoma cells by intraperitoneal injection to determine whether VES would inhibit melanoma growth in vivo.

The mechanism by which VES inhibits tumor growth is poorly understood. The ester linkage that attaches succinic acid to vitamin E eliminates the hydroxyl moiety, which mediates vitamin E's classical antioxidant properties. Thus, it is generally believed that the inhibitory effects of VES are not mediated by its antioxidant property.[5,9,18,19] Previous in vitro melanoma studies, including ours, have shown that VES inhibits cancer cell growth by a variety of mechanisms. These mechanisms include modulation of adenylate cyclase, inhibition

of protein kinase C activity, regulation of transforming growth factor beta (TGF-β) protein products, enhanced expression of TGF-β type II receptors, G_1 cell cycle blockage, DNA synthesis arrest, and the induction of apoptosis.[6-20] The role of these processes in the antitumor effects of VES in melanoma growth in vivo is yet to be elucidated.

Inhibition of angiogenesis could be implicated in the antitumor effects of VES. In our previous in vivo study where VES inhibited breast cancer growth in nude mice, we also found that VES inhibited vascular endothelial growth factor (VEGF) gene expression in the cancer cell line that constitutively express VEGF.[6] In the same study, we found that angiopoietin 1, which is not constitutively expressed by the breast cancer cell line MDA-MB-231, was induced by VES treatment. The results suggested that VES modulates the gene expression of some angiogenic factors. The mechanism of VES modulation of VEGF transcription is under investigation in our laboratory.

Because the processes of proliferation and apoptosis generally modulate in vivo tumor growth, we investigated the modulation of these processes by VES in our tumor model. Despite the significant antiproliferative effect of VES in vitro, we only observed a marginal difference in the antiproliferative effect of VES in vivo. The discrepancy between the in vitro and in vivo effects of VES on the proliferation of melanoma cells is intriguing. The reason for this discrepancy may be because of the sensitivity of the assay. It is also possible that the effect that was observed (VES 33% ± 8% vs control 57% ± 3%, $P = .0686$, Fig 3, *A*), although not statistically significant, is biologically significant.

In contrast, we observed a significant induction of apoptosis in melanoma cells both in vitro and in vivo. Several other in vitro studies have shown that VES triggers apoptosis in many cell lines, including breast, prostate, intestine, liver, tongue, and heart.[5,24-41] The mechanisms underlying VES induction of tumor cell apoptosis is not fully understood. Previous studies of VES-mediated apoptosis have implicated the modulation of Fas signaling, caspase-3 activation, lysosomal and mitochondrial destabilization, induction of c-jun expression, TGF-β signaling, induction of activator protein-1 transcriptional activity, and DNA synthesis arrest.[6,24-41] It is not known whether these VES in vitro apoptotic mechanisms occur in vivo. Our study supports the process of apoptosis as an important mechanism of VES antitumor effect in vivo. The implication of these observations is that VES may be used in synergy with other anti-proliferative agents against melanoma. It is likely that the mechanism of VES antitumor action involves multiple processes. In our model, we have observed the prominence of the apoptosis process in the antitumor action. We are actively exploring other processes.

In summary, we have demonstrated for the first time that VES, a derivative of vitamin E, is capable of inhibiting melanoma growth in vivo. The mechanism of VES's antitumor effects involved the promotion of melanoma apoptosis. Further studies of the antitumor effects of VES in vivo will aid in the design of clinical trials to incorporate the use of this micronutrient in human melanoma cancer prevention and treatment.

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