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Inhibition of Angiogenesis and Promotion of Melanoma Dormancy by Vitamin E Succinate

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Background: Relapse of melanoma after surgical treatment remains a significant clinical problem in need of novel therapies. Vitamin E succinate (VES) is a promising antitumor micronutrient. We evaluated the effect of VES on melanoma dormancy and angiogenesis.

Methods: B16F10 melanoma cells were allografted in mice. The effect of VES on melanoma dormancy was measured by monitoring tumor volume. Tumor vascularity was quantitated with CD31 immunostaining. The expression of vascular endothelial growth factor (VEGF), VEGF receptor 1, and VEGF receptor 2 in tumors was assessed by the intensity of immunostaining. VES effect on secreted VEGF protein and VEGF promoter activity was measured with enzyme-linked immunosorbent assay and transient transfection assay, respectively. Significance was determined by analysis of variance.

Results: VES promoted melanoma dormancy ($P = .0019$) and inhibited melanoma angiogenesis ($P < .0001$). VES also significantly suppressed the expression of VEGF, VEGF receptor 1, and VEGF receptor 2 in melanoma tumors ($P < .0001$). Melanoma VEGF secretion ($P = .0077$) and melanoma VEGF promoter activity ($P < .05$) were significantly inhibited by VES.

Conclusions: VES promotes melanoma dormancy and inhibits melanoma angiogenesis. The mechanism of the VES antiangiogenesis effect involves the inhibition of VEGF gene transcription. These findings support future studies of VES in the prevention of melanoma metastasis.

Key Words: Melanoma—Vitamin E succinate—Tumor dormancy—Angiogenesis—VEGF.

Melanoma is the leading cause of death from cutaneous malignancies.[1] Relapse after curative surgical treatment of melanoma remains a significant clinical challenge and accounts for most of the mortality from this disease.[2,3] Since 1970, a number of approaches have been tried for postsurgical adjuvant therapy for melanoma. Except for the recent success of high-dose interferon alfa-2, no other treatment modality has demonstrated reproducible increases in relapse-free or overall survival in randomized, controlled trials. Despite the success of interferon alfa-2, problems remain with this agent.[4–6] Prevention of relapse is not complete, and toxicity is significant. Therefore, new therapeutic strategies are needed.

Whereas the current strategy of adjuvant therapy aims at eliminating occult microscopic metastatic tumor cells, a novel treatment paradigm is chronic suppression of occult microscopic metastatic tumor cells so that they never become clinically relevant. This concept is termed the *promotion of tumor dormancy* because the microscopic tumor cells are maintained in a dormant state.[7] The transition from subclinical metastasis to obvious clinical metastasis represents a pathologic process whereby dormant micrometastases become active macrometastases. Little is known about the mechanisms controlling the melanoma switch from micrometastasis to macrometastasis. Recent work suggests that melanoma micrometastasis may eventually escape dormancy by means of a subset of tumor cells within the micrometastasis switching to an angiogenic phenotype. Barnhill et al.[8] and Barnhill[9] found that melanoma micrometastases have lower tumor proliferation rates and less angiogenesis than melanoma macrometastases. Therefore, the an-

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giogenesis switch is important in melanoma progression and clinical relapse. This observation confirms previous work from our laboratory and that of other investigators, who have observed the correlation between tumor angiogenesis and the ability of melanoma to metastasize.[10–14]

After the original hypothesis by Folkman,[15] it has been well established that tumors require proper vascularization (angiogenesis) for growth and metastasis.[16–18] Angiogenesis is regulated by a variety of positive and negative factors. The balance hypothesis for the angiogenesis switch, as advocated by Folkman, suggests that changes in the relative balance of inducers and inhibitors of angiogenesis can activate the angiogenesis switch.[19] Vascular endothelial growth factor (VEGF) is a crucial, positive regulator of angiogenesis in tumors, including melanoma. Although other tumor angiogenic factors have been identified, VEGF seems to be the most potent and the most specific.[20] Work from us and many other investigators has demonstrated that tumor angiogenesis and VEGF expression in melanoma are predictive of both metastasis and relapse.[12,21–26] The importance of VEGF as a tumor angiogenic factor in melanoma has been directly substantiated by experiments which show that VEGF expression is a prerequisite for melanoma tumor growth and metastasis.[27,28] Taken together, the prevailing evidence suggests that VEGF is a key regulator of melanoma angiogenesis and suggests that an inhibitor of VEGF-mediated angiogenesis may have therapeutic benefit in melanoma patients.

The connection between angiogenesis and melanoma progression has become an attractive target for new melanoma therapies. Drugs that interfere with angiogenesis and that can be feasibly combined with the current adjuvant agent, interferon alfa-2, would be extremely desirable.[29] Interferon has been shown to have antiangiogenesis activity that is mediated through the downregulation of basic fibroblast growth factor.[30]

Since 1982, many studies have demonstrated that the succinate derivative of vitamin E, vitamin E succinate (VES), is an important inhibitor of neoplastic cells in vitro.[31] Recent preclinical data suggest that VES is a promising metastasis prevention agent, because it has been demonstrated to have the ability to inhibit tumor cell proliferation, promote tumor cell apoptosis, and potentially inhibit angiogenesis.[32] We recently demonstrated that VES inhibits melanoma growth.[33] We showed that the inhibition of melanoma growth was associated with significant induction of tumor cell apoptosis, rather than inhibition of tumor cell proliferation. In a previous study of VES antitumor action in a model of squamous cell cancer of the oral cavity, Shklar and Schwartz[34] showed that VES inhibited tumor angiogenesis. Also, we recently observed that VES inhibits colorectal cancer metastases to the liver.[35] Also, we showed that VES inhibits the expression of VEGF in breast cancer cells.[32] In this study, we tested the hypothesis that VES will promote melanoma dormancy and inhibit melanoma angiogenesis. We also examined the effect of VES on VEGF and VEGF receptors (VEGF-Rs).

MATERIALS AND METHODS

Chemicals

(+)-α-Tocopherol acid succinate (d-form of VES) was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Lines

B16F10 murine melanoma cells and culture conditions were kindly provided by Isaiah Fidler (M. D. Anderson Cancer Center, Houston, TX). B16F10, a very aggressively growing and metastatic melanoma cell line, was maintained as monolayer cultures in RPMI medium supplemented with 10% fetal bovine serum, 100 μg/mL of streptomycin, 100 U/mL of penicillin, and 100 mM of sodium pyruvate (all from Life Technologies, Inc., Grand Island, NY). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Animals

Female athymic nude mice at 6 to 7 weeks of age, purchased from Harlan Sprague Dawley (Madison, WI), were housed under specific pathogen–free conditions. The mice were allowed to acclimatize for 1 week. We carefully observed the mice and adhered to the principles of humane laboratory animal practices. When the tumor burden became too large for the animals, we killed them. The Southern Illinois University Laboratory Animal Care and Use Committee, accredited by the American Association for Accreditation of Laboratory Animal Care, approved the housing, care, and use of animals, as well as procedures to minimize discomfort.

Tumor Cell Inoculation in Nude Mice

The B16F10 murine melanoma cells were harvested by trypsinization, centrifuged, resuspended in phosphate-buffered saline at a density of 1×10^6 cells per milliliter, and kept on ice. All mice were inoculated subcutaneously on the right flank with a .1-mL mixture (10^5 cells per 100 μl of phosphate-buffered saline) of murine melanoma cells by using a 27-gauge needle.

VES Administration

The mice received 50-μl intraperitoneal daily injections of VES (60 mg/mL in sesame oil, 100 mg/kg/day) or vehicle (sesame oil). Two groups of five mice were administered VES (group A) or vehicle (group B) from day 1 to day 17 after tumor cell inoculation and were killed on day 17. To determine the effect of VES on established exponentially growing tumors, another two groups of five mice were administered VES (group C) or vehicle (group D) from day 17 to day 25 after tumor cell inoculation and were killed on day 25. Primary tumor volumes were monitored biweekly with caliper measurements according to the formula

$$T_{vol} = (L + W)/2 \times (L \times W) \times .5236, \qquad (1)$$

where L was the maximum length of the tumor and W was the minimum length. At death, final tumor volumes were recorded. Tumors were fixed in 4% paraformaldehyde, pH 7.2, and embedded in paraffin for immunohistochemical detection.

Quantitation of Tumor Angiogenesis

Endothelial cells were stained with a monoclonal antibody against CD31 antigen (BD PharMingen, San Diego, CA) to highlight microvessels. Formalin-fixed, paraffin-embedded sections (4 μm) of the melanoma tumors were incubated at 60°C for 30 minutes, deparaffinized with Hemo-De™ (Fisher Scientific, Pittsburgh), and rehydrated through a graded series of alcohol and water. Antigen unmasking was performed on the deparaffinized slides by incubating in 10 mM of citrate buffer, pH 6.0, for 5 minutes at 95°C. To block endogenous peroxidase activity and permeabilize the cells, the sections were incubated in .3% methanol for 30 minutes and rinsed with triethanolamine-buffered saline for 5 minutes. Sections were incubated with rabbit serum for 30 minutes. They were then incubated with MEC 13.3 monoclonal rat antimouse CD31 antibody (PharMingen) at a dilution of 20 μg/mL overnight at 4°C. Sections of fetal liver were processed as described previously and used as a positive control. Mouse immunoglobulin (Ig)G_1 (Dako, Carpinteria, CA) was used as a negative control. Sections were incubated with biotin-conjugated rabbit anti-rat Ig (Dako) at a dilution of 1.5 μg/mL for 30 minutes, washed, and incubated with alkaline phosphatase–conjugated streptavidin (Roche, Summerville, NJ) at a dilution of 1/200 (1.5 μg/mL) for 30 minutes. As a substrate for the alkaline phosphatase reaction, we used freshly prepared Fast Red Substrate System™ (Roche), followed by a 10-minute wash in tap water. Sections were counterstained with hematoxylin and mounted with aqueous mounting media (Polysciences, Inc., Warrington, PA).

Vessel density was recorded as the number of point counts of CD31-positive vessels per high-power field ($\times$200) from the areas of highest vascularization, viewed through a Micromaster™ microscope (Fisher). Vessel densities were independently recorded by two observers for three different sections from each tumor and averaged.

Immunohistochemistry of VEGF, VEGF-R1, and VEGF-R2

Immunohistochemical staining for VEGF, VEGF-R1, and VEGF-R2 was performed on formalin-fixed, paraffin-embedded sections (4 μm) of melanoma tumors by using the streptavidin-peroxidase technique, as described previously. Antigen unmasking was performed on the deparaffinized slides by incubating in 10 mM of citrate buffer, pH 6.0, for 5 minutes at 95°C. Sections were incubated with a 1/50 dilution of rabbit polyclonal IgG antibody for VEGF, rabbit polyclonal IgG for VEGF-R1, or rabbit polyclonal IgG for VEGF-R2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Positive controls consisted of previous positively staining breast cancer tumors (MD-MB-231 cells xenografted in mice). Replacement of the primary antibody with a mouse IgG_{2a} isotype was performed as a negative control (Sigma). Sections were then incubated with a biotin-labeled secondary antibody (goat anti-rabbit IgG; KPL, Gaithersburg, MD). Counterstaining with hematoxylin for 2 minutes followed subsequent reactions with streptavidin-peroxidase conjugate (KPL) and diaminobenzidine (Research Genetics, Huntsville, AL). Immunostains were scored on a scale of 1 (least) to 4 (most) of positively stained cells for VEGF, VEGF-R1, and VEGF-R2. One evaluator with no prior knowledge of VES-treated slides scored the slides. Three different areas of each slide were scored at three different time periods to avoid scorer's bias.

VEGF Enzyme-Linked Immunosorbent Assay

The B16F10 cells (1×10^6/mL), plated in triplicate in 12-well plates, were incubated for 24 hours in media containing 5 to 100 μg/mL of VES, .5% ethanol (vehicle), or media with no additions (control). Cells were grown to 50% to 70% confluence and harvested by trypsinization. Cell number was determined with a hemacytometer and trypan blue exclusion analysis. Supernatants (1 mL) were treated with 100 mM of phenylmethyl sulfonyl fluoride. The effect of VES on secreted VEGF was determined by using the Accucyte™ murine VEGF enzyme-linked immunosorbent assay (Cytimmune Sciences, Inc., College Park, MD) according to the manufacturer's instructions.

Transient Transfection Assays

The B16F10 melanoma cells plated in triplicate in 12-well plates in 2 runs were grown to 100% confluence. The cells were transfected with a secreted alkaline phosphatase reporter plasmid (pSEAP2; Clontect Laboratories, Inc., Palo Alto, CA) containing the VEGF promoter and 5' untranslated region between −1810 and +1038 base pairs (essentially the full-length promoter). The VEGF promoter was obtained from Dr. Brian Seed (Edwin L. Steele Laboratory, Massachusetts General Hospital, Boston, MA). It is the human VEGF promoter, which was isolated from normal human vascular smooth muscle cells.

The cells were incubated for 24 hours in media containing 12.5 to 100 μg/mL of VES or in media with no additions (control). The dose-dependent effect of VES on VEGF promoter activity was determined by chemiluminescence secreted alkaline phosphatase assay (Great EscAPe™; Clontect Laboratories, Inc.) according to the manufacturer's instructions. Chemiluminescence was detected by using a plate luminometer (TD-20/20™ luminometer; Turner Designs, Sunnyvale, CA).

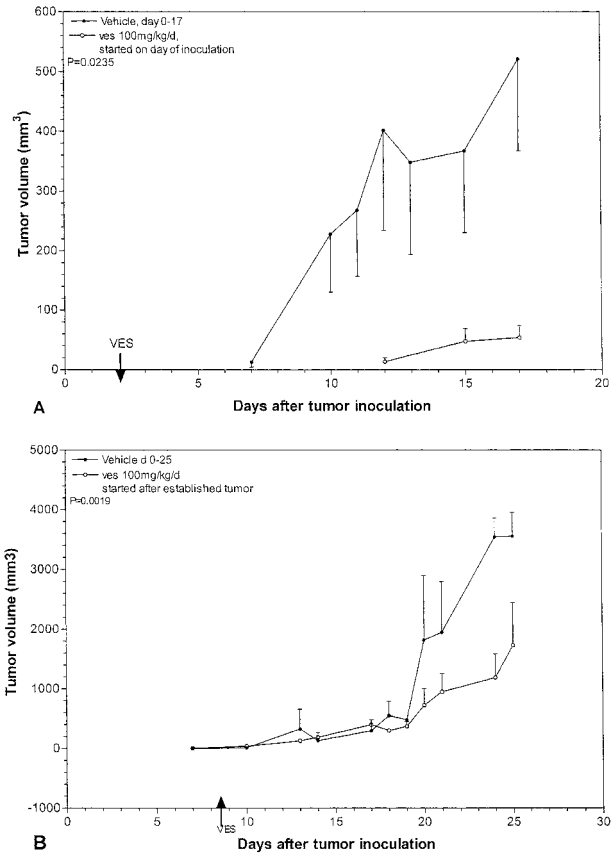

FIG. 1. Vitamin E succinate (VES) promotes melanoma dormancy. Athymic nude mice allografted with B16F10 cells were administered VES either at the time of melanoma cell inoculation or at day 17 after inoculation. After 17 days of melanoma cell inoculation in athymic nude mice, the inhibition of melanoma growth in mice administered VES at the time of tumor cell inoculation was profound, with tumor volume remaining at an average of 54 ± 20 mm^3, compared with control, with a volume of 521 ± 154 (**A**) ($P = .0235$). Similarly, 25 days after melanoma allograft in nude mice, VES administered at day 17 after melanoma cell inoculation in athymic nude mice significantly promoted melanoma tumor dormancy (**B**) (VES, 1734 ± 713 mm^3 vs. control, 3558 ± 398 mm^3; $P = .0019$). Significance was set at $P < .05$ for overall analysis of variance.

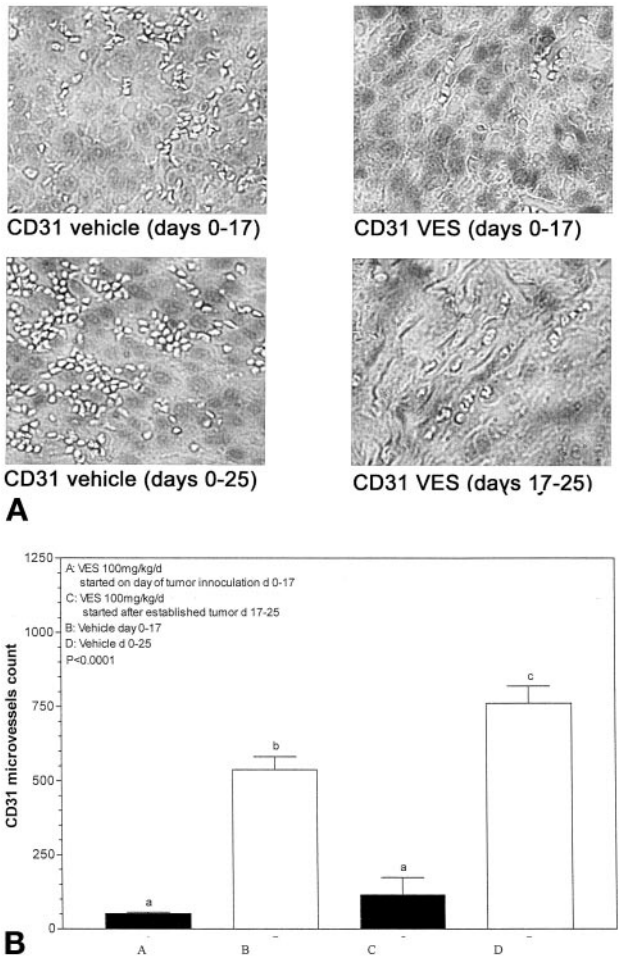

FIG. 2. Vitamin E succinate (VES) inhibits melanoma angiogenesis. (**A**) Representative photomicrographs of CD31 from formalin-fixed, paraffin-embedded sections of murine melanoma tumors obtained from athymic nude mice treated with vehicle or VES. Sections were incubated with MEC 13.3 monoclonal rat anti-mouse CD31 antibody followed by biotin-conjugated rabbit anti-rat immunoglobulin and alkaline phosphatase–conjugated streptavidin. Sections were visualized using freshly prepared Fast Red Substrate System and counterstained with hematoxylin (original magnification, ×200). The number of microvessels in tumors treated with VES was significantly lower ($P < .0001$) compared with control (**B**). Significance was set at $P < .05$ for overall analysis of variance. Columns with different superscripted lowercase letters significantly differ and denote pairwise comparisons of CD31 in VES-treated melanoma tumor groups.

Statistical Analysis

Split-plot analysis of variance was used to examine changes in tumor volume between the control and VES-treated groups of mice. One-way analysis of variance was used to compute immunohistochemistry data for microvessel density counts, VEGF, VEGF-R1, and VEGF-R2. Correlation coefficients were computed for the VES dose response and for the dose-dependent effects of VES on VEGF and VEGF promoter activity. Results were considered significant for $P < .05$. In the figures, superscripted lowercase letters denote pairwise comparisons of experimental groups with certain control groups. The P values listed in the legends are for the overall analysis of variance.

RESULTS

VES Promotes Melanoma Dormancy

Athymic nude mice allografted with B16F10 melanoma cells were divided into two cohorts of control and VES treated groups. One cohort had treatment started on the day of inoculation, and the second cohort had treatment started 17 days after tumor inoculation. The first cohorts were treated for 17 days and killed on day 17 of the treatment. The second cohort of mice was treated from day 17 after tumor inoculations until day 25 after tumor inoculation. Inhibition of melanoma growth in mice that received VES treatment at the time of tumor cell inoculation was profound, with tumor volume remaining at an average volume of 54 ± 20 mm^3, compared with the control volume of 521 ± 154 mm^3 ($P = .0235$; Fig. 1A). Also, when treatment was started 17 days after melanoma cell inoculation, the mice receiving VES had significant inhibition of tumor growth, with a volume of 1734 ± 713 mm^3, compared with control mice, which had a volume of 3558 ± 398 mm^3 ($P = .0019$; Fig. 1B). These results indicate that VES significantly inhibited the progression of melanoma growth (promoted tumor dormancy) whether it was administered at the time of tumor inoculation or after the establishment of tumor growth. The sizes of tumors treated with VES were similar to those reported previously, when the time to the tumor's increasing 10-fold was approximately 10 days.[33]

VES Inhibits Melanoma Angiogenesis

Because tumor growth is dependent on angiogenesis and VES inhibits melanoma tumor growth, the VES effect on melanoma angiogenesis was assessed. Tumor

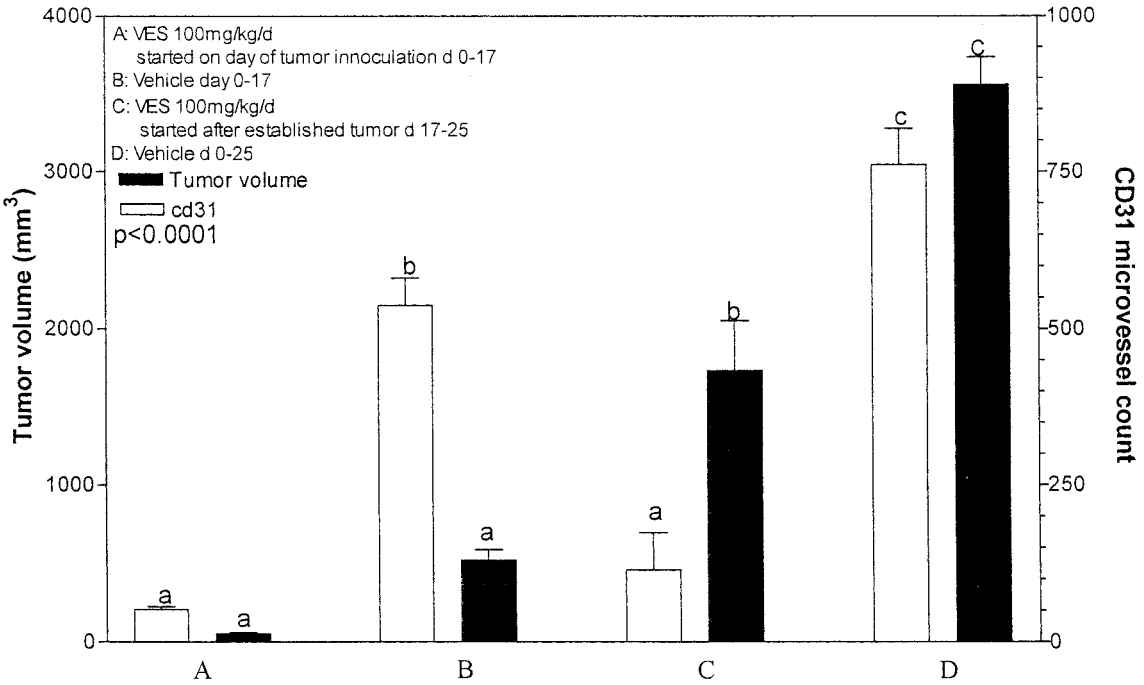

FIG. 3. Inhibition of angiogenesis was not an artifact of tumor size. Tumors of control mice killed on day 17 after tumor cell inoculation (521 ± 154 mm^3) were smaller than vitamin E succinate (VES)-treated tumors from day 17 to day 25 after tumor cell inoculation (1734 ± 713 mm^3). Yet the number of microvessels in the larger VES-treated tumors was significantly less than in the control ($P < .0001$). On average, the smaller control tumors had twice as many vessels as the larger VES-treated tumors. The inhibition of melanoma angiogenesis was specific for the effect of VES rather than tumor size. Significance was set at $P < .05$ for overall analysis of variance. Columns with different superscripted lowercase letters significantly differ and denote pairwise comparisons of tumor size and microvessels of VES-treated melanoma tumor groups.

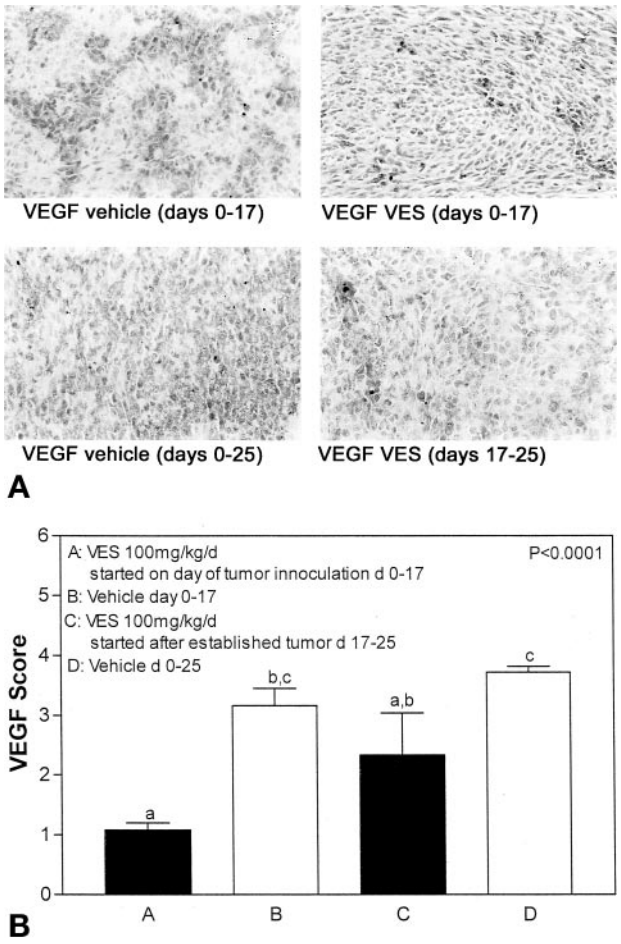

FIG. 4. Vitamin E succinate (VES) suppresses vascular endothelial growth factor (VEGF) protein expression. (**A**) Representative photomicrographs of VEGF from formalin-fixed, paraffin-embedded sections of murine melanoma tumors obtained from athymic nude mice treated with vehicle or VES. The level of VEGF protein in tumors treated with VES from the time of melanoma cell inoculation in nude mice was significantly lower compared with control (**B**). Similarly, a significant downregulation of VEGF protein by VES was observed in tumors treated with VES 17 days after tumor allograft in nude mice. These findings suggest that VES significantly suppressed the expression of VEGF protein in melanoma tumors whether it was administered at the time of or after melanoma cell inoculation in nude mice ($P < .0001$). Significance was set at $P < .05$ for overall analysis of variance. Columns with different superscripted lowercase letters significantly differ and denote pairwise comparisons of VEGF protein expression of VES-treated melanoma tumor groups.

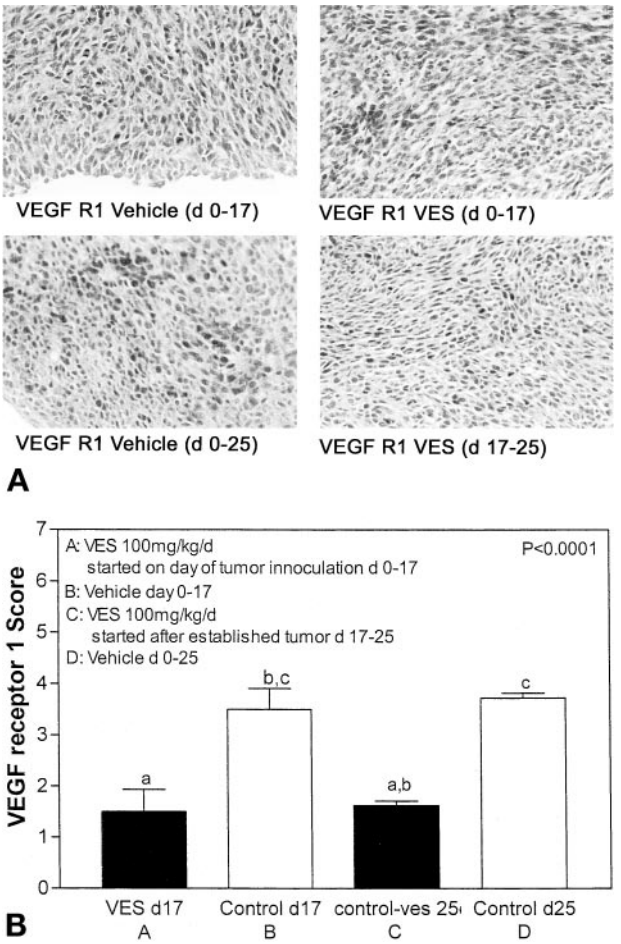

FIG. 5. The antiangiogenic effect of vitamin E succinate (VES) on vascular endothelial growth factor (VEGF) receptor 1 (VEGF-R1) expression in tumors. (**A**) Representative photomicrographs of VEGF-R1 from formalin-fixed, paraffin-embedded sections of murine melanoma tumors obtained from athymic nude mice treated with vehicle or VES. The number of VEGF-R1s in tumors treated with VES from the time of melanoma cell inoculation in nude mice was significantly lower compared with the control (**B**). Similarly, a significant downregulation of VEGF-R1 by VES was observed in tumors treated with VES 17 days after tumor allograft in nude mice. These findings suggest that VES significantly reduced the number of VEGF-R1s present in melanoma tumors whether it was administered at the time of or after melanoma cell inoculation in nude mice ($P < .0001$). Significance was set at $P < .05$ for overall analysis of variance. Columns with different superscripted lowercase letters significantly differ and denote pairwise comparisons of VEGF-R1 in VES-treated melanoma tumor groups.

sections were stained with CD31 to identify microvessel density.

Representative photomicrographs of CD31 staining of the tumors showed that tumors treated with VES had significantly fewer microvessels compared with the control ($P < .0001$; Fig. 2A and 2B). The number of microvessels in tumors treated with VES from the time of melanoma cell inoculation in nude mice was significantly lower compared with the control. Similarly, a significant downregulation of microvessels by VES was observed in tumors treated with VES from day 17 after tumor inoculation. These findings suggest that VES significantly suppressed the expression of microvessels in melanoma tumors, whether administered early or late

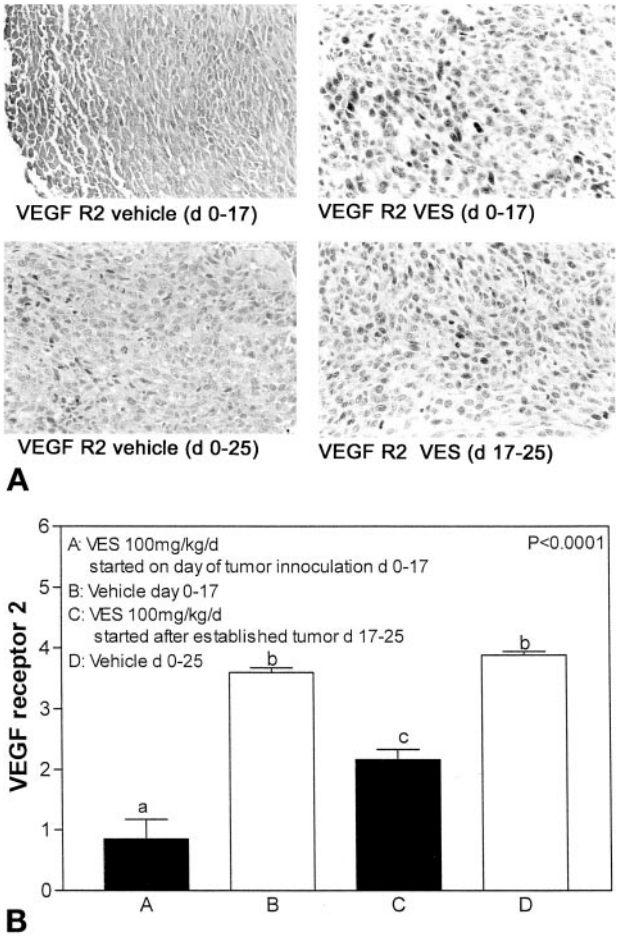

FIG. 6. The antiangiogenic effect of vitamin E succinate (VES) on vascular endothelial growth factor (VEGF) receptor 2 (VEGF-R2) expression in tumors. (**A**) Representative photomicrographs of VEGF-R1 from formalin-fixed, paraffin-embedded sections of murine melanoma tumors obtained from athymic nude mice treated with vehicle or VES. The number of VEGF-R2s in tumors treated with VES from the time of melanoma cell inoculation in nude mice was significantly lower compared with the control (**B**). Similarly, a significant downregulation of VEGF-R2 by VES was observed in tumors treated with VES 17 days after tumor allograft in nude mice. These findings suggest that VES significantly reduced the number of VEGF-R2s present in melanoma tumors whether it was administered at time of or after melanoma cell inoculation in nude mice ($P < .0001$). Significance was set at $P < .05$ for overall analysis of variance. Columns with different superscripted lowercase letters significantly differ and denote pairwise comparisons of VEGF-R2 in VES-treated melanoma tumor groups.

during melanoma growth. On the average, tumors from mice treated with VES had 87% fewer vessels than tumors from the control mice.

Furthermore, it is clear that the inhibition of angiogenesis was not an artifact of tumor size. Tumors of control mice killed on day 17 after tumor cell inoculation (521 ± 154 mm^3) were smaller than VES-treated tumors

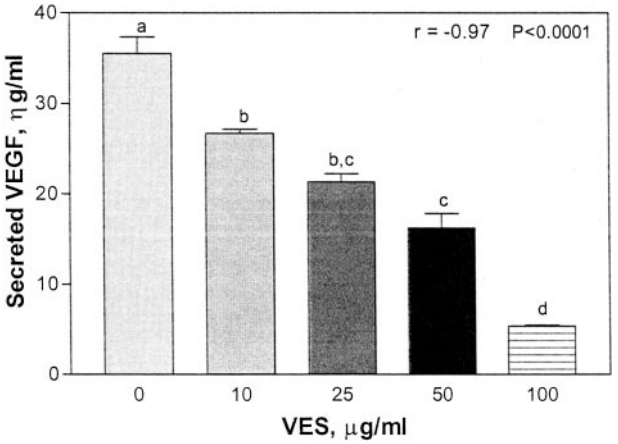

FIG. 7. Vitamin E succinate (VES) inhibition of and 24-hour dose-dependent effect of VES on secreted vascular endothelial growth factor (VEGF) protein from melanoma cells. The mechanism of VES VEGF inhibition was analyzed by measuring the dose effect of VES on secreted VEGF protein by enzyme-linked immunosorbent assay. VEGF protein decreased linearly as VES dose increased ($r = -.97$; $P < .0001$). [a,b,c,d]Columns with different superscripted lowercase letters differ significantly at $P < .05$. Significance was set at $P < .05$ for overall analysis of variance. [a,b,c]Columns with different superscripted lowercase letters significantly differ and denote pairwise comparisons of secreted VEGF protein in VES-treated melanoma cells.

from day 17 to day 25 after tumor cell inoculation (1734 ± 713 mm^3). Yet the number of microvessels in the larger VES-treated tumors was significantly less than in control ($P < .0001$; Fig. 3). On average, the smaller control tumors had twice the number of vessels than the larger VES-treated tumors.

VES Inhibits the Expression of Melanoma Tumor VEGF, VEGF-R1, and VEGF-R2

VEGF is a key cytokine in melanoma angiogenesis.[24,26–28] VEGF biological activities are mediated by tyrosine kinase receptors associated with endothelial cells. Three structurally related tyrosine kinases have been identified as putative VEGF-Rs (VEGF-R1, VEGF-R2, and VEGF-R3).[36–39] VEGF-R2 seems to be the major transducer of VEGF signals in endothelial cells.[20,40–42] Because VES significantly inhibited angiogenesis, we evaluated the effects of VES on VEGF, VEGF-R1, and VEGF-R2 by scoring the intensity of immunostaining of these molecules in VES-treated and untreated mice tumors. The levels of VEGF, VEGF-R1, and VEGF-R2 proteins in tumors treated with VES from the time of melanoma cell inoculation in nude mice were significantly lower compared with control. Similarly, a significant downregulation of VEGF, VEGF-R1, and VEGF-R2 proteins by VES was observed in tumors treated with VES 17 days after tumor allograft in nude mice. These findings suggest that VES significantly

suppressed the expression of VEGF, VEGF-R1, and VEGF-R2 proteins in melanoma tumors, whether administered early or late after melanoma cell inoculation in nude mice ($P < .0001$; Figs. 4–6).

VES Inhibits Melanoma VEGF Protein Secretion

The mechanism of VES inhibition of VEGF expression in melanoma cells was further substantiated by measuring the dose effect of VES on secreted VEGF protein by enzyme-linked immunosorbent assay. VES inhibited the expression of secreted VEGF protein of B16F10 melanoma cells in a dose-dependent manner. The amount of secreted VEGF decreased linearly with increasing VES dose ($P = .0077$; $r = -.9654$). After 24 hours of incubation, 10 μg/mL of VES was able to significantly decrease the level of secreted VEGF protein (Fig. 7).

VES Inhibits VEGF Promoter Activity in Melanoma Cells

Because VEGF expression in tumor cells is known to be regulated, at least in part, by transcription,[43] we measured the dose effect of VES on VEGF promoter activity by using transient transfection assays. The chemiluminescence assay detected the amount of secreted alkaline phosphatase as a measure of the effect of VES on VEGF promoter activity. After 24 hours of incubation, VEGF promoter activity decreased linearly as VES dose increased ($r = -.9731$, $P = .0053$; Fig. 8).

DISCUSSION

Cancer relapse after apparent successful initial treatment is a significant clinical problem, which is responsible for most of the mortality from cancer.[2,3] The pathogenesis of cancer relapse is beginning to be understood. It seems that activation of dormant micrometastasis to obvious clinical metastasis or macrometastasis is an important theme in this process. Recent work suggests that in melanoma, the mechanism of activation of dormant micrometastasis involves the triggering of angiogenesis and the shift to tumor cell proliferation relative to apoptosis.[9,44]

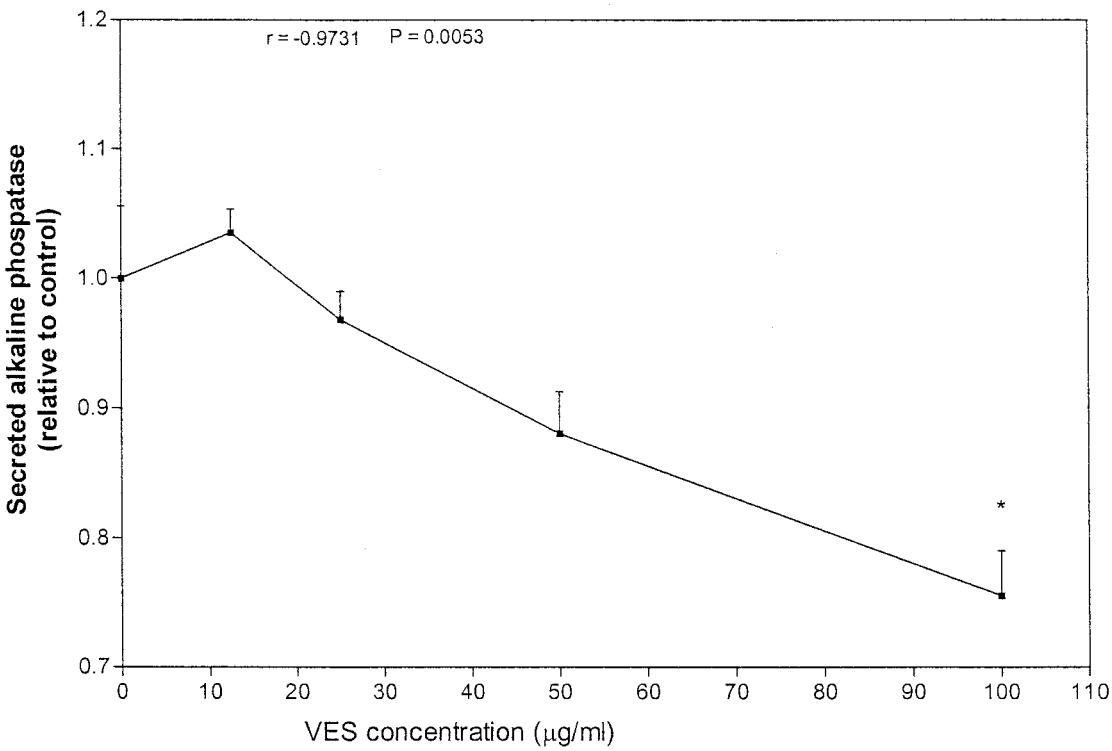

FIG. 8. Vitamin E succinate (VES) inhibits melanoma vascular endothelial growth factor (VEGF) promoter activity. The 24-hour dose-dependent effect of VES on VEGF promoter activity in melanoma cells is shown. The mechanism of VES VEGF inhibition was analyzed by measuring the dose effect of VES on VEGF promoter activity by using transient transfection assays. VEGF promoter activity decreased linearly as VES dose increased ($r = -.9731$; $P = .0053$). Significance was set at $P < .05$ for overall analysis of variance. Columns with different superscripted lowercase letters significantly differ and denote pairwise comparisons of VEGF promoter activity in VES-treated melanoma cells.

The definition of tumor dormancy varies among investigators. From a clinical standpoint, tumor dormancy represents undetectable tumor cells, which are quiescent for various periods of time. Biologically, the time period between microscopic tumor deposition in metastatic sites and its development into macroscopic metastasis is the period of dormancy. By logical extension, if a macroscopic tumor is inhibited from progressing in size, it has also been induced to be dormant. Therefore, inhibition of tumor growth is essentially promotion of tumor dormancy. In our model of tumor dormancy, we implanted the B16F10 melanoma cell line into the subcutaneous space and measured the time from implantation (microtumor) to the time of macroscopic tumor growth. We demonstrated that VES significantly inhibited tumor growth. It is interesting to note that the inhibition of tumor growth was much more profound at tumor inoculation compared with after the establishment of the log growth phase of the tumor. This implies that VES would be more effective at promoting dormant microtumors than at actively growing macrotumors. Clinical trials of VES in cancer should therefore be effective in the adjuvant setting rather than in the setting of clinical metastatic disease.

Our previous melanoma study showed that VES inhibited melanoma growth by induction of apoptosis.[33] In this study, we extend our understanding of the mechanism of VES antitumor effects by demonstrating significant inhibition of melanoma angiogenesis by VES. This is the second report of VES antiangiogenesis effect in cancer. Shklar and Schwartz[34] observed VES antiangiogenesis effects in a squamous cell cancer of the oral cavity model in hamsters.

The connection between angiogenesis and melanoma progression has become an attractive target for new melanoma therapies. Clinical trials with antiangiogenesis agents are under way. VES is an attractive chemoprevention agent because of its very low clinical toxicity.[45] We previously noted that VES inhibited VEGF expression in breast cancer.[35] In this study, we show that VES is also a potent inhibitor of VEGF expression in melanoma cells. It is of considerable interest that VES also inhibits VEGF-R1 and -R2 expression in melanoma. Our observations about VES inhibition of VEGF-R1 and -R2 are based on qualitative immunohistochemical techniques. This preliminary observation needs to be confirmed by other techniques. These intriguing data suggest that VES effectively inhibits VEGF action at both the tumor cell compartment and the endothelial compartment of the tumor. The mechanism of VEGF regulation in tumor cells is under intense study. Experimental evidence suggests that VEGF regulation could be at the level of transcription or RNA stabilization.[46] Although some cancer cells require stress, such as hypoxia, to induce VEGF expression, many cancer cells constitutively express VEGF. The B16F10 melanoma cell line constitutively expresses VEGF. The regulation of VEGF expression in the B16F10 melanoma cell line is unknown. In this study, we observed inhibition of VEGF protein secretion in association with inhibition of VEGF promoter activity. This suggests that VES inhibition of VEGF expression might involve inhibition of VEGF transcription. The contribution of messenger RNA transcription versus stability in VES regulation of VEGF expression is an intriguing question, which is the subject of ongoing experiments in our laboratory. Given the central role of VEGF in melanoma angiogenesis and progression, further studies into the molecular basis of VES inhibition of VEGF expression in melanoma cells will shed light on the biology of melanoma angiogenesis.

In summary, these studies demonstrate VES inhibition of VEGF expression in melanoma cells and indicate that the mechanism of inhibition is, at least in part, by the inhibition of VEGF transcription. It is of significant interest that VES also inhibits VEGF-Rs, suggesting a VES antiangiogenesis effect in the host compartment (endothelium) of the tumor. This clearly raises the possibility that VES may modulate other molecules in the tumor angiogenesis process. This study demonstrates that VES inhibits melanoma growth and angiogenesis. This inhibitory effect is associated with inhibition of VEGF and VEGF-R expression. These findings support future clinical studies of VES in the prevention of melanoma relapse.

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