PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **174**, 302–307 (1983)

Study on the Specificity of α-Tocopheryl (Vitamin E) Acid Succinate Effects on Melanoma, Glioma and Neuroblastoma Cells in Culture[1,2] (41741)

BHOLA N. RAMA AND KEDAR N. PRASAD

Center for Vitamins and Cancer Research, Department of Radiology, School of Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80262

Abstract. *d-* and *dl-α*-tocopheryl succinate inhibited growth and caused morphological changes in mouse melanoma (B-16), mouse neuroblastoma (NBP_2), and rat glioma (C-6) cells in culture. To study whether the effects of α-tocopheryl (vitamin E) succinate on tumor cells are mediated by antioxidant mechanisms, the effects of lipid-soluble antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were compared with those of vitamin E succinate. Results showed that these antioxidants produced alterations on the growth and morphology of neuroblastoma, melanoma, and glioma cells which are similar to those produced by vitamin E succinate; however, the extent of the effect depended upon the type of antioxidant and the form of tumor cells. These data suggest that the effects of vitamin E succinate on tumor cells may be mediated, in part, by antioxidant mechanisms.

Several animal experiments and human epidemiological studies suggest (1–6) that the high intake of tocopherol (vitamin E) reduces the risk of cancer. Recent *in vitro* studies show that tocopheryl esters (succinate and acetate) inhibit growth and cause morphological changes in mouse (7) and human neuroblastoma cells (8), mouse melanoma (9), and rat glioma cells (10) in culture; whereas, vitamin E succinate at a similar concentration produces no significant effect on mouse fibroblasts (L-cells). *d-α*-tocopheryl acid succinate is more effective than *dl-α*-tocopheryl acetate, *dl-α*-tocopherol-free alcohol, and *dl-α*-tocopheryl nicotinate on melanoma cells in culture (9). Vitamin E appears to induce differentiation without affecting the growth rate in mouse myeloid leukemia (11). In the present study we have compared the effects of *d-* and *dl-* forms of α-tocopheryl succinate on melanoma (B-16) and neuroblastoma (NBP_2)[3] cells in culture. One of the well-known effects of vitamin E is its antioxidant property (12–14). To study whether the effects of vitamin E on tumor cells in culture are mediated, in part, by antioxidant mechanisms, we have compared the effects of vitamin E succinate with those of lipid-soluble antioxidants, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT), and water-soluble antioxidant, vitamin C on tumor cells. In addition, the effects of trolox C, a lipid-soluble antioxidant, which contains only the tocopherol ring, are also compared with those of vitamin E succinate. We now report that *d-* and *dl-* forms of vitamin E succinate are equally effective on mouse melanoma (B-16) and mouse neuroblastoma (NBP_2) cells in culture. Vitamin E succinate also inhibits the growth of glioma cells in culture. The effects of vitamin E succinate on tumor cells are similar to those produced by other lipid-soluble antioxidants, but the latter require higher concentrations.

Materials and Methods. *Cell culture.* Mouse melanoma cells (B-16), mouse neuroblastoma cells (NBP_2), and rat glioma cells (C-6) were used for this study. Glioma cells of passages 28–39 were used, because cells of the older passages change their certain responses (15). NBP_2 clone has been developed and characterized in our laboratory (16). Glioma cells initially developed by Benda *et al.* (17) were obtained from Dr. Vernadakis, of University of Colorado Health Sciences Center, Denver,

[1] This work was supported by a grant from Hoffmann-La Roche, Inc. We thank Dr. Hemmy Bhagavan of Hoffmann-La Roche, Inc. and EM Laboratories, Inc., Elmsford, for supplying trolox C and *dl-α*-tocopheryl acid succinate; respectively.

[2] Part of this work was presented at the University of Chicago symposium on Etiology and Prevention of Cancer, October 2–4, 1982.

[3] Abbreviations used: neuroblastoma, NB; butylated hydroxyanisole, BHA; butylated hydroxytoluene, BHT.

0037-9727/83 $1.50

and B-16 melanoma cells were obtained from American Type Culture Collection. Melanoma and glioma cells were grown in F12 medium containing 5% fetal calf serum, whereas neuroblastoma cells were grown in F12 medium containing 10% agammaglobulin newborn calf serum. However, all experiments were carried out in F12 medium containing 5% fetal calf serum, because the different types of serum are known to modify the response of cells to various drugs (18). All media contained streptomycin (100 μg/ml) and penicillin (100 units/ml). All tumor cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. The doubling times of melanoma and glioma cells were 24 and 30 hr, respectively, whereas the doubling time of neuroblastoma cells was about 18 hr.

Forms of vitamins. *d*-α-tocopheryl acid succinate (Sigma Chemical Co., St. Louis, Mo.), and *dl*-α-tocopheryl acid succinate (EM Laboratories, Inc., Elmsford, N.Y.) were dissolved in ethanol. Succinic acid disodium was dissolved in water, because it was insoluble in ethanol. Other lipid-soluble antioxidants, butylated hydroxyanisole and butylated hydroxytoluene (Sigma Chemical Co., St. Louis, Mo.) and trolox C (Hoffmann–LaRoche, Inc., Nutley, N.J.) were dissolved in ethanol. Ascorbic acid was dissolved in water, and the pH was adjusted to 7 by the addition of sodium hydroxide. All solutions were stored at 4°C and protected from light. Fresh solutions were made every 2 weeks.

Assay of growth inhibition. The growth inhibition was assayed on the criteria of colony formation for melanoma cells, and the number of cells per dish for glioma and neuroblastoma cells. The colony formation criterion for growth inhibition could not be used for glioma and NB cells, because the plating efficiencies for glioma and NB cells were less than 10%.

To determine the growth inhibition on the basis of the number of cells per dish, cells (10^5 melanoma, 0.5×10^5 NB and glioma cells) were plated in Lux culture dishes (60 mm), and various agents were added separately to cultures 24 hr after plating. One set of control cultures received the same amount of solvent (ethanol 0.5%) alone or in combination with sodium succinate. Another set of control cultures was untreated. Drugs and growth medium were changed 2 days after treatment, and the number of cells per dish was determined 3 days (melanoma and NB cells) or 4 days (glioma cells) after treatment.

To determine the number of viable cells, the number of trypan blue (0.2% in 0.9% NaCl solution)-stained cells among attached cell population was determined. The stained cells were considered dead.

To determine the number of cells per dish, the melanoma and glioma cells were washed twice with phosphate-buffered saline (pH 7.0), and then incubated in the presence of 0.25% trypsin solution (in calcium-free modified Eagle's medium containing 1.0 m*M* EDTA) for 10–15 min. The NB cells were incubated in the presence of 0.25% pancreatin solution (in calcium-free modified Eagle's medium) for 8 min. A single cell suspension was prepared, and the number of cells was counted by a Coulter counter.

To determine colony formation, 100 cells were plated in Lux culture dishes (60 mm), and drugs were added separately to cultures 24 hr after plating. The control cultures were treated in the manner described in the previous paragraph. Twelve days after plating, the colonies were fixed in 5% buffered Formalin and stained with 0.5% cresyl violet. The colonies containing 50 cells or more were scored. The plating efficiency of melanoma cells was about 55%.

The average value of the cell number in untreated controls was considered 100%, and the growth inhibition in treated cultures was expressed as percentage of untreated controls.

Morphology. The changes in morphology of drug-treated melanoma cells were examined under light inverted microscope, and they were documented by photomicrographs.

Results. *Comparative effects of d- and dl-forms of vitamin E succinate.* Although the effects of vitamin E succinate on melanoma cells were studied (11), the relative potency of *d*- and *dl*- forms of vitamin E acid succinate was not known. Our results show that *d*- and *dl*- forms of vitamin E succinate were equally effective in inhibiting the growth of melanoma cells in culture (Table I). The above observation was found to be true when the growth inhibition was assayed either on the basis of colony formation or on the number of cells per dish (Table I). The morphological changes produced by *d*- (11) and *dl*- forms of vitamin

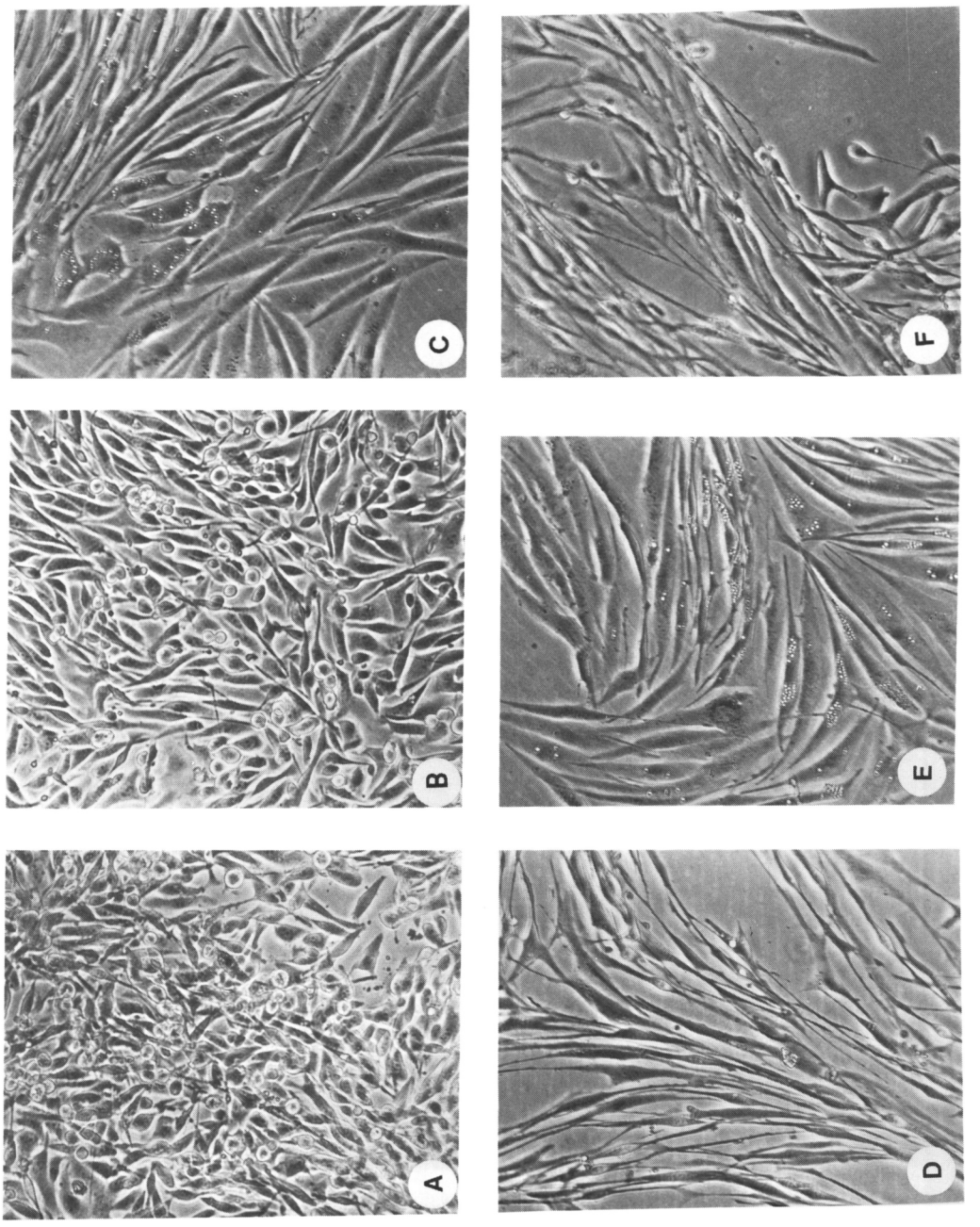
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TABLE I. EFFECT OF *d*- AND *dl*- FORMS OF VITAMIN E SUCCINATE AND OTHER LIPID-SOLUBLE ANTIOXIDANTS ON THE GROWTH INHIBITION OF MOUSE MELANOMA (B-16) CELLS IN CULTURE

Treatments	Growth inhibition (% of untreated controls)	
	Cell No.	Colony formation
Sodium succinate (5 μg/ml) + ethanol (0.5%)	109 ± 7[a]	96 ± 6[a]
dl-Vitamin E succinate (5 μg/ml)	59 ± 6	42 ± 4
d-Vitamin E succinate (5 μg/ml)	55 ± 5	36 ± 5
Butylated hydroxytoluene (5 μg/ml)	85 ± 5	66 ± 5
Butylated hydroxyanisole (5 μg/ml)	73 ± 4	76 ± 5
Trolox C (5 μg/ml)	83 ± 6	66 ± 6

Note. Melanoma cells (10^5 cells for counting cell number; 100 cells for colony formation) were plated in Lux culture dishes (60 mm). The growth inhibition was determined on the basis of number of cells per dish and number of colonies per dish. The values of untreated control cultures ($62 \pm 6 \times 10^4$; plating efficiency 55%) were considered to be 100%. The growth inhibition was determined as percentage of untreated controls. Each value represents an average of nine samples.

[a] Mean ± SD.

E succinate were also similar (Fig. 1C). The number of trypan blue-stained cells in control culture was less than 1%, whereas in vitamin E succinate-treated cells it was no more than 3%. At a high concentration (10 μg/ml), vitamin E succinate was lethal (data not shown).

The effect of vitamin E succinate was also studied on mouse neuroblastoma (NB) cells in culture. The untreated NB cells exhibited mostly round cell morphology and they grew in clumps. However, the NB cells treated with *d*- and *dl*- forms of vitamin E succinate became large and polygonal with short neurites, and did not form clumps. Both *d*- and *dl*- forms of vitamin E succinate were equally effective in inhibiting the growth of NB cells (Table II). The number of trypan blue-stained cells in control culture was less than 1%, whereas in vitamin E succinate-treated culture it was no more than 5%. At a higher concentration (8 μg/ml), vitamin E succinate was lethal.

Vitamin E succinate (5 μg/ml) also reduced the growth of glioma cells by about 34% of control, but at a higher concentration (10 μg/ml) it was lethal (data not shown). Glioma cells treated with vitamin E succinate became spindle-shaped with reduced cytoplasmic area in comparison to untreated cells which maintain fibroblastic appearance with bipolar cytoplasmic processes (data not shown). The number of trypan blue-stained cells in control culture was less than 1%, whereas in vitamin E succinate-treated culture, it was less than 2%.

Sodium succinate (5–8 μg/ml) with or without the same amount of ethanol (0.25–0.5%, final concentration) did not affect the growth or morphology of melanoma, NB or glioma cells in culture.

Effects of other lipid soluble antioxidants. To determine whether the effects of vitamin E acid succinate on the growth or the mor-

FIG. 1. Melanoma cells (10^5) were plated in Lux tissue culture dishes (60 mm), and drugs were added separately 24 hr after plating. Drugs and medium were changed at 2 and 3 days after treatment. Photomicrographs were taken 4 days after treatment. Control culture contains cells of fibroblastic morphology as well as round cells which grow in clumps (A). Cultures treated with ethanol (0.5%) alone or in combination with sodium succinate (6 μg/ml) also exhibited fibroblastic morphology with fewer round cells (B). *dl*-α-tocopheryl succinate-treated culture (6 μg/ml) showed a marked change in morphology (C). Cells treated with the same concentration (6 μg/ml) of butylated hydroxyanisole also showed morphological changes (D). However, cells treated with a high concentration of butylated hydroxyanisole (10 μg/ml) (E) appear similar to those found at lower concentration (6 μg/ml) of vitamin E succinate-treated culture (C). The morphological changes in cultures treated with a higher concentration (10 μg/ml) of trolox C were less pronounced (F) than those found in butylated hydroxyanisole treated cultures ×380 (final magnification for all).

TABLE II. EFFECT OF *d*- AND *dl*- FORMS OF VITAMIN E SUCCINATE AND OTHER LIPID-SOLUBLE ANTIOXIDANTS ON GROWTH INHIBITION OF NEUROBLASTOMA CELLS (NBP_2) IN CULTURE

Treatments	Growth inhibition (% of untreated controls)
Sodium succinate (5 μg/ml) plus ethanol (0.5%)	86 ± 6[a]
dl-Vitamin E succinate (5 μg/ml)	54 ± 7
d-Vitamin E succinate (5 μg/ml)	52 ± 6
Butylated hydroxytoluene (5 μg/ml)	37 ± 4
Butylated hydroxyanisole (5 μg/ml)	31 ± 4
Trolox C (5 μg/ml)	65 ± 5

Note. Cells (50,000) were plated in Lux culture dishes (60 mm), and the drugs were added separately 24 hr later. The drugs and medium were changed 2 days after treatment. The number of cells per dish was determined 3 days after treatment. Each experiment was repeated three times involving three samples per treatment. The average value of untreated controls ($162 \pm 13 \times 10^4$) was considered 100%, and the growth inhibition in treated culture was expressed as percentage of untreated controls. Each value represents an average of nine samples.

[a] Mean ± SD.

phology of tumor cells in culture were mediated by antioxidation; the effects of two well-known lipid-soluble antioxidants, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were compared with those of vitamin E succinate. The effects of trolox, a lipid soluble antioxidant which contains only tocopherol ring, were also studied.

The inhibition of growth after treatment of melanoma cells with BHT, BHA, and trolox C was less than that produced by vitamin E acid succinate (Table I). The morphological changes produced by a concentration of 6 μg/ml of BHT (not shown) and trolox (Fig. 1E) were less pronounced than those produced by BHA (Fig. 1D). The BHA-treated melanoma cells became very elongated and arranged in parallel in comparison to untreated (Fig. 1A) and ethanol-treated (Fig. 1B) controls. When melanoma cells were treated with a higher concentration (10 μg/ml) of BHA, (Fig. 1D) the morphological changes resemble those observed at 6 μg/ml of *dl*-α-tocopheryl succinate (Fig. 1C). The number of trypan blue-stained cells in antioxidant treated cultures was less than 4%.

The treatment of mouse neuroblastoma cells with BHT, BHA, and trolox C caused morphological changes and growth inhibition similar to those produced by vitamin E succinate. BHT and BHA were equally effective. The extent of growth inhibition produced by these antioxidants was slightly more than that produced by a similar concentration of vitamin E succinate. The number of trypan blue-stained cells in BHA- and BHT-treated NB and glioma cells was not more than 2–4%. Trolox C was least effective. Vitamin C at a concentration of 100 μg/ml did not affect growth rate or morphology of melanoma or neuroblastoma cells in culture (data not shown).

Discussion. Our previous study (9) showed that *d*-α-tocopheryl acid succinate was more potent than *dl*-α-tocopheryl acetate, *dl*-α-tocopherol-free alcohol and *dl*-α-tocopheryl nicotinate in causing growth inhibition and morphological changes in mouse melanoma cells in culture. Our present results show that *d*- and *dl*- forms of α-tocopheryl succinate are equally effective in causing above changes in mouse melanoma and mouse neuroblastoma cells in culture.

Our results show that BHT and BHA produce growth inhibition and morphological changes in mouse tumor cells similar to those produced by vitamin E succinate; however, they require higher concentrations. This suggests that the effects of vitamin E succinate on tumor cells are mediated, in part, by antioxidant mechanisms.

dl-α-tocopherol-free alcohol is being used in Phase I trial (after patients have become unresponsive to all known therapeutic modalities) for the treatment of metastatic human neuroblastomas, primitive neuroectodermal tumor, and retinoblastoma (19). Vitamin E was given intravenously over 3 to 6 hr twice weekly, in dosages of 450 to 2300 mg/m^2 or daily 24-hr infusions for 9 days. Some antitumor- and analgesic-effects were observed. If one considers that the biology of tumor cells in Phase I patients is very complex as a result of extensive therapy, and that these cells have become unresponsive to all therapeutic agents, even a partial response by the infusion of vitamin E alone can be considered encouraging. The major untoward side effect of high doses of vitamin E was an increased bleeding ten-

dency which was effectively counteracted with vitamin K infusions. It should be pointed out that *dl*-α-tocopherol-free alcohol which is being used in the treatment of human tumor is much less potent than *dl*-α-tocopheryl succinate (9); however, the latter cannot be used in the treatment until the pharmacology and toxicology of this form of vitamin E have been defined.

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Received March 31, 1983. P.S.E.B.M. 1983, Vol. 174.