Effects of Tocopherol (Vitamin E) Acid Succinate on Morphological Alterations and Growth Inhibition in Melanoma Cells in Culture

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INTRODUCTION

The role of vitamins in the prevention and management of neoplasms is becoming increasingly evident. The beneficial effects of vitamin C in the prevention (3) and management (3, 17) of neoplasms have been extensively reviewed. Retinoid, an analog of vitamin A, also exhibits anticancer properties (9, 10, 14, 22) and is being used clinically for a variety of neoplasms (13). We have reported earlier (18, 20) that Aquosol dL-α-tocopherol acetate induces morphological differentiation in a certain clone (NB P2) of mouse neuroblastoma cells and enhances the growth inhibitory effect of tumor-therapeutic agents on neuroblastoma and rat glioma (C-6) cells in culture. The extent of modification of the effect of tumor-therapeutic agents by vitamin E depends upon the cell type and the particular agent. We report here for the first time that d-α-tocopherol (vitamin E) acid succinate induces morphological changes and growth inhibition in mouse melanoma (B-16) cells in culture.

MATERIALS AND METHODS

Cell Culture. Mouse melanoma cells (B-16) and mouse fibroblasts (L-cells) were used for this study. Cells were grown in F-12 medium containing 5% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 units/ml) and were maintained at 37°C in a humidified atmosphere of 5% CO₂. The doubling time of both melanoma cells and L-cells in culture under the above growth conditions was 24 hr.

Forms of Vitamin E. Four different forms of vitamin E were used in this study. d-α-Tocopherol acid succinate and dL-α-tocopherol nicotinate (Sigma Chemical Co., St. Louis, Mo.) were dissolved in ethanol. Succinic acid disodium salt (Sigma) was dissolved in water because it was insoluble in ethanol. DL-α-Tocopherol free alcohol (Hoffmann-La Roche Inc., Nutley, N. J.) was dissolved in a specialized solvent (1 ml solvent contains 0.1 ml ethyl alcohol, 0.1 ml propylene glycol, 0.1 ml Emulphor EL-820, 0.01 ml benzyl alcohol, 0.3 mg sodium acetate trihydrate, 2.5 mg glacial acetic acid, 9.0 mg sodium chloride, 0.1 mg sodium edetate, and water; the pH was adjusted to 4 with sodium hydroxide), and the solution was further diluted with ethanol. Aquasol vitamin E (DL-α-tocopherol acetate; USV Laboratories, Tuckahoe, N. Y.) was also dissolved in a specialized solvent (the contents of this solvent could not be obtained because of trade secrets), and the solution was further diluted with water. The solvents for both dL-α-tocopherol free alcohol and dL-α-tocopherol acetate were obtained separately from Hoffmann-La Roche and USV Laboratories, respectively. All solutions were stored at 4°C and protected from light.

Assay of Growth Inhibition. The effects of various forms of vitamin E on growth inhibition were determined by finding the protein content per dish; however, the effect of vitamin E acid succinate on growth inhibition was determined by using 2 additional techniques, namely, the number of viable cells per dish and colony formation. To determine the growth inhibition on the basis of protein content per dish or the number of viable cells per dish, cells (10⁵) were plated in Lux tissue culture dishes (60 mm), and d-α-tocopherol acid succinate (1 to 10 μg/ml), dL-α-tocopherol acetate (1 to 100 μg/ml) were added separately to cultures 24 hr after plating. Control cultures received an equivalent volume of solvent. Another set of control cultures was untreated. Whenever the effect of d-α-tocopherol acetate succinate was studied, sodium succinate (1 to 10 μg/ml) in combination with an equivalent volume of ethanol was added to separate cultures as an additional control. Drugs and growth medium were changed 2 days after treatment, and the growth inhibition was determined the following day.

To determine the protein, cells were washed twice with phosphate-buffered saline (sodium chloride, 8 g; potassium chloride, 2 g; sodium phosphate, 1.15 g; and potassium phosphate, 2 g/liter; pH 7.0), and 1 ml of cold 5% trichloroacetic acid was added. After storage at 4°C for 4 to 24 hr, an equivalent volume of 1 m NaOH was added to neutralize the contents. Cells were removed from the dishes by use of a rubber policeman and homogenized. The amount of protein was determined using the method of Lowry et al. (11).

To determine the number of viable cells, the number of trypan blue...
(0.2% in 0.9% NaCl solution) stained cells among attached cell populations was determined, and then all cells were removed from the dish surface. For counting purposes, the cells were washed twice with phosphate-buffered saline and then incubated in the presence of trypsin solution (0.25% in calcium-free modified Eagle’s medium containing 1 mM EDTA) for 10 min. A single-cell suspension was prepared, and the number of cells was counted by a Coulter Counter. The stained cells were considered dead and were subtracted from the total in order to obtain the number of viable cells per dish.

To determine colony formation, 100 cells were plated in Lux tissue culture dishes (60 mm), and vitamin E acid succinate at various concentrations was added 24 hr later. The control cultures received the same amount of sodium succinate and an equivalent volume of ethanol. Another set of control cultures was untreated. Twelve days after plating, the colonies were fixed in 5% formalin and stained in 0.5% cresyl violet. The colonies containing 50 cells or more were scored. The plating efficiency of melanoma cells was 62 ± 4% (S.D.).

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

Morphology. The changes in morphology in cultured melanoma cells were documented by photomicrographs.

Effect of o-a-Tocopherol Acid Succinate on Melanoma Cells Cultured in SFM. Serum contains complex factors, many of which are unknown. The amounts of known factors vary from one batch of serum to another (27). Therefore, it is possible that serum factors may modify the effect of vitamin E. Hence, the effect of vitamin E acid succinate on melanoma cells cultured in SFM (2) was studied. Cells (10^6) were plated in serum-supplemented medium. Twenty-four hr after plating, cells were washed twice with F-12 medium without serum, and then SFM was added. After 15 min of incubation at 37°, vitamin E acid succinate at various concentrations was added. Drug and medium were changed at 2 days after treatment, and the morphology and growth inhibition (cell number/dish) were determined 3 days after treatment according to the method described previously.

Assay of Melanin Content. The content of melanin was assayed according to the procedure described by Mayskens (13). Cells (0.25 × 10^6 cells for control; 0.5 × 10^6 cells for vitamin E acid succinate treatment) were plated in Lux tissue culture dishes (100 mm) containing 20 ml of growth medium. Twenty-four hr after plating, vitamin E acid succinate (6 μg/ml) or sodium succinate (6 μg/ml) with an equivalent volume of ethanol was added. Drug and medium were changed after 3 days of treatment. Cells were removed from the dish according to the procedure described in the previous section. Cells were washed twice with phosphate-buffered saline (pH 7.0) and then dissolved in 1.0 ml of 1 N NaOH and 10% dimethyl sulfoxide for 30 min. The absorbance was determined at 470 nm. The average absorbance value of untreated controls was 0.048 ± 0.008/million cells. The absorbance was linear up to 6 x 10^6 cells/ml of reaction mixture. The average absorbance value of untreated controls was considered 100%, and the melanin content in treated cultures was expressed as percentage of untreated controls.

Assay of cAMP Level. Cells (10^6) were plated in Lux tissue culture dishes (60 mm). After 24 hr of plating, vitamin E acid succinate (6 μg/ml) was added. The medium and vitamin E were changed after 2 days of treatment. The control cultures received sodium succinate (6 μg/ml) and an equivalent volume of ethanol (final concentration 0.6%). Another set of cultures was untreated. After 3 days of treatment, the growth medium was changed, and cells were incubated for 30 min before processing for the determination of the level of cAMP. In another set of similarly plated cultures, cells were incubated in the presence of vitamin E acid succinate for a period of 15 min, and then the level of cAMP was determined. To measure the level of cAMP, the cells were washed twice with phosphate-buffered saline (pH 7.0), and 2 ml of 5% trichloroacetic acid were added. The contents were removed by use of a rubber policeman, and the level of cAMP was determined by the method of Gilman (5).

RESULTS

Effect on Morphology. Control melanoma cells in culture exhibited primarily a fibroblastic appearance during exponential growth; however, many clumps of round cells were observed at confluency (Fig. 1A). The treatment of melanoma cultures with o-a-tocopherol acid succinate (soluble in ethanol) caused dramatic changes in morphology which were dose-dependent, and this effect became visible as early as 24 hr after beginning treatment. At a concentration of 5 μg/ml, cells appeared large and elongated and were arranged in parallel at several locations (Fig. 1C). At a higher concentration of vitamin E acid succinate (6 μg/ml), cells were much larger (Fig. 1D) than those found at the lower concentration (5 μg/ml). Control cultures treated with similar amounts of sodium succinate and an equivalent amount of ethanol (Fig. 1B) or similar amounts of other forms of vitamin E such as DL-a-tocopherol nicotinate (soluble in ethanol), DL-a-tocopherol free alcohol (soluble in a specialized solvent provided by Hoffmann-La Roche), Aquasol DL-a-tocopherol acetate (soluble in a specialized solvent supplied by USV Laboratories), or their respective solvents did not cause any significant change in morphology (data not shown). In contrast to melanoma cells, mouse fibroblasts, at vitamin E acid succinate concentrations ranging from 2 to 6 μg/ml, did not exhibit any significant morphological alterations. At a high concentration (10 μg/ml) of vitamin E acid succinate, most of the fibroblastic cells became round and granulated. This was due to the toxicity of vitamin E acid succinate.

When vitamin E acid succinate was removed after 4 days of treatment, the morphological change did not reverse during the period of observation (2 days after removal). Vitamin E-resistant cells (approximately less than 10%), which have the appearance of untreated cells, were present in treated cultures. Partially affected cells may also exist in vitamin E acid succinate-treated cultures.

Effect on Growth. The effect of vitamin E acid succinate was measured by 3 different methods: number of viable cells per dish; protein content per dish; and colony formation.

Vitamin E acid succinate inhibited the growth (measured by the reduction of protein content per dish) of melanoma cells in culture in a dose-dependent fashion (Chart 1). When vitamin E acid succinate was removed after 4 days of treatment, the growth did not begin for a period of 24 hr; however, a slight increase in growth was observed at 2 days after removal of vitamin E (Chart 2). The concentration of vitamin E acid succinate needed to inhibit growth by 50% was similar on the basis of the 3 different techniques of assaying growth inhibition mentioned above (Table 1). Sodium succinate with an equivalent volume of ethanol, DL-a-tocopherol nicotinate, DL-a-tocopherol free alcohol, and Aquasol DL-a-tocopherol acetate each at a concentration of 10 μg/ml, or an equivalent volume of their respective solvents, did not significantly affect the growth of melanoma cells in culture (Chart 1). The concentrations of vitamin E acid succinate needed to inhibit the growth...
of melanoma cells and fibroblasts by 50% were 6 and 10 μg/ml, respectively (Table 1).

The solvents of dl-α-tocopherol free alcohol and Aquasol dl-α-tocopherol acetate at higher concentrations (50 and 100 μg/ml) by themselves markedly inhibited the growth of melanoma cells, and the presence of the vitamin E in the solvents reduced the solvent-induced growth inhibition (Chart 1).

**Effect of Vitamin E Acid Succinate in SFM.** When melanoma cells were grown in SFM for a period of 4 days, the average number of cells per dish represented 70 to 80% of that found in serum-supplemented medium. Vitamin E acid succinate produced growth inhibition and caused morphological changes in melanoma cells cultured in SFM in a manner similar to that found in serum-supplemented medium; however, the concentration requirement was about 5 times less (data not shown). The concentration of vitamin E acid succinate needed to inhibit the growth by 50% in SFM was 1.2 μg/ml (Table 1), which is about 5-fold lower than that obtained in serum-supplemented medium (5.5 μg/ml).

**Effect on Melanin Content.** The melanin content in untreated cultures increased in some round cells which were located in areas of high cell density (data not shown). Vitamin E acid succinate-treated melanoma cells contained about 2-fold more melanin than did those found in untreated culture (Table 2); however, a similar increase in melanin content was found in cultures treated with sodium succinate and an equivalent volume of ethanol. Further studies revealed that ethanol alone was sufficient to increase the melanin content in melanoma cells in culture (Table 2).

**Effect on cAMP Level.** The level of cAMP did not change in vitamin E-treated cultures after either 15 min or 3 days of treatment. The level of cAMP in untreated melanoma cultures was 13 ± 1.3 pmol/mg protein.

**DISCUSSION**

Vitamin E acid succinate induced dramatic changes in morphology and markedly inhibited the growth of melanoma cells in culture. This effect of vitamin E acid succinate was due to its vitamin E property because sodium succinate at a similar concentration, with an equivalent volume of ethanol, neither inhibited the growth nor induced morphological changes in melanoma cells, in culture. When melanoma cells were grown in SFM, vitamin E acid succinate produced changes in morphology and growth similar to those produced in serum-supplemented medium; however, the concentration requirement was about 5 times less. The exact mechanisms of the higher degree of sensitivity of melanoma cells to vitamin E acid succinate in SFM are unknown. However, it is possible that serum factors may in some way make vitamin E less available to melanoma cells, and/or selenite, which is a necessary component of SFM, may interact with vitamin E in a way that results in enhanced growth inhibition. Melanoma cells were more sensitive to vitamin E acid succinate than to vitamin E nicotinate, vitamin E free alcohol, or Aquasol vitamin E acetate. The exact reasons for this are unknown; however, the following possibilities can be suggested: (a) vitamin E acid succinate may be relatively more stable in solution; (b) it may easily cross the cell membrane; and (c) it may undergo slow degradation within the cell. The relative effectiveness of dl and dl forms of vitamin E acid succinate remains to be evaluated. Melanoma cells were more sensitive to vitamin E acid succinate than were fibroblasts. In addition, vitamin E acid succinate at similar
Vitamin E acid succinate (6 μg/ml) 250 ± 53 (d)
Sodium succinate alone (6 μg/ml) 98 ± 6 (c)
Ethanol alone (0.6%) 195 ± 40 (b)

The mechanisms of solvent-induced toxicity or the growth inhibition was markedly reduced in the presence of vitamin E. The culture eventually became confluent. This may account for the occurrence of vitamin E acid succinate-resistant cells as well as only partially affected melanoma cells.

The growth inhibition was determined 3 days after treatment. The growth inhibition was also determined by the method of colony formation. Treatment untreated controls was considered 100%. The melanin content in both dividing and nondividing melanoma cells in culture. The present study also suggests that the expression of this differentiated phenotype is not necessarily linked with growth rate or malignancy.

Since cAMP-stimulating agents inhibit growth and cause morphological changes in melanoma cells in culture (6–8), the question arises as to whether the effect of vitamin E acid succinate on melanoma cells is mediated by cAMP. The present study shows that the effect of vitamin E on melanoma cells is not mediated by an increase in the level of cAMP.

The exact mechanism of vitamin E acid succinate on melanoma cells is unknown. We have reported (19) that vitamin E is bound to proteins (M.W. 20,000) which are located in the cytosol, pellet, and nuclear fractions of melanoma cells. It is speculated that these proteins may modulate the effect of vitamin E on mammalian cells. In addition, vitamin E-binding proteins (M.W. 20,000) are also present in human serum (19). These proteins may act as carrier proteins for vitamin E. Other functions of vitamin E may include stimulation of the host’s immune system (24); stabilization of the cell membrane, probably by participating in enzyme-lipid interactions (4, 15); antioxidative functions (23); and inhibition of the synthesis of prostaglandins (12).

If the present and previous (18, 20) results can be applied to in vivo conditions, then vitamin E acid succinate may be a useful anticancer agent for melanoma cells and possibly for other neoplasms. Indeed, a clinical study (1) has shown that the administration of vitamin E produces a beneficial effect in patients with chronic cystic mastitis, the most common benign lesion of the female breast. These studies suggest that vitamin E could induce multiple effects during the management of tumors such as cell death (1, 20), differentiation (20), inhibition of cell division (20), potentiation of the effect of tumor-therapeutic agents (18), reduction of the toxic effects of certain chemotherapeutic agents (16, 21, 25, 26), and stimulation of the host’s immune system (24). Since the above effects of vitamin E are dependent upon the type of tumor and the type of therapeutic agent, the use of vitamin E in the management of tumors must be based on a biological rationale for a maximum benefit.
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REFERENCES

Melanoma cells ($10^6$) were plated in Lux tissue culture dishes (60 mm), and d-tocopherol (vitamin E) acid succinate (soluble in ethanol) and sodium succinate plus ethanol were added to separate cultures 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 fibroblastic cells as well as round cells in clumps (A). Cultures treated with ethanol (1%) and sodium acid succinate (5 to 6 μg/ml) also exhibited fibroblastic morphology with fewer round cells (B). Vitamin E acid succinate-treated cultures [5 μg/ml (C); 6 μg/ml (D)] showed a dramatic change in morphology. × 450.
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