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dl-α-Tocopheryl Succinate Enhances the Effect of γ-Irradiation on Neuroblastoma Cells in Culture[1,2] (41772)

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Abstract. The effect of *dl*-α-tocopheryl (vitamin E) succinate in modifying the radiation response of mouse neuroblastoma (NBP_2) and mouse fibroblast (L-cells) cells in culture was studied on the criterion of growth inhibition (due to cell death and inhibition of cell division). Results show that vitamin E succinate markedly enhanced the effect of ^{60}CO-γ-irradiation on NB cells, but it did not significantly modify the effect of irradiation on mouse fibroblasts. Sodium succinate plus ethanol (0.25% final concentration) did not modify the radiation response of NB cells or fibroblasts. Butylated hydroxyanisole, a lipid soluble antioxidant, also enhanced the effect of irradiation on NB cells, indicating that the effect of vitamin E in modifying the radiation response may be mediated, in part, by antioxidation mechanisms.

Some studies have shown that α-tocopherol (vitamin E) protects normal tissue *in vitro* and *in vivo* (1–8) against radiation damage, whereas others have reported that vitamin E is ineffective in protecting normal or tumor tissue (9–12). A few studies have shown that vitamin E enhances the effect of irradiation on tumor cells *in vivo* (13, 14) and *in vitro* (15). The exact reasons for these discrepancies are unknown; however, the difference in the form of vitamin E, radiation doses, route of administration, type of cells, and time of administration may, in part, be responsible for the above difference. Recently, it was noted (16) that the specialized solvent of *dl*-α-tocopheryl acetate at higher concentrations (dilution of 1000-fold) was toxic to tumor cells in culture. Therefore, vitamin E acetate-induced enhancement of radiation effect on neuroblastoma cells may have been in part due to the effect of solvent. Hence, the role of vitamin E in modifying the radiation response of tumor cells requires reevaluation. In addition, it is not known whether vitamin E would modify the effect of irradiation on fibroblasts in culture.

In a recent study (16) it was found that *d*-α-tocopheryl acid succinate (readily soluble in ethanol) was more potent than *dl*-α-tocopheryl acetate, *dl*-α-tocopherol free alcohol, and *dl*-α-tocopheryl nicotinate in causing the growth inhibition and the morphological alterations on tumor cells in culture. D- and *dl*-forms of vitamin E succinate were equally effective on neuroblastoma cells in culture (unpublished observation). Therefore, vitamin E succinate appears to be the most suitable form of vitamin E in evaluating its role in modifying the radiation response of tumor cells in culture. We report here that *dl*-α-tocopheryl succinate markedly enhances the growth inhibitory effect of γ-irradiation on mouse neuroblastoma (NBP_2) cells in culture, but it does not produce such an effect on mouse fibroblasts (L-cells) in culture.

Materials and Methods. *Cell culture.* Mouse neuroblastoma cells and mouse fibroblasts were used for this study. The NBP_2 clone has been developed and characterized in our laboratory (17). The L-cells were purchased from American Tissue Culture Collections. Cells were grown in F12 medium containing 5% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 U/ml), and were maintained at 37°C in a humidified atmosphere of 5% CO_2. The doubling times of both NBP_2 and L-cells in culture, under the above conditions, were 18 and 30 hr, respectively.

Forms of vitamins. *dl*-α-Tocopheryl succinate (EM Laboratories, Inc., Elmsford, N.Y.)

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was dissolved in ethanol. Succinic acid disodium was dissolved in water, because it was insoluble in ethanol. Butylated hydroxyanisole (Sigma Chemical Co., St. Louis, Mo.) was dissolved in ethanol. All solutions were stored at 4°C and protected from light. Fresh solutions were made every week.

^{60}Co-γ-irradiation. Cells were γ-irradiated at room temperature at a dose rate of 23 rad/min. Vitamin E succinate was added immediately before irradiation and was present during the entire period of observation. Vitamin E succinate and fresh growth medium were changed 2 days after irradiation and the number of cells per dish was determined 3 days after irradiation. One set of the irradiated control cultures received the same amount of solvent (0.25% ethanol) and sodium succinate; whereas another set of irradiated control cultures were left untreated. Sham-irradiated control cultures were treated similarly.

Viability of cells. The viability of cells was determined 3 days after treatment. To determine the number of viable cells, the number of trypan blue-stained (0.2% in 0.9% NaCl solution) cells among attached cell population was determined, and then cells were removed from the dish surface for the purpose of counting. The stained cells were considered dead. In addition, many of the morphologically differentiated cells do not pick up trypan blue, but they do not divide. Therefore, such differentiated cells on the criterion of reproductive death, can be considered dead. The colony formation criterion for viability could not be used because of poor plating efficiency (<10%).

Growth inhibition. The growth inhibition (due to cell death and inhibition of cell division) was determined on the basis of number of cells per dish, 3 days after treatment. To determine the number of cells per dish, the cells were incubated in the presence of 0.25% pancreatin solution for 8–10 min. A single-cell suspension was prepared, and the number of cells was counted by a Coulter Counter. 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.

Results. γ-Irradiation and vitamin E succinate inhibited the growth (due to cell death and inhibition of cell division) of mouse NB[3] cells in culture (Fig. 1). The radiation dose, which inhibited the growth of NB cells by about 50% of controls, was 400 rad; and the concentration of vitamin E succinate which inhibited the growth of NB[3] cells by about 50% of controls, was about 5 μg/ml. The number of trypan blue-stained cells in irradiated (400 rad) NB cell culture was <1% of attached cell population, whereas, in vitamin E succinate treated (5 μg/ml) culture it was also <1% of attached cell population. The solvent of vitamin E succinate (ethanol, final concentration 0.25%) and sodium succinate (5 μg/ml) did not affect the growth or the morphology of NB cells.

When vitamin E succinate (5 μg/ml) was added before or immediately after irradiation (400 rad), the growth was reduced to about 18% of controls (Fig. 1). The number of trypan blue-stained cells in culture treated with 400 rad plus vitamin E succinate was about 2%. The same amount of ethanol (0.25% final concentration) plus sodium succinate (5 μg/ml) did not modify the radiation response of NB cells in culture (Table I). At higher radiation doses (500–800 rad), the effect of vitamin E succinate was less pronounced (Fig. 1). This was due to the fact that γ-irradiation by itself at higher doses markedly reduced the growth of NB cells in culture.

γ-irradiation and vitamin E succinate also reduced the growth of L-cells in culture in a dose-dependent manner (data not shown). The fibroblasts were less sensitive to γ-irradiation and vitamin E succinate than NB cells in culture. A concentration (5 μg/ml) of vitamin E succinate which inhibited the growth of NB cells in culture, had no significant effect on the growth of L-cells. Similarly, a dose of 400 rad which inhibits the growth of NB cells by about 50% of controls, reduced the growth of fibroblasts by about 35% of controls. When vitamin E succinate (5 μg/ml) was added before γ-irradiation (500 rad), the growth was inhibited to about 59 ± 4.9% of untreated controls which was similar to that observed after treatment of cells with irradiation alone

[3] Abbreviations used: Neuroblastoma, NB; butylated hydroxyanisole (BHA).

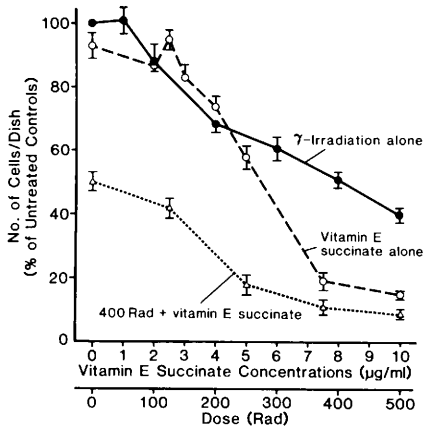

FIG. 1. Neuroblastoma cells (NBP_2) were plated in Lux culture dishes (60 mm), and the cells were γ-irradiated 24 hr after plating. Vitamin E succinate or the solvent (ethanol 0.25% and sodium succinate 5 μg/ml) was added immediately before irradiation. The drugs and medium were changed 2 days after treatment. The number of cells per dish was determined 3 days after treatment. Each experiment was repeated at least twice involving three samples per treatment. The average value ($172 \pm 7 \times 10^4$) of untreated control NB cells was considered 100%, and the growth in treated cultures was expressed as percentage of untreated controls. The bar at each point is the standard error of the mean.

(61 ± 2.7% of untreated controls). The number of trypan blue-stained cells in cultures treated with γ-radiation alone or in combination with vitamin E succinate was less than 1% of attached cell population. The ethanol (0.25%) and sodium succinate (5 μg/ml) also did not modify the radiation response of mouse fibroblasts in culture.

To establish whether the effect of vitamin E succinate in modifying the radiation response of NB cells in culture is mediated by antioxidation mechanisms, butylated hydroxyanisole (BHA), a well-known lipid-soluble antioxidant, was used in combination with γ-irradiation. The concentration (2 μg/ml) of BHA, which inhibited the growth of mouse NB cells in culture by about 50% of controls, also enhanced the radiation response of neuroblastoma cells in culture (Table I).

Discussion. The present study shows that vitamin E succinate enhances the growth inhibitory effect of γ-irradiation on mouse NB cells in culture; however, it does not enhance the effect of irradiation on mouse fibroblasts in culture. The fact that ethanol (solvent of vitamin E succinate) plus sodium succinate do not modify the radiation response of NB cells in culture suggests that the effect of vitamin E succinate on irradiated NB cells in culture is due to the effect of vitamin E. Although a previous study shows that *dl*-α-tocopheryl acetate (Aquasol, vitamin E) enhances the effect of radiation on mouse NB cells and rat glioma cells in culture, it was not conclusive, due to the fact that the solvent of

TABLE I. MODIFICATION OF THE RADIATION RESPONSE OF MOUSE NEUROBLASTOMA (NBP_2) BY *dl*-α-TOCOPHERYL SUCCINATE

Treatments	No. of cells/dish (% of untreated controls)	Trypan blue-stained cells (% of attached cells)
400 rad	51 ± 2.7[a]	<1
Vitamin E succinate (5 μg/ml)	58 ± 3.5	<1
400 rad plus vitamin E succinate	18 ± 3.0	<2
No radiation, sodium succinate (5 μg/ml) and ethanol (0.25%, final concentration)	94 ± 6	<1
400 rad plus sodium succinate and ethanol	59 ± 2.1	<1
Butylated hydroxyanisole (2 μg/ml)	56 ± 2.5	<1
400 rad plus butylated hydroxyanisole	31 ± 1.8	<1

Note. Neuroblastoma cells (50,000 cells) were plated in Lux culture dishes (60 mm), and the cells were irradiated 24 hr after plating. Vitamin E succinate or the solvent was added immediately before irradiation. 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 at least twice involving three samples per treatment. The average value ($172 \pm 7 \times 10^4$) of untreated control NB cells was considered 100%, and the growth in treated cultures was expressed as percentage of untreated controls.

[a] Standard error of the mean.

Aquasol vitamin E acetate was found to be toxic (16). The latter study raised the question whether Aquasol vitamin E acetate-induced enhancement of radiation effects on tumor cells might have been, at least in part, due to the effect of solvent. The present study conclusively shows that vitamin E markedly enhances the effect of irradiation on NB cells in culture.

The exact mechanisms of the effect of vitamin E in modifying the radiation response of tumor cells are unknown. One of the well-established mechanisms of vitamin E is its antioxidant mechanisms (18–20). In order to ascertain whether vitamin E-induced enhancement of radiation effects is due to its antioxidant mechanisms, the effect of butylated hydroxyanisole (BHA), a lipid-soluble antioxidant, was studied. Results show that BHA also enhances the effect of irradiation, but to a lesser degree than that produced by vitamin E succinate. This suggests that the effect of vitamin E in modifying the radiation response of tumor cells may be mediated, in part, by antioxidant mechanisms. The fact that vitamin C, a water-soluble antioxidant, also increases the radiation response of neuroblastoma cells in culture (21), supports the above hypothesis. However, this hypothesis may be applicable only to certain tumor cells. On the other hand, the commonly accepted view that an antioxidant should protect tissue against radiation damage, may be applicable to certain normal tissues. The fact that the administration of vitamin E before irradiation protects normal tissues (1–8), supports this view. However, some studies have failed to observe any radioprotective value of vitamin E on normal tissues (9–12). The exact reasons for this difference in results are unknown, but the difference in dose, route of administration, form of vitamin E, and tissue accumulation of vitamin E may account for this variation in radiation response. The reasons for the differential effect of vitamin E in modifying the radiation response of tumor cells and normal cells are also unknown; however, the following speculations can be made: The presence of an excess amount of antioxidant inside the cells may be lethal. Normal cells may have stringent homeostatic control for the uptake of vitamin E; therefore, they do not pick up vitamin E more than the cellular requirement. Ionizing radiation may change the membranes of normal cells, but it may not impair the uptake mechanism of vitamin E in a way which enhances the transport of vitamin E. If this is the case, vitamin E would protect the normal tissue by reducing the effect of radiation-induced free radicals. On the other hand, the tumor cells may have lost the homeostatic control for the uptake of vitamin E which allows the entry of an excess amount of vitamin E inside the cells. Ionizing radiation further impairs this control and, thereby, increased amounts of vitamin E enter the cells. This may account, in part, for the vitamin E-induced enhancement effect of irradiation on tumor cells.

The present results cannot be readily extrapolated to *in vivo* conditions; however, the mouse neuroblastoma cells in culture have proven to be of good predictive value for human NB tumors *in vivo*. For example, the effects of cyclic nucleotides on mouse neuroblastoma cells in culture (22, 23) are similar to those found in human neuroblastoma tumors *in vivo* (24–26).

The initial observation that vitamin E inhibits the growth of mouse neuroblastoma cells in culture (16–18, 27) has led to the phase I trial of *dl*-α-tocopherol free alcohol in the treatment of metastatic neuroblastomas, primitive neuroectodermal tumors, and retinoblastomas (28). 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 during phase I trial is very complex as a result of extensive therapy, and that these cells have become unresponsive to all therapeutic agents, even a partial response in some patients (5/13) 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 tendency, 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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