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# Vitamin E Induces Morphological Differentiation and Increases the Effect of Ionizing Radiation on Neuroblastoma Cells in Culture (40598)[1]

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Vitamin E possesses antioxidant properties and therefore has been presumed to participate only in antioxidative activities of cells *in vivo.* However, the cellular and molecular mechanisms of vitamin E are not understood. Sodium ascorbate (vitamin C), which also exhibits antioxidant properties, shows antitumor activity *in vivo* (1) and *in vitro* (2, 3). In addition, sodium ascorbate potentiates the effect of ionizing radiation and certain chemotherapeutic agents on neuroblastoma (NB) cells in culture. This property of sodium L-ascorbate is shared by sodium D-ascorbate, but is not shared by another antioxidant, glutathione (2). We, therefore, wondered if vitamin E would exhibit antitumor activity *in vitro.* We now report that vitamin E causes dramatic increase in the formation of neurites in NB cells, but it does not induce the formation of cytoplasmic processes in glioma cells. Neuroblastoma cells are more sensitive to vitamin E than glioma cells for the criterion of growth inhibition. Vitamin E increases the effect of ionizing radiation NB and glioma cells.

*Materials and Methods.* Mouse neuroblastoma clone $NBP_2$, which contains both tyrosine hydroxylase and choline acetyltransferase activities, was used in this study (4). Rat glioma clone (C-6) (5) of passages 30–42 (6) was used in this investigation. Neuroblastoma cells were grown in F12-medium containing 10% agammaglobulin newborn calf serum, whereas glioma cells were grown in F12 medium containing 10% fetal calf serum. Both types of media contained antibiotics (penicillin 100 U/ml and streptomycin 100 $\mu$g/ml). These cells were maintained at 37° in a humidified atmosphere of 5% $CO_2$.

Aquasol vitamin E (DL-alphatochopherylacetate water solubilized with polysorbate 80; 50 IU/ml) was diluted with water. All solutions were protected from light and stored at 4°. To study the effect on morphological changes, cells (10,000) were plated in Lux tissue culture dishes (60 mm), and vitamin E (0.01–0.1 IU/ml) was added 24 hr later. No medium or drug was changed during the period of the experiment. The number of morphologically differentiated cells was determined 3 days after treatment. Cells with cytoplasmic processes greater than 50 $\mu$m in length were considered morphologically differentiated. The number of morphologically differentiated cells was also determined 1, 2, and 3 days after the addition of vitamin E (0.06 IU/ml). To study the effect of vitamin E on growth rate, cells ($5 \times 10^4$ NB cells; $10^5$ glioma) were plated in Lux tissue culture dishes (60 mm), and vitamin E at various concentrations (0.01–0.1 IU/ml) was added 24 hr later. Cell number was counted 3 days after treatment.

To investigate whether vitamin E enhances the effect of ionizing radiation, cells (50,000 NB; 100,000 glioma) were plated in Lux tissue culture dishes (60 mm), and irradiated at room temperature with $^{60}Co$-$\gamma$-irradiation at the dose rate of 41 rad/min 24 hr after plating. Vitamin E (0.05 IU/ml) was added immediately after irradiation. The drug and medium were changed 2 and 3 days after irradiation, and the cell number was counted 4 days after irradiation.

Cell viability was determined by counting the number of cells stained with trypan blue (0.2% in saline) among attached cell population before removing for counting. For counting purposes NB cells were removed from the dish by incubating them in the presence of pancreatin solution (0.25% in Ca-free MEM) for 10 min; whereas glioma cells were washed twice with phosphate-buffered saline and then incubated in the presence of trypsin solution (0.25% in Ca-free MEM) for 40 min.

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Cells were counted by a Coulter counter. The stained cells were considered dead and subtracted from the total in order to obtain the number of viable cells/dish.

To determine the level of cyclic AMP, cells ($10^5$, NB; $2 \times 10^5$, glioma) were plated in Lux tissue culture dishes (60 mm) and vitamin E (0.06 IU/ml for NB, 0.1 IU/ml for glioma) was added 24 hr later. Media and drug were changed 2 days after treatment, and the cyclic AMP level was determined 5 min and 3 days after treatment according to Gilman's method (7). Protein was determined according to the method of Lowry, *et al.* (8). Data were expressed as pmoles of cyclic AMP/mg proteins.

*Results and discussion.* Treatment of NB cells with vitamin E caused dramatic increase in morphological differentiation as evidenced by the formation of long neurites and increases in the size of soma and nucleus. A significant increase in morphological differentiation of NB cells was observed at a concentration of 0.05 IU/ml (Fig. 1). The concentration range which caused a maximal increase in the formation of neurites was very narrow (0.06–0.08 IU/ml). The cell death was marked after treatment of the cultures with higher concentrations (0.07–0.08 IU/ml) of vitamin E (Fig. 1). If the formed neurites retract after the removal of stimulus, the effect is referred to as reversible; if they maintain their phenotype after the removal of stimulus, the effect is referred to as irreversible. The reversibility of neurites was dependent upon cell density at the time of treatment and on concentrations of vitamin E. Treatment of cultures of lower cell density ($10^4$/dish) with vitamin E caused irreversible morphological differentiation 3 days after treatment. This is shown by the fact that the neurites did not retract 1 day after the removal of vitamin E. When the cultures were examined 3 days after removal of vitamin E, colonies of highly differentiated, partially differentiated, and mixture of differentiated and undifferentiated NB cells were found. There were relatively more colonies of differentiated cells in NB cultures treated with higher concentrations of vitamin E (0.07–0.08 IU/ml). Many of the differentiated NB cells died during a 6-day period. These data show that cells resistant to vitamin E exist in the NB cell culture even after treatment with high concentrations of vitamin E. We do not know the reasons for the existence of vitamin E-resistant cells in NB culture. The resistance of cells to vitamin E does not depend upon the cell cycle, since the resistant cells were observed in cultures which were continuously treated with vitamin E for at least three generation times. Cells are resistant to vitamin E probably due to heritable mutation. Since the vitamin E-resistant cells (undifferentiated and partly differentiated) were not subcultured, we do not know if the acquired differentiated features are heritable for a prolonged period of time.

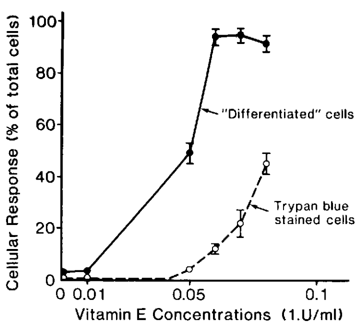


FIG. 1. Neuroblastoma cells ($10^4$) were plated in Lux tissue culture dishes (60 mm), and vitamin E was added 24 h later. Drug and medium were changed at 2 days after treatment, and the number of morphologically differentiated cells was counted 3 days after treatment. The number of trypan blue-stained cells among attached cell population was also determined. Each value represents an average of at least six samples. The bar at each point is standard deviation. The bars not shown in figure were equal to sizes of symbols.

The cultures of higher cell density ($5 \times 10^4$/dish) required higher concentrations (0.07–0.08 IU/ml) of vitamin E for a similar effect on morphological differentiation (Table I). The changing of medium and vitamin E (0.06 IU/ml) daily produced more differentiated cells in cultures plated at high cell density ($5 \times 10^4$/dish) than those in which medium and drug were changed only once during a 3-day period (Table I). A significant increase in morphological differentiation was observed 1 day after treatment; however, a maximal increase was found 3 days after treatment (Fig. 2).

Vitamin E failed to cause morphological differentiation in another neuroblastoma clone, $NBA_{2(1)}$ (contains only tyrosine hy-

TABLE I. EFFECT OF CELL DENSITY ON MORPHOLOGICAL DIFFERENTIATION OF NEUROBLASTOMA CELLS IN CULTURE[a]

| Cell density (No. × $10^4$/ dish) at plating | Vitamin E (IU/ ml) | Morphological differentiation (% of total cells) |
|---|---|---|
| 1–10 | Control | 3 ± 0.5[b] |
| 1 | 0.06 | 85 ± 4 |
| 5 | 0.06 | 70 ± 3 |
| 10 | 0.06 | 23 ± 2 |
| 5 | 0.06 (changed | 92 ± 2 |
| 10 | daily) | 84 ± 3 |
| | 0.08 | |

[a] Cells ($NBP_2$) were plated in Lux tissue culture dishes (60 mm). Vitamin E was added 24 hr after plating. Medium and drug change 2 days after treatment except where indicated otherwise, and the morphological differentiation was determined 3 days after treatment. Each sample represents an average of at least six samples.
[b] Standard deviation.

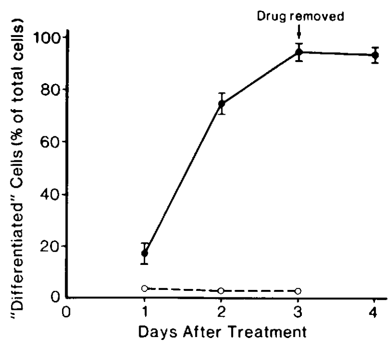


FIG. 2. Neuroblastoma cells ($10^4$) were plated in Lux tissue culture dishes (60 mm), and vitamin (0.06 IU/ml) was added 24 hr later. No drug or medium was changed. The number of morphologically differentiated cells was determined 3 days after treatment. To examine the reversibility of drug effect, vitamin E was removed, fresh growth medium was added, and the number of morphologically differentiated was determined 1 day later. Each value represents an average of six to eight samples. The bar at each point is standard deviation. The bars not shown in figure were equal to sizes of symbols.

droxylase but no choline acetyltransferase) (4), even though it increased the size of soma and nucleus and caused growth inhibition similar to those observed in $NBP_2$ clone (data not shown).

Glioma cells did not form cytoplasmic processes after treatment with vitamin E (0.05–0.1 IU/ml). This was also true when glioma cells were grown and treated with vitamin E in the same growth medium in which NB cells were grown. At a high concentration of vitamin E (0.1 IU/ml), glioma cells became flattened and large, and cytoplasms contained numerous dark granules. These changes were mostly reversible at 24 hr after the removal of drug.

Neuroblastoma cells were more sensitive to vitamin E than glioma cells for the criterion of growth inhibition (Fig. 3). This was also true when both cell types were grown in F12 medium containing 10% agammaglobulin newborn calf serum. We found that glioma cells grown in NB medium exhibited greater sensitivity to vitamin E in comparison to those grown in glioma medium. The growth inhibition in NB cells is due to inhibition of cell division as well as cell death, whereas in glioma cells it is primarily due to inhibition of cell division. The number of floaters or trypan blue stained cells in vitamin E-treated glioma cell culture was 7%.

Treatment of NB cells with vitamin E (0.06 IU/ml) for a period of 5 min or 3 days did not change the intracellular level of cyclic AMP in comparison to control (12 ± 1.6 pmole/mg proteins); however, glioma cells revealed an increase of about three to four fold in the cellular cyclic AMP (40 ± 13 pmole/mg proteins) in comparison to control (11 ± 3.5 pmole/mg proteins) 3 days after treatment with vitamin E (0.1 U/ml). Since

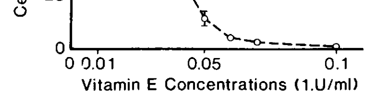


FIG. 3. Neuroblastoma cells (5 × $10^4$) and glioma cells ($10^5$) were plated in Lux tissue culture dishes (60 mm), and vitamin E was added 24 hr later. Drug and medium were changed 2 days after treatment. The cell number and the number of trypan blue-stained cells were determined 3 days after treatment. The number of stained cells was subtracted from total number of cells to obtain viable cells per dish. The average value of control cultures was considered 100%. Each value represents an average of at least six samples. The bar at each point is standard deviation. The bars not shown in figure were equal to sizes of symbols.

no change in the cellular cyclic AMP was observed at 5 min after treatment, the rise in cyclic AMP level in vitamin E-treated glioma cells may be due to an indirect effect of vitamin E, possibly either by stimulating membrane-bound adenylate cyclase or by inhibiting cyclic AMP phosphodiesterase activity.

The combination of vitamin E and ionizing radiation produced an additive effect on both glioma cells and NB cells for the criterion of growth inhibition (Table II). The trypan blue-stained cells in irradiated or irradiated plus vitamin E was about 5–10%. X irradiation has been known to cause morphological differentiation in NB cell culture (9). The combination of vitamin E and ionizing radiation produced an additive effect on morphological differentiation of NB cells (Table II). Neither vitamin E nor γ-irradiation produced morphological changes in glioma cells.

The mechanisms of the effect of vitamin E on mammalian tumor cells are unknown. However, the induction of morphological differentiation in NB cells by vitamin E may not be due to its antioxidant property, because other antioxidants such as sodium ascorbate and glutathione do not produce morphological changes in NB cells (2). It is also not due to a change in the intracellular level of cyclic AMP. Thus vitamin E at certain concentration ranges exhibits antitumor activity *in vitro* by inducing differentiation (certain clone of NB), and by inhibiting growth. The significance of this finding *in vivo* remains to be ascertained.

TABLE II. EFFECT OF VITAMIN E IN COMBINATION WITH IONIZING RADIATION ON NEUROBLASTOMA CELLS IN CULTURE[a]

| Cell type | Treatments | Cell No. (% of control) | Morphological differentiation (% of total cells) |
|---|---|---|---|
| Neuroblastoma | Control | 100 | 3 ± 5[b] |
| Neuroblastoma | 400 rads | 26 ± 3 | 20 ± 2 |
| Neuroblastoma | Vit. E 0.05 IU/ml | 32 ± 4 | 43 ± 3 |
| Neuroblastoma | Vit. E + 400 rads | 7 ± 1 | 72 ± 3 |
| Glioma | Control | 100 | No change |
| Glioma | 400 rads | 37 ± 3 | No change |
| Glioma | Vit. E 0.05 IU/ml | 80 ± 3 | No change |
| Glioma | Vit. E + 400 rads | 29 ± 4 | No change |

[a] Cells ($5 \times 10^4$ NB; $10^5$ glioma) were plated in Lux tissue culture dishes (60 mm). Drug and irradiation were given 24 hr after plating. Fresh medium and drug were added 2 and 3 days after treatment, and the cell number and morphological differentiation were determined 4 days after treatment. Each value represents an average of six samples.

[b] Standard deviation.

*Summary.* Vitamin E increased the expression of morphological differentiation in mouse neuroblastoma ($NBP_2$) cells in culture, but it did not produce a similar effect in glioma cell culture (C-6). The extent of vitamin E-induced changes on NB cells was dependent upon cell density, drug concentration, and the frequency of change of growth medium and drug. Neuroblastoma cells were more sensitive to vitamin E than glioma cells for the criterion of growth inhibition (due to cell death and reduction in cell division). The combination of vitamin E and X-irradiation produced an additive effect on both NB and glioma cells for the criterion of growth inhibition; however, it produced an additive effect for the criterion of morphological differentiation only on NB cells.

1. Cameron, E., and Pauling, L., Proc. Nat. Acad. Sci. USA **73**, 3685 (1976).
2. Prasad, K. N., Sinha, P. K., Ramanujam, M., and Sakamoto, A., Proc. Nat. Acad. Sci. USA, **76**, 829, (1979).
3. Prasad, K. N., and Sinha, P. K., *in* "Cell Differentiation and Neoplasia" (G. F. Saunders, ed.), p. 111. Raven Press, New York (1978).
4. Prasad, K. N., Mandal, B., Waymire, J. C., Lees, G. J., Vernadakis, A., and Weiner, N., Nature New Biol. **241**, 117 (1973).
5. Benda, P., Lightbody, J., Sato, G., Levine, L., and Sweet, W., Science **161**, 370 (1968).
6. Devellis, J., Inglish, D., and Galey, F., *in* "Cellular Aspects of Growth and Differentiation in Nervous Tissue" (D. Pease, ed.), p. 23. Univ. of California Press, Los Angeles (1970).
7. Gilman, A., Proc. Nat. Acad. Sci. USA **67**, 305 (1970).
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).
9. Prasad, K. N., *Nature* (London) **234**, 471–473 (1971).

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