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Combined Effects of Vitamin E (Alpha-tocopherol) and Cisplatin on the Growth of Murine Neuroblastoma *In Vivo*

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Abstract—*Combined effects of vitamin E (alpha-tocopherol) and cisplatin on the growth of two murine neuroblastomas (C1300, NS-20) was investigated* in vivo. *Five groups of mice were prepared; group 1 were fed the control diet, group 2 were fed a vitamin E-deficient diet, group 3 were fed a vitamin E-supplemented diet, group 4 were fed the control diet and plus vitamin E solution given intraperitoneally during the treatment (solvent i.p. group), and group 5 were given vitamin E in the same manner (20 mg/kg/day; vitamin E i.p. group). Cisplatin (6 mg/kg) was injected intraperitoneally into the mice of each group during the treatment.*

In case of the C1300 neuroblastoma, the antitumor activity of cisplatin was most enhanced in the mice receiving vitamin E i.p., and the intra-tumor vitamin E and platinum levels were significantly higher in this group than in the other groups ($P < 0.01$, and $P < 0.05$ respectively).

In contrast, in animals transplanted with the NS-20 murine neuroblastoma, which proved to be a cisplatin-tolerant tumor in separate experiments, no combined effect of those drugs was observed, although the intra-tumor level of platinum was elevated.

The possibility was that vitamin E increases the influx of cisplatin into the tumor cells and acts after incorporation of cisplatin through the plasma membrane. Vitamin E did not accentuate the cisplatin-induced renal impairment in vitamin E-loaded groups. Those results suggested that vitamin E should be considered as a co-agent of cisplatin for the treatment of neuroblastoma.

INTRODUCTION

Cis-DIAMMINE-DICHLOROPLATINUM (cisplatin, CDDP) is one of the most effective anti-tumor agents against advanced neuroblastoma in children. However, because of the nephrotoxicity and ototoxicity, the long term clinical administration is limited [1, 2]. Jordan *et al.* [3] reported that one of the mechanisms of cisplatin-induced renal damage might be induced by oxygen free-radical reaction.

Vitamin E is a potent antioxidant which scavenges oxygen free radicals. On the other hand, recent reports by Prasad and Rama [4] showed that vitamin E induced both morphological differentiation and growth inhibition of murine and human neuroblastoma cells in culture, at least, in part, by an antioxidant mechanism. They also observed the additive or synergistic effect of vitamin E and several anti-tumor agents on the growth inhibition of murine neuroblastoma cells *in vitro* [5].

We examined the combined effect of vitamin E (alpha-tocopherol) and cisplatin given to neuroblastoma-bearing mice in order to observe whether the administration of vitamin E might enhance tumor growth inhibition and protect against renal damage induced by cisplatin.

MATERIALS AND METHODS

Chemicals

Cisplatin (Briplatin, 10 mg/20 ml) was obtained from Bristol Myers Co. Ltd., Japan. Vitamin E (*dl*-α-tocopherol) and its solvent (HCO60; polyoxyethlene hydrogenated castor oil derivatives 60 mole ether, and propylene glycol) as a control were obtained from Eisai Co. Ltd., Tokyo, Japan. The control, vitamin E-deficient, and vitamin E-supplemented diets were also kindly provided by Eisai Co. Ltd., Tokyo, Japan. The composition of the vitamin E deficient basal diet (in w/w) for animals was as follows (Masugi and Nakamura [6]): corn

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starch 38.0%, α-malt starch 10.0%, granulated sugar 5.0%, vitamin-free casein 25.0%, purified lard 6.0%, mineral mixture 6.0%, powdered filter paper 8.0%, and vitamin mixture 2%, containing vitamin A acetate 1000 IU, vitamin D_3 200 IU, thiamine-HCl 1.6 mg, cyanocobalamin 1.0 μg, L-ascorbic acid 60.0 mg, vitamin K_3 10.4 mg, D-biotin 0.04 mg, folic acid 0.4 mg, Ca-pantothenate 10.0 mg, *p*-aminobenzoic acid 10.0 mg, niacin 12.0 mg, inositol 12.0 mg, and choline 4000 mg/100 g diet. For the control diet, 2 mg of vitamin E per 100 g diet was added to the vitamin E-deficient basal diet. The vitamin E-supplemented diet contained 40 mg of vitamin E per 100 g diet.

Animals and diets

Weanling female A/Jax mice weighing 15–20 g, purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan, were divided randomly into three groups: (a) fed the control diet; (b) those fed the vitamin E-deficient diet, and (c) fed the vitamin E-supplemented diet. These mice were housed five to six animals per cage and were fed the diets for over 8 weeks. Food and water were available *ad libitum*.

Treatment schedule

For treatment with vitamin E, the mice were divided into five groups: group 1 were fed the control diet (control), group 2 were given the vitamin E-deficient diet [vitamin E(−) p.o.], group 3 were given the vitamin E-supplemented diet [vitamin E(+) p.o.), group 4 were given control diet and given only the solvent of vitamin E solution intraperitoneally (solvent i.p.), and group 5 vitamin E in the same manner (vitamin E i.p.). Vitamin E (20 mg/kg/day) or its solvent (same volume of vitamin E) was injected intraperitoneally for 9 days during the treatment.

Cisplatin (6 mg/kg) was injected intraperitoneally three times daily for 4 days during the vitamin treatment (Figs. 2, 3).

Tumor growth

A C1300 neuroblastoma cell line, which had been maintained in A/Jax mice, was established in our laboratory. The NS-20 cholinergic clonal cells of neuroblastoma were kindly provided by Dr. T. Amano (Mitsubishi Kasei Life Science Institute, Machida, Tokyo, Japan).

The C1300 or NS-20 murine neuroblastoma cells (5×10^5 cells) were transplanted into the left thigh of all mice in each group. When the tumor had grown to about 1 cm in diameter, the treatment was initiated. The tumor volume was measured every 3 days. Three dimensions were measured and the volume was calculated using the following formula:

$$V = \pi/6 \times \text{length} \times \text{width} \times \text{height}.$$

All the mice were killed 8 days after termination of the treatment and various normal tissues and the tumor were taken for the assay of vitamin E and platinum content, and for histological examination. The histological specimens were fixed in formalin, and embedded in paraffin, and stained with hematoxylin–eosin.

In the case of NS-20 neuroblastoma, all the mice were killed 4 days after termination of the treatment (cisplatin was injected twice, and vitamin E [or solvent] was given for 5 days during the treatment), since this clone of cells grows rapidly, compared with the C1300 neuroblastoma cells.

Assay of vitamin E and platinum

Blood (1 ml) was taken by cutting the axillary artery and the plasma vitamin E (alpha-tocopherol) concentration was assayed. Tumor, liver, and kidney were also excised for microscopic examination and for the assay of vitamin E content. The concentration of vitamin E in the plasma and tissues were assayed by the fluorometric method of Abe and Katui [7].

Platinum concentration in the plasma and tissues were measured by the method of Leroy *et al.* [8], using flameless atomic absorption spectrophotometry.

Statistics

Student's *t* test was used to determine the statistical significance of the results.

RESULTS

Vitamin E concentration in various tissues

The vitamin E (alpha tocopherol) concentrations of blood, tumor, kidney, and liver of the control mice bearing C1300 neuroblastoma were 1.66 ± 0.18 μg/ml (mean ± S.D., $n = 4$), 5.42 ± 1.29 μg/g ($n = 4$), 10.59 ± 2.41 μg/g ($n = 4$), and 45.48 ± 4.89 μg/g ($n = 4$), respectively (Fig. 1). The administration of cisplatin had no significant effect on the concentration of these tissues. Vitamin E concentration in the tissues in the vitamin E(−) p.o. group showed low values, while values in the vitamin E p.o. and vitamin E i.p. groups were markedly elevated (Fig. 1).

Platinum concentration of the C1300 neuroblastoma

The tumour platinum concentration on the 8th day after the administration of 6 mg/kg × 3 cisplatin was measured in each group on the vitamin E treatment. The concentration of platinum in the tumor in the group given only cisplatin was

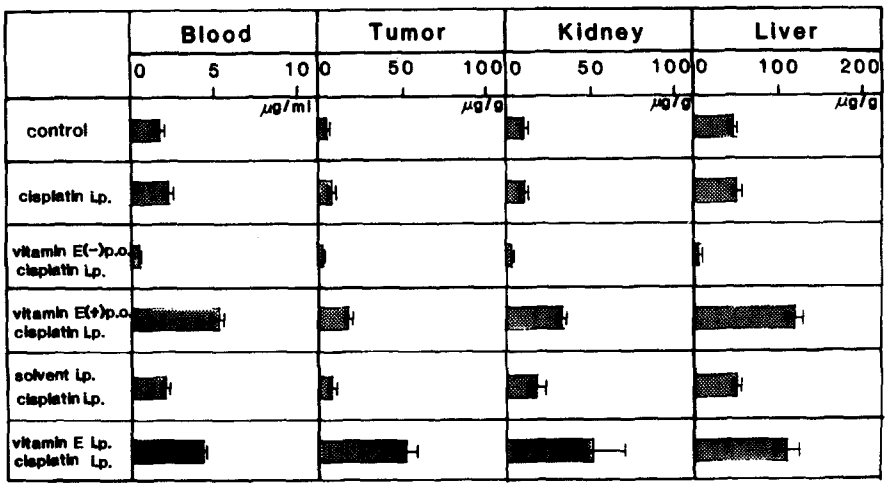

Fig. 1. Vitamin E (dl-α-*tocopherol*) *concentration of blood, tumor* (C1300), *kidney, and liver after the treatment of cisplatin and vitamin E. Each value represents an average of four samples. The bar at each point shows standard deviation of the mean. The concentrations of vitamin E of those tissues in the vitamin E(+) p.o. + cisplatin i.p. and vitamin E i.p. + cisplatin i.p. groups was significantly higher than those of other groups* ($P < 0.01$).

Table 1. Platinum accumulation in tumor and kidney of mice bearing C1300 neuroblastoma

Group	*n*	Platinum content (μg/g, dry wt) Tumor	Kidney
CDDP	4	0.79 ± 0.48*	20.34 ± 6.35
Vitamin E(−) p.o. + CDDP	4	0.80 ± 0.41	ND
Vitamin E(+) p.o. + CDDP	4	6.35 ± 3.63	ND
Solvent i.p. + CDDP	4	1.26 ± 0.51	18.48 ± 5.21
Vitamin E i.p. + CDDP	3	9.54 ± 3.70*	22.10 ± 3.93

*$P < 0.05$.

0.79 ± 0.48 μg/g dry weight (mean ± S.D.). In the group given vitamin E i.p., the platinum content was 10 times greater than that of the control. A similar increase was seen in the group of vitamin E(+) p.o. (Table 1).

Combined effect of vitamin E and cisplatin on C1300 neuroblastoma growth and the kidney

The treatment of mice with vitamin E alone had no significant effect on the growth inhibition of neuroblastoma 16 days after the initiation of vitamin E treatment (Fig. 2). When the mice were given cisplatin (6 mg/kg i.p. × 3), there was a marked inhibition of tumor growth (Fig. 3), especially in the groups receiving vitamin E(+) p.o. and vitamin E i.p. In the latter group, compared with those of solvent i.p. + cisplatin i.p., vitamin E(−) p.o.+ cisplatin i.p., and cisplatin i.p. groups, the tumor volume was significantly small (mean ± S.D.; 0.02 ± 0.04, $P < 0.05$) at least 16 days after the initiation of the therapy (Table 2).

Histologically, tumor cells of the vitamin E i.p.+ cisplatin i.p. group degenerated to a greater extent than in the tumors grown in other groups (Fig. 4).

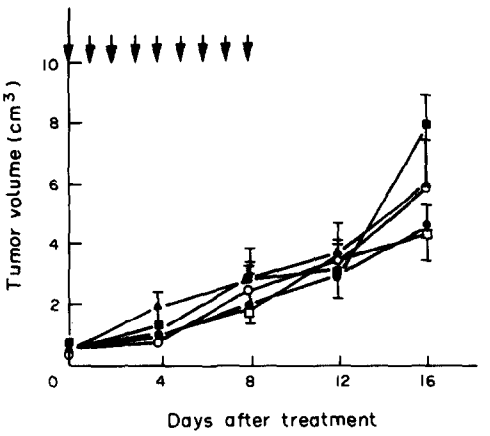

Fig. 2. The effect of vitamin E on the tumor growth of C1300 murine neuroblastoma. ●; *Control,* □; *vitamin E i.p.* (20 *mg/kg*), ○; *solvent i.p.,* ■; *vitamin E(+) p.o.,* ▲; *vitamin E(−) p.o.,* ↓ = *single injection of vitamin E. Each value represents an average of four to six mice. The bar at each point shows standard deviation of the mean. There were no significant differences in the tumor growth of each group* 16 *days after the initiation of the treatment.*

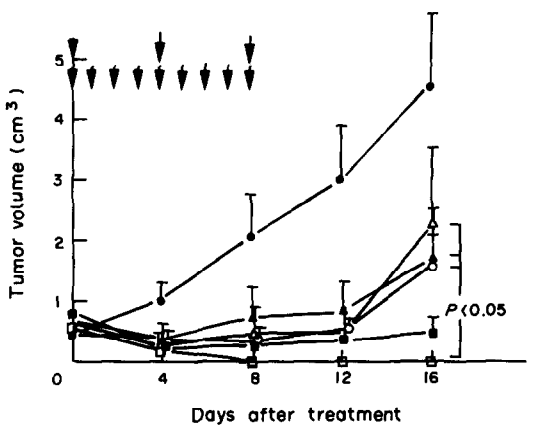

Fig. 3. The combined effect of vitamin E and cisplatin on the tumor growth of C1300 murine neuroblastoma. ●; Control, △; cisplatin i.p., □; vitamin E i.p. (20 mg/kg) + cisplatin i.p. (6 mg/kg), ○; solvent i.p. + cisplatin i.p., ■; vitamin E(+) p.o. + cisplatin i.p., ▲; vitamin E(−) p.o. + cisplatin i.p., ↓ = single injection of vitamin E and cisplatin respectively. Each value represents an average of four to six mice. The bar at each point shows standard deviation of the mean. The tumor growth of vitamin E i.p. + cisplatin i.p. group was most inhibited 16 days after the initiation of the treatment.

However, renal damage in the mice loaded with vitamin E was similar to that in the controls, cisplatin alone and vitamin E(−) p.o. groups; only slight glomerular change and tubular dilatation. The platinum content was not elevated in the kidney, even in the group given vitamin E i.p. (Table 1).

Effect of vitamin E and cisplatin on NS-20 neuroblastoma growth

The effect of vitamin E and cisplatin on the growth of NS-20 murine neuroblastoma cells, which is a cholinergic clone and found to be cisplatin-tolerant *in vivo* (Okuzono *et al.* personal communication), was also examined in this study. With this cell line, the tumor growth was not significantly inhibited by the combined treatment with vitamin E and cisplatin (Fig. 5), although the concentration vitamin E and cisplatin were both increased in the tumors (data not shown).

DISCUSSION

The relation of vitamin E to cancer has been attributed to its antioxidant properties such as the potential for preventing toxic effects of free radicals or conversion of precarcinogens to carcinogens. However, there are no definitive published studies demonstrating a direct effect on cancer. Prasad and colleagues [9, 10] observed that mouse neuroblastoma and melanoma cells were sensitive to vitamin E *in vitro*, and reported that vitamin E increased the growth inhibitory and differentiating effects of cancer therapeutic agents on neuroblastoma *in vitro* [5]. Helson *et al.* [11] reported the growth inhibitory effect of vitamin E, especially vitamin E succinate (50 mg/kg/day i.p. × 5 days) and Ephanyl (*dl*-α-tocopherol in a micellar type dispersion, 400 mg/kg/day i.p. × 5 days) on nude mice bearing human neuroblastoma (SK-N-MC) [12]. They also performed a phase I clinical study of vitamin E in the treatment of neuroblastoma [13], but the result was not encouraging.

Our present study using mice bearing C1300 neuroblastoma showed no effect of an oral or intraperitoneal single administration of vitamin E (*dl*-α-tocopherol) at a dose of 20 mg/kg/day × 9 on the growth of the tumor, although the concentrations of vitamin E were elevated in tissues and blood (data not shown). However, when combined with cisplatin, an increase in the intra-tumor vitamin E content significantly enhanced the antitumor activity of cisplatin, without morphologically enhancing the renal impairment.

The mechanism of this co-operative action of vitamin E and cisplatin against neuroblastoma *in vivo* cannot be readily explained by the *in vitro* observations by Prasad *et al.* [5]; since there was no morphological differentiation of the tumor cells in this study.

One of the causes of the enhancement of the

Table 2. Combined effects of vitamin E and cisplatin on the growth of C1300 murine neuroblastoma

Group	*n*	Tumor volume (cm³)*	
		− cisplatin	+ cisplatin
control	4	4.58 ± 1.20	2.26 ± 1.30**
vitamin E(−) p.o.	4	6.10 ± 1.81	1.72 ± 0.83**
vitamin E(+) p.o.	4	8.01 ± 0.87	0.48 ± 0.22
solvent i.p.	4	5.89 ± 1.50	1.54 ± 0.63**
vitamin E i.p.	4	4.30 ± 0.99	0.02 ± 0.04**

*Tumor volume 16 days after initiation of treatment. The treatment of mice with vitamin E alone had no significant effect on the growth inhibition of neuroblastoma 16 days after the initiation of vitamin E treatment.

**$P < 0.05$ (mean ± S.D.).

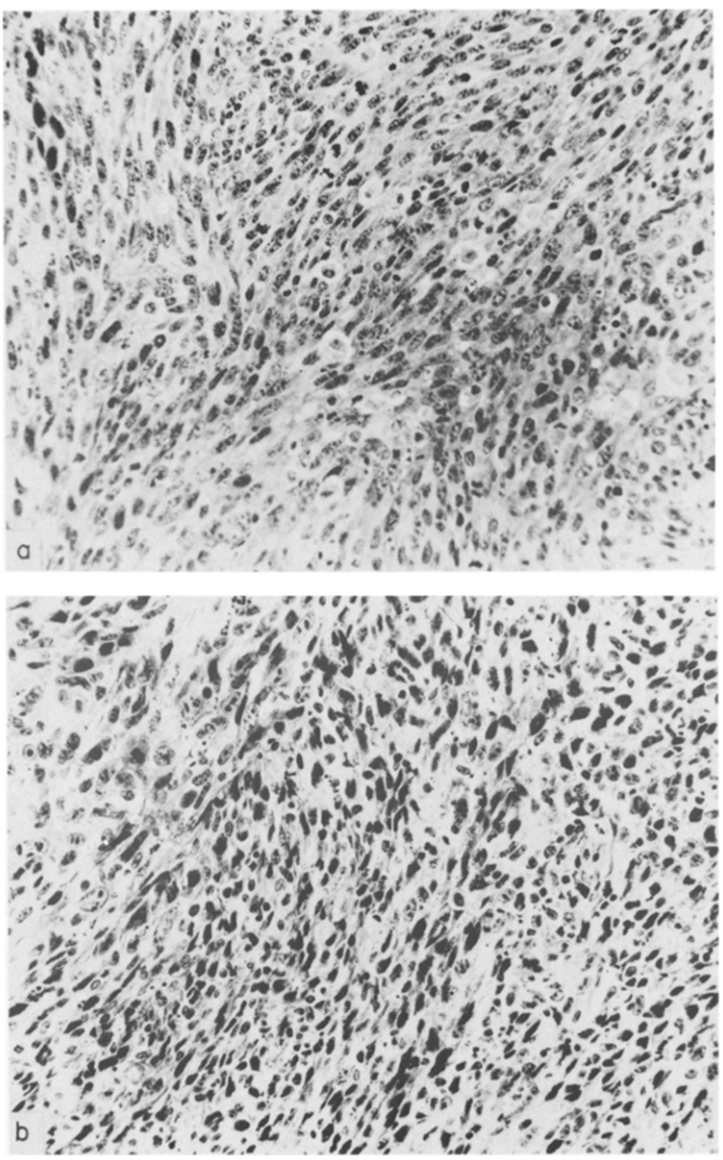

Fig. 4. (*a*) C1300 *murine neuroblastoma treated with cisplatin i.p.* (*b*) C1300 *murine neuroblastoma treated with vitamine E i.p. + cisplatin i.p.; compared with* (*a*), *in* (*b*) *necrotic or degenerated tumor cells are more apparent.*

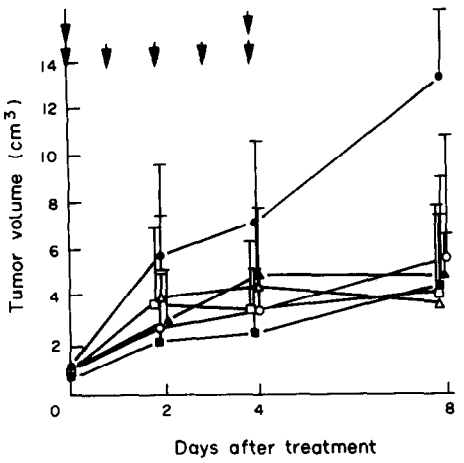

Fig. 5. The combined effect of vitamin E and cisplatin on the tumor growth of NS-20 murine neuroblastoma. ●; *Control,* ▲; *cisplatin i.p.,* □; *vitamin E i.p.* (20 mg/kg) + *cisplatin i.p.* (6 mg/kg), ○; *solvent i.p.* + *cisplatin i.p.,* ■; *vitamin E*(+) *p.o.* + *cisplatin i.p.,* ▲; *vitamin E*(−) *p.o.* + *cisplatin i.p.,* ↓ ◆ = *single injection of vitamin E and cisplatin, respectively. Each value represents an average of four to six mice. The bar at each point shows standard deviation of the mean. There was no significant difference in the tumor growth of each group 8 days after the initiation of the treatment.*

cisplatin-induced tumor growth inhibition of vitamin E might be the increased uptake of cisplatin into tumor cells. This might be specific because the cisplatin concentration increased only in the tumor and not in the kidney. Prasad and Rama [4] suggested that vitamin E might modulate permeability of the tumor cell membrane by altering the level of peroxidation. Vitamin A, another lipid-soluble vitamin, has been reported to inhibit the growth of neuroblastoma cells and to increase the influx of some anticancer agents [14–17].

Another possible mechanism is stimulation by vitamin E of antitumor immunity. Stimulation of helper T cells might enhance the antitumor activity of cisplatin. Yasunaga *et al.* [18, 19] reported that the administration of vitamin E (20 IU/kg × 7 days) in mice enhanced both lymphoproliferative reactions and the antitumor effects of adriamycin.

These inferences can hardly be extrapolated to the case of NS-20 neuroblastoma, because the treatment of A/Jax mice with vitamin E did not have an effect on the activity of cisplatin, regardless of the elevation of the platinum concentration in the tumor. Mouse NS-20 neuroblastoma was established by cloning from cultured C1300 neuroblastoma cells by Amano *et al.* [20]. Our recent experiments have shown that NS-20 clone cells are cisplatin-tolerant, both *in vivo* and *in vitro*. The present result of NS-20 neuroblastoma suggests that another active site of vitamin E is in the process after passage of cisplatin through the plasma membrane. Amatruda *et al.* [21] reported that vitamin A induced *in vitro* morphological differentiation of human neuroblastoma cells accompanied by a decrease of the expression of N-myc oncogene. Vitamin E may act similarly at the site on DNA or RNA where cisplatin binds (Prasad *et al.* 1981).

Vitamin E did not increase the uptake of cisplatin into the kidney and there were no effects seen microscopically. The present results suggest that cisplatin and vitamin E should be tried as a combined chemotherapy of neuroblastoma.

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