Jpn. J. Cancer Res. 82, 511–517, May 1991

Induction of Transplantable Tumors by Repeated Subcutaneous Injections of Natural and Synthetic Vitamin E in Mice and Rats

Yumiko Nitta, Kenji Kamiya, Masanori Tanimoto, Seiji Sadamoto, Ohtsura Niwa and Kenjiro Yokoro[1]

Department of Pathology, Research Institute for Nuclear Medicine and Biology, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima 734

Natural vitamin E and synthetic vitamin E (*dl*-α-tocopheryl acetate) were tested for their tumorigenicity in rodents. Transplantable tumors, at the site of injection, were induced by repeated injections of these compounds in two strains of mice, NFS/N and C57BL/6N × C3H/He F1, and in a strain of rats, Fischer 344. Natural vitamin E was tumorigenic in both strains of female mice only when injected with soya oil. In contrast, *dl*-α-tocopheryl acetate alone was capable of inducing tumors in Fischer 344 rats. Only one out of 5 male NFS/N mice given *dl*-α-tocopheryl acetate developed a tumor. Therefore, Fischer 344 rats were more susceptible to tumor formation by *dl*-α-tocopheryl acetate than NFS/N mice. *dl*-α-Tocopheryl acetate with soya oil or with palm oil also resulted in the formation of transplantable tumors in NFS/N mice and Fischer 344 rats. There was no difference in the tumor incidence between mice treated with *dl*-α-tocopheryl acetate alone and *dl*-α-tocopheryl acetate plus soya oil or palm oil. However, in rats, the incidence was lower for a group treated with *dl*-α-tocopheryl acetate plus palm oil than for those with *dl*-α-tocopheryl acetate alone and with *dl*-α-tocopheryl acetate plus soya oil.

Key words: Vitamin E — Tumorigenicity — Soft tissue tumor

There are a variety of synthetic and naturally-occurring antioxidants in our environment. Some of the antioxidants, such as vitamin E, butylated hydroxyanisole, butylated hydroxytoluene and ethoxiquin, inhibit chemical carcinogenesis in various organs of rats and mice when given orally.[1-4] However, evidence has been presented recently on the tumorigenicity of some antioxidants. Butylated hydroxyanisole was the first to be shown to induce squamous cell carcinomas in the forestomach of Fischer 344 (F344) rats.[5] Caffeic acid, sesamol and catechol are also antioxidants and have been shown to be carcinogenic in some experimental animals.[6]

In addition, natural vitamin E (α-tocopherol) was demonstrated to be tumorigenic in mice when given orally or by direct injection.[7] In that experiment, repeated subcutaneous injections of either α-tocopherol or soya oil alone were not tumorigenic, while the combination of the two resulted in the development of fibrosarcomas at the site of injections. We were intrigued by these observations and designed experiments to elucidate the mechanism of tumor induction by vitamin E. We report here our findings on tumor induction in mice and rats by repeated injections of a mixture of natural tocopherols and *dl*-α-tocopheryl acetate (*dl*-α-TA), a synthetic α-tocopherol, with or without vegetable oils.

[1] To whom correspondence should be addressed.

MATERIALS AND METHODS

Chemicals Natural vitamin E (E-mix 80) was obtained from Eisai Co. Ltd., Tokyo. The composition of tocopherols in E-mix 80 was determined by HPLC analysis. Total concentration of tocopherols was 73.9%, which included α-tocopherol (8.9%), β-tocopherol (0.9%), γ-tocopherol (41.0%) and δ-tocopherol (49.2%). *dl*-α-TA, a synthetic vitamin E, was also obtained from Eisai Co. Ltd. The purity of the agent was 98.3% as determined by HPLC analysis. Soya oil and palm oil were purchased from Wako Pure Chemical Co. Ltd., Osaka, and from Fuji Co. Ltd., Osaka, respectively. Soya oil contains polyunsaturated fatty acids and palm oil contains saturated fatty acids. No antioxidant (including natural and synthetic vitamin E) had been added to either of the oils.

Animals C57BL/6N × C3H/He F1 (6HF1) mice were purchased from Charles River Japan, Inc., Atsugi. NFS/N mice and F344 rats were bred in the animal facility of our institute. These animals were housed in an air-conditioned animal room kept at 24±2°C.

Experimental designs

Experiment 1; tumor induction in mice by E-mix 80: Female mice of NSF/N and 6HF1 strain were used. The experimental schedules, which involved 6 groups of mice, are shown in Table I. Twenty mg of E-mix 80 with or without 0.1 ml of soya oil, or 0.1 ml of soya oil was injected once a week at 4 independent sites on the dorsum in rotation. Injections were initiated at 8 weeks of age,

and were repeated 52 times. When a tumor reached more than 10 mm in diameter, mice were killed for complete autopsy. Other animals were killed at the age of 60 weeks.

Experiment 2; tumor induction in mice by *dl*-α-TA: Both sexes of NSF/N mice were used. This experiment consisted of 5 groups as summarized in Table II. Twenty mg of *dl*-α-TA mixed with 0.1 ml of soya oil, 20 mg of *dl*-α-TA mixed with 0.1 ml of palm oil, 0.1 ml of soya oil or 0.1 ml of palm oil was injected once a week. Injections were started at 8 weeks of age with the same rotation schedule as in experiment 1, and were repeated until the mice were 60 weeks old. When tumors grew more than 10 mm in diameter, mice were killed for complete autopsy. Other mice were observed for 8 weeks after the last injections, and were killed at the age of 68 weeks.

Experiment 3; tumor induction in rat by *dl*-α-TA: Male F344 rats were used. Five groups were employed in this study as shown in Table III. Forty mg of *dl*-α-TA mixed with 0.2 ml of soya oil, 40 mg of *dl*-α-TA mixed with 0.2 ml of palm oil, 0.2 ml of soya oil, 0.2 ml of palm oil or 40 mg of *dl*-α-TA was injected into each rat once a week. Injections were started at 9 to 11 weeks of age, and repeated 52 times in the same rotation schedule as in experiment 1. Autopsies were performed on rats with tumors of more than 20 mm in diameter. Other rats were autopsied after an 8-week observation period following the last injection.

Test for tumor transplantability Six out of the 8 subcutaneous tumors developed in NFS/N mice (experiment 2) and 23 out of 35 subcutaneous tumors developed in F344 rats (experiment 3) were tested for transplantability in syngeneic animals. When tumor-bearing animals were killed, a piece of each tumor was removed and subcutaneously implanted into the dorsum of recipients. These recipients were examined for tumor take at about one month after the transplantations.

Autopsy, and light and electron microscopic observation Every mouse and rat was subjected to complete autopsy. All injected sites and other tissues were thoroughly examined. Tissues for microscopic examination were fixed in 10% neutral formaldehyde and paraffin-embedded sections were stained with hematoxylin and eosin (HE). For electron microscopic examination, tumor tissues were fixed and embedded by the conventional method. Sections were doubly stained with uranyl acetate and lead citrate, and were observed under an electron microscope (JEOL 1200-EX).

Purification of DNA and Southern blotting hybridization All the procedures for molecular analysis of mouse tumors were as described previously.[8] Briefly, tumor tissues were frozen quickly by immersing the sample in liquid nitrogen and ground to a fine powder in a mortar. The tissues were then lysed in a buffer containing 1% sodium dodecyl sulfate, 0.1 *M* NaCl, 5 m*M* EDTA, 20 m*M* Tris-HCl (pH 8.0) and 100 μg/ml RNase A. After incubation at 37°C for 1 h, proteinase K was added to 100 μg/ml and the lysate was further incubated. DNA was recovered by ethanol precipitation after phenol-chloroform extraction of the lysate. DNA was digested with appropriate restriction enzymes and processed for Southern blotting hybridization. ^{32}P-Labeled probe was prepared by the oligonucleotide labeling method.[9]

RESULTS

Tumor induction in mice by E-mix 80 (experiment 1) Incidences of tumors in various groups are shown in Table I. In both strains of mice, E-mix 80 mixed with soya oil induced tumors at the site of injections. There

Table I. Induction of Tumors by Injection of E-mix 80 with or without Soya Oil in Two Strains of Female Mice

Group	Strain	Treatment[a]	Incidence[b] (%)	Amplification of c-*myc* gene[c]
1	NFS/N	E-mix 80 with soya oil	6/7 (85.6)	1/1
2	NFS/N	Soya oil	0/7	NT[d]
3	NFS/N	E-mix 80	0/7	NT
4	6HF1	E-mix 80 with soya oil	6/10 (60.0)	2/6
5	6HF1	Soya oil	0/10	NT
6	6HF1	E-mix 80	0/10	NT

a) Twenty mg of E-mix 80 mixed with 0.1 ml of soya oil (E-mix 80 with soya oil), 0.1 ml of soya oil alone (soya oil), or 20 mg of E-mix 80 alone (E-mix 80) was injected once a week at four separate sites on the dorsum in rotation.
b) Number of tumors/number of mice (%).
c) Number of amplified cases/number of tumors tested.
d) Not tested.

Table II. Induction of Tumors by Injection of *dl*-α-Tocopheryl Acetate (*dl*-α-TA) with or without Vegetable Oils in NFS/N Mice

Group	Treatment[a]	Incidence[b] (%)		Transplantability[c]
		Male	Female	
1	*dl*-α-TA with soya oil	2/10 (20.0)	4/10 (40.0)	4/4
2	*dl*-α-TA with palm oil	1/5 (20.0)	0/5	1/1
3	Soya oil	0/5	0/5	NT[d]
4	Palm oil	0/5	0/5	NT
5	*dl*-α-TA	1/5 (20.0)	0/5	1/1

a) Twenty mg of *dl*-α-TA mixed with 0.1 ml of vegetable oil (*dl*-α-TA with soya oil or with palm oil), 0.1 ml of vegetable oil (soya oil or palm oil), or 20 mg of *dl*-α-TA (*dl*-α-TA) was injected into each mouse once a week at four independent sites on the dorsum in rotation.
b) Number of tumors/number of mice (%).
c) Number of transplantable tumors/number of tumors tested.
d) Not tested.

Table III. Induction of Tumors by Injection of *dl*-α-Tocopheryl Acetate (*dl*-α-TA) with or without Vegetable Oils in Fischer 344 Rats

Group	Treatment[a]	Incidence (%)		Transplantability[d]
1	*dl*-α-TA with soya oil	10/15[b] (66.7)[f]	10/60[c]	10/10
2	*dl*-α-TA with palm oil	4/18 (22.2)[g]	4/72	3/3
3	Soya oil	0/12	0/48	NT[e]
4	Palm oil	0/12	0/48	NT
5	*dl*-α-TA	14/17 (82.4)	21/68	10/10

a) Forty mg of *dl*-α-TA mixed with 0.2 ml of vegetable oil (*dl*-α-TA with soya oil or with palm oil), 0.2 ml of vegetable oil (soya oil or palm oil), or 40 mg of *dl*-α-TA (*dl*-α-TA) was injected into each rat once a week at four independent sites on the dorsum in rotation.
b) Number of tumor-bearing rats/numbr of rats (%).
c) Number of tumors/injected sites.
d) Number of transplantable tumors/number of tumors tested.
e) Not tested.
f) $P<0.01$ (when compared with group 2 by χ^2-test).
g) $P<0.01$ (when compared with group 5 by χ^2-test).

was no strain difference in tumor incidences between NSF/N mice and 6HF1 mice (85.7% for NFS/N mice and 60.0% for 6HF1 mice, statistically not significant). All of the tumor-bearing mice bore one tumor. Injections of E-mix 80 alone did not induce tumors, nor did vegetable oil alone.

Tumor induction in mice and rats by *dl*-α-TA (experiments 2 and 3) In NFS/N mice, injection of *dl*-α-TA with soya oil or with palm oil resulted in the formation of subcutaneous tumors (Table II). There was no sex difference of susceptibility in the incidence of tumor induced by *dl*-α-TA with soya oil-treated groups. The tumor incidence of group 1 was as low as that of group 2 (statistically not significant). There was no difference in tumor incidence between the two types of oils. Furthermore, injections of *dl*-α-TA alone also induced tumors in subcutis (20% for males and 0% for females).

In F344 rats, *dl*-α-TA alone induced tumors at a high frequency (14 out of 17 rats or 82.4%, and 21 out of 68 sites)(Table III). Out of these 14 rats, seven animals bore tumors at two distinct sites. *dl*-α-TA mixed with soya oil or palm oil also induced tumors, and all tumor-bearing rats in these groups carried only one tumor. The incidence in the *dl*-α-TA with palm oil-treated group (4 out of 18 rats or 22.2%) was lower than that of the *dl*-α-TA alone group as well as the *dl*-α-TA with soya oil-treated group (10 out of 15 rats or 66.7%) ($P<0.01$). Palm oil seems to suppress the tumorigenicity of *dl*-α-TA in rats.

Transplantability of the tumors was confirmed by grafting the tumors in syngeneic recipient mice and rats

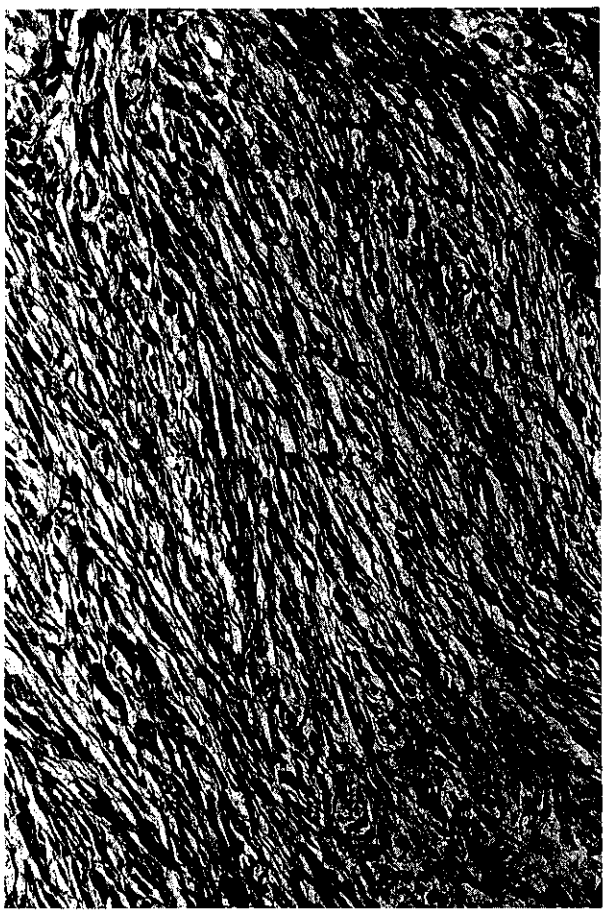

Fig. 1. Fibrosarcoma induced in a male F344 rat by repeated subcutaneous injections of *dl*-α-TA with soya oil. Densely proliferating fusiform cells are seen ($\times$200 HE stain).

(Tables II and III). All of the tumors examined were transplantable.

Histopathological observations The tumors induced in soft tissues were classified into 2 types: fibrosarcoma (Fig. 1) and malignant fibrous histiocytoma (MFH). MFH tumors were further divided into 3 types: fibrous, myxoid and giant cell (Fig. 2 and Fig. 3). Their incidences in each group are summarized in Table IV. Fibrosarcomas were frequent in mice treated with E-mix 80 with soya oil (4 out of 6 tumors in NFS/N strain and 5 out of 6 tumors in 6HF1 strain). MFHs were observed frequently in *dl*-α-TA-treated rats (all tumors in *dl*-α-TA with soya oil-treated group, 2 out of 4 tumors in *dl*-α-TA with palm oil-treated group and 20 out of 21 tumors in *dl*-α-TA alone-treated group). In experiment 1, injections of soya oil alone of E-mix 80 alone induced cysts with inflammatory fibrosis at injected sites. Cholesterin-like structures and post necrotic calcifications were observed in the group injected with E-mix 80 alone. In

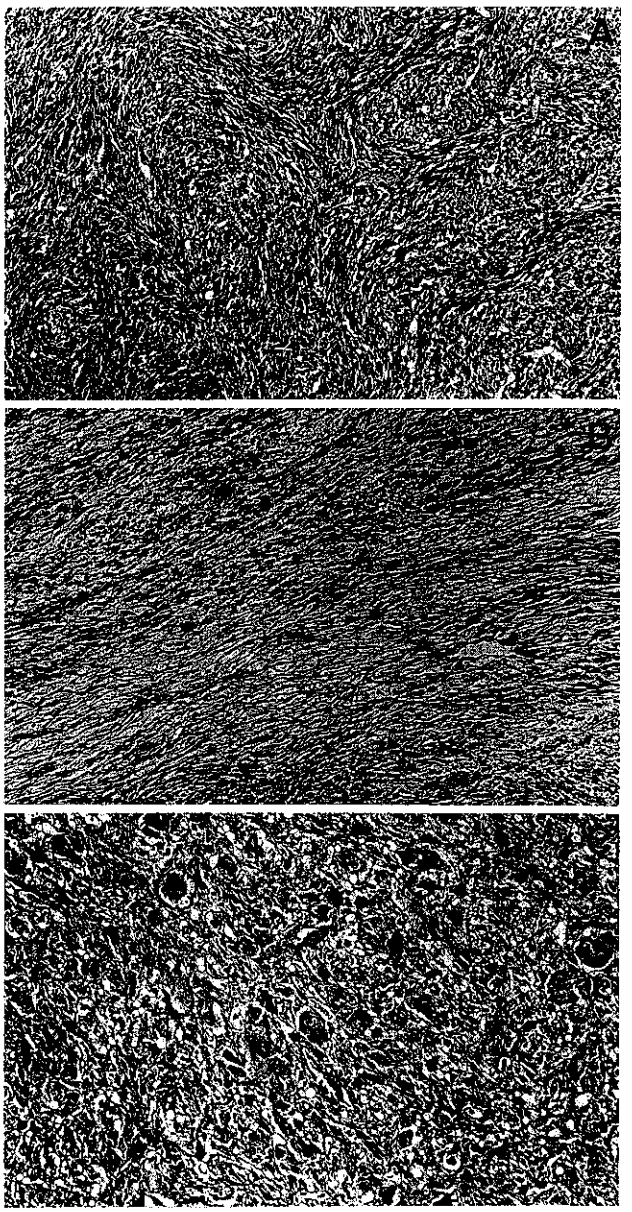

Fig. 2. (A) Fibrous subtype of MFH induced in a male NFS/N mouse by injections of *dl*-α-TA with soya oil. Spindle tumor cells form a storiform pattern ($\times$100 HE stain). (B) Myxoid subtype of MFH induced in a male F344 rat by injections of *dl*-α-TA with soya oil. Polygonal cells are loosely arranged in the myxoid matrix ($\times$100 HE stain). (C) Giant-cell subtype of MFH induced in a male F344 rat by injections of *dl*-α-TA with soya oil. Irregularly arranged spindle cells as well as polynucleic giant cells are prominent ($\times$100 HE stain).

experiment 2, *dl*-α-TA with vegetable oils caused fibrosis and *dl*-α-TA alone also caused chronic inflammation. In experiment 3, *dl*-α-TA alone as well as *dl*-α-TA with vegetable oils caused chronic inflammation and formed

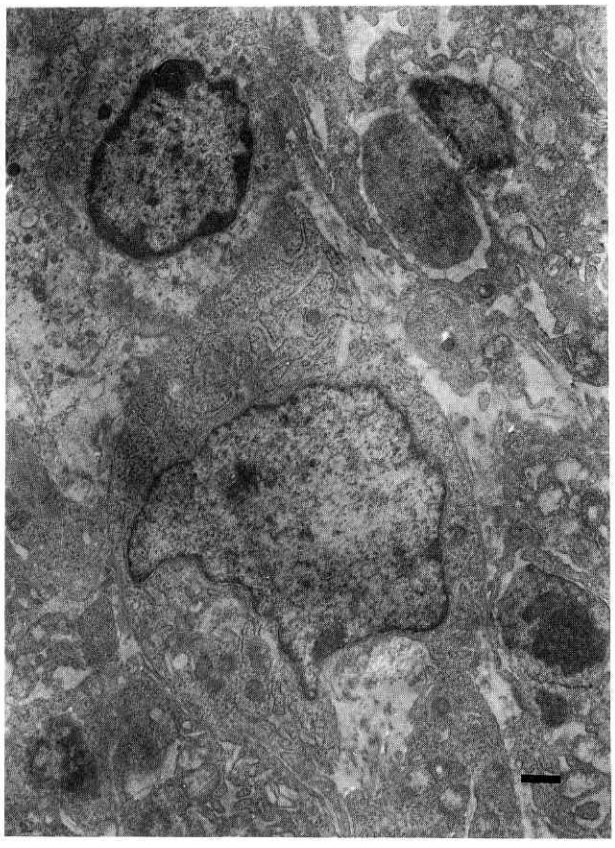

Fig. 3. Ultrastructures of Fig. 2(A). Undifferentiated cells were composed of a large nucleus, mitochondrias and free ribosomes. The bar represents 1 μm.

large granulomas at injection sites, while soya oil alone or palm oil alone did not induce any tumor or cause inflammation. There was no injection-related lesion except at subcutaneous sites.

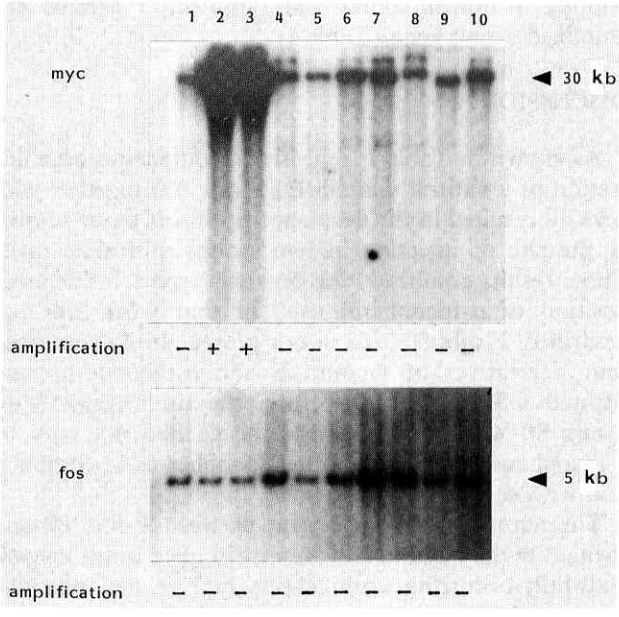

Fig. 4. Amplification of the c-*myc* gene. DNAs purified from E-mix 80 plus soya oil-induced fibrosarcomas (lanes 1, 2, 3, 4 and 7), neutron-induced sarcoma (lane 5) and gamma-ray-induced sarcomas (lanes 8, 9 and 10) were digested with *Eco*RI and electrophoresed. The *fos* gene was used as an internal control (unamplified copy). kb represents kilobases.

Table IV. Histological Types of Soft Tissue Tumors

Experiment	Animals	Treatment[a]	Incidence[b]	FS[c]	MFH[d]		
					fibrous	myxoid	giant cell
1	NFS/N	E-mix 80 with soya oil	6/7 (6/28)	4	1	0	1
1	6HF1	E-mix 80 with soya oil	6/10 (6/40)	5	1	0	0
2	NFS/N	*dl*-α-TA with soya oil	6/20 (6/80)	2	2	0	2
2	NFS/N	*dl*-α-TA with palm oil	1/10 (1/40)	0	0	0	1
2	NFS/N	*dl*-α-TA	1/10 (1/40)	0	1	0	0
3	F344	*dl*-α-TA with soya oil	10/15 (1/60)	0	8	2	0
3	F344	*dl*-α-TA with palm oil	4/18 (4/72)	2	2	0	0
3	F344	*dl*-α-TA	21/17 (21/68)	1	18	2	0

a) In experiment 1, 20 mg of E-mix 80 mixed with 0.1 ml of soya oil was injected into each mouse once a week at four independent sites on the dorsum in rotation. In experiment 2, 20 mg of *dl*-α-TA with or without 0.1 ml of vegetable oil was injected into each mouse in the same schedule as for experiment 1. In experiment 3, 40 mg of *dl*-α-TA with or without 0.2 ml of vegetable oil was injected into each rat in the same schedule as for experiment 1.
b) Number of tumors/number of animals (number of tumors/injected sites).
c) Fibrosarcoma.
d) Malignant fibrous histiocytoma.

Amplification of the c-*myc* gene DNA was extracted from tumors induced by injections of E-mix 80 with soya oil. Southern blotting of the tumor DNA was performed on *Eco*RI-digested DNA. The blot was probed with the mouse c-*myc* gene. The intensity of the bands was normalized to that of the *fos* gene which served as an internal marker for the non-amplified sequence (Fig. 4). Among 7 fibrosarcomas thus studied, 3 carried the amplified c-*myc* gene (Table I).[10]

DISCUSSION

As shown in Table I, repeated subcutaneous administration of a natural vitamin E (E-mix 80) together with soya oil resulted in the development of soft tissue tumors at the site of injection in two strains of female mice. These results confirmed the previous report.[7] The composition of α-tocopherol used in that work was not described. E-mix 80 used in our present study contained four derivatives of vitamin E and α-tocopherol constituted 6.5% of it. Therefore, the tumorigenicity of E-mix 80 with soya oil might be elicited not only by α-tocopherol but also by other tocopherols in combination with soya oil components.

The nature of the synergism between E-mix 80 and soya oil in the induction of tumors in mice is not known. Naturally-occurring antioxidants and related phenolic compounds are present in vegetable oils. They are known to act as modifiers of chemical carcinogenesis as well as being carcinogens themselves. Caffeic acid, a phenolic compound in soybeans and cereals,[11] inhibits benzo[*a*]-pyrene-induced forestomach carcinogenesis[12] and mouse skin tumor promotion by 12-O-tetradecanoyl-phorbol-13-acetate,[13] but promotes rat forestomach carcinogenesis initiated by 7,12-dimethylbenz[*a*]anthracene.[14] Furthermore, caffeic acid is carcinogenic by itself for rat forestomach epithelium, and sesamol, a phenolic compound contained in vegetable oils, can induce forestomach carcinomas in male rats as well as male and female mice when given orally.[6, 15] Although the concentrations of these ingredients in soya oil or palm oil used in our experiments were not determined, no antioxidant (including natural and synthetic vitamin E) had been added to them. It is conceivable that ingredients in vegetable oils and E-mix 80 may interact in the induction of tumors.

In our present work, *dl*-α-TA alone was shown to be tumorigenic in rats when given by repeated subcutaneous injections. Since the tumor incidence in mice was very low, the tumorigenicity of this compound in mice was not assessed in detail. Although the dose of *dl*-α-TA used in rats (40 mg/rat) was smaller than that in mice (20 mg/mouse) on a body weight basis, the tumor incidence was higher in rats than in mice. The difference might be attributed to higher susceptibility of F344 rats to *dl*-α-TA than NFS/N mice. The incidence of tumors was lower when rats were treated with *dl*-α-TA plus palm oil than that with *dl*-α-TA alone as well as with *dl*-α-TA plus soya oil. Thus, the tumorigenicity of *dl*-α-TA was suppressed by palm oil in rats ($P<0.01$). Although soya oil, which was composed more of polyunsaturated fatty acid might have a suppressive action on the tumorigenic effect of *dl*-α-TA, the effect was not statistically significant. Polyunsaturated fatty acids in cellular membranes are easily attacked by free radicals. The oxidative destruction of unsaturated fatty acids leads to a chain reaction of reproduction of free radicals.[16] Tocopherols in cellular membranes have protective activities as radical scavengers. The level of saturation of fatty acids coinjected with *dl*-α-TA may affect the amount of free radicals, but the mechanism of suppression of *dl*-α-TA action by palm oil is not known at present.

Another important factor in the induction of tumors is the inflammation at the site of injection. Histopathologically, vegetable oils were absorbed soon after injection when applied alone and did not induce inflammation. Natural and synthetic vitamin E by themselves, as well as in combination with vegetable oils, effectively induced local inflammatory reactions. They were absorbed poorly, if at all, and were recognized as foreign bodies to be rejected. The infiltration of polymorphonuclear leukocytes was prominent at inflammation sites. The leukocytes are known to release superoxides and they may participate in tumor promotion. For example, phorbol-12-myristate-13-acetate, a mouse skin tumor promoter, binds specifically to protein kinase C,[17] and causes rapid intake of oxygen and formation of oxygen radicals in macrophages.[18] This reaction was inhibited by anti-inflammatory agents which block the formation of radicals.[19] Oxygen radicals may also act directly as a mutagen or indirectly to convert mixtures of vitamin E and vegetable oils to tumorigenic agents.

All the α-tocopherol-induced tumors were fibrosarcomas in the previous report.[7] Here, we observed MFHs as well as fibrosarcomas both in E-mix 80-treated and in synthetic *dl*-α-TA-treated groups. MFH has been induced in rats by subcutaneous injections of a variety of agents.[20, 21] In our present experiments, repeated subcutaneous injections of vitamin E and vegetable oils induced MFHs as well as fibrosarcomas. It is likely that the replicative or phagocytic activities of not only fibroblasts but also histiocytes were stimulated in the inflammatory environment.

Examination of the fibrosarcomas induced by E-mix 80 mixed with soya oil revealed that tumor cells frequently had an amplified c-*myc* gene (3 out of 7 cases; 43%).[10] Such gene amplification is thought to result from the overreplication of DNA sequences following DNA

damage. The mechanism of c-*myc* gene amplification in tumors induced by E-mix 80 plus soya oil is not known. We suspect that DNA damage caused by local inflammation may trigger the process of amplification.

We examined the long-term effect in mice of dietary E-mix 80 and found that it enhanced the spontaneous hepatocarcinogenesis in mice.[22] Though the doses employed in our experiments were rather high and the route of administration in the present experiments was different from that of practical usage in man, the results of the present studies together with our previous data[22] suggest that possible adverse effects of natural as well as synthetic vitamin E should not be overlooked.

ACKNOWLEDGMENTS

The authors are obliged to T. Nishioka, A. Kinomura, K. Mizuno and T. Matsuura for their excellent technical assistance. This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan.

(Received December 11, 1990/Accepted February 26, 1991)

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