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Initiation of a transplantable fibrosarcoma by the synergism of two non-initiators, alpha-tocopherol and soya oil*

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Summary. When, in the course of an ageing study, α-tocopherol (vitamin E), dissolved in soya oil, was given to 22 Balb/c mice once a week subcutaneously for 10 months, it caused the development of vigorously growing fibrosarcomata at the site of the injections in 17 (77.3%) of the animals. The tumors produced in this manner proved eminently transplantable into syngeneic Balb/c hosts, and have been serially transplanted every 3–4 weeks for over 3 years in such recipients, having reached their 44th transplantation cycle at the present time; upon transplantation, they now exhibit a 100% "take" incidence and proliferate extremely rapidly, growing from pin-head size to up to half the weight of a whole recipient mouse within 3 weeks. All fibrosarcomata showed marked mitotic activity, invasion of adjacent tissues and extensive necrotic areas, and they became more undifferentiated after the third transplantation cycle.

Neither pure α-tocopherol alone nor soya oil alone produced any tumors when given subcutaneously once a week, for 10 months to groups of 22 Balb/c mice each.

It is concluded that the two agents α-tocopherol and soya oil which proved non-carcinogenic when injected alone, developed a powerful carcinogenic effect when they acted on subcutaneous connective tissue simultaneously. The possible mechanisms of this phenomenon are discussed.

Key words: Tocopherol – Anti-oxidants – Soya oil – Polyunsaturated fatty acids – Neoplasia

Introduction

Several years ago, while attempting to study the effect of a chronic treatment of subcutaneous injections of a soya oil solution of Vitamin E (α-tocopherol)

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on the development of lipofuscin in neurons and myocardium of ageing mice, we unexpectedly observed that most of the animals so treated developed rapidly growing, transplantable fibrosarcomata at the site of the injections.

To find out whether the observed carcinogenic effect was due to the α-tocopherol or to its soya oil solvent, we subsequently performed an additional experiment in which we studied the effects of chronic injections of pure α-tocopherol alone and pure soya oil alone – under conditions of injection dosage, frequency, mode of administration and total duration that were identical with those in the original experiment in which the combination of the two agents proved carcinogenic. Since neither α-tocopherol alone nor soya oil alone produced any neoplasms we concluded that the carcinogenic effect was a result of a synergy of these two substances.

This paper presents the gross, light microscopic and electron-microscopic findings of the tumors produced in mice by the chronic tocopherol-soya oil treatment, and of their transplants. A separate publication will discuss the effects of pure tocopherol treatment on lipofuscin development in ageing mice.

Materials and methods

The following 4 groups of 22 male Balb/c mice each were employed in this study:

Group 1. Combined Vitamin E-soya oil injections. Starting 1 month after birth, (at an average body weight of 20 grams) the animals of this group were injected subcutaneously into the dorsum once a week with 16 milligrams α-tocopherol (Vitamin E) dissolved in 0.1 ml soya oil for 10 full months. The injections were then discontinued, because in the 9th month of treatment the mice started, one after another, to develop tumors at the injection sites. As each animal developed a tumor it was allowed to live for an average of 3–4 weeks from the first detection of the neoplasm (until the latter reached at least cherry size) and then it was sacrificed and its tumor and main viscera removed for histological examination. By the beginning of the 17th month (the 7th after stopping the injections), when 17 of the 22 mice had developed tumors and been killed, the surviving 5 still tumor-free animals were also sacrificed and thus the group terminated.

The soya oil solution of Vitamin E was prepared by dissolving 160 mg of pure crystallized α-tocopherol acetate (an oily liquid at room temperature, obtained from Sigma, St. Louis, MO.) in each ml. of pure, cold-pressed soya oil ("Golden Harvest" brand obtained from Natural Sales Co. Pittsburg, PA.).

Group 2. Untreated controls. Starting 1 month after birth these mice were injected subcutaneously once a week with 0.1 ml. physiological saline, for 10 months. The injections were then stopped and the animals were killed 7 months later.

Group 3. Vitamin E injections alone. Starting 1 month after birth, these animals were injected once a week with 16 mg pure crystallised α-tocopherol (0.02 ml of the oily liquid form of this vitamin obtained from Sigma, St. Louis, MO. USA) for 10 months. The injections were then discontinued, the animals kept alive for another 7 months and sacrificed.

Group 4. Soya oil injections alone. Starting 1 month after birth, these mice were injected subcutaneously once a week with 0.1 ml pure cold-pressed soya oil ("Golden Harvest" brand, obtained from Natural Sales Co., Pittsburg, PA. USA) for 10 months, kept for another 7 months after stopping the injections and then killed.

Groups 3 and 4 were begun after groups 1 and 2 were terminated.

All injections were given to the dorsum, rotating the injection sites every week so as to spread the injected materials evenly over the entire dorsal surface of the animals.

Upon sacrifice, every animal was weighed, autopsied carefully and standard samples of 12 organs from each of 6 mice per group were formalin-fixed and processed for light microscopic study; hematoxyline-eosine stained paraffin sections were thus examined from brain, spinal cord, heart, lung, liver, kidney, pancreas, testis, spleen, thymus, adrenal and skeletal muscle of 6 animals in each group. In addition, samples of spinal cord and heart were post-fixed in glutaraldehyde-osmic acid and processed for a separate electron microscopic study of lipofuscin development. Finally, all tumors produced in group 1 were fixed in 10% buffered formaldehyde and weighed and multiple blocks from them were processed for light microscopic study and – after glutaraldehyde-osmic acid post-fixation – electron microscopic examination.

Specimens from six of the tumors that developed originally in group 1 were transplanted into 6 normal adult male Balb/c mice. When these transplants had grown in their hosts for 4 weeks, specimens from them were transplanted into a fresh set of 6 normal male Balb/c recipients and the tumors have been maintained in this manner by serial transplantation into isogeneic hosts every 3–4 weeks for more than 3 years; they are in their 44th transplantation cycle at the moment of writing. It should be noted that since after the 10th cycle the percentage of "takes" was consistently 100%, only 4 recipients were used for each cycle after the 17th generation of tumors and the ratio of donor: host mice was reduced at that time from 1:1 to 1:2.

The procedure of transplantation was as follows: The tumorbearing donor mouse was anesthetised with an intraperitoneal injection of 0.05 ml of a 50 mg/ml nembutal solution; a tiny piece of viable tumor was then removed from it through incisional biopsy, quickly trimmed to a roughly 1 mm^3 cube-shaped specimen under cacodylate buffer in a petri dish, and implanted subcutaneously through a small incision into the dorsum of an ether-anesthetised recipient mouse. In all cases, the tumor donors were killed after yielding their tumor specimens, while the tumor recipients were kept alive for 3–4 weeks and became, in turn tumor donors to the next set of recipients.

The two largest tumors in each transplantation cycle were fixed in 10% buffered formaldehyde and weighed after fixation. Some blocks from them were then embedded in paraffin and processed into H&E stained sections for light microscopic study, while other blocks were post fixed in buffered 5% glutaraldehyde followed by 2% osmic acid, and embedded in epon for electron microscopic examination.

Results

A. Original development of the fibrosarcomata

Seventeen of the 22 mice that were injected with the Vitamin E-soya oil mixture developed tumors in the subcutaneous space of the dorsum. The first tumors became noticeable as small, lentil-sized nodules during the 9th month of injections, and they kept erupting one after another even after discontinuation of the injections at the end of the 10th month. All 17 tumors grew to oval or spherical masses of a weight of at least 7 g and a size of at least roughly $1.5 \times 1.5 \times 1.5$ cm during the 7 additional months the mice were kept alive for observation.

Grossly, the tumors exhibited apparently well demarcated boundaries, a firm rubbery consistency and white fish-flesh like cut surface, and most of them developed necrotic soft areas in their central portions as they grew in size; often the skin over the largest tumors became necrotic as well (Fig. 1).

Light microscopically, the neoplasms proved very cellular. They consisted primarily of sheets of tightly packed, reticulin producing spindle cells

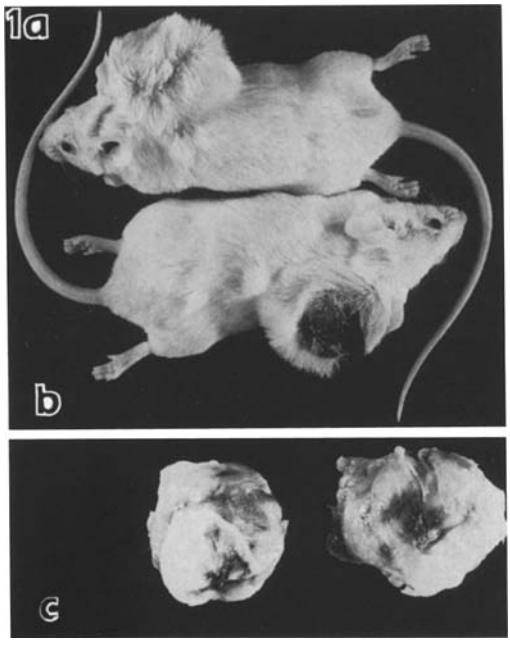

Fig. 1. a Typical fibrosarcoma developing in the dorsum of a tocopherol-soya oil injected mouse. 75% of actual size. **b** Typical necrosis and erosion of skin over a fibrosarcoma in a tocopherol-soya oil injected mouse. 75% of actual size. **c** Two dissected fibrosarcomata showing non-necrotic pale and necrotic dark areas. Actual size

with dark, cigar-shaped nuclei and numerous mitoses (Fig. 2). The necrotic areas showed nuclear pycnosis, caryorhexis and cellular debris, and they were frequently associated with venule thrombosis, and often massive polymorphonuclear infiltration (Fig. 3).

Electron microscopically, the cells of the neoplasm exhibited the features of fibroblasts. They possessed no basement membrane, marginal pinocytic vesicles or myofilaments, they usually showed long attenuated processes, and immature collagen fibrils were sometimes intimately associated with their surfaces (Fig. 4).

No metastases into any viscera of the tumor-bearing (Vitamin E-soya oil injected) animals were observed during their autopsies or the routine histological examination of their main organs. However, most tumors clearly invaded tissues of their local environment such as the striated muscle of the dorsal body wall and the subcutaneous adipose tissue (Fig. 5).

No tumors whatsoever developed in any of the control (saline injected) mice or the animals injected with Vitamin E or soya oil alone. No significant leucocytic response developed locally at the sites injected with Vitamin E alone, but most injection sites of soya oil alone were massively infiltrated by polymorphonuclear (neutrophil) leucocytes.

B. Transplantation of the fibrosarcomata

The transplanted tumors "took" in the great majority of the implanted hosts of most of the first 10 transplantation cycles, and in 100% of the recipients of all subsequent cycles. They seemed to proliferate faster than the original neoplasms, reaching usually one third and sometimes more

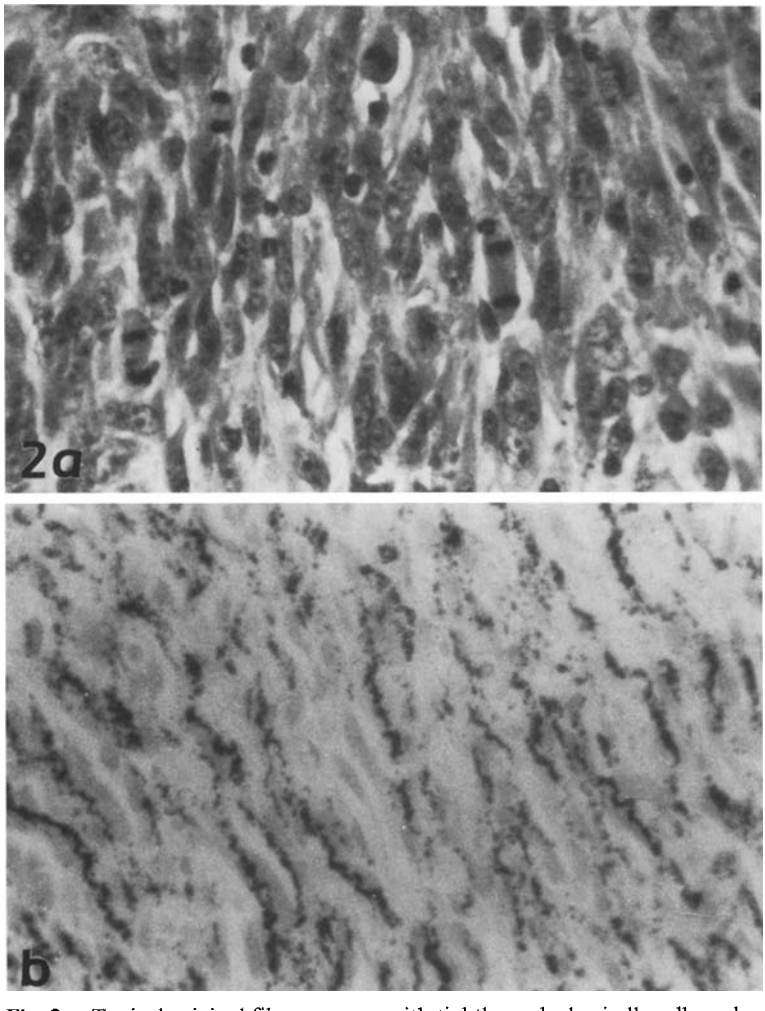

Fig. 2. a Typical original fibrosarcoma with tightly packed spindle cells and numerous mitoses. Hematoxyline-Eosine. High power. **b** Black staining reticulin scattered among the fibrosarcoma cells. Snook's reticulin stain. High power

than half the weight of their host (up to 19 grams in a 35 gram mouse), as well as relatively huge sizes (up to $5 \times 5 \times 3$ cm) within 3–4 weeks (Fig. 6).

The neoplasms of the first 2 transplantation cycles proved histologically similar to the original tumors, but those of the subsequent cycles developed rather suddenly (from the third cycle on) the following significant morphological changes: (*a*) they became much more undifferentiated, i.e. much more pleomorphic, with occasional multinucleated giant cells, and their nuclei expanded, became vesicular and acquired prominent, usually multiple nucleoli, and (*b*) their mitoses increased further in number (often reaching a concentration of as many as 18 per high power field) and they were frequently bizarre-shaped (Figs. 7 and 8).

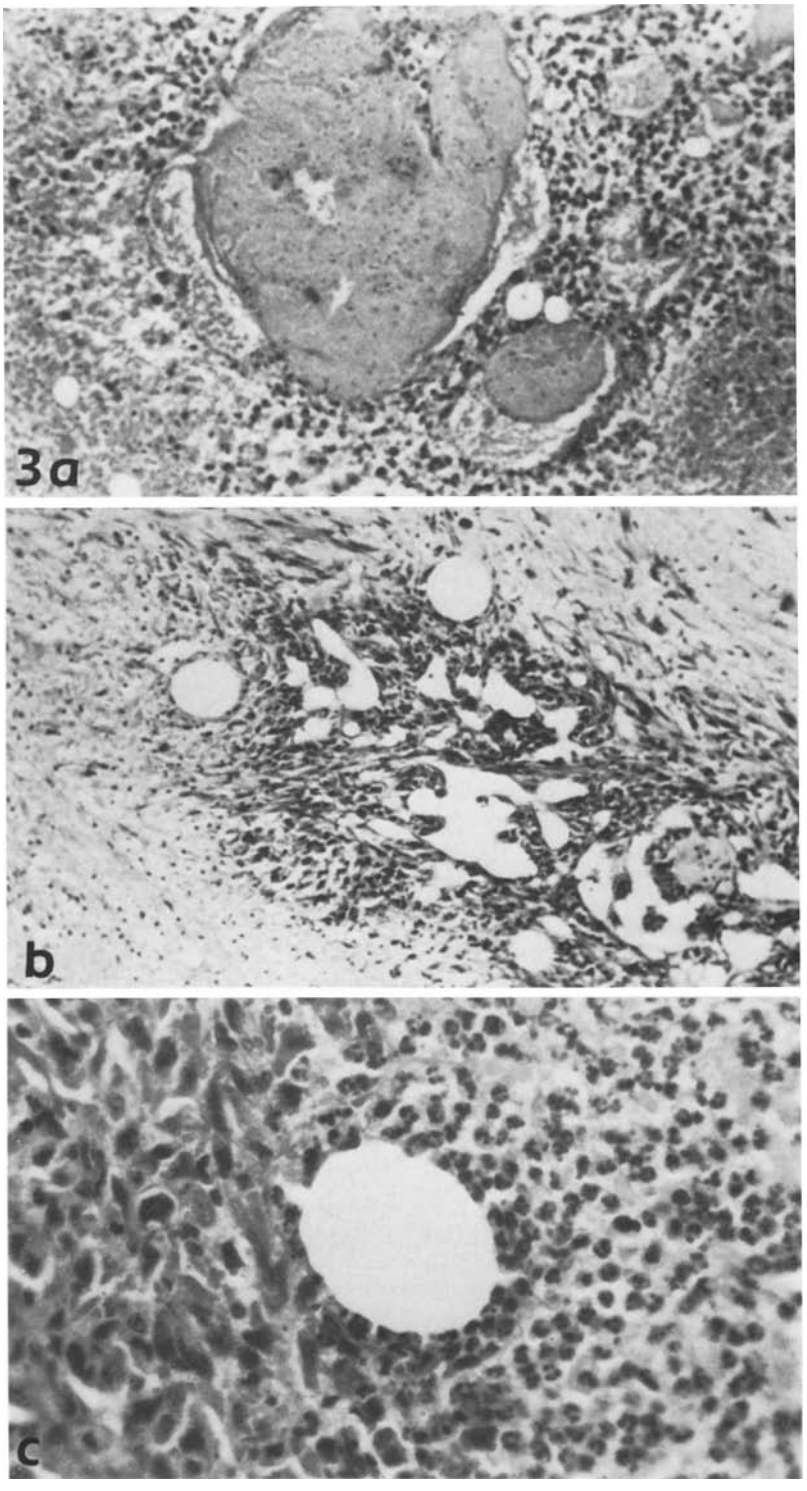

Fig. 3. a Necrotic-hemorrhagic areas of fibrosarcoma associated with two venules occluded and distended by thrombi. Hematoxyline-Eosine. Low power. **b** A viable island of tumor cells clustered around a group of patent microvessels and surrounded by necrotic tumor. Hematoxyline-Eosine. Low power. **c** Viable fibrosarcoma to the left and massive polymorphonuclear infiltration to the right of a tocopherol-soya oil vacuole. Hematoxyline-Eosine. High power

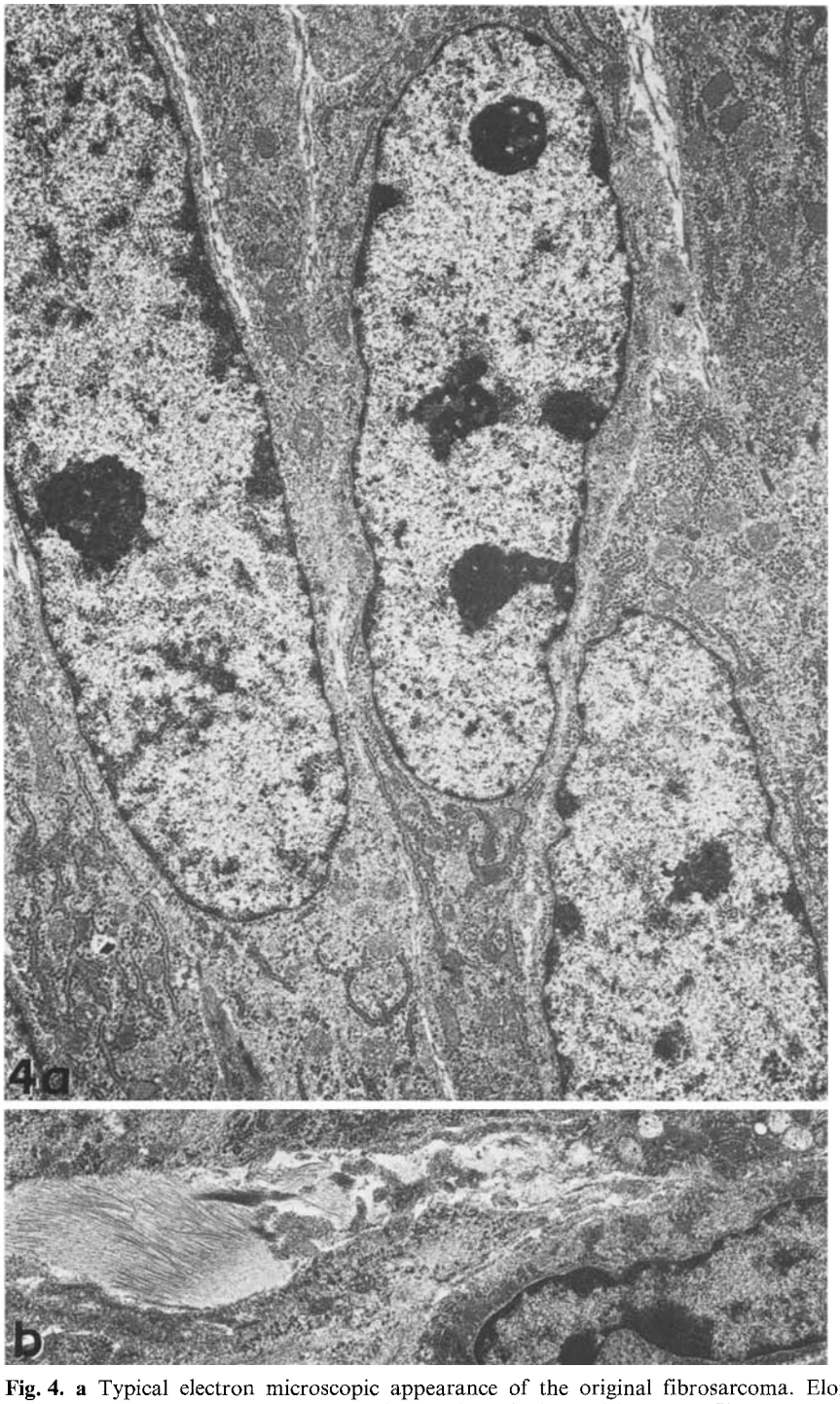

Fig. 4. **a** Typical electron microscopic appearance of the original fibrosarcoma. Elongated cells rich in ribosomes and rough endoplasmic reticulum without myofilaments, pinocytic vesicles or basement membrane. × 3000. **b** A bundle of delicate collagen fibrils lying among tumor cells. × 3000

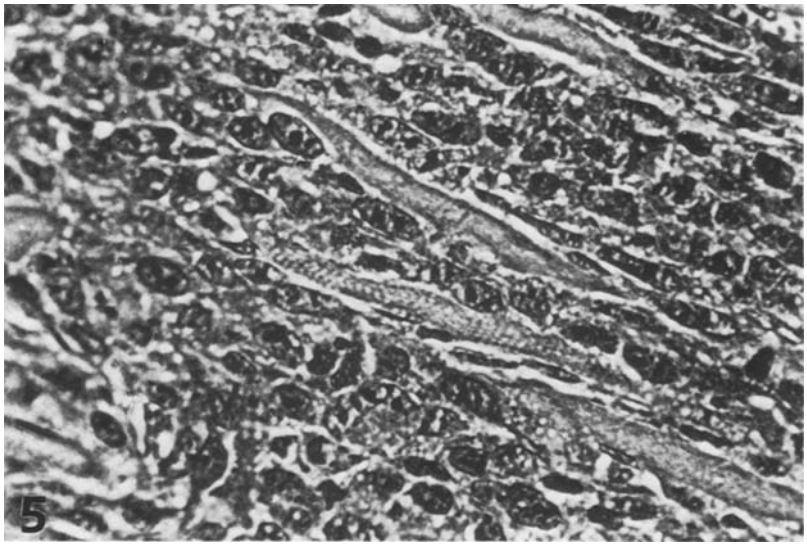

Fig. 5. Invasion of dorsal body wall by fibrosarcoma. Tumor cells are pouring between striated muscle cells, one of which still shows striations. Phase contrast on a Hematoxyline-eosine stained section. High power

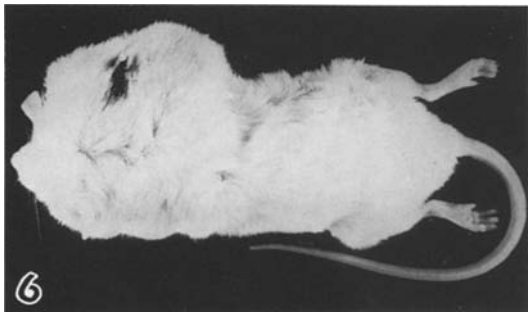

Fig. 6. Typical transplanted fibrosarcoma, with beginning necrosis of the over-lying skin, growing in the dorsum of a normal (non-injected) Balb/c recipient mouse. Actual size

In addition, starting with the first transplant cycle the necrotic areas were practically always associated with extensive venule thromboses and hemorrhages as well as some polymorph infiltration, whereas viable tumor areas were almost invariably associated with patent vessels.

None of the transplanted tumors of any cycle has yet (up to the 44th cycle) metastasized into the viscera of its host during the 3–4 week period between its transplantation and the killing of its recipient mouse, but most of them have invaded neighboring tissues (muscle, fat), just like the original tumors.

Discussion

The results of this study show that prolonged subcutaneous injections of either the natural anti-oxidant Vitamin E (tocopherol) or soya oil alone

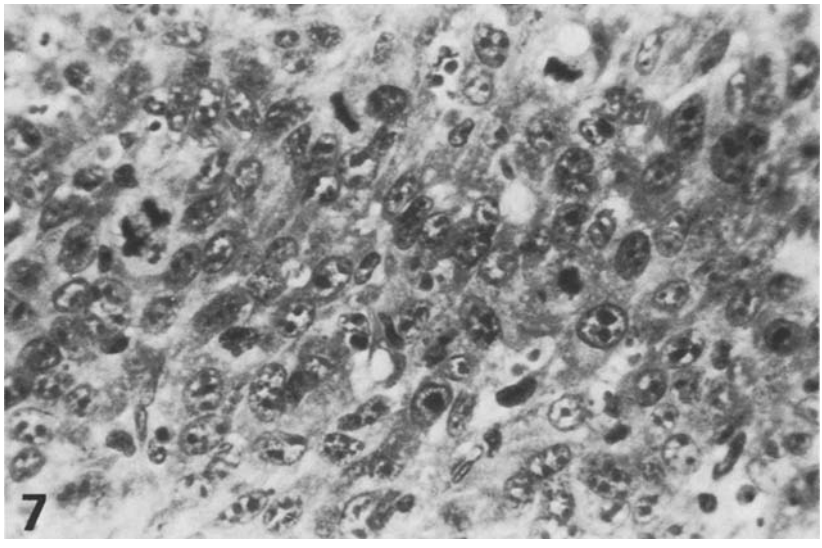

Fig. 7. Typical changed histological appearance of a transplanted fibrosarcoma after the third transplantation cycle. Pleomorphic vesicular nuclei with multiple, often unusually big nucleoli and sometimes bizarre mitoses. Compare with Fig. 2a. Hematoxyline-Eosine. High power

are not carcinogenic but that protracted injections of a combination of these two agents will initiate neoplasms of connective tissue in the majority of the animals so treated. The tumors produced in this manner exhibit all characteristics of true neoplasms: extremely vigorous mitotic activity, bizarre mitoses, anaplasia, pleomorphism, local invasion, necrosis, and inexhaustible serial transplantability far beyond the potential of non-neoplastic tissues.

The literature data accumulated so far on the relationship of soya oil and anti-oxidants to neoplasia reveal that both agents can act as either *promoters* of carcinogens or *anti-carcinogens,* depending on the circumstances, but that neither is an *initiator* of neoplasia – at least at the dosages and treatment durations applied to date.

As far as soya products are concerned, prolonged high dietary intake of *whole soya* has been found to be without carcinogenic effect on the stomach of rats (Macdonald and Dueck 1976) and humans (Hirayama 1982) and to protect against human gastric cancer (Hirayama 1982) and radiation-induced carcinogenesis in rodents (Yavelow et al. 1983), but it has been shown to be a pancreatic mitogen and a promoter of the pancreatic carcinogens dihydroxypropylnitrosamine (DHPN) and azaserine in animals (Levison et al. 1979; McGuiness et al. 1980; Oates and Morgan 1982). The mitogenic (hyperplasia-inducing) effect on the pancreas has been found to be due to the trypsin inhibitor in whole soya flour which inactivates pancreatic trypsin (an inhibitor of cholecystokinin), and therefore activates cholecystokinin, a proven pancreatic mitogen. The promotion of the pancreatic carcinogens DHPN and azaserine, in turn, has been attributed to an in-

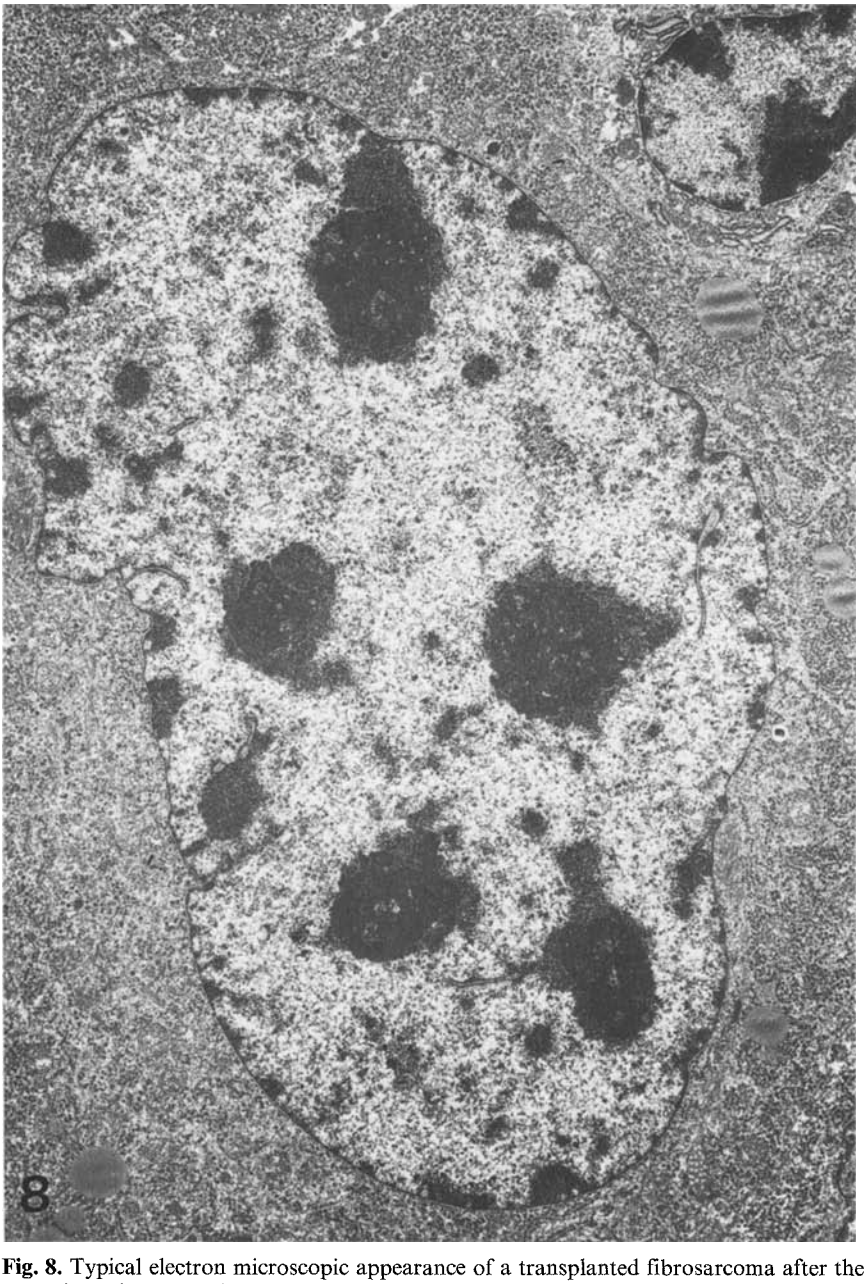

Fig. 8. Typical electron microscopic appearance of a transplanted fibrosarcoma after the third transplantation cycle. A very large nucleus with expanded nuclear surface due to several narrow inpouchings of the inner nuclear membrane and multiple big nucleoli. The abundant surrounding cytoplasm contains some lipid globules, and the small nucleus in the upper right attests to the marked pleomorphism of such tumor transplants. Compare with Fig. 4a. × 3000

creased carcinogen susceptibility of pancreatic tissue because of its cholecystokinin-induced enhanced mitotic activity.

High dietary intakes of vegetable oils rich in polyunsaturated fatty acids (PUFA), including *soya oil*, on the other hand, have been known to promote the mammary and colon carcinogenesis that is induced by dimethylbenzanthracene and related hydrocarbons (King and McCay 1983; Rogers 1983), apparently because they give rise to lipid peroxides which help activate those hydrocarbons oxidatively and because some of them are biotransformed into carcinogenesis-promoting prostaglandin molecules. It should be noted, nevertheless, that while PUFA enhance the "takes" of some experimental sarcoma transplants such as the AK-3T3, they have been found to inhibit the "takes" of other transplants, such as the FK-3T3 (Kurek and Corwin 1982).

Finally, some – though not all – *anti-oxidants* inhibit the neoplasia that is induced by chemical carcinogens such as dimethylbenzanthracene which are activated by metabolic oxidations (King and McCay 1983; Medina et al. 1983), but under certain conditions they promote the neoplasia which is induced by other agents such as the lung adenomata induced by urethane (King and McCay 1983), and they can promote the mutagenic effect of cyclophosphamide on bone marrow cells (Kodytkova et al. 1983).

Thus, the mechanism of the observed carcinogenic synergy between tocopherol and soya oil is unknown and can only be the subject of speculation at present. Since neither tocopherol nor soya oil given alone proved carcinogenic initiators in our study (or in the relevant literature), the possibility has to be considered that either tocopherol turned a soya oil component into a carcinogen or a soya oil component turned tocopherol into a carcinogen, in the special environement of local inflammation with marked polymorph infiltration and the pronounced leucocytic release of oxidising radicals that is now known to accompany such infiltrations (Weiss and LoBuglio 1982).

Regarding the first possibility, since it is well known that anti-oxidants such as vitamin E inhibit the oxidation of PUFA (Dam 1970), tocopherol could have acted on some of the PUFA molecules of soya oil, preventing their complete oxidative degradation by leucocytic oxidising radicals and allowing only their partial oxidation. Such a partial oxidation could have developed carcinogenic epoxides out of their aliphatic carbon chains, epoxides similar to the known carcinogens diepoxybutane and propiolactone (Florey 1970). The leucocytic radicals alone evidently did not produce any carcinogenic agents out of the PUFA of soy oil since no tumors whatsoever developed from the interaction of PUFA and polymorph infiltrates in the animals injected with soya oil alone.

Regarding the second possibility, some lipoperoxide derivatives of soya oil PUFA and some leucocytic oxidising radicals could conceivably have acted on tocopherol and turned it into a carcinogen. This is at least a theoretical possibility, especially in view of the recent finding that tocopherols can be turned into peroxides by their exposure to PUFA that have been heated to 70° C, aerated at 100 ml/hour and, therefore oxidized them-

selves (Chow and Draper 1974). And while we did not aerate the soya oil at 100 ml/hour or heat it to 70° C, the possibility remains that its chronic exposure in the mouse body to 38° C and to leucocytic peroxides could have been sufficient to oxidise some of its PUFA molecules.

Further research is evidently needed to unravel the mechanism of the carcinogenic synergy of tocopherol and soya oil that was observed in this study.

The following questions about the fibrosarcoma we produced also remain open and will have to be tackled by separate investigations: What caused the cytologic change of this tumor in the direction of markedly increased anaplasia during transplantation? Why has it not been observed to metastasize so far? What is the cause of the necrosis of some of its portions?

In closing, this study seems to have created one more convenient tool for in vivo carcinogenesis research, since the tumor it produced grows extremely rapidly in mormal (non-immunosuppressed, non-irradiated, non-thymectomised) Balb/c mice and exhibits a transplantation "take" rate of 100%; it has also opened up certain interesting theoretical questions about the nature of the carcinogenic synergism of polyunsaturated fatty acids and anti-oxidants when given parenterally together and acting in a setting of polymorph exudation. We must emphasize, of course, that our results do not in any way ascribe carcinogenic properties to either Vitamin E or soya oil when these substances are given orally and separately.

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