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# Vitamin E Inhibits Experimental Carcinogenesis and Tumour Angiogenesis

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**In an experiment in which vitamin E inhibited carcinogenesis, it was found that tumour angiogenesis and tumour growth-factor alpha (TGFα) expression were also inhibited. Forty male golden hamsters were divided into four equal groups. Group 1 animals had the left buccal pouches painted three times weekly with 7,12-dimethylbenz(a)anthracene (DMBA) for 14 weeks. Group 2 animals had the same procedure of DMBA applications but also received alpha tocopherol. Groups 3 and 4 were vitamin E and untreated controls. Angiogenesis was studied with factor 8-related antigen (F8-RA) which identifies endothelial cells. TGFα was studied with the appropriate antibody. Staining was effected by the standard avidin–biotin horseradish peroxidase system. Mean tumour volume was significantly lower in the DMBA–vitamin E group compared to the tumour control group. Angiogenesis was significantly inhibited in the DMBA–vitamin E group and TGFα expression was also inhibited. It is suggested that inhibition of tumour angiogenesis by vitamin E may be an additional mechanism for the anticancer action of vitamin E. Copyright © 1996 Elsevier Science Ltd**

**Keywords: vitamin E, carcinogenesis, angiogenesis, TGFα**

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## INTRODUCTION

A number of antioxidant nutrients, such as vitamin E (alpha tocopherol) and β-carotene have been shown to be capable of the inhibition [1, 2], prevention [3] and regression [4, 5] of experimental oral carcinogenesis. Vitamin A and other retinoids have also been shown to inhibit the development of oral cancer in experimental animals [6, 7]. Vitamin C has been reported to inhibit carcinogenesis [8], but it has been shown to have no appreciable effect on experimental oral carcinogenesis [9].

Among the mechanisms suggested for tumour inhibition by antioxidant micronutrients, based upon experimental evidence, have been immunoenhancement and modifications in the *p53* tumour regulating structure—the gene and its protein product that can act to suppress tumour development. An enhancement of *p53* expression would enhance the tumour suppressor activity of the gene. A decrease in the expression of its mutant form, or oncogene, would also serve to depress tumour development.

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Vitamin E has been found to be a potent immunoenhancer [10]. Studies with experimental carcinogenesis of the hamster buccal pouch revealed that vitamin E stimulated the migration of cytotoxic macrophages and lymphocytes to the developing tumour site. These cells were laden with tumour necrosis factors alpha (in macrophages) and beta (in lymphocytes) and the cytokines were deposited at the developing tumour site to destroy the tumour cells [11]. This was a clear demonstration of the concept of immunosurveillance [12], originally postulated by Ehrlich [13] in 1909, and further elaborated by Burnet [14].

It was also found that a variety of molecular mechanisms appeared to control tumour development and growth in the hamster model. The expression of "wild type" *p53*, the tumour suppressor gene, was found to be significantly enhanced in animals receiving vitamin E as well as having the chemical carcinogen applied to the buccal pouch mucosa [15]. There was also a significant decrease in the expression of mutant *p53*, the oncogene often found in a large variety of malignant tumours [16], including human oral cancer [17].

Antioxidant micronutrients other than vitamin E, such as beta carotene, and antioxidants such as glutathione have

shown similar effects on carcinogenesis and the growth of cancer cells in culture. This has led to the concept of a "common pathway" for the destruction of cancer cells by antioxidants through immunoenhancement and stabilization of the *p53* tumour suppressor mechanism [18]. A synergism between vitamin E and other antioxidants has also been demonstrated in experiments dealing with cancer inhibition and regression [19, 20].

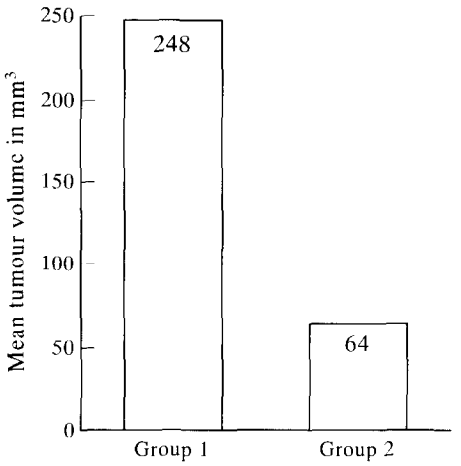


**Fig. 1. Graphic illustration of mean tumour volume in left buccal pouches of animals in groups 1 (tumour control) and 2 (vitamin E experimental group). The tumour yield in the group 2 animals is significantly lower than that in the group 1 animals.**

**Fig. 2. Illustration of epidermoid carcinomas in right buccal pouch of tumour control animal (group 1) after 14 weeks. Multiple exophytic tumours are evident, and are of large size.**

**Fig. 3. One papillary tumour of right buccal pouch in group 2 animal after 14 weeks. Several other tumours are just beginning to develop and appear as extremely small projections.**

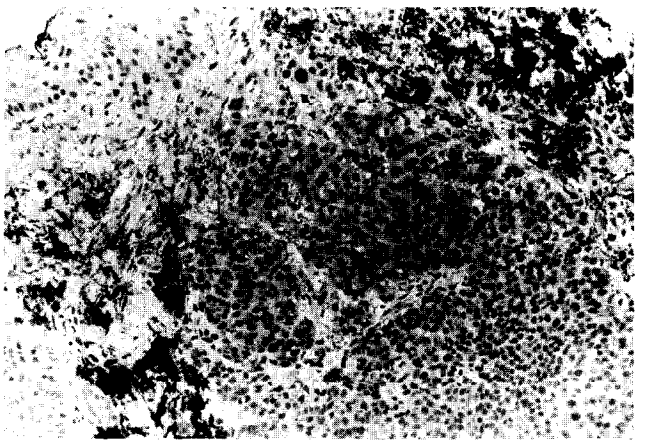

**Fig. 4. Invasive squamous cell carcinoma of hamster buccal pouch in tumour control animal showing intense stain for F8-RA in the connective tissue at the margins of the malignancy. Immunoperoxidase technique with haematoxylin counterstain × 150.**

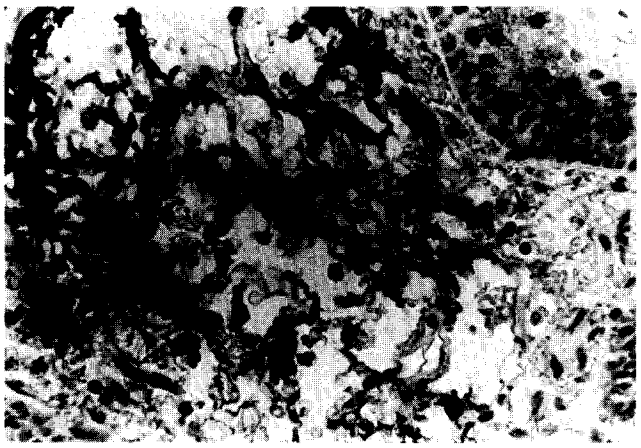

**Fig. 5. High power view showing intense stain for endothelial cells forming small blood vessels. Immunoperoxidase technique with haematoxylin counterstain ×300.**

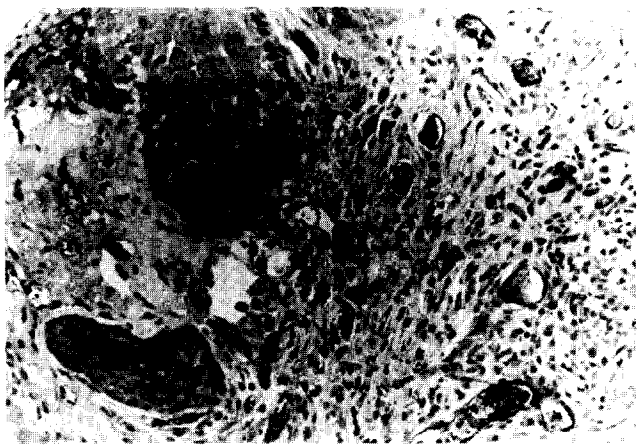

**Fig. 6. Invasive squamous cell carcinoma in vitamin E animal, showing well-outlined small blood vessels staining for F8-RA, but without the intense 'hot spots' for angiogenesis seen in the tumour control animals. Immunoperoxidase technique with haematoxylin counterstain ×300.**

Since malignant tumours require vascular proliferation for their nutrition, and send out angiogenic peptides such as tumour growth factor alpha (TGFα) to induce endothelial proliferation, it was thought that an inhibition of tumour angiogenesis could be another part of this common pathway of cancer inhibition by antioxidants. TGFα is a potent stimulator of angiogenesis but is one of many growth factors and cytokines that are angiogenic. The concept of tumour angiogenesis, originally outlined by Folkman and associates [21], has received support from a considerable body of experimental evidence [22–26] as well as clinical–pathological studies in humans [27]. Initial studies have demonstrated that both vitamin E and glutathione can inhibit tumour angiogenesis as part of their inhibitory action on carcinogenesis [28, 29].

An experiment was designed to investigate whether vitamin E would inhibit tumour angiogenesis as well as inhibiting carcinogenesis, and whether angiogenic cytokine activity was also inhibited. The experimental cancer model used was the hamster buccal pouch, an excellent model for oral cancer as

**Fig. 7. Leucoplakic area in buccal pouch of tumour control animal, showing several capillaries. Immunoperoxidase technique with haematoxylin counterstain ×200.**

well as for other squamous cell carcinomas. This model has been widely used in experimental carcinogenesis and resembles human oral cancer very closely [30]. The tumours develop slowly over a 12 week period and start with a keratotic and dysplastic lesion similar to human oral leukoplakia [31]. The tumours are moderately well differentiated but eventually may metastasise to regional lymph nodes in the neck. These tumours express many oncogenes expressed in human oral cancer and also express many metabolic markers that are found in human oral cancer [32]. Tumour angiogenesis and TGFα expression were studied by the appropriate immunohistochemical techniques.

## MATERIALS AND METHODS

*Animals*

Forty golden Syrian young adult male hamsters (*Mesocricetus auratus*) were divided into four equal groups of ten animals:

Group 1: 7, 12 dimethylbenz(a)anthracene (DMBA) treated (tumour control)
Group 2: DMBA treated and vitamin E administered systemically by mouth
Group 3: untreated control
Group 4: vitamin E control.

The animals were randomly bred (Lakeview Strain LVG, Charles River Breeding Laboratories, Wilmington, Massa-

**Fig. 8. Leucoplakic area in buccal pouch of vitamin E animal, showing several capillaries. Immunoperoxidase technique with haematoxylin counterstain ×200.**

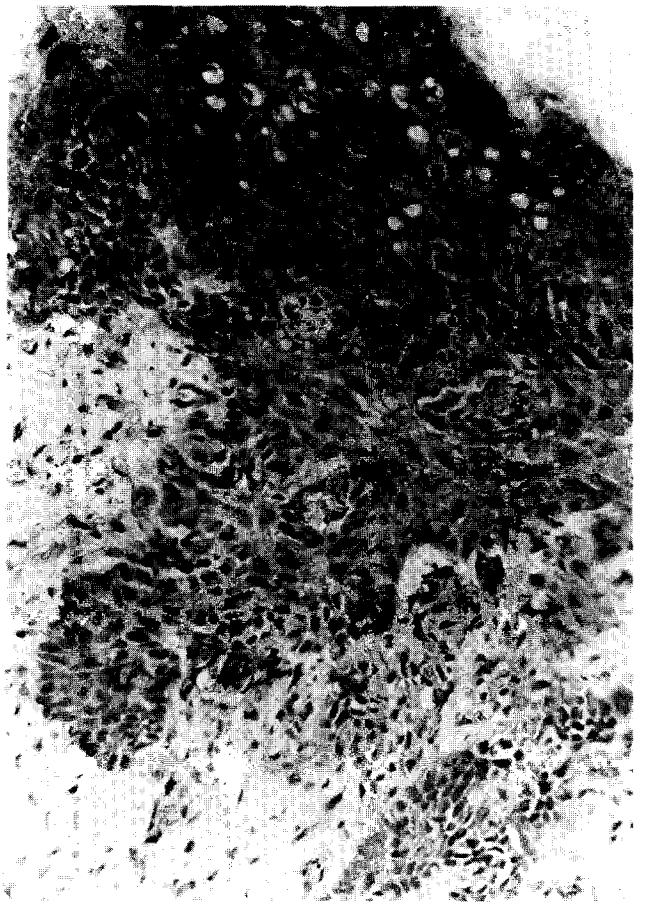

**Fig. 9. Carcinoma in tumour control animal stained for TGFα. There is intense staining of the epithelium. Immunoperoxidase technique with haematoxylin counterstain ×250.**

chusetts, U.S.A.), housed five per cage, and fed standard Ralston Purina Laboratory pellets and water *ad libitum*. The animals were maintained in a controlled environment under standardised conditions of temperature and humidity with an alternating 12:12 hour light–dark cycle. The animals were 60–90 days old and weighed 95–125 g at the start of the experiment.

The animals in groups 1 and 2 had the right buccal pouches painted three times per week with a 0.5% solution of 7, 12 dimethylbenz(a)anthracene (DMBA) (Sigma Chemical Co. St Louis, Missouri, U.S.A.) in heavy mineral oil (USP), using a number 4 sable brush. Each application leaves approximately 0.6 mg DMBA on the mucosal surface of the buccal pouch, as determined previously by the use of radioactive $^{14}C$ DMBA. The animals in group 2 (experimental group), in addition to the DMBA applications, received 10 mg/kg alpha tocopherol acid succinate (Sigma Chemical Company) in 0.5 ml mineral oil delivered orally by pipette three times per week on days alternate to the DMBA applications. At the termination of the 14 week experimental period the animals were euthanised in a carbon dioxide chamber. Tumours of the right buccal pouches were photographed, counted, and measured. Figures were obtained for mean tumour volume and overall tumour burden. Both right and left pouches were excised, fixed in 10% formalin, sectioned in paraffin, and stained with haematoxylin and eosin. Serial sections were stained with the peroxidase–antiperoxidase technique for immunohistochemical studies, using the localisation of factor VIII-related antigen to demonstrate the endothelial cells comprising the blood vessels [33]. The immunohistochemical techniques were carried out on formalin-fixed, paraffin-embedded sections.

*Histochemical techniques*

Tumour angiogenesis was studied by the immunohistochemical technique for factor VIII-related antigen (DAKO L1809) (clone F8-RA). Staining was by standard immunoperoxidase technique (DAKO quick staining kit, peroxidase K686). Tissue controls included various normal organs and malignant tumours. Three sections per pouch were used for the staining procedure and were blindly read by two oral pathologists. An attempt was made to count the number of blood vessels on the histological sections. Only rough figures could be obtained, since the tumours often presented massive proliferations of endothelial cells that were developing into vessels.

TGFα expression was studied with the appropriate antibody (clone 213-4.4 dilution 1:200-Oncogene Science, Manhasset, New York, U.S.A.). An automated immune enzymatic staining system (Biotech 1000) was used. This consisted of a standard avidin–biotin horseradish peroxidase secondary detection staining system.

Appropriate controls were used for immunohistochemical

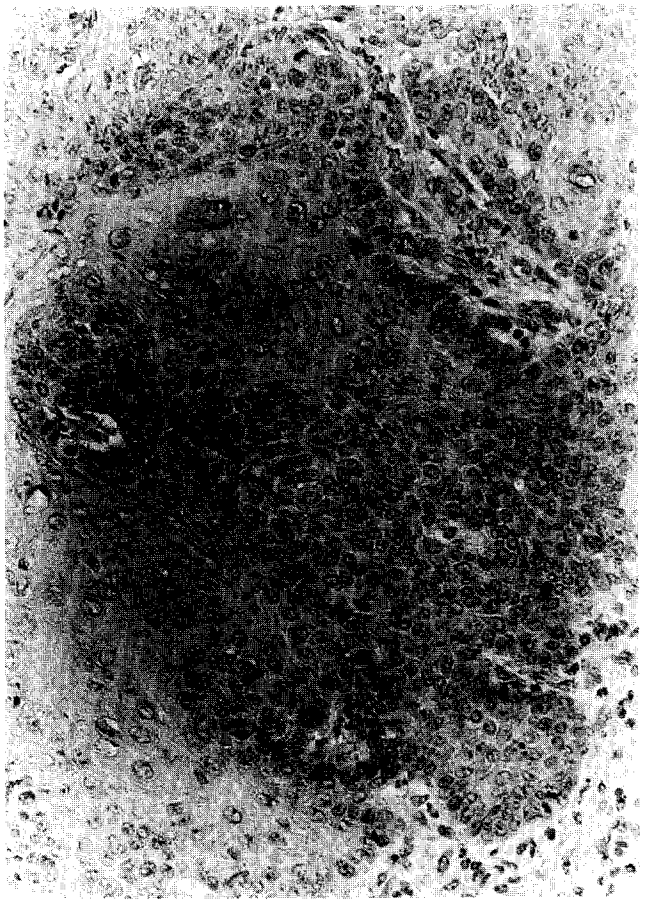

**Fig. 10. Carcinoma in vitamin E animal demonstrating a relative absence of epithelial staining for TGFα. Only the light counterstain is seen. Immunoperoxidase technique with haematoxylin counterstain ×250.**

staining. Negative controls for the antibody consisted of serial paraffin-embedded sections of the same tissues and the omission of the primary antibody in the staining procedures. Positive controls consisted of a series of paraffin-embedded tissues known to be strongly positive for TGFα expression. These were normal tissues and malignant tumours, both carcinomas rich in the cytokine and sarcomas that had little TGFα expression. The use of the robotic Biotech 1000 computerised system for staining offered reassurance that every experimental and control slide was stained and washed for exactly the same time period.

## RESULTS

*Gross and microscopic pathology*

At the 14 week termination of the carcinogenesis experiment, the animals receiving vitamin E systemically (group 2) demonstrated a significant inhibition of tumour development compared with the tumour control animals (group 1). Mean tumour burden was 248 $mm^3$ in group 1 compared with 64 $mm^3$ in group 2 ($P$ value—0.001 by Student's $t$-test) (Figs 1–3). The range of tumour burden was 196–322 $mm^3$ in the group 1 animals and 34–86 $mm^3$ in the group 2 animals. The tumours were all epidermoid carcinomas of well-to-moderate differentiation.

*Immunohistochemical findings for angiogenesis (F8-RA)*

The connective tissue adjacent to the epidermoid carcinomas in the tumour control group 1 animals demonstrated intense staining for proliferating endothelial cells, particularly in localised areas of the connective tissue near the boundary of the invading carcinoma (Figs 4, 5). In the experimental group receiving vitamin E, the carcinomas tended to be smaller in size, and the underlying connective tissue demonstrated considerably fewer capillaries (Fig. 6). In the leucoplakic lesions, the tumour control animals demonstrated capillaries in the underlying connective tissue, but not in abnormally significant numbers, and not significantly different from the numbers seen in the vitamin E experimental animals (Figs 7, 8).

*Immunohistochemical findings for TGFα*

In the tumour control animals, the epidermoid carcinomas stained strongly for TGFα expression, particularly in the nuclei. In the vitamin E animals, the tumours stained less intensely and the nuclei were relatively free of TGFα expression (Figs 9, 10).

## DISCUSSION

It is apparent that the anticancer activity of vitamin E is also associated with a depression of angiogenesis activity and a decrease in TGFα expression. Since TGFα is a well documented stimulator of angiogenesis [34], the depression of TGFα activity may serve as a mechanism for the inhibition of tumour angiogenesis, and angiogenesis inhibition may represent an additional mechanism whereby vitamin E and other antioxidant micronutrients can exert their anticancer activity. It is of interest that the invasive carcinomas of the hamster buccal pouch epithelium showed an intense reaction to factor VIII staining ahead of tumour invasion, but very little activity related to the precancerous leucoplakic lesions. This may be somewhat analogous to the findings of Weidner and associates in invasive prostate carcinoma, where tumour angiogenesis correlated with the metastatic lesions [35]. Since vitamin E inhibited both tumour angiogenesis and TGFα expression, its primary effect may be on the TGFα, which then affects tumour angiogenesis. TGFα expression has been shown by Wong and associates to be enhanced during experimental carcinogenesis of the hamster buccal pouch [36, 37], both in epithelial cells and in eosinophil leucocytes. TGFα may be a major stimulator of the resulting angiogenesis, and its inhibition by vitamin E may depress angiogenic activity. However, there may be other angiogenic stimulators that are affected by vitamin E, such as epidemal growth factor (EGF). Schwartz has recently demonstrated that there was a reduction of both EGF and TGFα expression during inhibition of cancer cell development induced by beta-carotene [38].

It is apparent that vitamin E inhibits carcinogenesis through a variety of mechanisms. One mechanism is immunoenhancement through cytotoxic macrophages and lymphocytes secreting tumour necrosis factors alpha and beta at the developing tumour site. Another mechanism is regulation of the *p53* tumour suppressor gene and resultant inhibition of oncogene activity. A third mechanism may be inhibition of angiogenesis, so that the developing tumour has an insufficient blood supply.

Since a number of antioxidant micronutrients act through the first two mechanisms, it has been postulated that there is a "common pathway" through which retinoids, carotenoids, tocopherols and glutathione exert their anticancer effect. It has been suggested that inhibition of angiogenesis may be part of this common pathway. A relationship between angiogenesis and the *p53* pathway has recently been demonstrated through control of thrombospondin-1 [39]. Additional research will help to further clarify this overall concept of a *common pathway* for cancer inhibition by antioxidant nutrients.


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