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p53 in the Anticancer Mechanism of Vitamin E

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Immunohistochemical techniques were used to study the expression of "wild type" p53 and "mutant" p53 in experimental cancer inhibition by vitamin E. The cancer model used was the squamous cell carcinoma of hamster buccal pouch induced by the carcinogen 7,12 dimethylbenz(a)anthracene (DMBA). Cancer development was studied sequentially for 8–14 weeks and specimens prepared for histological and immunohistochemical interpretation. Primary antibodies used were monoclonal antibodies for "wild type" and "mutant" p53. Specificity of antibodies was confirmed by flow cytometry. Peroxidase–antiperoxidase staining was used on the tissue specimens. In those animals receiving vitamin E the buccal pouch tumour development was significantly inhibited and there was a notable expression of "wild type" p53. There was also a relative absence of "mutant" p53 in the buccal pouch lesions of animals receiving vitamin E. These observations suggest that vitamin E may inhibit cancer formation by stimulating the expression of a cancer suppressor gene.
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INTRODUCTION

p53 IS A GENE that has received wide attention as a tumour suppressor gene [1]. It encodes a 53 kD protein that acts within the nucleus of cancer cells and appears to regulate the replication of DNA. This "wild type" of p53 may be converted by mutation to a "mutant type" of p53 that can act as an oncogene, possibly alone or together with other oncogenes. The mutant type of p53 is expressed in many different types of malignant tumours [2] including carcinomas of breast [3–6], lung [7–9], colon [10–12], stomach [13], oral mucous membrane [14, 15] and osteogenic sarcoma [16]. Differences have been found in p53 expression between primary cancers and their metastatic lesions. Yamada *et al.* [13] found no p53 mutations in 19 primary lesions of gastric cancer, but found p53 mutations in a number of metastatic lesions from gastric cancer, suggesting that the p53 gene mutations preferentially occur in the advanced stages of gastric cancer.

The possible roles of a number of oncogenes have been studied in oral cancer. c-*erb*B1 is over expressed in some human oral cancers and was found to be expressed in experimental oral cancer [17]. Increased expression of other oncogenes has also been reported in oral cancer, including Ki-*ras* [18] and Ha-*ras* [19] in experimental oral cancer and Ha-*ras* in human oral carcinomas [20]. Field and associates have indicated an increased expression of p53 in oral malignancies in patients who were heavy smokers [21]. Somers and associates have demonstrated p53 mutations to be the most common genetic alteration detected in head and neck cancers [22]. The cooperation of p53 with other oncogenes such as Ha-*ras* has been demonstrated [23].

p53 was first identified in 1979 as a host cell protein binding to the DNA virus, SV40 [24]. Early studies presented evidence that elevated levels of p53 could cooperate with other oncogenes to produce transformed cells [26]. Although initial studies conceived of p53 being an oncogene with dominant transforming capabilities, studies by Ben-David and associates found that inactivation of p53 could lead to transformation and that p53 appeared to act as a tumour-suppressor gene rather than a dominant transforming gene [27]. Further studies revealed that p53 has no ability to transform cells but mutant forms of p53 do possess this ability, often in cooperation with other well-known oncogenes. For activity as a tumour-suppressor gene, the natural or "wild type" of p53 must be present in substantial amounts so that it can over balance the expression of mutant forms [28, 29].

Vitamin E has a low toxicity [30] and potent anticancer action, first demonstrated in 1969 in experimental animals [30]. A significant inhibitory effect on oral cancer was demonstrated in the hamster pouch model by Shklar in 1982 with vitamin E administered orally [32].

The development of experimental oral carcinomas could also be retarded by the topical application of vitamin E on days alternate to the carcinogen application [33]. Using a less potent carcinogen it was possible to demonstrate that vitamin E could completely prevent the development of the carcinomas [34]. Vitamin E was also shown to be capable of regressing established carcinomas of hamster buccal pouch when injected close to the tumour site [35]. One aspect of the mechanism of vitamin E prevention and regression is an immunoenhancement [36], with the migration of cytotoxic lymphocytes and cytotoxic macrophages rich in tumour necrosis factor alpha to initial dysplastic lesions or tumour foci [37, 38]. Vitamin E, as a very potent antioxidant, is a well known trapper of free oxygen radicals. It has been found to protect cells from carcinogenic chemicals by inhibiting lipid peroxidation and its damaging free-radical-mediated consequences [39].

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Vitamin E and beta carotene have been found to be synergistic in their anticancer effect [40]. An explanation may be that while both these nutrients are antioxidants, they may function as antioxidants in a different, but complementary manner. Beta carotene functions as a very potent quenching agent of superoxide ion in a low partial pressure of oxygen. Alpha tocopherol is a very potent chain-breaking antioxidant at a high partial pressure of oxygen.

Several human studies have confirmed the animal studies with vitamin E as a anticancer agent. Menkes and associates [41], in a retrospective epidemiological study, found that low plasma levels of beta carotene and tocopherol bore a relationship to the subsequent development of lung cancer. Palan and associates [42] found an inverse relationship between plasma levels of beta carotene and alpha tocopherol and dysplasia and cancer of the uterine cervix.

Since vitamin E has a potent activity in cancer inhibition, and since the concept of cancer suppressor genes has been established, it was engaging to seek the mechanism of vitamin E's anticancer action in the stimulation of a cancer suppressor gene or inhibition of related oncogenes. An experiment was undertaken to study the anticancer activity of vitamin E, using the well-established and excellent hamster buccal pouch experimental model for epidermoid carcinoma [43–47] and the expression and location of p53 by standard immunohistochemical techniques, since antibodies to both "wild type" and "mutant" p53 are available. An experiment was carried out to demonstrate the possible role of p53 in the inhibition of experimental oral cancer by vitamin E.

MATERIALS AND METHODS

Methods

Eighty golden Syrian young adult male hamsters (*Mesocricetus auratus*) were divided into four equal groups of 20 animals:

Group 1—DMBA treated (tumour control).

Group 2—DMBA treated and vitamin E administered systemically by mouth.

Group 3—untreated control.

Group 4—vitamin E control.

Animals in groups 1 and 2 had the right buccal pouch painted three times per week with a 0.5% solution of 7,12 dimethylbenz(a)anthracene (DMBA) (Sigma Chemical Co.) in heavy mineral oil USP, using a number 4 sable brush. Each application consisted of 0.4 mg DMBA in 0.25 cc oil, as confirmed in previous studies by ^{14}C labelling. The left buccal pouches remained untreated as an internal control for the DMBA application.

Animals in groups 2 and 4 had vitamin E acid succinate (Sigma Chemical Co.) administered orally by pipette three times weekly on days alternate to the DMBA application. The vitamin E was deposited into the animals' oropharynx by pipette in a dose of 10 mg three times per week and this technique resulted in very little vitamin E reaching the local pouch environment. Previous studies have confirmed this.

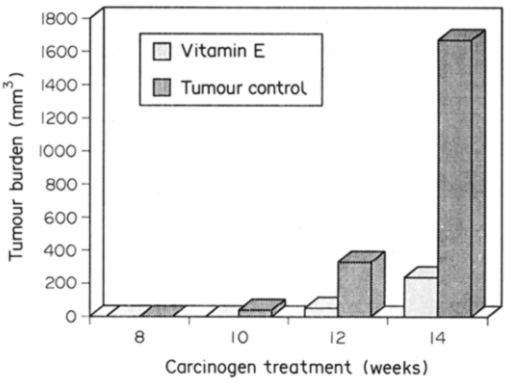

Fig. 1. Graphic illustration of tumour inhibition by vitamin E in hamster buccal pouch experimental cancer model.

The hamsters (Lakeview Strain LVG) were obtained from Charles River Breeding Laboratories (Wilmington, Massachusetts) and were kept in a 12 h interval light–dark environment. The hamsters were fed a normal diet (Ralston Purina Chow No 5012) containing 22% protein and water ad libitum.

The right pouches were everted and examined weekly after 6 weeks. Five animals in each of the four groups were euthanised in a CO_2 chamber at 8, 10, 12 and 14 weeks. The buccal pouches were photographed, the tumours counted and measured, and leukoplakic areas scored. Figures were obtained for tumour burden (number of tumours $\times 4/3\pi r^3$ where r represents 1/2 average diameter of tumours) in each cheek pouch and statistical analysis was carried out comparing average tumour burden in tumour control group and vitamin E group. The pouches were excised, fixed in 10% formalin for 96 h, and sectioned in paraffin for histological and immunohistochemical studies. Cervical lymph nodes, salivary glands and major organs were also fixed in formalin for histological studies.

Animals were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and with the guidelines of the Institute of Laboratory Animal Resources, National Research Council.

Immunohistochemical technique

The relative levels of protein expression (p53) were assessed following avidin–biotin, peroxidase–antiperoxidase immunohistochemical staining of the buccal pouch. The primary antibodies used were p53 monoclonal antibodies, PAb240 (mammalian mutant p53, recognises amino acid sequences 206–211) and PAb246 (rodent wild type recognises amino acid sequences 88–109) (Oncogene Science, Uniondale, New York). Controls and staining procedure: The specificity of

Fig. 2. (Facing page). Tissue sections of hamster buccal pouches obtained from animal groups used in the inhibition of oral carcinogenesis study are presented. The sections were stained immunohistochemically using an avidin–biotin peroxidase–anti-peroxidase technique. The monoclonal antibodies allowed us to separate "wild type" from the "mutant" form of p53. (a) Normal hamster buccal pouch showing relative absence of p53 wild type expression, (200×). (b) Hyperplastic, dysplastic mucosa in animals receiving vitamin E demonstrating increased p53 wild type expression (400×). (c) Invasive epidermoid carcinoma in animals that received vitamin E treatment. High levels of p53 wild type expression was noted (150×). (d) High power view of c (400×).(e) Epidermoid carcinoma in tumour control animal showing absence of p53 wild type expression (200×). (f) Normal hamster buccal pouch showing absence of p53 mutant expression in the epithelium (arrow) (80×). (g) Epidermoid carcinoma in tumour control animal showing increased expression of mutant p53 (150×). (h) High power view of e (400×). (i) Epidermoid carcinoma in a animal receiving vitamin E showing a relative absence of mutant p53 (150×).

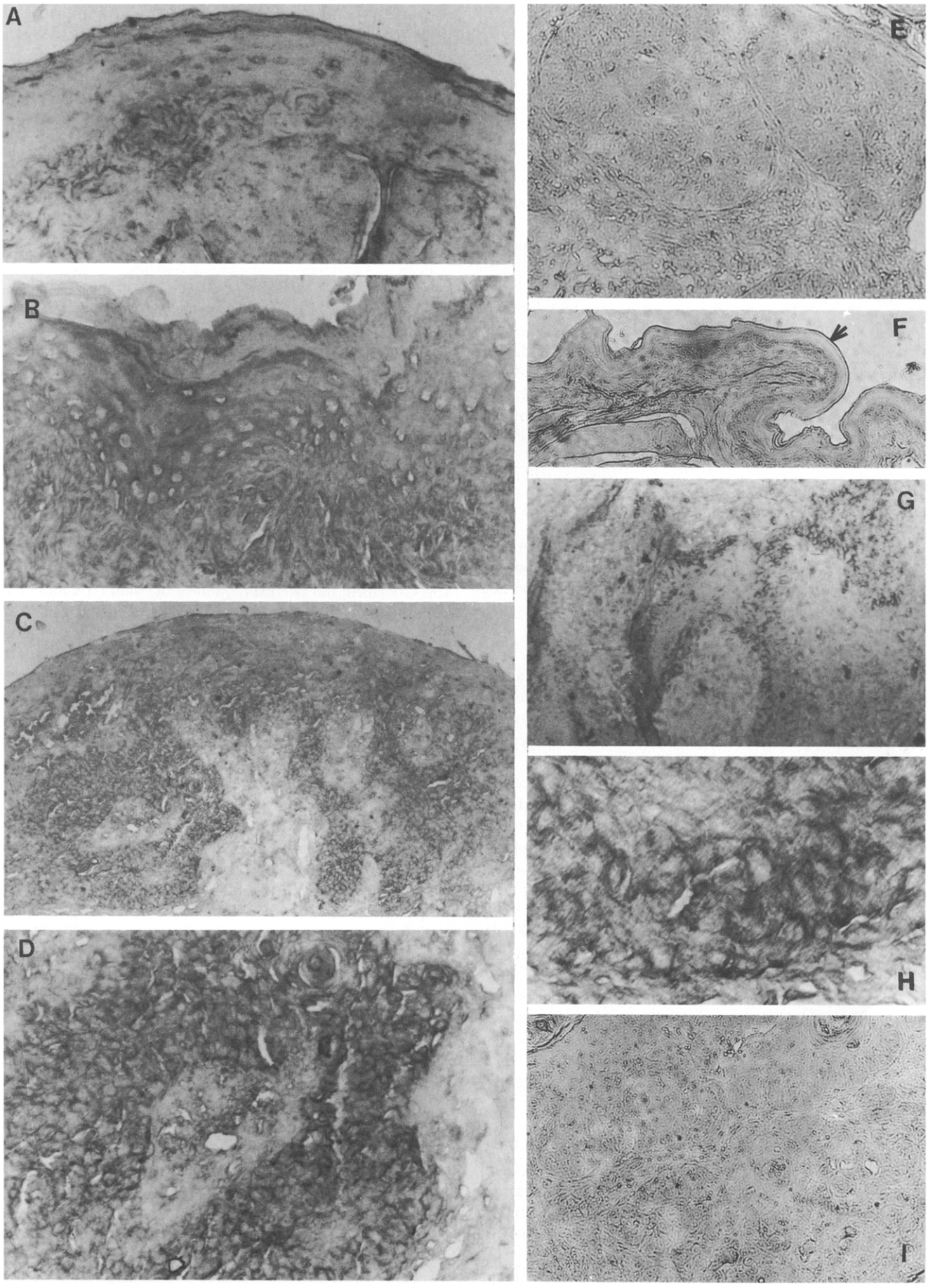
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Table 1. Table showing relative expression of "wild type" and "mutant" p53 in normal, tumour control and vitamin E animals

Groups	Wild type	Mutant
Normal (untreated)	+	−
Tumour control (DMBA)	+	+ + +
Normal + vitamin E	+ +	−
DMBA + vitamin E	+ + +	+

each antibody was confirmed by flow cytometry, and the level of antibody recognition for p53 expressed in HCPC-1 cells was quantitated following *in vitro* treatment. Negative controls for the primary antibody binding was the elimination of the primary antibody, the incubation of the tissue with diluted mouse serum (1:300) and the use of an antibody with an identical isotype (mouse antihuman antibody). An immunoenzymatic staining kit was used to visualise the binding of the primary antibody in the tissue sections (DAKO-Quick Staining System 40, K686). Three sections per pouch were used for each staining procedure and blindly read by two oral pathologists. At least three areas of normal, dysplasia, or carcinoma *in situ*/invasive carcinoma were scored.

RESULTS

Gross findings

There was a significant inhibition of tumour development in the animals receiving vitamin E. The tumour inhibition could be observed at all stages after 8 weeks and was particularly notable at the end of the 14 week experiment (Fig. 1).

Immunohistochemistry

"Wild type" p53 was relatively absent in normal hamster buccal pouch mucosa (Fig. 2a). Its expression was found to increase in dysplastic mucosa in animals receiving vitamin E (Fig. 2b). There was a notable expression in invasive epidermoid carcinoma in animals treated with vitamin E. (Fig. 2c, d) with the staining prominently seen. Localisation of p53 appeared to be in nuclear membranes, in cytoplasm adjacent to nuclei and, to a lesser extent, in both cytoplasm and nuclei. Carcinomas in animals untreated with vitamin E did not stain for p53 "wild type" (Fig. 2f).

"Mutant" p53 was seen to have a strong expression in the carcinomas of the tumour control animals (Fig. 2g, 2h) but was relatively absent in the tumours of the animals receiving vitamin E (Fig. 2i). It was also absent in normal epithelium (Fig. 2f).

A summary of the overall immunohistochemical results is presented and indicates that vitamin E treated animals showed increased levels of wild type p53. The increase in wild type p53 was observed in normal, dysplastic and invasive oral carcinoma (Table 1).

DISCUSSION

In addition to stimulation of a variety of immune responses [26–38], vitamin E acts directly upon cancer cells to destroy them. Schwartz and Shklar [48] found a selective cytotoxic effect of both beta carotene and alpha tocopherol on human cancer cell lines *in vitro*, at relatively high concentration (>70 μmol/l). Seven different cell lines were studied (two oral carcinomas, two breast carcinomas, two lung carcinomas and one malignant melanoma). A consistent morphological change was shown in cancer cells after treatment with beta carotene or vitamin E—a rounding of tumour cells and lifting off from the culture plate. There were also quantitative reductions in proliferations ([^{3}H]thymidine) and succinic dehydrogenase activity (MTT Assay). In addition, there was a change in protein expression, specifically following β-carotene treatment, which produced an increased expression of heat shock protein 70, which has been related to p53 in numerous recent investigations [49–51].

At lower concentrations an increased differentiation has been noted to be induced by treatment with β-carotene or vitamin E. Specifically, β-carotene induced increased cell to cell communication and the increased expression of connexin-43 [52].

Both p53 and hsp 70 have characteristics in common. Both proteins are expressed at high levels in $G_1 \rightarrow S$ of the cell cycle, and are signals of a cellular "SOS" response to either exogenous or endogenous oxidative stress. The result of their enhanced expression was the development of apoptosis (programmed cell death) [53, 54]. Vitamin E could suppress tumour cell proliferation, resulting in the accumulation of the tumor cells in G_1, followed by differentiation [48, 55], a process ascribed to the development of apoptosis [56].

A model for the function of p53 was proposed by Lane [57]. In normal cell division, p53 is not required. Normal cell response to DNA damage would involve a genome guarding function of p53. If this response is not successful, the overexpression or dysregulation of wild type (tumour suppressor form) p53 may be overexpressed. As demonstrated by Tuck and Crawford, the overexpression of the wild type p53 could initiate tumorgenic transformation [58]. The premalignant cell may attempt to stabilise the p53 complex through enhanced hsp 70 constitutive expression or viral oncoprotein expression [59, 60]. The completion of the transformation process may involve the induction of oxidants that promote instability and mutational damage to the DNA. The enhanced expression of mutant p53 may be a cellular response to dysregulated wild type gene expression by acting as a dominant negative regulator of the wild type p53. The gross manifestation of this cellular response, we suggest, would be the death of some malignant clones due to mitotic failure, while viable tumour clones will arise from the surviving genetically altered cells.

Vitamin E, acting as a powerful inhibitor of cellular peroxidation and hydroxyl radical formation, could reduce the triggering of signal inductive pathways involving NF-kB [61]. The NF-kB signal system has been shown to be controlled through the level of cellular antioxidant activity [62]. Vitamin E treatment of cells has not only resulted in the reduction in reactive oxidants, but a marked reduction in transcription factor induction, such as N-*myc* [63], and an increased DNA repair [64] has been identified. Associated with an oxidative state change, there may also be the reduction in hsp or adenovirus E1A protein binding to mutant p53, resulting in the destabilisation of mutant p53, and mutant p53 protein–protein interactions, particularly with promotor gene regions, such as, hsp 70 [65]. The final result would be the reduction in mutant p53 with an enhanced expression for wild type p53.

Since "wild type" p53 is stimulated by vitamin E, and "mutant" p53 expression is diminished by vitamin E, it could be suggested that vitamin E exerts its anticancer effect by:

1. stimulating a cancer repressor (cancer suppressor) gene to prevent the action of carcinogenic influences.

2. Preventing the mutation of p53 to oncogenic forms by promoting DNA repair. This process may occur as vitamin E inhibits oxidant production.
3. Preventing the mutation of other protooncogenes that may function together with "mutant" p53.

An understanding of the basic mechanism of vitamin E anticancer activity may result in more effective agents to prevent and control cancer in humans. The effective control of cancer in humans by non-toxic agents is the major thrust and hope of this research. This is the first report showing that a non-toxic micronutrient, vitamin E was capable of modulating the activity of a cancer suppressor gene.

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