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Oral Cancer Inhibition by Micronutrients. The Experimental Basis for Clinical Trials

Gerald Shklar and Joel Schwartz

Extensive research has been carried out in experimental animals to demonstrate the anticancer activity of retinoids, carotenoids and tocopherol on oral cancer and oral precancerous leukoplakia. The anticancer properties of these micronutrients have been studied in experiments dealing with inhibition of carcinogenesis, prevention of oral cancer development and regression of established oral carcinoma. Synergism has been demonstrated in the anticancer activity of beta carotene and alpha tocopherol. Synergism has also been demonstrated between beta carotene and anticancer alkylating agents such as melphalan and cyclophosphamide. Micronutrients such as beta carotene have been found to inhibit both major phases of carcinogenesis—initiation and promotion. Animal studies of oral cancer inhibition, prevention and regression have been substantiated by tissue culture studies, using animal and human derived oral cancer cell lines and normal epithelial cells. Mechanisms of the anticancer activity of the micronutrients on experimental oral cancer have been explored. They include stimulation of elements of the immune system to kill cancer cells, and enhanced expression of heat-shock proteins and repressor genes such as P 53.
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INTRODUCTION

A NUMBER OF antioxidant micronutrients have been shown to be potent inhibitors of experimental oral cancer and oral precancerous leukoplakia. Although several oral cancer experimental models have been studied, including rat lingual carcinoma, the simplest and most effective model system is that of the hamster buccal pouch. Originally described by Salley in 1954 [1], the model was further developed by Morris [2] and by Shklar and associates [3, 4]. In the experimental cancer model, epidermoid carcinomas are induced in the buccal pouch of the Syrian hamster (*Mesocricetus auratus*) by painting the pouch mucosa with a potent chemical carcinogen, usually the carcinogenic hydrocarbon 7,12 dimethylbenz(a)anthracene (DMBA). The carcinogen is applied topically three times per week in a 0.5% solution in heavy mineral oil, using a number 4 sable brush. Each application delivers approximately 0.4 mg of DMBA as measured by ^{14}C-labelled DMBA. With the 0.5% solution, precancerous leukoplakia (hyperkeratosis and dysplasia) develops at 6–8 weeks, carcinoma *in situ* at 8–10 weeks, early proliferative epidermoid carcinomas at 10–12 weeks, and large exophytic and invasive tumours at 12–14 weeks [5]. Metastasis to regional lymph nodes occurs late, usually after 16 weeks [6]. If a 0.1% solution of DMBA is used, the entire sequence of events is delayed so that the dysplasic lesions occur at 14–18 weeks, the *in situ* carcinomas at 18–22 weeks, the early carcinomas at 22–26 weeks and the large papillary and invasive lesions at 26–30 weeks [7]. This extended period of carcinogenesis may be more comparable with human oral cancer, which usually develops slowly and as a response to relatively mild carcinogenic influences. The extended experimental period of carcinogenesis is also necessary for the study of initiating influences as compared with promotion influences [8].

The hamster pouch model is an excellent experimental model for oral carcinogenesis, and is receiving wide acceptance as one of the better overall models for carcinogenesis, based on its many advantages. While the buccal pouch is a unique oral structure, its epithelium is that of oral mucosa of the keratinising type, such as palate or gingiva.

(1) The technique is simple to use and the tumours develop slowly as in human cancer [9].
(2) The cancers are preceded by a keratotic and dysplastic lesion comparable to human oral leukoplakia [10].
(3) The lesions are grossly visible at all stages. They can be recorded and measured at time of euthanasia so that a figure for tumour burden [11] can be developed by multiplying the number of tumours by the average tumour volume (1/2 mean diameter $3 \times 4/3\ \pi$) in mm^3.
(4) All procedures, painting with carcinogen and observations can be carried out without anaesthesia. Oral administration of micronutrients or other agents can also be carried out without anaesthesia. Thus very few animals are lost during the experimental periods.
(5) The epidermoid carcinomas of the hamster buccal pouch are indistinguishable from human oral epidermoid carcinomas of the well-to-moderately differentiated variety at both light microscopic and ultrastructural levels of study [12].
(6) Both hamster and human lesions have similar metabolic markers such as the enzyme gamma glutamyl transpeptidase (GGT) [13–14]. Increased levels of lactic dehydrogenase are found in both hamster and human oral cancers, indicating an alteration from aerobic respiration to anaerobic glycolysis [15]. Increased levels of

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TFG alpha are also found in both hamster and human cancer [16, 17].

(7) The hamster carcinoma develops with the activation and expression of the c-*erb*-B1 oncogene [18, 19] and many human oral cancers express the same oncogene [20]. Increased expression of H *ras* and K *ras* also occur in both the animal model and human oral cancer [21, 22].

(8) The hamster carcinogenesis model is under immune control as in human cancer. The tumours develop more rapidly when the animal's immune system is depressed by immunosuppressive drugs such as cortisone [23] or methotrexate [24] or by specific antilymphocyte serum [25, 26].

(9) The hamster buccal pouch can be used as a site for the transplantation of tumour cells, but only if the animal is appropriately immunosuppressed [27].

Tumour development can be delayed by immunoenhancing agents such as BCG [28] or levamisole [29, 30]. The outdated and erroneous concept of the hamster buccal pouch as an "immunologically privileged" site [31] can now be rejected in the light of this research and further investigations showing the depression or modification of activity of Langerhans cells, macrophages, T lymphocytes and other immune cells during buccal pouch carcinogenesis [32]. Langerhans cells, with Fc receptors and Ia cell surface antigens, are macrophage equivalents associated with antigen processing, delayed hypersensitivity and interaction of immunological reactions [33]. They are normally present in hamster buccal pouch [34]. Antoniades and associates [35] have also shown that during DMBA carcinogenesis, there was an inhibitory effect on peritoneal derived macrophages and their cytolysis of tumour target cells. There was also a reduced number of monocytes in the tumour bearing hamsters.

Recently the hamster cheek pouch model has been used for the study of changes in keratin expression during carcinogenesis [36] and various metabolic pathways [37].

The ultimate goal of studies on buccal pouch carcinogenesis has always been the development of agents that could regress the carcinomas, once established, or prevent their development. Antimetabolites, such as methotrexate or azathioprine, could induce tumour regression, but were highly toxic and strongly immunosuppressive [38]. Many animals died during these experiments. Non-toxic agents were sought that could exert a potent anticancer effect. To date, studies have been carried out with retinoids, carotenoids, tocopherol and glutathione.

RETINOIDS

Chu and Malmgren first reported in 1965 that vitamin A had an inhibitory effect on the development of tumours in the fore-stomach and cervix of the Syrian hamster [39]. Bollag found that retinoic acid could inhibit the development of chemically induced papillomas and carcinomas of mouse skin [40]. Sporn and associates found that a synthetic retinoid, 13-*cis*-retinoic acid, inhibited bladder carcinogenesis in the rat [41].

Based on this research, Shklar and associates found that 13-*cis*-retinoic acid significantly inhibited the development of carcinomas in the hamster buccal pouch model [42]. This finding was also confirmed in the rat tongue carcinoma model

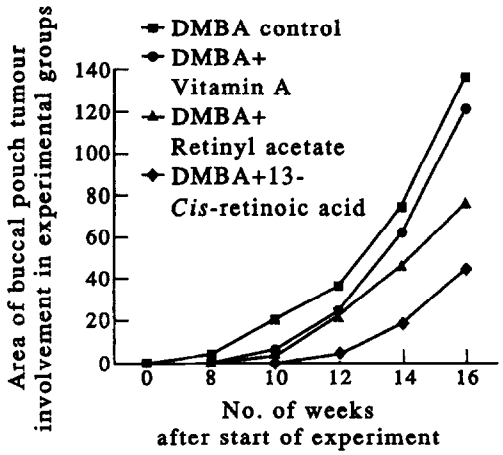

Fig. 1. Retinoids and oral carcinogenesis. Experiments demonstrating the effectiveness of oral administration of natural Vitamin A, retinyl acetate and 13-*cis*-retinoic acid in oral carcinogenesis inhibition. The synthetic retinoid is the most effective of the three.

[43, 44]. Retinyl acetate was also found to delay carcinogenesis in the hamster buccal pouch model (Fig. 1) even after the precancerous leukoplakia had developed [45]. Sonis and Shklar demonstrated that 13-*cis*-retinoic acid enhanced cell mediated immunity in the hamster and this could counter the immunodepression induced by developing tumours [46]. While these investigations demonstrated a significant anticancer effect of 13-*cis*-retinoic acid, there was considerable toxicity, when the dose level of the retinoid was increased for greater antitumour activity. Degeneratic alterations in both liver and kidney were noted and there were many deaths among the experimental and control animals receiving the drug [47].

While human trials have been carried out with 13-*cis*-retinoic acid on oral leukoplakia, the success of the drug in reversing the precancerous pathology was somewhat mitigated by its toxicity. The onset and severity of toxic effects was found, by Hong and associates, to vary considerably according to the dose of the retinoic acid [48]. This retinoid was also found to be capable of preventing secondary primary tumours in patients who had squamous cell carcinomas of the head and neck [49]. Retinoids appear to exert their anticancer activity by their major activity on cell differentiation [50] and by acting to stimulate immune cells that are depressed during carcinogenesis [51].

CAROTENOIDS

Carotenoids are far less toxic than retinoids and both beta carotene and canthaxanthin were shown to have anticancer activity by Matthews-Roth [52] in 1982. Schwartz *et al.* demonstrated that beta carotene could regress chemically induced epidermoid carcinomas of hamster buccal pouch (Figs 2–4) and that macrophages rich in tumour necrosis factor alpha appeared to be associated with the regressing tumours [53]. Suda *et al.* followed up this initial observation on oral cancer by demonstrating an inhibition of experimental oral carcinogenesis by the topical application of beta carotene [54]. The inhibition of cancer development was found to be effective during both initiation and promotion phases of carcinogenesis [54]. Beta carotene was also found to reduce the activity of gamma glutamyl transpeptidase (GGT) during the inhibition

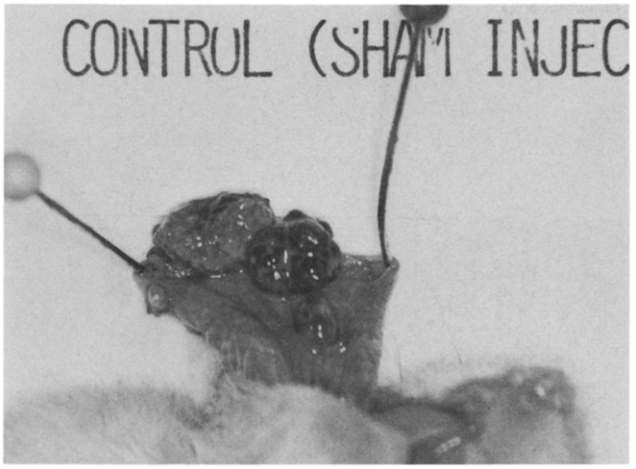

Fig. 2. Established epidermoid carcinoma of hamster buccal pouch.

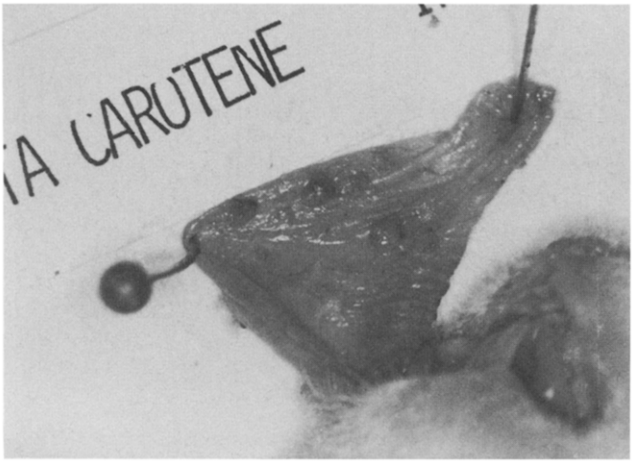

Fig. 3. Regression after 2 weeks of injections of beta carotene into pouch.

of tumour development [55], suggesting that beta carotene was acting to depress some of the metabolic pathways involved in carcinogenesis. Solt had previously demonstrated the localisation of GGT in areas of hamster buccal pouch mucosa undergoing malignant transformation [56] and suggested this as a histochemical marker similar to that disclosed in other well-studied carcinogenesis models [57]. Since beta carotene may be partially metabolised to vitamin A, buccal pouch carcinogenesis was studied with another carotenoid, canthaxanthin, which does not convert to vitamin A. A similar regression of oral carcinogenesis was found, following injection of canthaxanthin into the local sites of developing tumours [58]. The studies with canthaxanthin indicated that the potent anticancer activity of beta carotene was primarily carotenoid, rather than a retinoid effect due to conversion of beta carotene to vitamin A. Furthermore, vitamin A does not have this potent effect on experimental oral carcinomas.

The mechanism of beta carotene's anticancer activity is being explored, using the techniques of immunology and molecular biology. A notable enhancement of immune response is achieved by the administration of beta carotene during oral carcinogenesis [59–61] involving cytotoxic T-lymphocytes and macrophages rich in tumour necrosis factor

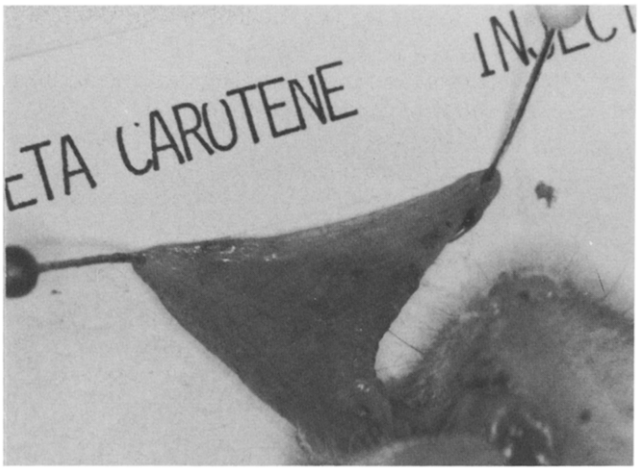

Fig. 4. Regression after 4 weeks of injection of beta carotene.

alpha. The effect of beta carotene and canthaxanthin on the immune response has also been shown by Bendich and Shapiro in the rat [62].

In addition to immunostimulation, there is a direct effect of beta carotene on tumour cells. Schwartz and associates with the use of liposomes, have shown that beta carotene penetrates into cancer cells and this is a selective effect, since normal epithelial cells are unaffected. Using electron microscopy the beta carotene liposomes can be seen within the experimental cancer cells and result in the destruction of nuclei as well as organelles [63]. The cancer cells appear to have a "leaky" membrane allowing the beta carotene liposomes to enter. Schwartz and associates have also found that beta carotene induces a 70 kD protein within the cancer cells. It appears to be a "heat shock" protein and is associated with the cytotoxic activity of beta carotene [64].

VITAMIN E (ALPHA TOCOPHEROL)

Vitamin E has a very low toxicity [65] or can be said to be non-toxic, since even massive daily doses are not lethal to experimental animals. Vitamin E has a potent anticancer action. This was first demonstrated in 1969 by Harman [66] in experimental animals. A significant inhibitory effect on oral cancer was demonstrated in the hamster pouch model by Shklar in 1982. The vitamin E was administered systemically by oral route in these initial experiments [67]. The development of the experimental oral carcinomas could also be retarded by the topical application of vitamin E on days alternate to the carcinogen application [68]. Using a carcinogen less potent than the standard 0.5% solution (0.1%) it was possible to demonstrate that vitamin E could completely prevent the development of the carcinomas (Figs 5–8) [69]. Vitamin E was also shown to be capable of regressing established carcinomas of hamster buccal pouch when injected close to the tumour site [70]. However, as with beta carotene, tumour regression could not be accomplished by oral administration of vitamin E. The mechanism of vitamin E prevention is an immunoenhancement similar to that induced by beta carotene [71] with the migration to initial tumour foci or dysplastic lesions by cytotoxic lymphocytes and cytotoxic macrophages with tumour necrosis factor alpha. The tumour necrosis factor-laden macrophages are also found at the tumour sites during cancer regression induced by injected vitamin E [72, 73]. Vitamin E, as a very potent antioxidant,

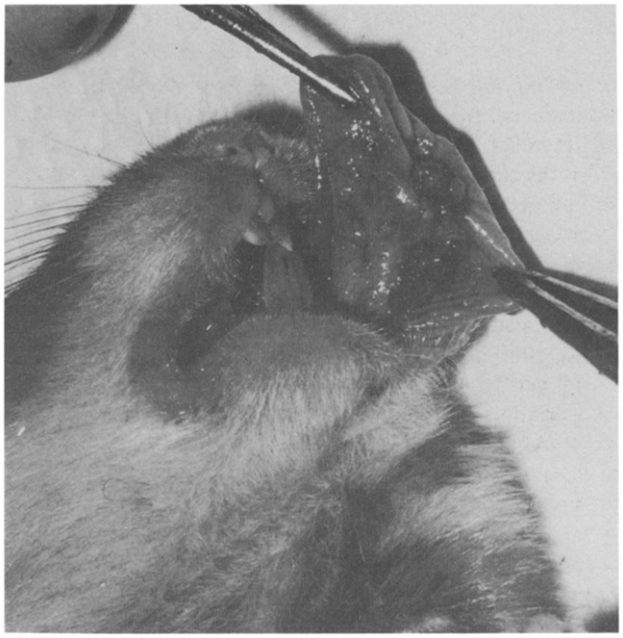

Fig. 5. Large cancer after 30 weeks of DMBA applications (0.1% solution thrice weekly), to hamster buccal pouch.

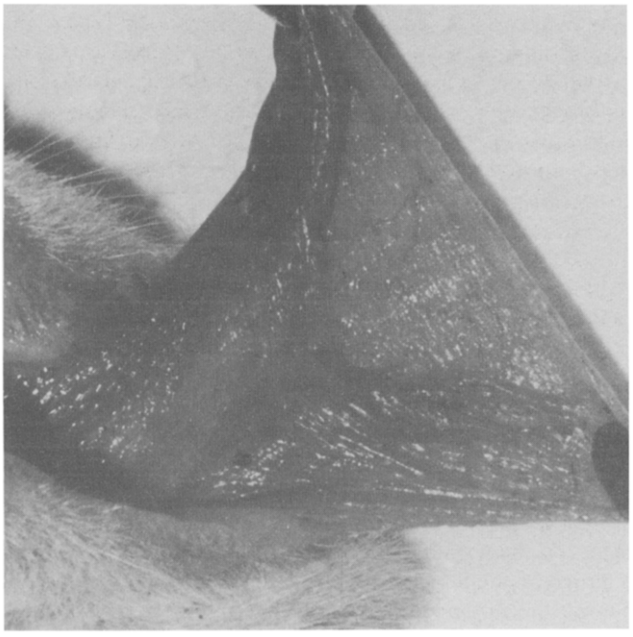

Fig. 6. Prevention of tumours by oral administration of vitamin E. Carcinogen was applied as in the control animals in Fig. 5.

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 [74].

COMBINATIONS OF MICRONUTRIENTS

Vitamin E and beta carotene have been found to be synergistic in their anticancer effect [75]. While vitamin E or beta carotene, separately, are not capable of regressing

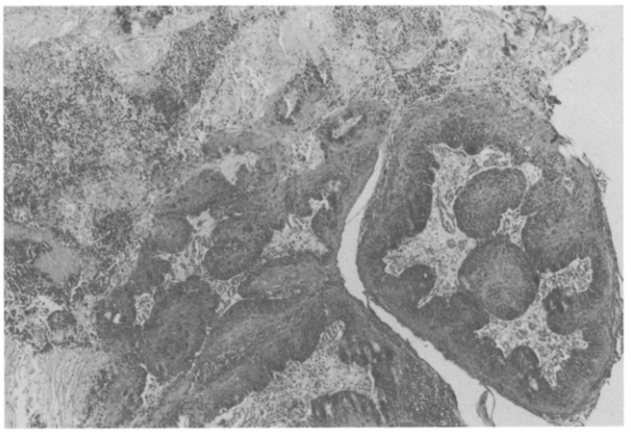

Fig. 7. Microscopic features of cancer in Fig. 5, an epidermoid carcinoma with ulceration and inflammation.

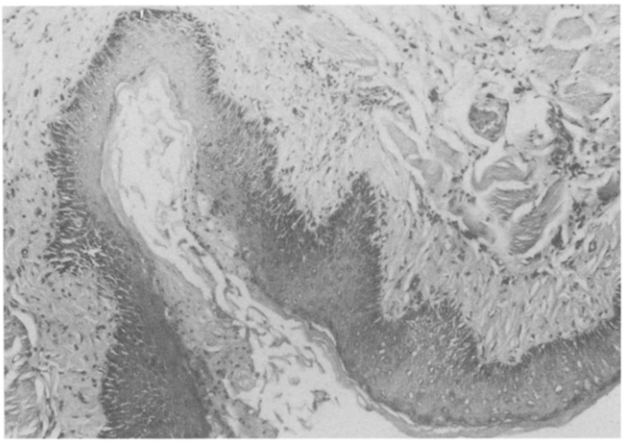

Fig. 8. Microscopic features of pouch mucosa in Fig. 5. There is hyperkeratosis and some dysplasia but no tumour.

established oral cancer of hamster buccal pouch when given orally on a daily basis, they are capable of cancer regression when combined and given orally on a daily basis. This was the first demonstration that an orally administered mixture of vitamin E and beta carotene could regress established cancers (Fig. 9). An explanation may be that while both these

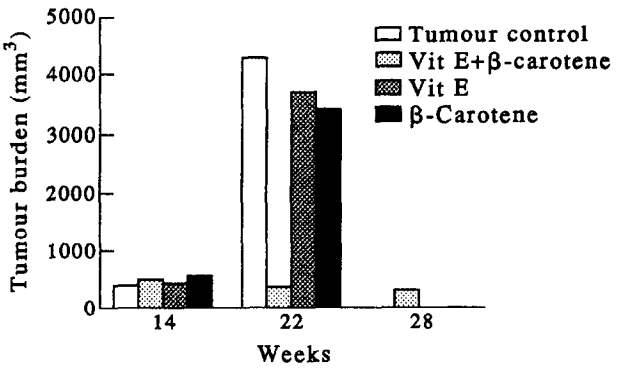

Fig. 9. Tumour burden 14–28 weeks. The effect of oral administration of combined beta carotene and vitamin E in the regression of established oral cancers of hamster pouch (14 weeks). Beta carotene and vitamin E separately do not regress the tumours and those animals were killed at 22 weeks because of the very large size of the tumours.

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.

NATURAL FOODS

An extract of *Spirulina* and *Dunaliella* algae was found to be a potent inhibitor of hamster buccal pouch carcinogenesis. This extract contains beta carotene and other carotenoids as well as vitamin E. The mixture was found to be more effective than beta carotene alone [76]. Onion extract has also been shown to retard experimental oral carcinogenesis [11, 77], but the anticancer constituent or constituents of onion have not yet been established. Garlic extract has also been shown to inhibit oral carcinogenesis [78].

BETA CAROTENE AND VITAMIN E IN HUMAN STUDIES

Several human studies have confirmed the animal studies with beta carotene and vitamin E as anticancer or cancer preventive agents. Menkes and associates [79], 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 [80] found an inverse relationship between plasma levels of beta carotene and alpha tocopherol and dysplasia and cancer of uterine cervix. Remission of oral leukoplakia has been reported with the use of retinoids [48] and carotenoids [81] Garewal and Meyskens have carried out a critical appraisal of the use of retinoids and carotenoids in the prevention of oral cancer by the management of oral precancerous leukoplakia [82]. Both beta carotene and vitamin E have been found to be cytotoxic to a variety of human cancer cells in tissue culture, including several lines of oral epidermoid carcinoma, breast carcinoma, lung carcinoma and malignant melanoma. Furthermore, the cytotoxic effect on cancer was selective, with normal control cells for each cancer line showing no effect [83]. Initial studies for the cancer cytotoxicity of the nutrients were carried out on a hamster oral carcinoma cell line derived from a DMBA-induced carcinoma of hamster buccal pouch [84].

The experimental and epidemiological basis is now well established for human trials on the effect of combination of beta carotene and vitamin E on the prevention and regression of human cancer (particularly oral cancer) and precancerous states (particularly oral leukoplakia).

BETA CAROTENE, VITAMIN E AND CANCER CHEMOTHERAPY DRUGS

Synergism has been demonstrated between beta carotene and anticancer alkylating agents such as melphalan, BCNU, and cyclophosphamide. Beta carotene also enhances cisplatin cytotoxicity toward SCC-25 oral cancer cells. Vitamin E was found to enhance the cytotoxicity of melphalan but not cisplatin. Beta carotene was found to be selectively cytotoxic toward normally oxygenated cells and at normal pH. Vitamin E was selectively cytotoxic toward both normally oxygenated cells as well as hypoxic cells [85].

MECHANISMS OF ANTICANCER ACTIVITY OF RETINOIDS, CAROTENOIDS, AND TOCOPHEROLS

Antioxidant and pro-oxidant control of tumour cell growth

Ultrastructural and *in vivo* observations have demonstrated that retinoids and carotenoids, such as β-carotene can alter the morphology of target cells (86–88). Following the treatment of tumour cells with the carotenoid, β-carotene, or retinoids, there are dose-dependent changes in the cell membranes and organelles of the treated tumour cells. Retinoid treatment, in general, increases differentiation and attachment of tumour cells or transformed cells *in vivo* or *in vitro*, perhaps through the induction of extracellular matrix proteins, such as laminin [89]. β-carotene at low doses also produces an increased differentiation of cells in culture and is associated with an increased gap junction communication, and the expression of the gap junctional protein connexin-43 [90]. Higher doses of β-carotene, result in a loss of pseudopodia, and cell attachments, associated with the reduction of the extracellular matrix protein, fibronectin [88]. β-carotene was also found to produce a swelling of mitochondria, and endoplasmic reticulum, increased vacuolisation, and chromatic clumping. These changes are consistent with the triggering of programmed cell death which has been noted following retinoid treatment [91].

Experimental data suggests that there may be common pathways involved in the control of tumour cell growth. The carotenoid β-carotene, is carried in the serum with low density lipoproteins [92]. Unlike the retinoids, or tocopherols, the carotenoids do not appear to have nuclear binding proteins or membrane binding proteins to facilitate their uptake and biological function [93]. Phase and electronmicrographic analysis of liposomes and micellar constructs of β-carotene have indicated that β-carotene is taken into the cell through endocytosis and vacuolarisation [93].

Although retinoids, carotenoids, and tocopherols probably all modify membrane phospholipid metabolism because of their intrinsic hydrophobicity, the evidence indicates that there are some differences in cellular responses.

We suggest that β-carotene becomes a reactive pro-oxidant molecule, signified by the reduction in the activity of superoxide dismutase, glutathione-*S*-transferase, and non-protein sulphydryls [94]. Retinoids have not been shown to induce these changes. β-carotene has been shown to act as a pro-oxidant and become an auto-oxidiser at high partial pressures of oxygen [94]. This type of oxygen environment could be associated with malignant transformation [95]. At lower partial pressures of oxygen associated with early transformation or normal cells, β-carotene will act as an antioxidant and an inhibitor of peroxidation [96]. In contrast, vitamin E, is a strong antioxidant and inhibitor of peroxidation at high oxygen pressures [97]. β-carotene could also inhibit early malignant transformation, as an antioxidant and peroxidation inhibitor. The antioxidant cellular response would be distinguished by increased cellular communication and differentiation [90]. Once the transformation process has progressed towards completion (promotion), or is fully completed, β-carotene, at relatively higher concentrations, in its pro-oxidant form, would be required to inhibit tumour cell growth. The significance attributed to these observations, has been the determination that β-carotene and alpha tocopherol can function synergistically, enhancing their capacity to inhibit tumour cells in hypoxic environments [75].

The cellular signal for the pro-oxidant effect of β-carotene was found to be a 70 kD heat shock protein (hsp) [64]. Additional assays reversing the β-carotene induced suppression of tumour cell growth required the use of the iron chelator, o-phenoanthroline [98]. It can therefore be suggested that reactive β-carotene would bind to membrane associated enzymes that possess an iron binding site and in its pro-oxidant state produce an oxidised protein. The oxidised proteins would bind to hsp 70 and trigger the enhanced expression of hsp 70 through a protein-hsp complex [99, 100]. The enhanced expression of the hsp 70 gene has been linked to other genes such as tumour necrosis factor (TNF) [101]. β-carotene and vitamin E stimulated an enhanced expression of TNF in macrophages and T-lymphocytes [71]. Hsp 70 has also been identified with p53 and been shown to bind to mutant p53, stabilising and increasing the half life of the dominant negative control of the mutant over the tumour suppressor (wild type) p53 [102, 103]. Following treatment with β-carotene hsp 70 expression was associated with the suppression of tumour cell growth, while tumour cells (oral carcinoma, SCC-25) accumulated in G_1 of their cell cycle [104]. An enhanced expression of wild type p53, with a decreased expression of the mutant form have been found to be stimulated by both β-carotene and vitamin E. Both hsp 70 and p53 have been designated signals of programmed cell death, and are expressed predominantly during G_1 [105, 106].

Another genetic response produced by β-carotene is suggested by Stich and colleagues. They have shown a decreased genotoxic effect on the DNA and the reduction in micronuclei in oral mucosal cells, perhaps indicating an increase in DNA repair [81]. During the inhibition of oral carcinogenesis with β-carotene, Hibino *et al.* have shown a reduction of polyamine levels, perhaps indicating a reduction in DNA synthesis [107]. Alpha tocopherol has also shown the ability to decrease sister chromatid exchanges resulting from oxygen radicals, while retinoic acid may also decrease DNA cleavage in response to X-radiation or bleomycin treatment [108, 109].

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