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Reviews

Chemoprevention of Oral Carcinogenesis

Takuji Tanaka

INTRODUCTION

CANCER, a malignant epithelial neoplasm, is a collective term for at least a hundred different diseases. These can be quite unique from the point of view of cause, histologic appearances, symptoms and prognosis. However, several common characteristics can be seen: (a) cancer cells are derived from the body's own cells; (b) cancer cells generally continue to grow in an unlimited fashion, at least when they have reached a size that leads to their being diagnosed; (c) they often infiltrate, i.e. they grow into and destroy the surrounding normal tissue; and (d) they can spread via blood and/or lymphatic vessels and cause secondary growths—known as metastases—in other organs. In general, malignant neoplasms in humans and rodents develop through multistep processes [1]. During such processes, several genetic alterations occur [2]. These have been clearly indicated by the findings in many rodent and human carcinogenesis studies.

In the oral cavity, the most frequent cancers are histologically squamous cell carcinomas. Their incidence varies from region to region in the world [3]. Some of the highest rates are reported to be in developing countries, particularly in southern Asia (India, Sri Lanka, South Vietnam, Papua New Guinea or the Philippines), China and parts of Brazil [4–7]. In the southern Asian countries, up to 25% of all malignancies originate in the oral cavity [3, 6, 7] and tobacco and betel nut chewing are responsible for this malignancy [8]. In Europe, France has the highest incidence of oral and pharyngeal cancer. This variation in incidence is related to exposure to known aetiological agents [9]. There have been recent increases in some types of oral cancer in Scandinavia, the United States, and Scotland, especially among younger males with increased use of chewing or smokeless tobacco being implicated as a potential cause [5, 10]. In the United States there are approximately 43 000 new cases annually, resulting in about 11 600 deaths [11]. Although Japan has one of the lowest incidences of oral and pharyngeal cancer in the world, the patients with these malignancies have been increasing, accounting for 4900 new cases and 1825 deaths in 1980, and 11 000 new cases and 2607 deaths in 1990 [12, 13]. Epidemiological data provided strong support for exogenous factors such as tobacco and alcohol use as being major causative agents [14, 15]. There is increased risk for those whose occupation is related to the liquor trade [16], although there is no clear indication as to the differential effects of various forms of alcohol consumption [9, 17]. Ethanol is reported to act as a promoter in hamster oral carcinogenesis, in association with lipid peroxidation [18]. Viral infection [19, 20] and contaminants such as nitrosamines [21], polycyclic hydrocarbons [22] or urethanes [23] are also suggested to be causative factors. In oral carcinogenesis, the precursor lesions are considered to be leukoplakia and/or dysplasia [24–26]. It has been reported that patients with oral cancer have an increased incidence of second primary tumours of oral cavity [27–30]. In fact, patients with early lesions have a high cure rate of their primary tumours, but go on to succumb to the second malignancy. Approximately 10–40% of such patients will develop second primary tumours, a rate that is related to continued exposure to carcinogens and/or promoters [31]. This is considered to be the result of a diffuse mucosal abnormality, often referred as "field cancerisation" [32]. Thus, oral cancer is a multifocal disease and experimental studies indicated that such lesions develop through a multistage process [26, 33]. Because of easy accessibility for examination and follow-up of the lesions in the oral cavity, oral cancer is an excellent target organ for clinical and experimental chemoprevention studies. Cancer chemoprevention is defined as intervention with chemical agents before invasion to halt or slow the carcinogenic process. It is clear that the most convincing approach to proving efficacy of a chemopreventive agent would be to conduct a clinical trial that shows reduction in the frequency of the malignancy of a target organ in an intervention group versus a control group, based on a body of evidence from a variety of other types of studies in order to draw conclusions pertaining to cancer preventive activity. Such a study includes epidemiological data, laboratory evidence and animal models.

The purpose of this article is to provide a balanced review of the importance of several factors that influence the incidence of human oral cancer, potential mechanisms of its development and cancer chemoprevention by natural and synthetic chemicals against oral cancer that may help to reduce its progression in the high-risk and general population, with emphasis on recent clinical intervention trials.

AETIOLOGICAL FACTORS FOR ORAL CANCER

The factors which confer increased risk can be categorised as lifestyle, occupational exposure, disease state and genetic susceptibility. Oral cavity cancer has been related to both pipe and cigarette smoking and tobacco chewing, and there is a

Correspondence to T. Tanaka at the 1st Department of Pathology, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu City 500, Japan.
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synergistic effect with alcohol consumption [9, 15, 34]. An additional lifestyle factor which influences the development of oral cancer is diet. The risk for oral cancer is increased with increasing meat and animal fat consumption [35]. Also, the use of red chilli powder has been reported as a risk factor for upper aerodigestive tract cancers, including oral cancer [36]. As is true for several other malignancies, a low intake of vegetables and fruits has been associated with increased risk for head and neck cancers, including that of the oral cavity [35, 37–43]. Thus, dietary factors affect the occurrence of oral cancer: there is an inverse relationship between fruit, vegetable or fish intake and oral cancer [35, 36, 41]. Carotenoid, vitamin E, vitamin C and/or fibre in fruits or vegetables may have a protective effect on oral malignancy, although correlations with individual nutrients are difficult from epidemiological trials [31, 42, 44–46]. Carcinogens (nitrosamines, polycyclic hydrocarbons [22] and urethanes) are also major aetiological factors for oral cancer [21–23]. Other factors like sunlight (for lip cancer), tobacco (smoking and chewing), alcohol, dental factors (dental sepsis, poor oral hygiene, and chronic irritation), nutritional deficiencies (iron or vitamin A deficiency) and infections (candida, syphilis, herpes simplex virus, human papilloma virus and human immunodeficiency virus) may play some role in oral carcinogenesis [15, 47, 48]. Also, yeasts play a causal role in oral cancer by means of endogenous nitrosamine production [49]. The use of marijuana is also of concern [50]. Thus, both intrinsic and extrinsic factors could work together in an interrelated way to produce oral malignancies.

BIOLOGICAL ASPECTS OF ORAL CANCER DEVELOPMENT

In oral carcinogenesis in both humans and animals, sequential pathological alterations from hyperplasia through dysplasia to neoplasms (benign and malignant) can be observed [26, 51]. During hamster oral carcinogenesis, γ-glutamyltranspeptidase (GGT) that is normally not expressed in the buccal pouch is histochemically detectable in buccal pouch epithelium [52–54]. Similar findings were observed in the 4-nitroquinoline 1-oxide(4-NQO)-model in rats (Tanaka T, unpublished data). Such phenotypic alteration is also found in dysplastic and neoplastic lesions. Unlike in mouse skin carcinogenesis [55], GGT expression in the buccal pouch can be detected at a very early stage [as early as 3 days after the first dosing of 7,12-dimethylbenz(a)anthracene (DMBA)] in hamster buccal pouch carcinogenesis. GGT is a presumptive tumour marker in hamster buccal pouch carcinogenesis and has been found to be increased during preneoplastic stages of carcinogenesis but drop sharply with formation of overt neoplasms [56]. On the other hand, recent work by Zhang demonstrated that glutathione S-transferase placental form (GST-P) is another tumour marker and showed that, with continuous carcinogen (DMBA) application, a progressive increase in the number and size of GST-P-stained foci was present [57]. Such findings were also seen in the 4-NQO-model (Tanaka T, unpublished data). All the tumours were positive for GST-P staining, but only 62.5% of tumours stained for GGT. Such phenotypic alterations are important in detecting those preneoplastic lesions which have the greatest propensity to progress to carcinomas and in screening for different carcinogens, promoters and progressors. In addition, epidermal growth factor (EGF) receptor and transglutaminase type I, polyamine (putrescine, spermidine and spermine) levels, ornithine decarboxylase (ODC) activity and micronuclei incidence were increased during the carcinogenic process in the hamster model [58]. Recently, Zenklusen *et al.* have reported the expression of transforming growth factor-β1 in Syrian hamster cheek pouch carcinogenesis using immunohistochemical methods and northern blot analysis [59]. They speculated that the expression of TGF-β1 seems to have two formal stages—the first being an overexpression step as a reaction to the uncontrolled growth, the second being a step in which tumours have no internal expression of TGF-β1, but in which external protein accumulates in the surrounding stroma. The normal pattern of keratin in cheek pouch epithelium is consistent with that of human oral mucosa. The human oral premalignant lesions express K1 keratin (67 kD) [60]. This may suggest that such lesions may be a necessary step in the pathway to squamous cell carcinoma development. Immunohistochemical and immunoblotting patterns of two differentiation-associated keratins, K1 and K13 (47 kD) and a proliferation-associated keratin K14 (55 kD) during pouch carcinogenesis were as follows: although K1, which is not normally expressed in internal epithelium, was present in most hyperplastic stages, it was essentially absent in the most dysplastic areas as well as carcinomas, except for small foci in well-differentiated squamous cell carcinoma [61]. Normal expression of K13 was preserved during all stages of the DMBA complete carcinogenesis protocol. Expression of the proliferation-associated keratin K14 was topographically altered during the process of carcinogenesis. Nucleolar organiser regions (NORs) that reflect the nucleolar activity of cells (rDNA transcription) were also altered during the carcinogenesis process [26]. Their number per nucleus increases with increases in atypia and with progression of tumour development. AgNORs enumeration is considered a useful estimate for the development of oral cancer [26].

Expression of several cancer-related genes at different stages of DMBA-induced tumour development in the hamster cheek pouch model have also been described. These include c-Ha-*ras* and c-*erb*B. Overexpression of the c-Ha-*ras* gene was observed at a very early stage of tumour development, while expression of c-*erb*B was detected after 8–10 weeks of DMBA exposure and increased with progression of the disease [62, 63]. The c-*erb*B proto-oncogene that is the cellular gene coding for EGF-R has been found to be overexpressed in DMBA-treated pouch epithelium and in tumours [64]. Moreover, this gene appears to be amplified in cultured cell lines derived from DMBA-induced squamous cell carcinoma [65]. Expression of c-*myc* and c-*sis* was detected at control levels, while c-*fos* gene activity could not be detected at any stage of carcinogenesis. Thus, it is considered that imcreased expression of the *ras* gene is correlated with the initial transformation of hamster cheek pouch epithelium, whereas activation of the c-*erb*B gene is correlated with the extensive proliferative as well as malignant phenotype of these epithelial cells in the intact animal [66]. Mutated Ha-*ras* alleles, that is an A to T transversion in the second position of codon 61, resulting in an amino acid change from glycine to leucine have been observed [63]. p53 mutations are also suspected to play a role in the hamster model [67]. In humans, certain premalignant oral lesions are described. These include erythroplasia, submucous fibrosis, erosive lichen planus, discoid lupus erythematosus, dyskeratosis congenita, syphilis and candidal leukoplakia [24, 25, 68, 69]. Several studies such as those of the expression of supurabasal keratin-19 (K19), blood group substances, β2

microglobulin, human leukocyte antigen (HLA), growth factor receptor and oncogenes will help to determine which premalignant lesions will progress to malignancies [70–75]. The expressions of EGF-R and *ras* 21 in squamous cell carcinoma of the oral cavity were recently reported [76, 77]. Using the chromosome *in situ* hybridisation, premalignant oral lesions (leukoplakia; histological hyperplasia or dysplasia) have chromosomal alterations (chromosomes 7 and 17), the extent of which appeared to be associated with the degree of malignant transformation [78, 79]. Changes in both oncogenes and tumour-suppressor genes has been identified during the course of oral cancer development [80, 81]. As in most other tumours, expression of mutated or phenotypically altered p53 is a common occurrence in head and neck carcinogenesis, including oral carcinoma [82, 83]. A recent report indicates that aberrant p53 occurs very early, being detectable in epithelium at a considerable distance from the primary tumours and preceding signs of overt neoplasia [84]. Such evidence shows that at both the protein and the DNA levels, multifocal and discontinuous changes in p53 provide the molecular basis for a multifocal development of multiple tumours and second primary cancers in head and neck regions [85, 86]. Also, there is evidence from several studies on spontaneous, as well as experimentally-induced malignant and premalignant epithelial lesions, that neoplastic and dysplastic changes, but not reversible pathological processes such as gingivitis, are associated with a neo-expression of simple epithelial-type cytokeratins (cytokeratins 8 and 18) [87]. They also postulated that the p53 immunopositivity, together with the expression of the histone H3 gene and of at least one of the simple epithelial cytokeratins, represents a significant change of these cells towards neoplasia. Recent work by Maestro *et al.* [88] indicated that at least three oncosuppressor genes mapping on 3p (3p24-ter, 3p21.3 and 3p14-cen) may be involved in head and neck cancer development and gives support for a common oncogenic pathway with squamous cell lung cancer, in which a similar pattern of 3p deletion has been described recently.

ORAL CANCER INHIBITION BY MICRONUTRIENT, NON-NUTRIENTS AND SYNTHETIC CHEMICALS: EXPERIMENTAL EVIDENCE

Until recently, cancer prevention has consisted of attempts to eliminate carcinogenic substances and to detect and remove precancerous lesions. However, with increasing knowledge of the events involved in carcinogenesis and the factors that modulate the carcinogenesis processes (initiation, promotion, conversion and progression), efforts have increasingly focused on interrupting and/or reversing the neoplastic process. Such attempts at cancer chemoprevention or the reversal of carcinogenesis in the premalignant phase have derived from the study by Sporn *et al.* [89]. Since then a number of chemopreventive agents have been introduced from *in vitro* and/or *in vivo* animal studies. These include natural or synthetic compounds, micronutrients or non-nutrients. Such chemopreventives can broadly be classified into two categories: (i) blocking agents that inhibit tumour initiation; and (ii) suppressing agents that inhibit tumour promotion and/or progression [90]. Recently, Morse and Stoner have further differentiated both blocking agents and suppressing agents, based on mechanistic principles [91]. Among them, several chemopreventives in oral carcinogenesis have been identified (Tables 1 and 2) based on epidemiological and experimental data [35–37, 41–45, 92, 93].

(*a*) *Micronutrients*

Micronutrients including vitamin A have been reported to possess suppressing potency in oral tumorigenesis in rodents (Table 1). Among the chemopreventives, retinoids are the best studied compounds. Vitamin A (or retinol) has physiological roles in growth, epithelial differentiation and proliferation, in addition to vision and reproduction. It is ingested in milk, eggs and meat in the form of retinol, retinaldehyde and their esters and in plants in the form of carotenoids. Vitamin A is stored in the liver while the unmetabolised carotenoids go to fat and other tissues. Deprivation of vitamin A from a decreased intake of vegetables has been linked with a 2-fold increase in oral, pharyngeal and laryngeal cancers [38], whereas a high retinol intake is associated with a 50–60% decrease in the incidence of laryngeal and tongue cancer [94]. Plasma vitamin A and carotene levels in patients with cancer of the oral cavity and oro-pharynx were found to be low compared to controls [95], suggesting that vitamin A deficiency may act as a promoting factor. Retinol deficiency resulted in hyperkeratatosis and hyperplasia of the oral mucosa, similar to the premalignant condition caused by chemical carcinogenesis in animal models [96]. Extensive work by Shklar's group has found a significant anticancer effect of 13-*cis*-retinoic acid using the hamster buccal pouch model and the rat tongue carcinogenesis model [97–99]. Retinyl acetate could also delay carcinogenesis, even after the leukoplakia had developed [100]. Recent work using the 4-NQO-induced rat tongue carcinogenesis model has demonstrated that dietary administration of vitamin A (0.1 g, 100 000 IU/kg) diet during carcinogen administration (4 or 7 months) decreased cancer development [101]. In humans who are tobacco chewers, vitamin A or β-carotene caused remission of precancerous lesions (leukoplakia) in the oral cavity [102]. 13-*cis*-retinoid was also found to be capable of preventing secondary primary tumours in patients who had squamous cell carcinomas of the head and neck [103]. How retinoids modulate carcinogenesis, differentiation and proliferation is not fully understood, but there is strong evidence of direct interaction at the transcription level of gene expression, as reviewed by several authors [104–106]. The retinoid molecules bind to a specific cytosolic receptor protein (cellular retinol or retinoic acid-binding protein, CRBP or CRABP) that exists in normal tissues including oral mucosa, and such proteins transport the retinoid to the nucleus. Nuclear retinoic acid receptors (RAR-α,β,γ) complex with the retinoids and then undergo a conformational change that allows binding to DNA sequences that can induce or suppress gene transcription by transactivation [107]. Other mechanisms by which retinoids act as chemopreventives include their ability to induce cell differentiation, inhibit cell proliferation and cause immunomodulation. The observed effects of retinoids on cell proliferation and differentiation are based mainly on investigations using human transformed tumour cell lines. 13-*cis*-retinoic acid enhanced cell-mediated immunity in the hamsters and this could counter the immunodepression induced by developing tumours [108]. Thus, several retinoids (a class of chemical compounds structurally related to vitamin A and comprised of natural and synthetic analogues) have suppressing effect on chemically-

Table 1. Natural chemopreventive compounds in oral carcinogenesis

Agent	Species	Carcinogen	Author	Reference
Retinoids (natural or synthetic)	Hamster	DMBA	Shklar *et al.* 1980	[97]
	Hamster	DMBA	Burge-Bottenbley and Shklar 1983	[100]
	Hamster	DMBA	Goodwin *et al.* 1986	[188]
	Hamster	DMBA	Gijare *et al.* 1990	[147]
	Rat	4-NQO	Inoue *et al.* 1993	[101]
β-Carotene	Hamster	DMBA	Mathews-Roth 1982	[126]
	Hamster	DMBA	Suda *et al.* 1986	[128]
	Hamster	DMBA	Suda *et al.* 1987	[129]
	Hamster	DMBA	Schwartz and Shklar 1988	[136]
	Hamster	DMBA	Gijare *et al.* 1990	[147]
	Rat	4-NQO	Tanaka *et al.* 1994	[172]
Vitamin E	Hamster	DMBA	Shklar 1982	[150]
	Hamster	DMBA	Okukoya *et al.* 1984	[151]
	Hamster	DMBA	Shklar *et al.* 1987	[153]
	Hamster	DMBA	Trickler and Shklar 1987	[152]
	Hamster	DMBA	Shklar and Schwartz 1988	[130]
Selenium	Hamster	DMBA	Goodwin *et al.* 1986	[188]
	Rat	4-NQO	Inoue *et al.* 1993	[101]
Canthaxanthin	Hamster	DMBA	Mathews-Roth 1982	[126]
	Hamster	DMBA	Shklar and Schwartz 1988	[130]
	Hamster	DMBA	Schwartz and Shklar 1988	[136]
	Rat	4-NQO	Tanaka *et al.* 1994	[135]
Astaxanthin	Rat	4-NQO	Tanaka *et al.* 1994	[135]
Phytoene	Hamster	DMBA	Mathews-Roth 1982	[126]
Spirulina dunaliella	Hamster	DMBA	Schwarz and Shklar 1987	[158]
			Schwartz *et al.* 1988	[159]
Calmette–Guerin bacillus	Hamster	DMBA	Giunta *et al.* 1974	[180]
Bowman–Birk inhibitor	Hamster	DMBA	Messadi *et al.* 1986	[176]
	Hamster	DMBA	Kennedy *et al.* 1993	[177]
Soybean trypsin inhibitor	Hamster	DMBA	Messadi *et al.* 1986	[176]
Onion extract	Hamster	DMBA	Niukian *et al.* 1987	[160]
Garlic extract	Hamster	DMBA	Meng and Shyu 1990	[162]
Green coffee beans	Hamster	DMBA	Miller *et al.* 1988	[163]
Indole-3-carbinol	Rat	4-NQO	Tanaka *et al.* 1992	[166]
Sinigrin	Rat	4-NQO	Tanaka *et al.* 1992	[166]
Limonin 17-β-o-glucopyranoside	Hamster	DMBA	Miller *et al.* 1992	[165]
Kahwehol	Hamster	DMBA	Miller *et al.* 1991	[164]
Cafestol	Hamster	DMBA	Miller *et al.* 1991	[164]
Caffeic acid	Rat	4-NQO	Tanaka *et al.* 1993	[167]
Ferulic acid	Rat	4-NQO	Tanaka *et al.* 1993	[167]
Chlorogenic acid	Rat	4-NQO	Tanaka *et al.* 1993	[167]
Ellagic acid	Rat	4-NQO	Tanaka *et al.* 1993	[167]
Protocatechuic acid	Rat	4-NQO	Tanaka *et al.* 1994	[168]
Hesperidine	Rat	4-NQO	Tanaka *et al.* 1994	[172]
Curcumin	Rat	4-NQO	Tanaka *et al.* 1994	[172]

induced tumours in several organs and on human neoplasms [104, 105, 109, 110]. In animal models however, dietary retinol or retinoids have variously been reported to inhibit, have no effect or to enhance carcinogenesis [110–113]. These modulating effects are considered to depend on the model system, carcinogen dose and type of retinoids. As reviewed by Birt [112], retinol or retinoids enhanced carcinogenesis in eight of 32 experiments reported in various organs (skin, lung, trachea, liver, colon, and pancreas). In addition, dietary retinol enhanced tumours in the hamster cheek pouch induced by DMBA [114]. Although the synthetic retinoids have higher therapeutic effects than those of the natural vitamin A compounds, there are side-effects including chronic toxicities of liver, serum lipids, skeletal and teratogenic effects [115]. Since most retinoid-responsive neoplastic processes require long-term therapy to prevent tumours, the future of retinoids may depend less on screening for efficacy than on screening for toxic effects, as investigators search for drugs with high therapeutic indices for long-term, low-dose therapy studies. To avoid toxicity and enhance the favourable effects, over 2000 retinoids have been synthesised and four of these retinoids (vitamin A or retinol, β-all-trans-retinoic acid or tretinoin, 13-*cis*-retinoic acid or isotretinoin, and an aromatic ethyl ester derivative or etretinate) have been used in clinical trials. The retinamide (e.g. 4-HRP) and highly potent arotenoids, which are third-generation retinoids, are extremely promising candidates for cancer chemoprevention, as well as primary treatment. 4-Fenretinide (4-HPR) deserves special mention, being utilised now in several ongoing chemoprevention trials in skin, breast and oral cancer [116]. It is remarkable for its lack of skin and mucosal toxicity: its major side-effect is night blindness [117]. Our recent study demonstrated that a newly

Table 2. Synthetic chemopreventive compounds in oral carcinogenesis

Agent	Species	Carcinogen	Author	Reference
Levamisole	Hamster	DMBA	Eisenberg and Shklar 1977	[181]
Phenanthrene	Hamster	DMBA	Malament and Shklar 1981	[182]
1,4-Dimethyl-naphthalene	Hamster	DMBA	Malament and Shklar 1981	[182]
Aspirin	Hamster	DMBA	Perkins and Shklar 1982	[178]
Indomethacin	Hamster	DMBA	Perkins and Shklar 1982	[178]
	Rat	4-NQO	Tanaka *et al.* 1989	[184]
Ibuprofen	Hamster	DMBA	Cornwall *et al.* 1983	[179]
Butylated hydroxytoluene	Rat	4-NQO	Tanaka *et al.* 1987	[183]
	Rat	4-NQO	Inoue *et al.* 1993	[101]
Butylated hydroxyanisole	Rat	4-NQO	Tanaka *et al.* 1987	[183]
Disulfiram	Rat	4-NQO	Tanaka *et al.* 1987	[183]
Piroxicam	Rat	4-NQO	Tanaka *et al.* 1989	[184]
D,L-α-Difluoromethyl ornithine	Rat	4-NQO	Tanaka *et al.* 1993	[185]
KYN-45	Rat	4-NQO	Tanaka *et al.* 1994	[118]
Arotinoid	Rat	4-NQO	Tanaka *et al.* 1994	[118]

synthesised retinoid KYN-54 and arotenoid (Ro 40-8757) clearly inhibited 4-NQO-induced tongue neoplasms without any toxicity [118]. These synthetic retinoids were also able to inhibit colon carcinogenesis [119]. Further direction to clinical research is being provided by several *in vitro* studies on the additive and synergistic activity of retinoids with hormones, DNA synthesis inhibitors, irradiation, other biological modifiers or other micronutrients [109, 120].

Carotenoids with or without provitamin A activity are far less toxic than retinoids. A review by Bertram *et al.* indicated that the dietary intake of β-carotene, the carotenoid with the highest provitamin A activity, rather than the intake of vitamin A itself, best correlated with decreased cancer incidence [121]. β-Carotene is a naturally occurring pigment in many dark-green and yellow/orange leafy vegetables and fruits [122]. Of about 600 carotenoids that exist in nature and are contained in vegetables, fish and sea algae, possibly 10% serve as precursors of vitamin A. Besides serving as the major dietary source of retinol for humans, β-carotene and other carotenoids have been found to possess common biological functions (photoprotection, anti-oxidant properties including singlet oxygen quenching, immunomodulation and anticancer activity) in both humans and rodents [44, 123–125]. β-Carotene and canthaxanthin (which has no provitamin A activity) have been shown to have anticancer activity in the oral cavity [126–128]. Such inhibition by carotenoids was found to be effective during both the initiation and promotion phases of carcinogenesis [128]. β-Carotene could reduce the GGT activity during tumour development [129], suggesting that β-carotene was acting to depress some of the metabolic pathways involved in carcinogenesis since phenotypic alteration of GGT expression occurs during hamster buccal pouch carcinogenesis [52]. The mechanisms by which carotenoids exert anticancer properties may include immunostimulation [130, 131], inhibition of cell proliferation [132, 133], or enhancement of gap junctional communication by increasing the expression of the major gap junction protein (connexin 43) [134]. Carotenoids are capable of interacting with the free radicals that can cause extensive cellular damage and/or initiate a chain reaction to lipid peroxidation, which in turn may lead to alterations of membranes, enzymes and nucleic acids [123, 125]. Our recent studies have demonstrated that astaxanthin and canthaxanthin can be chemopreventive on 4-NQO-induced rat tongue carcinogenesis [135]. Thus, it is now considered that the antitumour effects of carotenoids are independent of their provitamin A activities. Recent data suggest that carotenoids as well as retinoids may be effective in reversing a putative "field cancerisation" defect in the epithelium at risk for oral cancer [31, 102, 136]. With the sole toxicity being a yellowing of the skin despite prolonged use at high dose, carotenoids have been an attractive choice for clinical chemoprevention studies [31].

Vitamin C is one of the antioxidant vitamins that appears to inhibit carcinogenesis in rodents [112]. The most convincing evidence for the involvement of vitamin C in cancer prevention is the ability of ascorbic acid to prevent *N*-nitroso compound formation [137, 138]. Despite the fact that in the hamster buccal pouch carcinogenesis model, vitamin C enhanced the development of carcinoma [139], β-carotene, vitamin E and vitamin C have been used against oral premalignant leukoplakia [44]. Early results show a response rate of about 60% in an ongoing trial using a combination of antioxidant vitamins and β-carotene [140]. A need to test combinations of such agents has been postulated by several investigators [141–143], in order to obtain better chemopreventive efficacy with low or no toxicity than occurs with the administration of a single chemopreventive agent.

Toxicity of vitamin E is minimal despite ingestion of large doses for extended periods [144]. Eight tocopherols, of which α-tocopherol is the most active, constitute vitamin E. Absorbed from a wide variety of foods, especially vegetable oils, as a lipid-soluble vitamin, it correlates strongly with serum lipoprotein levels. Since it is deposited diffusely in all tissues, depletion may take months. Physiologically it is important in enzyme activation of haematopoiesis, drug metabolism, and pollutant detoxification. Low serum vitamin E levels or deficient diets have been associated with increased toxicity from lead and oxidant gases. Activity against carcinogenesis has been suggested by epidemiological and laboratory studies. Certain cancers are inversely correlated with vitamin E levels [145], but studies are limited by vitamin E's wide distribution in food substances. In 1969 Harman first demonstrated a potent anticancer action of vitamin E in an experimental animal model [146]. Circumstantial evidence of chemoprevention comes from its direct inactivation of the cigarette carcinogen nitrosodimethylamine and indirectly

via effects upon liver enzymes that prevent *in vivo* formation of *N*-nitroso compounds [148, 149]. A significant inhibitory effect or tumour regression was demonstrated by administration of vitamin E systematically [150], topical application [151, 152] or by injection close to the tumour site [153]. However, as with β-carotene, tumour regression could not be accomplished by oral administration of vitamin E. Although nitrate inactivation might be a major mechanism, most have attributed the chemoprevention property of vitamin E to its very potent anti-oxidant actions. Like the carotenes, it is a well known trapper of free oxygen radicals. It has been found to protect cells from carcinogens by inhibiting lipid peroxidation and the damaging free-radical mediated consequences [154]. Prevention of DNA adduct formation [155] and immunoenhancement [52, 156] have also been suggested as mechanisms of cancer prevention by vitamin E. As with the carotenoids, the few side effects make it an attractive agent for use in chemoprevention [157].

(b) Natural products

Table 1 lists several natural products that may inhibit experimental oral carcinogenesis in hamsters and rats. An extract that contains carotenoid including β-carotene and vitamin E from *Spirulina dunaliella* algae was found to inhibit hamster buccal pouch carcinogenesis [158, 159] and had more suppressing activity than that of β-carotene alone [158]. Onion extract can retard experimental oral carcinogenesis [160, 161], although the constituent(s) that are responsible for the anticancer activity have to be established. Garlic extract also possesses the ability to inhibit oral carcinogenesis [162]. Green coffee beans and their constituents have been shown to inhibit hamster buccal pouch carcinogenesis [163, 164]. Limonin 17-β-D-glucopyranoside, which is a limonoid glucoside isolated from oranges, inhibited DMBA-induced hamster buccal pouch carcinogenesis [165]. Our search for natural products with chemopreventive property using a rat oral carcinogenesis model induced by 4-NQO has revealed that indole-3-carbinol, sinigrin, and phenolic compounds (ferulic, chlorogenic, caffeic and ellagic acids) have very potent tumour-preventive activity [166, 167]. Recently, we have found a new potent chemopreventive agent protocatechuic acid (PCA) derived from citrus fruits [168]. PCA is a simple phenolic acid with very potent antioxidative potential [169]. When this compound was administered in the diet during the initiation and postinitiation phases of 4-NQO-induced rat carcinogenesis model, PCA clearly inhibited oral cancer development in a dose-dependent manner when given in either phases of carcinogenesis, without producing any toxicity. Such inhibition may be due to an antiproliferative action in the target organ [168]. PCA also possesses chemopreventive potential in other animal carcinogenesis models (liver, colon and glandular stomach) [170, 171]. More recently, curcumin and hesperidine were shown to reduce cancer development in 4-NQO-induced rat oral carcinogenesis: the order of inhibitory potencies was curcumin > β-carotene > hesperidine [172]. Selenium use in chemoprevention has been limited by its complex and poorly understood pharmacology. Epidemiologic evidence linking carcinogenesis and selenium includes tumours of the oral cavity and the oropharynx but is strong especially with the breast the breast and colon [173]. Animal models show that selenium inhibits cancer in a species-specific, time-dependent and very dose-related way, but with many contradictory results [174]. Selenium actions may arise from its role as a cofactor for glutathione peroxidase, an enzyme fundamental to the natural antioxidant defence system of each cell [175]. Toxic levels of selenium are little different from therapeutic levels, resulting in weight loss, hepatotoxicity and cirrhosis in animals, results which have forestalled its application in humans [121]. A soyabean extract containing the Bowman–Birk protease inhibitor has been reported to be capable of suppressing DMBA-induced oral carcinogenesis in hamsters [176, 177] and its effect was irreversible [177].

(c) Synthetic chemicals other than the retinoids

Several drugs have been reported to have chemopreventive activity in the rodent carcinogenesis model (Table 2). These include non-steroidal anti-inflammatory drugs like aspirin [178], indomethacin [178] and ibuprofen [179], and immunostimulators like BCG [180] and levamisole [181]. Other chemicals like phenanthrene and 1,4-dimethylnaphthalene were also reported to inhibit hamster buccal pouch carcinogenesis [182]. Disulfiram, indomethacin, piroxicam and D,L-α-difluoromethylornithine (DFMO), an ornithine decarboxylase (ODC) inhibitor have been demonstrated using a 4-NQO-induced rat oral carcinogenesis model in our laboratory [183–185]. *N*-acetyl-L-cysteine [186] and oltipraz [187] are considered to be possible chemopreventive agents including in the oral cavity, but available evidence has not yet been accumulated. Synthetic antioxidants such as butylated hydroxyanisole and hydroxytoluene used as food additives have been demonstrated to have inhibitory effects on oral carcinogenesis [183]. Their inhibitory action on oral carcinogenesis might be due to alterations in ODC activity, polyamine levels, or cell proliferation and/or to activity in detoxifying drugs or metabolising enzymes.

(d) Combinations of micronutrients

There have been few experimental studies estimating combined exposure to chemopreventives that exert synergistically inhibiting effects on oral carcinogenesis (Table 3). Goodwin *et al.* reported that DMBA-induced tongue carcinomas in hamsters were inhibited by 13-*cis*-retinoic acid with and without selenium [188]. Vitamin E and β-carotene have been found to be synergistic in their anticancer effect [189]. While vitamin E or β-carotene separately appear incapable of regressing established oral cancer in the hamster buccal pouch, they are capable of inducing cancer regression when combined. This may be explained by the fact that both are antioxidants acting in a different, but complementary manner [190]. Recent work by Inoue *et al.* indicated that combined treatment with sodium selenite + butylated hydroxytoluene or sodium selenite + vitamin A acetate + butylated hydroxytoluene inhibited tongue carcinogenesis induced by 4-NQO in Sprague–Dawley rats, although the inhibitory effect of these combined chemopreventives was not always superior to that of each chemopreventive agent used alone [110]. Chemoprevention strategies using combinations of inhibitors (e.g. β-carotene plus α-tocopherol) based on these *in vivo* studies have led to the recent design of clinical trials.

Table 3. Chemoprevention of oral carcinogenesis by combination treatment

Agents	Species	Carcinogen	Author	Reference
13-*cis*-retinoic acid + selenium	Hamster	DMBA	Goodwin *et al.* 1986	[188]
Vitamin E + β-carotene	Hamster	DMBA	Shklar *et al.* 1989	[189]
Sodium selenate + BHT	Rat	4-NQO	Inoue *et al.* 1993	[101]
Sodium selenate + vitamin A acetate	Rat	4-NQO	Inoue *et al.* 1993	[101]
BHT + vitamin A acetate	Rat	4-NQO	Inoue *et al.* 1993	[101]
Sodium selenate + BHT + vitamin A acetate	Rat	4-NQO	Inoue *et al.* 1993	[101]

BHT, butylated hydroxytoluene.

Table 4. Oral premalignancy trial by retinoids, β-carotene or vitamin E

Agent	CR (%)	PR (%)	OR (%)	Author	Reference
Isotretinoin	0	87	87	Koch 1978	[205]
Etretinate	0	91	91	Koch 1978	[205]
Tretinoin	0	59	59	Koch 1978	[205]
Etretinate (topical and oral)	29	54	83	Koch 1981	[208]
Etretinate	24	48	71	Koch 1981	[208]
Isotretinoin	8	58	66	Hong *et al.* 1986	[207]
Retinol	57	0	57	Stich *et al.* 1988	[204]
β-Carotene	15	ND	ND	Stich *et al.* 1988	[204]
β-Carotene + vitamin C	27	ND	ND	Stich *et al.* 1988	[204]
β-Carotene	8	63	71	Garewal *et al.* 1990	[209]
β-Carotene + vitamin E + vitamin C	ND	ND	60	Kaugers *et al.* 1990	[211]
β-Carotene	33	11	44	Toma *et al.* 1991	[212]
β-Carotene	28	22	50	Malaker *et al.* 1991	[210]
β-Carotene	ND	ND	56	Garewal *et al.* 1992	[220]
Vitamin E	ND	ND	65	Benner *et al.* 1993	[201]

CR, complete response; PR, partial response; OR, overall response; ND, not determined.

CURRENT CLINICAL TRIALS FOR ORAL CANCER CHEMOPREVENTION

Beginning in the 1980s, the National Cancer Institute (NCI) started chemoprevention research to identify and evaluate anticarcinogenic agents that may inhibit the multistage processes (initiation, promotion, conversion, and/or progression) of carcinogenesis either before or after the disease process has begun [191]. This strategy aims to prevent and control malignant transformation from a mechanistic approach derived from chemical and biological researches that include preclinical *in vitro* and *in vivo* studies, intermediate-endpoint biomarker studies, and clinical studies [192–197]. *In vitro* and animal *in vivo* studies are important for understanding the cellular and molecular evolution of epithelial carcinogenesis, establishing intermediate-endpoint biomarkers, screening candidate chemopreventive agents and combinations and dose-response studies. Thus, preclinical studies are fundamental to the multidisciplinary chemoprevention programme. The earliest trials were conducted in the treatment of oral leukoplakia, sometimes a premalignant lesion [198]. Since then, a number of trials (Table 4) have been conducted in the world [44, 102, 198–217]. Positive results in oral leukoplakia led to subsequent clinical trials in second primary malignancies.

(*a*) *Clinical trials for oral premalignancy (primary chemoprevention)*

Oral premalignant lesions are basically characterised clinically by white (leukoplakia) or red (erythroplasia) mucosal lesions in the oral cavity that cannot be otherwise characterised. Despite this non-specific definition, patients who have these lesions are at increased risk for the development of oral cavity cancer. When dysplasia is present, a worse prognosis ensues: dysplasia may be a direct precursor lesion for oral cancer. The development of these lesions is often associated with carcinogen and/or promoter exposure such as alcohol and tobacco use. Several studies have evaluated the efficacy of chemopreventive agents to reverse these premalignant lesions. These agents include retinoid, β-carotene, vitamin E, vitamin C, retinoids and/or selenium [44, 102, 198–217]. All these agents [202, 203] have produced promising results, although none have undergone randomised placebo-controlled testing to establish activity. Since the early 1960s, high doses of vitamin A have been shown to be effective in reversing the hyperkeratosis associated with leukoplakia. In the 1970s, the activity of synthetic retinoids (13-*cis*-retinoic acid, trans-retinoic acid and etretinate) was demonstrated with an approximately 60–90% response rate, but with toxicity [205–208]. In late 1982, Hong's group published the first randomised chemoprevention trial of primary therapy in oral leukoplakia, a short-term, placebo-controlled, double-blind study of high-dose 13-*cis*-retinoic acid (1–2 mg/kg/day) [207]. The clinical response rate was 67% (8% complete and 59% partial) with the retinoid treatment and 10% with placebo (Table 4). Dysplasia was reversed in 54% who received the retinoid and 10% who received placebo [207]. Thus, this trial confirmed activity of 13-*cis*-retinoic acid in leukoplakia, but demonstrated major toxicity that was unacceptable.

Subsequent trials conducted by this group resulted in the first randomised maintenance trial of less toxic therapy in patients with oral leukoplakia [213]. The trial was designed as follows: in the first phase of the study, 70 patients with leukoplakia underwent induction therapy with a high dose of isotretinion (1.5 mg/kg/day) for 3 months; in the second phase, patients with responses or stable lesions were randomly assigned to maintenance therapy with either β-carotene (30 mg/day) or a low dose of isotretinoin (0.5 mg/kg/day) for 9 months. When preceded by high-dose induction therapy, low-dose isotretinoin therapy was significantly more active against leukoplakia than was β-carotene, and was easily tolerated [213]. However, several problems occurred including the progression of the lesions in some patients and some toxicity from high-dose isotretinoin was still seen. Several other recent studies in leukoplakia using natural vitamin A or a synthetic retinamide achieved significantly higher complete remission [214, 215]. Preliminary results from an on-going randomised maintenance trial at the Istituto Nazionale Tumori of Milan in Italy in order to evaluate the effectiveness of fenretinide (4-HPR) in preventing relapses, new localisations and carcinomas in patients who have been surgically treated for oral leukoplakias indicated that 4-HPR is well-tolerated and appears to be effective in preventing relapses and new localisation during the treatment [216, 217].

In view of the toxicity of retinoids and the considerable preclinical evidence in support of a possible preventive role, attention has recently focused on the use of other micronutrients (β-carotene, vitamin E and vitamin C) in oral leukoplakia [44, 157, 218, 219]. In contrast to the retinoid studies, clinical trials with these agents have started more recently. Studies using vitamins E and C are currently ongoing with results likely to be available shortly. β-Carotene and vitamin E produce regression of oral leukoplakia. This has now been shown in seven clinical trials: five with β-carotene alone, one with vitamin E and one with a combination of both [201, 204, 209–211, 220]. The data are listed in Table 3. Combination therapy with β-carotene (30 mg/day), ascorbic acid (1000 mg/day), and α-tocopherol (800 U/day) for 9 months produced 55.7% clinical improvement in 79 patients with histologically confirmed leukoplakia or dysplasia and such improvement was most likely to occur in those who reduced their use of alcohol or tobacco and those with dysplasia [218]. Actual cancer incidence reduction trials in high risk groups have targeted the prevention of second malignancies in patients cured of an oral cancer. Such trials are now in progress. These data, together with the lack of any significant side effects, and an emerging role for these agents in the prevention of other chronic diseases such as atherosclerosis, are strongly supportive of a very significant disease-preventive role for these micronutrients, including a chemopreventive role in oral cancer [44, 157]. *N*-acetyl-L-cysteine (NAC), an antioxidant and nucleophile, is being tested in EUROSCAN, a European Organization for Research and Treatment of Cancer (EORTC) chemoprevention study of curatively treated patients with oral, laryngeal and lung cancer, which started in June 1988 [221]. In a 2×2 factorial design, retinyl palmitate (300 000 U/day for a year) and half this dose for the second year, or NAC (600 mg/day for 2 years), or both drugs in combination, versus no drugs, are being studied as chemopreventive agents. The endpoints of EUROSCAN are the number and time of occurrence of second tumours, local or regional recurrences and distant metastases. Preliminary data from EUROSCAN showed good compliance in treated patients and a low frequency of side-effects [186]. NAC is believed to act in the early stage of carcinogenesis, preceding and possibly shortly after the occurrence of DNA damage, while vitamin A acts later, in the promotion and progression phases. Thus, theoretically, the combination covers nearly the entire carcinogenic process, with no expected interaction with regard to side-effects.

(b) Prevention of second primary neoplasms

As mentioned above, patients with head and neck cancer including oral malignancies have an increased incidence of second primary malignancies of the upper aerodigestive tract [27–30]. If habits such as tobacco use continue, prospective second primary tumour rate approaches 3–5% per year [44]. Since a high cure rate of the primary tumour is usually achievable in patients who have early cancers, the second primary malignancies are often the cause of their mortality. Therefore, strategies for reducing the incidence of second primaries are of importance to such a patient group. Agents active in reversing premalignant lesions might indeed prove to be effective in reversing the field where the increased incidence of second malignancies occurs. Although non-toxic agents are to be preferred, a somewhat greater degree of toxicity is acceptable in this group since cancer risk is considerably higher than in the usual patients with leukoplakia. A recent report of an adjuvant trial using high-dose 13-*cis*-retinoic acid (isotretinoin, 50–10 mg/m^2/day) in 103 patients (49 patients treated with 13-*cis*-retinoic acid and 51 with placebo) with all stages (I–IV) of cancer showed a remarkable reduction in second primary malignancies, although retinoid therapy had had no impact on the recurrence of the primary cancer in the initial report after a follow-up of 32 months [103]. Benner *et al.* recently updated the findings for this trial, based on a median follow-up of 54.5 months. This again showed that there is no difference in the rates of recurrence of the original tumour between the isotretinoin- and the placebo-treated groups but a significant reduction in the rate of development of second primary tumours in the isotretinoin group [222]. In another trial, the Southwest Oncology Group planned second primary prevention trials using β-carotene in which only early stage (I or II) cancer patients who have entered remission after their primary treatment will be included [44].

FUTURE DIRECTIONS

Early results have been extremely encouraging, suggesting that there may be a real potential for using non-toxic chemopreventive agents in preventing oral cavity cancer. Although chemoprevention research is producing promising results with the retinoid 13-*cis*-retinoic acid in the head and neck neoplasms including oral cancer. If non-toxic natural products that are found as active as the retinoids possessing toxicity at a high dose, use of these natural agents is of interest. Even if these agents are half as active, they are much more likely to be usable as chemopreventives than are the toxic compounds. There is also a need to test combinations of agents including biological response modifiers [44, 120, 141, 196, 197] in order to obtain more active chemopreventive effects with less toxicity than a single agent. In this context, preliminary results of an ongoing trial using a combination of β-carotene, vitamin E and vitamin C [140] are important. Reliable biomarkers should also be established. Recent molecular research will introduce molecular biomarkers that

may serve ultimately as intermediate endpoints for chemopreventive trials. Moreover, there are cellular and genetic targets for chemopreventive agents including genistein, D-limonene, canventol, L-731,735 and benzodiazepine peptidomimetics [196, 197]. In addition, a model system for identification of progressor agents active in human carcinogenesis should be established in order to clarify the process of progression and find anti-progressor agents [223]. These approaches will provide a way to control carcinogenesis in the near future [224].

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