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Chemoprevention of esophageal squamous cell carcinoma

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Abstract

Esophageal squamous cell carcinoma (SCC) is responsible for approximately one-sixth of all cancer-related mortality worldwide. **This** malignancy has a multifactorial etiology involving several environmental, dietary and genetic factors. Since esophageal cancer has often metastasized at the time of diagnosis, current treatment modalities offer poor survival and cure rates. Chemoprevention offers a viable alternative that could well be effective against the disease. Clinical investigations have shown that primary chemoprevention of this disease is feasible if potent inhibitory agents are identified. The Fischer 344 (F-344) rat model of esophageal SCC has been used extensively to investigate the biology of the disease, and to identify chemopreventive agents that could be useful in human trials. Multiple compounds that inhibit tumor initiation by esophageal carcinogens have been identified using this model. These include several isothiocyanates, diallyl sulfide and polyphenolic compounds. These compounds influence the metabolic activation of esophageal carcinogens resulting in reduced genetic (DNA) damage. Recently, a few agents have been shown to inhibit the progression of preneoplastic lesions in the rat esophagus into tumors. These agents include inhibitors of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF) and c-Jun [a component of activator protein-1 (AP-1)]. Using a food based approach to cancer prevention, we have shown that freeze-dried berry preparations inhibit both the initiation and promotion/progression stages of esophageal SCC in F-344 rats. These observations have led to a clinical trial in China to evaluate the ability of freeze-dried strawberries to influence the progression of esophageal dysplasia to SCC.

Keywords

esophagus; squamous cell carcinoma; chemoprevention; rat; human

Introduction

Esophageal cancer in humans occurs worldwide with a variable geographic distribution and ranks sixth as a cause of cancer mortality (Parkin et al., 2001). There are two main types of esophageal cancer with distinct etiological and pathological characteristics, squamous cell carcinoma (SCC) and adenocarcina. Esophageal SCC is the predominant type of esophageal malignancy worldwide, although adenocarcinomas are more prevalent in the USA (Souza, 2002). Epithelial dysplasia, characterized by an accumulation of atypical cells with nuclear hyperchromasia, abnormally clumped chromatin and loss of polarity, is the principal precursor lesion of esophageal SCC (Krasna and Wolfer, 1996). Esophageal SCC develops through a

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progressive sequence from mild to severe dysplasia, carcinoma *in situ* and, finally, invasive carcinoma (Anani et al., 1991; Kuwano et al., 1993; Shu et al., 1981). The tumors present as fungating, ulcerating or infiltrating lesions in the esophageal epithelium. Most esophageal cancer patients present with advanced metastatic disease at the time of diagnosis (Layke and Lopez, 2006). This results in a poor prognosis; only 1 in 5 esophageal cancer patients survive more than 3 years after initial diagnosis (Polednak, 2003; Younes et al., 2002).

Epidemiology of esophageal SCC

The incidence of esophageal SCC shows marked variation in its geographic distribution and occurs at very high frequencies in certain parts of China, Iran, South Africa, Uruguay, France, Italy and Puerto Rico (Krasna and Wolfer, 1996; Rose, 1973; Schottenfeld, 1984; Sons, 1987; Stoner and Gupta, 2001; Yang, 1980). One-half of all esophageal SCC in the world occurs in China. Areas located in the southern parts of the Taihang mountains on the borders of Henan, Shansi and Hopei provinces have among the highest incidence and mortality rates for esophageal SCC in the world. In Linxian county in Henan province, the age-adjusted mortality rates from esophageal SCC have been reported **to be** as high as 151/100,000 for males and 115/100,000 for females annually (Munoz and Buiatti, 1996). Studies in these high-risk areas point to specific environmental factors as etiological agents of this disease. Esophageal SCC is infrequent in individuals less than 40 years of age but beyond this, the incidence increases with each decade of life (Pickens and Orringer, 2003). Males have a 3- to 4-fold greater risk for developing esophageal SCC than females and, in the USA, African Americans have more than a 5-fold higher incidence of esophageal SCC than Caucasians (Pickens and Orringer, 2003).

Etiology of esophageal SCC

There are several factors involved in the etiology of esophageal SCC (Stoner and Gupta, 2001). The excessive use of tobacco is a principal risk factor for **this** disease (Layke and Lopez, 2006). Several tobacco carcinogens, including certain nitrosamines, polycyclic aromatic hydrocarbons and aromatic amines, and toxins, including aldehydes and phenols, may be causally related to esophageal cancer (Hecht and Stoner, 1996; Tuyns, 1982; Wynder and Bross, 1961). Alcohol consumption has been shown to further increase the risk for SCC of the esophagus among tobacco users (Tuyns, 1980). Consumption of salt-cured, salt-pickled and moldy food is also implicated in the development of this disease, because these foods are frequently contaminated with *N*-nitrosamine carcinogens and/or fungal toxins (Ribeiro et al., 1996). Research in China and South Africa provides evidence that *N*-nitroso compounds and their precursors are etiological factors for esophageal SCC in these high incidence areas (Li et al, 1986; Lu et al., 1991). *N*-nitrosomethylbenzylamine (NMBA), a potent esophageal carcinogen in the rat, and other nitrosamines, have been identified in the diets and gastric juice collected from subjects in Henan province, China. The detection of O^6-methylguanine in the DNA of normal esophageal tissue taken from esophageal cancer patients in China further substantiates the role of methylating nitrosamines in the development of esophageal cancer (Umbenhauer et al., 1985; Yang et al., 1992). In addition, contaminated foods often contain nitrates, nitrites and secondary and tertiary amines which act as precursors for the formation of nitrosamine carcinogens in the acidic conditions of the stomach (Hecht and Stoner, 1996).

Other factors associated with **the** etiology of esophageal SCC include vitamin and trace mineral deficiencies (Li et al., 1986; Lu et al., 1991). Plasma levels of vitamins A, C and E tend to be lower in patients with esophageal cancer. Studies from high risk areas for esophageal SCC indicate an inverse relationship between esophageal cancer mortality and levels of zinc, selenium and other trace elements in foods (Stoner and Gupta, 2001). Lye ingestion, diets high in starch but low in fruits and vegetables, and radiation therapy have been linked to an increased

risk for esophageal SCC (Freeman, 2004; Layke and Lopez, 2006). In addition, consumption of temperature hot beverages, such as tea, and fungal invasion in esophageal tissues leading to localized inflammation and irritation may be additional promoting factors for esophageal SCC (Li et al., 1984). Furthermore, diseases affecting the esophagus and nearby structures such as achalasia, previous head and neck cancer, and Plummer-Vision syndrome are associated with an increased risk for esophageal SCC (Layke and Lopez, 2006). Finally, a role for human papilloma virus (HPV) has also been suggested in the etiology of SCC of the esophagus (Togawa et al., 1994). To date, however, the exact role of HPV infections in the development of the disease has not been elucidated.

It is clear that multiple environmental factors lead to the occurrence and development of esophageal SCC. Environmental carcinogens are able to affect the genetic material of host cells inducing aberrant regulation of multiple genes in esophageal cells leading to uncontrolled growth and, ultimately, esophageal cancer. Molecular studies of human esophageal tumors have identified numerous genetic abnormalities (Lam, 2000; Mandard et al., 2000). Stoner and Gupta, 2001, summarized genetic changes observed in esophageal SCCs including: (i) alterations in tumor suppressor genes leading to altered DNA repair, cell proliferation and apoptosis; (ii) disruption of the G_1/S cell cycle checkpoint and loss of cell cycle control; and, (iii) alterations in oncogene function leading to deregulation of cell signaling pathways. Unlike in tumors of the human lung, pancreas, skin and colon, mutational activation of the *ras* genes is a rare event in primary SCCs of human esophagus. Genetic alterations more commonly associated with esophageal SCCs include *p53* mutations (Gao et al., 1994; Hollstein et al., 1991), loss of p16MST1 and/or p15 (Xing et al., 1999) and/or RARß (Xu et al., 1999, 2005), amplification of INT-2, EGFR, cyclin D_1 and c-Myc (Guo et al., 1993; Hollstein et al., 1988; Jiang et al., 1993; Lu et al., 1988), and elevations in hTERT, BMP-6, iNOS, COX-2 (Hiyama et al., 1999; Raida et al., 1999; Tanaka et al., 1999; Zimmerman et al., 1999) and ß-catenin levels (Kimura et al., 1999). In addition, loss of heterozygosity on chromosomes 1p, 3p, 4, 5q, 9, 11q, 13q, 17q and 18 have frequently been observed in esophageal SCCs. These chromosome changes lead to a loss of putative tumor suppressor function (An et al., 2005; Mandard et al., 2000, Moodley et al., 2006).

More recently, a number of additional genetic alterations have been identified in human esophageal SCC including: enhanced expression of the transcription activator, NFκB (Yang et al., 2005); decreased expression of the tumor suppressor genes, loss of disabled-2 (DAB-2) (Anupam et al., 2006), N-myc downstream regulated gene-1 (NDRG1) (Ando et al., 2006), Smad 4 and E-cadherin; and, altered expression of apoptosis related genes including bcl-2, caspase 3, TRAIL, Fas-L and Fas (Chang et al., 2005). The epigenetic silencing of tumor suppressor genes such as RAS association domain family 1A (RASSF1A) and fragile histidine triad (FHIT) genes has also been reported (Kuroki et al., 2003). One or several of these genetic alterations undoubtedly contribute to the growth and invasive/metastatic potential of esophageal SCCs.

Prevention of Esophageal SCC

One approach to the prevention of esophageal SCC is through changes in lifestyle, especially the avoidance of alcohol and tobacco use. Additional benefits may be realized by the elimination of high salt foods that may be contaminated with microbial toxins, nitrosamines and their precursors. The increased consumption of vegetables and fruit throughout the world, and especially in the high-risk areas for esophageal SCC, might also be expected to reduce the incidence and mortality from the disease. Significant educational efforts are necessary to inform populations of the major risk factors for the disease and steps they might take to reduce their risk. Chemoprevention is another feasible approach, and it may have special relevance in high incidence areas of the world where carcinogen exposure is high. Animal models provide

an excellent opportunity to evaluate chemoprevention strategies against cancer. The rat has been used almost exclusively as an animal model for studies of the etiology, biology and chemoprevention of esophageal SCC. The remainder of this article discusses molecular alterations and chemoprevention approaches in the rat model and their relevance to human esophageal SCC.

Rat esophageal tumor model

Nitrosamine-induced tumorigenesis in the Fischer-344 rat has proven to be a valuable animal model for studies of the molecular biology and chemoprevention of esophageal SCC (Beer and Stoner, 1998; Hecht and Stoner, 1996; Stoner and Gupta, 2001). Several nitrosamines, including the food contaminant, *N*-nitrosomethylbenzylamine (NMBA), and the tobacco-specific nitrosamine, *N*-nitrosonornicotine (NNN), induce tumorigenesis in the rat esophagus (Figure 1) (Stoner and Gupta, 2001). NMBA is by far the most potent inducer of tumors in the rat esophagus. As for other nitrosamines, the first step in the metabolic activation of NMBA involves hydroxylation of the methylene carbon by esophageal cytochrome P450 enzymes (Figure 2) (Stoner and Gupta, 2001). This reaction produces an α-hydroxy derivative which spontaneously decomposes to methyldiazohydroxide and benzaldehyde. Methyldiazohydroxide leads to formation of a methylcarbonium ion, the ultimate electrophilic species that methylates guanine residues at the N^7 and O^6 positions. The O^6-methylguanine adduct is particularly important for carcinogenesis since it is poorly repaired and leads to single base mispairing in DNA. Repeat NMBA dosing results in esophageal tumor formation within 15-20 weeks (Figure 3). Several preneoplastic lesions produced in NMBA-treated rat esophagus closely mimic lesions observed in the human disease. These lesions include simple hyperplasia, leukoplakia and epithelial dysplasia (Figure 4). It should be noted that squamous papilloma is the predominant tumor type seen in the rat esophagus model. This differs from humans in that papillomas are rarely observed in the human esophagus. The incidence of SCC in the rat esophagus is rather low since the animals often succumb to the occlusive effects of large papillomas in their esophagi before carcinomas can develop. In a typical tumor bioassay, subcutaneous administration of NMBA at either 0.25 or 0.5 mg/kg body weight three times per week for 5 weeks, or once per week for 15 weeks, results in a 100% tumor incidence by 25 weeks (Stoner and Gupta, 2001). On average, these two doses of NMBA will produce from 2-4 or 4-8 tumors per esophagus, respectively, at 25 weeks. In the past several years our laboratory and others have used this model to develop surrogate end-point biomarkers, identify novel targets for intervention and therapy and evaluate putative chemoprevention agents against esophageal SCC.

Genetic analyses of NMBA-induced rat esophageal tumors have identified multiple molecular events in the conversion of normal esophagus to cancer (Figure 5) (Stoner and Gupta, 2001). Mutations in oncogenes and tumor suppressor genes in rat esophageal tumors are most likely due to formation of methylated guanine adducts in esophageal DNA. In contrast to human esophageal tumors, a large majority, between 60 and 100%, of NMBA-induced rat esophageal papillomas have a G:C→ A:T transition mutation in codon 12 of the H-*ras* gene (Lozano et al., 1994;Wang et al. 1990). One study indicated that G →A mutations in codon 12 of the gene occur at very low frequency in early premalignant lesions of the esophagus and increase proportionately in lesions that progress to papilloma (Liston et al., 2000). These data suggest that mutational activation of the H-*ras* gene is important for progression of the premalignant lesions to papillomas. Additional evidence in support of this concept is the observation that transgenic rats carrying the human c-Ha-*ras* proto-oncogene are highly susceptible to the induction of esophageal tumors with NMBA (Asamoto et al., 2002). As has been found in human esophageal tumors, G:C→ A:T transition mutations have been observed in the *p*53 tumor suppressor gene in ~30 % of rat esophageal papillomas (Lozano et al., 1994; Wang et al., 1996). These mutations have been found to be evenly distributed across the gene; no “hot-

spots" were found for these mutations in the gene. In other studies, cyclin D1 and cyclin E mRNA levels were found to be elevated in NMBA-induced rat esophageal papillomas, and immunohistochemical staining revealed extensive nuclear staining for both G_1 cyclins (Wang et al., 1996; Youssef et al., 1997). These observations suggest that cell cycle regulation is altered during rat esophageal tumorigenesis. Increased expression of EGFR and proliferating cell nuclear antigen (PCNA), deregulated expression of transforming growth factor ß1 and altered localization of E-cadherin and α-catenin have also been documented in these tumors (Khare et al., 1999;Stoner and Gupta, 2001; Wang et al., 1996).

Recent studies in our laboratory have demonstrated elevated levels of COX-2 mRNA and PGE_2 (Carlton et al., 2002), iNOS protein (Chen et al., 2004), and iNOS, COX-2, VEGF and c-Jun mRNA and protein (Chen et al., 2006; Chen et al., 2006) in preneoplastic lesions and **in** papillomas of NMBA-treated rat esophagus. The mRNA and protein levels of these genes increased with progression of the premalignant lesions to papillomas indicating that they play a functional role in esophageal tumorigenesis. This was further substantiated by direct correlations between COX-2, iNOS and VEGF mRNA and protein expression levels and the levels of PGE_2, nitrate/nitrite and the number of microvessels, respectively, in esophageal tissues.

Chemoprevention studies in rat esophagus

Mechanistically, chemopreventive agents have been classified as either "blocking" agents or "suppressing" agents (Wattenberg, 1985). Blocking agents act at the initiation stage of carcinogenesis through their influence on the metabolism of carcinogens leading, ultimately, to reduced damage to cellular DNA. Suppressing agents act on the promotion/progression stages of carcinogenesis by influencing cell proliferation rates, apoptosis, differentiation, angiogenesis, tissue invasion, etc. Several compounds have been shown to inhibit the initiation and promotion/progression stages of esophageal carcinogenesis in the rat. These compounds and their actions are described below. However, it should be recognized that many of the compounds to be discussed act on both the initiation and promotion/progression stages of carcinogenesis and, thus, the classification below is, in part, one of convenience.

Agents that inhibit tumor initiation in rat esophagus

Chemoprevention studies in the rat esophagus model have identified several agents that inhibit tumor initiation (Table 1). Ellagic acid (EA), a naturally occurring polyphenol, when given in the diet at concentrations of 0.4 and 4.0 g/kg, significantly inhibits esophageal tumor development (Mandal and Stoner, 1990). EA inhibits the metabolic activation of NMBA into electrophillic species, and stimulates the activities of Phase II detoxifying enzymes (Barch and Fox, 1989;Mandal et al., 1988). Addition of 13-*cis*-retinoic acid to the diet antagonized the preventive effects of EA (Daniel and Stoner, 1991). Diallyl sulfide, a component of garlic that acts principally by stimulation of Phase II enzymes, was also found to be an effective inhibitor of NMBA-induced tumorigenesis in the rat esophagus (Brady et al., 1988;Wargovich et al., 1988). The polyphenolic fraction of black tea (theaflavins), as well as the major catechin in green tea, [(-)-epigallocatechin 3-gallate (EGCG)], had a modest effect on tumor multiplicity when administered in the drinking water (de Boer JG et al., 2004; Li et al., 2002; Morse et al., 1997). The mechanisms by which tea inhibited tumor initiation in these studies was not determined however, EGCG down-regulates early expressed genes (COX-2, cyclin D1) in esophageal carcinogenesis probably during NMBA treatment. Curcumin, a component of the spice, tumeric, at 500 ppm in the diet inhibited both the initiation and post-initiation stages of NMBA-induced esophageal tumorigenesis (Ushida et al., 2000). Curcumin significantly decreased tumor development and the incidence and multiplicity of preneoplastic lesions. These effects correlated with reduced expression of cell proliferation markers (5-bromo-2′-deoxyuridine labeling index) in the non-lesional esophageal epithelium. In a later report by the

same laboratory, curcumin was shown to reduce the level of CYP2B1 in the rat esophagus and this was correlated with inhibition of the metabolic activation of NMBA (Mori et al., 2006). Thus, the modulating effect of curcumin on the initiation phase of esophageal carcinogenesis appears to be due, in part, to its effects on the metabolism of NMBA.

The most effective group of anti-initiating agents so far evaluated in the rat esophagus are the arylalkyl isothiocyanates (Figure 6). Phenethyl isothiocyanate (PEITC), found as a glucosinolate in many cruciferous vegetables, such as watercress, cauliflower, Brussels sprouts, cabbage, etc. is a potent inhibitor of the metabolic activation of nitrosamine carcinogens and DNA methylation *in vivo* and *in vitro* (Carlson et al., 1981;Hanley et al., 1983;Stoner et al., 1991). Dietary administration of PEITC at concentrations of 3.0 mmol/kg diet or greater completely inhibits NMBA-induced esophageal tumorigenesis in the rat (Stoner et al., 1991), and lower concentrations were also effective (Wilkinson et al., 1995). Since isothiocyanates of longer chain length were found to be more effective inhibitors of NNK tumorigenesis in the mouse lung (Morse et al., 1991), we examined the effect of alkyl chain length of isothiocyanates on inhibition of NMBA-induced tumorigenesis in rat esophagus. The inhibitory activity of the isothiocyanates was found to correlate with increasing side chain length. Phenylpropyl isothiocyanate (PPITC) was a more potent inhibitor than PEITC, whereas benzyl isothiocyanate (BITC), a shorter chain length isothiocyanate, was less active. Reductions in NMBA-induced O^6-methylguanine levels in esophageal DNA following dietary administration of the isothiocyanates correlated with their relative ability to inhibit tumor incidence and multiplicity (Wilkinson et al., 1995). PPITC was also able to effectively inhibit another important esophageal carcinogen, the tobacco-specific nitrosamine NNN, when provided in the rat diet (Stoner et al., 1998). Interestingly, phenylbutyl isothiocyanate (PBITC) was found to be less effective than PPITC and phenylhexyl isothiocyanate (PHITC) actually enhanced the tumor response to NMBA (Stoner and Morse, 1997; Stoner et al., 1995, 1999;Wilkinson et al., 1995). The mechanism of this enhancement appears to be due to a cytotoxic effect of PHITC in the rat esophagus that results in increased cell proliferation (Hudson et al., 2001), and not due to either a stimulatory effect of PHITC on NMBA activation or an inhibitory effect of PHITC on DNA repair (Morse et al., 1997).

Agents that inhibit tumor progression in rat esophagus

An effective chemopreventive agent against human esophageal SCC should possess significant inhibitory activity when administered after carcinogen exposure (i.e., post-initiation). The reason for this is that a major subject population for chemoprevention of human esophageal SCC includes individuals who possess dysplastic esophageal lesions which can progress to esophageal SCC (Wang et al., 2005). Effective chemopreventive agents for this cohort would either regress these lesions or extend the latency period for their progression to SCC. To date, very few single compounds have been found to be effective in inhibiting the promotion/progression stages of NMBA tumorigenesis in the rat esophagus (Table 2).

As indicated above, PEITC is a highly effective anti-initiation agent in the rat esophagus however, its usefulness for the human esophagus may be limited since it had no effects on NMBA tumorigenesis when administered post-initiation (Siglin et al., 1995). Similarly, EA had only a modest effect on esophageal tumorigenesis when administered post-initiation, and dietary sulindac, supplemental calciuim and selenium were ineffective (Hu et al., 1992; Siglin et al., 1995). Decaffeinated green tea and black tea were found to be effective in the post-initiation period, but only when given at very high concentrations (Wang et al., 1995). In more recent studies however, effective agents for preventing tumor progression in the rat esophagus when administered post-initiation have been identified. **The synthetic compound**, *S,S*'-1,4-phenylene-*bis* (1,2-ethanediyl)bis-isothiourea (PBIT), a selective iNOS inhibitor, reduced both tumor incidence and multiplicity in the rat esophagus when provided at 50 or 100 ppm in the

diet (Chen et al., 2004). PBIT reduced the production of NO in preneoplastic and papillomatous esophageal lesions when compared with comparable lesions in rats treated with NMBA only. L-748706, a selective COX-2 inhibitor, reduced tumor multiplicity in the rat esophagus when provided at 100 and 150 ppm in the diet (Stoner et al., 2005). The compound was effective only when it reduced PGE_2 levels in preneoplastic esophageal tissues approximately to levels found in normal, untreated esophagus. Resveratrol (Li et al., 2002), and JTE-522 (Li et al., 2001), a selective COX-2 inhibitor, both inhibited tumor development in NMBA-treated rat esophagus by reducing PGE_2 levels in the esophagus. Irinotecan hydrochloride (CPT-11), a potent anti-cancer drug with suppressive effects against gastric and colorectal cancers, exhibited anti-progression effects against esophageal tumorigenesis by reducing the proliferation rate of cells in NMBA-exposed squamous epithelium and preneoplastic lesions (Fujiwara et al., 2004).

Agents that either enhanced NMBA-tumorigenesis in the rat esophagus or had no effect—As indicated above, dietary PHITC enhanced NMBA-tumorigenesis in the rat esophagus and the mechanism appears to be due to a low-grade cytotoxic effect leading to increased proliferation of premalignant cells (Hudson et al., 2001). *N* -(4-hydroxyphenyl) retinamide (4-HPR), a synthetic amide of all-*trans*-retinoic acid, also significantly enhanced esophageal tumorigenesis (Gupta et al., 2001). At 0.4 and 0.8 gm/kg diet, 4-HPR increased tumor multiplicity by 2.4- and 3.7- fold, respectively. 4-HPR enhanced NMBA metabolism and DNA adduct formation, as well as increased tumor size. It also produced a marked stimulation in the growth rate of premalignant cells (data not published). Piroxicam, a potent COX inhibitor, was ineffective as an inhibitor of NMBA-tumorigenesis in the esophagus when administered in the diet at 200 and 400 ppm (Carlton et al., 2002). Interestingly, piroxicam reduced PGE_2 levels in NMBA-treated esophagus below those found in normal esophagus and still it was ineffective. The authors speculated that piroxicam may have failed to modulate additional biochemical pathways involved in NMBA-induced tumorigenesis. Perillyl alcohol, a monoterpene found in lavender, spearmint and fruit, had a weak promoting effect on NMBA-tumorigenesis when added to the diet at 0.5 and 1% (Liston et al., 2003). Although the monoterpene has the potential to inhibit Ha-*ras* farnesylation, it did not affect Ras membrane localization in this study, and it produced an increase in nuclear and cytoplasmic vacuolization in basal epithelial cells. It is possible that this local toxic effect resulted in increased cellular proliferation and an enhancement of postinitiation effects by perillyl alcohol.

Freeze-dried berries as preventative agents for the esophagus—Cancer chemoprevention using a “food-based” approach is emerging as an alternative to the use of single compounds. The addition of freeze-dried vegetables to the diet was shown to be effective in preventing tumor development in the rodent colon (Rijken et al., 1999). Other studies have documented the ability of whole foods, such as tomato juice, paprika juice, dry beans and soybeans, to inhibit carcinogenesis in animal models (Gotoh et al., 1998; Hughes et al., 1997; Melendez-Martinez et al., 2004; Narisawa et al., 2000, 1998; Okajima et al., 1988; Schaffer et al., 1996). Epidemiological studies suggest that the varied geographical distribution of human esophageal SCC throughout the world may be due, in part, to diets that are deficient in vegetables and fruit (Ribeiro et al., 1996). Based on these observations, our laboratory has developed a food-based approach for the prevention of esophageal cancer. While conducting studies with EA in the mid-1980’s, we decided to identify foods in which EA might be found. We tested a series of fruits for their content of EA and found high concentrations (520 - 1,800 μg/g dry weight) in black raspberries (BRB), red raspberries, strawberries (STRW) and cranberries (Daniel et al., 1989). Ellagic acid was found exclusively in the pulp and the seed of these fruits, it was not detected in the juice. Based upon these observations, we decided to freeze-dry (lyophilize) berries to increase the concentration of putative inhibitory agents in them since berries are composed of 85-90% water. After removal of the seeds through the use

of a sieve, the berry pulp is then ground into a fine powder for subsequent administration to animals. In a series of experiments, we observed that both freeze-dried STRW and BRB powders, at 5 and 10% of the diet, inhibited NMBA-induced tumor initiation in the rat esophagus (Table 3) (Carlton et al., 2001; Kresty et al., 2001; Stoner et al., 1999). At 10% of the diet, both STRW and BRB powders produced an approximate 50% reduction in tumor multiplicity in the esophagus. This inhibition was similar to that seen in earlier experiments with pure EA, which initially led us to believe that the EA in the berries was responsible for their inhibitory effect. Analysis of the STRW diet indicated, however, that the EA content in a 10% STRW diet is less than one-fifth of the EA we used in initial studies with pure EA. Thus, it became apparent that other components in berries; e.g., vitamins, minerals, other polyphenols, phytosterols, etc., might also be responsible for their cancer inhibitory effects (Stoner et al., 2006). The anti-initiation effects of berries correlated with their ability to reduce the formation of O^6-methylguanine adducts in esophageal DNA. In this regard, dietary berry powders and berry extracts have been shown to influence both the metabolic activation and detoxification of NMBA by the esophagus (Reen et al., 2006).

At the same dietary concentrations (5 and 10%), STRW and BRB powders significantly reduced tumor multiplicity by more than 30% when administered in a post-initiation scheme indicating the ability of the berries to inhibit tumor progression in the esophagus (Carlton et al., 2001; Kresty et al., 2001; Stoner et al., 1999). In one study, BRB were found to reduce the PCNA labeling index in NMBA-treated esophagus indicating their ability to reduce the growth rate of preneoplastic cells (Kresty et al., 2001). More recently, we have shown that BRB down-regulate NMBA-induced cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (i-NOS), c-Jun, and vascular endothelial growth factor (VEGF) in the esophagus, and this correlated with reduced levels of PGE_2, nitrate/nitrite, and microvessel density, respectively (Chen et al., 2006; Chen et al., 2006). The extent of COX-2 inhibition by BRB was similar to that observed with the specific COX-2 inhibitor, L-748706, indicating the potency of berries for down-regulating COX-2. A more extensive description of the cancer inhibitory effects of berries for the rat esophagus is presented in a recent review article (Stoner, et al., 2006). Additional food substances that have been shown to inhibit NMBA-induced tumorigenesis in the rat esophagus are fermented brown rice and rice bran (Kuno et al., 2004). These foods inhibited the post-initiation stage of esophageal tumorigenesis by reducing the proliferation rate of premalignant cells.

Chemoprevention of human esophageal squamous cell carcinoma

An important component in chemoprevention of human esophageal SCC is that of blocking the progression of premalignant lesions, such as epithelial dysplasia, to malignant SCC (Wang et al., 2005). With the availability of endoscopic and cytological screening techniques, the identification and follow-up of esophageal dysplasia among high-risk populations has become possible. The use of “balloon cytology” coupled with endoscopy in China has proven useful in identifying individuals with premalignant lesions and improving their survival by clinical interventions. Individuals with identified premalignant lesions have also been subjects for clinical chemoprevention trials.

As has been seen in the rat model of esophageal SCC, studies in human cancer have found a relationship between the rate of cell proliferation and risk for esophageal SCC. Individuals at higher risk for esophageal SCC have a more rapid rate of cell proliferation in the superficial and intermediate layers of the esophageal epithelium (Munoz et al., 1985). Moreover, there is a positive correlation between increasing rates of cell proliferation and histological progression of premalignant lesions from hyperplasia to mild and moderate dysplasia (Wang et al., 1990). Thus, clinical trials have been conducted with candidate chemopreventive agents that may inhibit cell proliferation rates in the esophagus. The results of these studies have not been too

encouraging. In a study amongst residents of Linxian, China, daily supplementation with vitamins and minerals were evaluated during a period of 30 months. Esophageal lesions previously diagnosed as acanthosis, esophagitis, squamous dysplasia and SCC were examined for rates of cell proliferation. At the end of the observation period, no treatment effect on the overall amount of squamous epithelial proliferation was found in any of the histological catagories (Rao et al., 1994). In another study, 200 subjects were given dietary supplementation of calcium (1200 mg/day) for 11 months (Wang, et al., 1993). This treatment did not result in reduced rates of cell proliferation in the esophageal epithelium in either hyperplastic or dysplastic lesions. Although cell proliferation rates were not measured, one study investigated the effects of antitumor-B (ATB; a mixture of Chinese herbs), a retinoid (4-ethoxycarbophenylretinamide; 4-ECPR) and riboflavin supplementation in the diet of subjects diagnosed with mild or marked esophageal dysplasia in Hunan, China (Lin et al., 1990). The results revealed a significant reduction in cancer development from pre-existing dysplasia. ATB treatment for 3-5 years reduced the cancer development rate by 52 and 47%, respectively. 4-ECPR lowered the clinical cancer rate by 37-43%. The overall incidence of cancer was not affected in subjects supplemented with riboflavin. The exact composition of ATB and its mechanism of action against esophageal SCC remains to be determined. More recently, a randomized, placebo-controlled, esophageal squamous cell cancer chemoprevention trial evaluated selenomethionine (200 μg daily) and celecoxib (200 mg twice daily) given for 10 months in high-risk populations (Limburg et al., 2005). One rationale for the use of celecoxib, a COX-2 inhibitor, is that both dysplastic lesions and esophageal SCC have been found to overexpress COX-2 (Yu et al., 2004). Subjects had histologically confirmed mild or moderate esophageal dysplasia at baseline. Per subject change in the worst dysplasia grade was defined as the primary end-point. Overall, selenomethionine treatment resulted in a trend toward increased dysplasia regression (43 vs 32%) and decreased dysplasia progression (14 vs 19%). Celecoxib, a specific COX-2 inhibitor, did not influence changes in dysplasia grade overall. Interestingly, results from nine epidemiological studies (2 cohort and 7 case control), involving 1813 cases of esophageal cancer, suggest a protective effect between aspirin and other NSAIDs use and the occurrence of esophageal cancer (SCC and adenocarcinoma) (Corley et al., 2003). These results suggest that agents which inhibit COX-1 or both COX-1 and COX-2 might be more effective against esophageal cancer than specific inhibitors of COX-2.

Green tea has been shown to reduce tumor multiplicity in the rat esophagus when administered in the drinking water (de Boer et al., 2004). However, epidemiological studies of the relationship between green tea consumption and the occurrence of esophageal SCC have been inconclusive. In a recent study in Japan, the effects of smoking, alcohol use and green tea consumption on the risk of esophageal cancer in Japanese men were investigated (Ishikawa et al., 2006). Results from this study indicated that men who smoked, and consumed alcohol and green tea, were at increased risk for the development of esophageal SCC compared with men who had never smoked and consumed little or no alcohol or green tea. Green tea consumption did not appear to reduce the risk for esophageal cancer in users of tobacco and alcohol. A clinical trial conducted in a high-incidence area for esophageal SCC in China found that green tea was not effective in reducing rates of cell proliferation or alleviating the development of dysplastic lesions in the esophagus (Wang et al., 2002).

Our laboratory conducted a phase I trial in 11 normal subjects to determine the safety and tolerability of oral consumption of 45 g per day of freeze-dried black raspberries (BRB) for 1 week (Stoner et al., 2005). We also determined, for this dosing regimen, whether the four anthocyanins and ellagic acid in BRB might have sufficient bioavailability to be measurable in plasma and urine. Results of this study indicated that the BRB were well tolerated clinically. Maximum concentrations of anthocyanins and ellagic acid in plasma occurred at 1 to 2 hours, and in urine from 0 to 4 hours, following berry consumption. We concluded that 45 g of freeze-dried BRB daily are well tolerated by humans and, although the anthocyanins and ellagic acid

are not well absorbed into the blood, their localized absorption into gastrointestinal tissues may be sufficient for protection against cancer. We have recently initiated a chemoprevention trial in a high-risk population in China to evaluate the ability of the berries to influence the development of dysplastic lesions.

Conclusions

The 5-year survival rate for esophageal SCC has not improved substantially in the past several decades in spite of advances in surgical techniques, radiotherapy and chemotherapy. Prevention is clearly and important approach to reduce the incidence and mortality from this disease. Lifestyle changes, especially the avoidance of tobacco and alcohol use, and the elimination of high salt and moldy foods, would reduce the incidence and mortality from the disease. In addition, the increased consumption of vegetables and fruit in the diet would provide sources of preventative agents and reduce known dietary deficiencies associated with development of the disease. Chemoprevention is another feasible approach. Special emphasis needs to be placed on the identification of additional molecular determinants in the development of esophageal SCC. Mechanistic studies using the F-344 rat model of esophageal carcinogenesis can provide important leads as to new targets for chemoprevention. In this regard, recent studies demonstrating the chemopreventive efficacy of agents that modulate the expression levels of iNOS, c-Jun (AP-1), COX-2 and VEGF in rat esophagus provide additional leads for agents that might be efficacious in humans. Studies in our laboratory indicate that compounds in freeze-dried berries modulate all of these molecular biomarkers, and probably many others. Thus, a food-based approach to the chemoprevention of this disease might also be effective.

Abbreviations

AP-1, activator protein-1; ATB, antitumor-B; BITC, benzyl isothiocyanate; BMP-6, bone morphogenetic protein 6; BRB, freeze-dried black raspberries; COX-2, cyclooxygenase-2; CYP2A3, cytochrome P450 2A3; CYP2E1, cytochrome P450 2E1; EA, ellagic acid; 4-ECPR, 4-ethoxycarbophenylretinamide; EGCG, (-)-epigallocatechin 3-gallate; EGFR, epidermal growth factor receptor; 4-HPR, *N*-(4-hydroxyphenyl)retinamide; HPV, human papilloma virus; hTERT, human telomerase reverse transcriptase; iNOS, inducible nitric oxide synthase; INT-2, fibroblast growth factor-3; NFκB, nuclear factor kappa B; NMBA, *N*-nitrosomethylbenzylamine; NNN, *N*-nitrosonornicotine; NO, nitric oxide; PBIT, *S*,*S*-1,4-phenylene-*bis*(1,2-ethanediyl)*bis*-isothiourea; PBITC, phenylbutyl isothiocyanate; PCNA, proliferating cell nuclear antigen; PEITC, phenethyl isothiocyanate; PHITC, phenylhexyl isothiocyanate; PPITC, phenylpropyl isothiocyanate; RARβ, retinoic acid receptor beta; SCC, squamous cell carcinoma; STRW, freeze-dried strawberries; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; VEGF, vascular endothelial growth factor.

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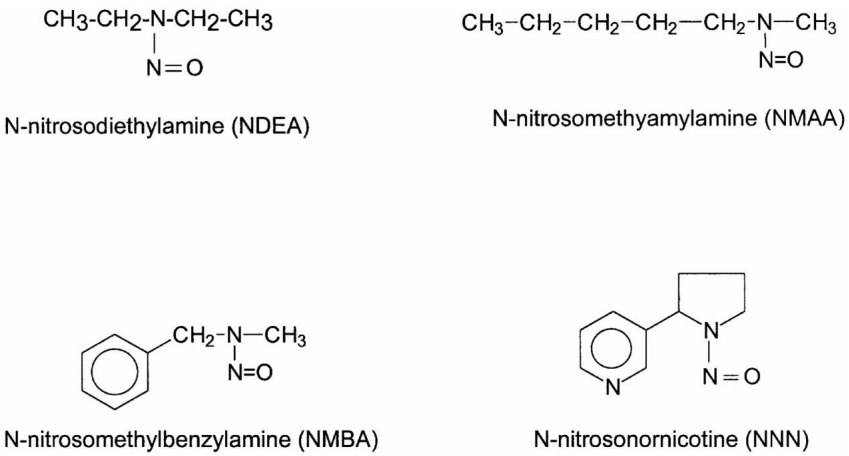

Figure 1.
Structures of some esophageal carcinogens (from Stoner and Gupta, 2001).

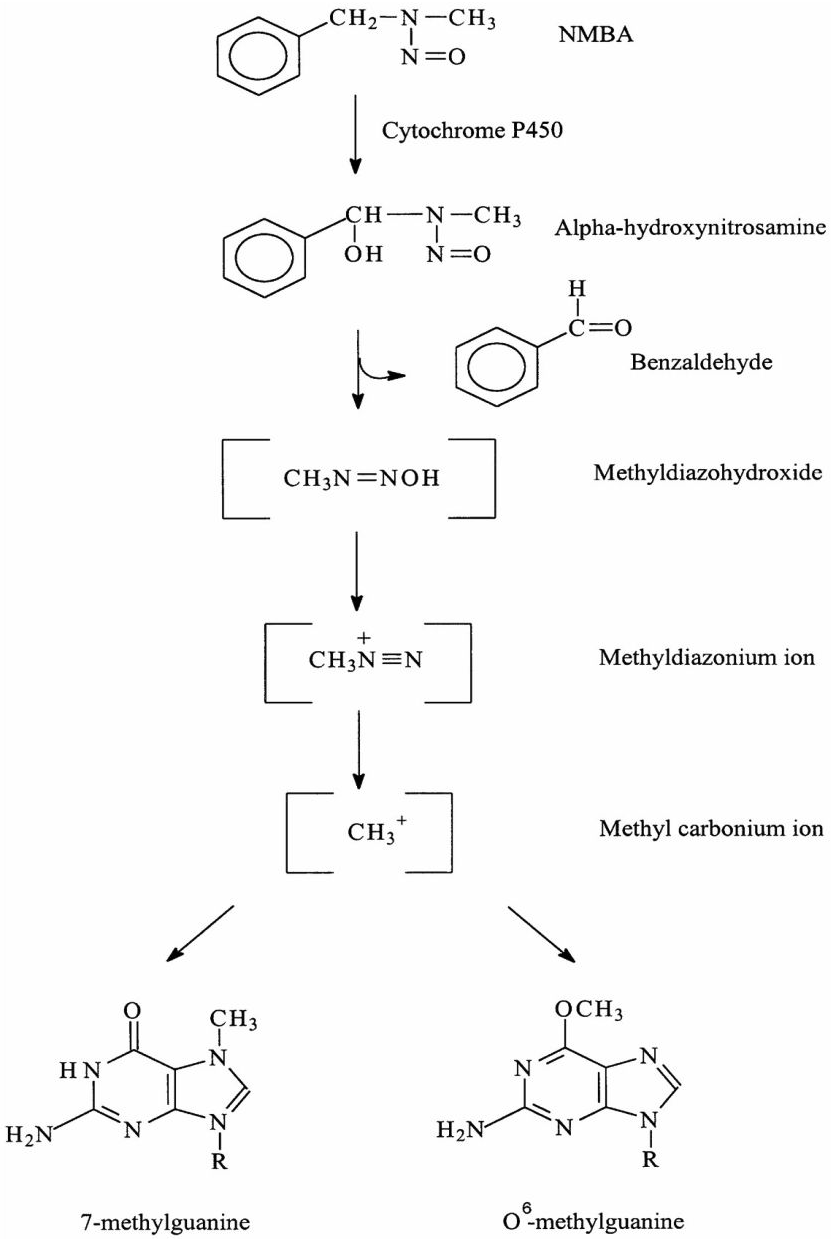

Figure 2.
Schema for metabolic activation of NMBA (from Stoner and Gupta, 2001).

Figure 3.
Appearance of rat esophageal lesions at the termination of a 25-wk bioassay. There are several papillomas on the surface of the esophagus. The lesion on the lower left was found to be a carcinoma upon histological analysis (from Stoner et al., 2006).

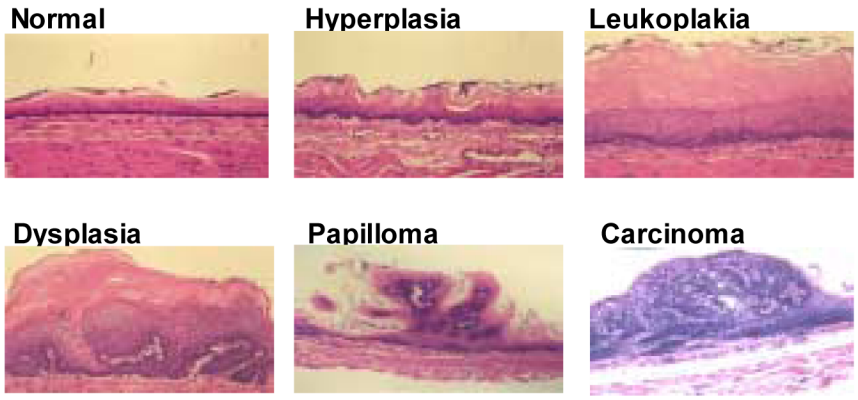

Figure 4.
Histopathology of NMBA-induced lesions in rat esophagus (from Stoner et al., 2006).

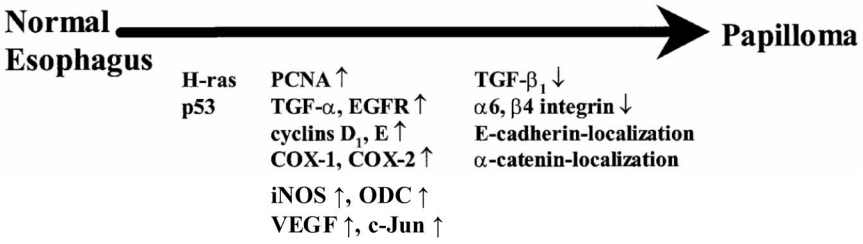

Figure 5.
Molecular events in papilloma development during NMBA-induced rat esophageal tumorigenesis (from Stoner and Gupta, 2001).

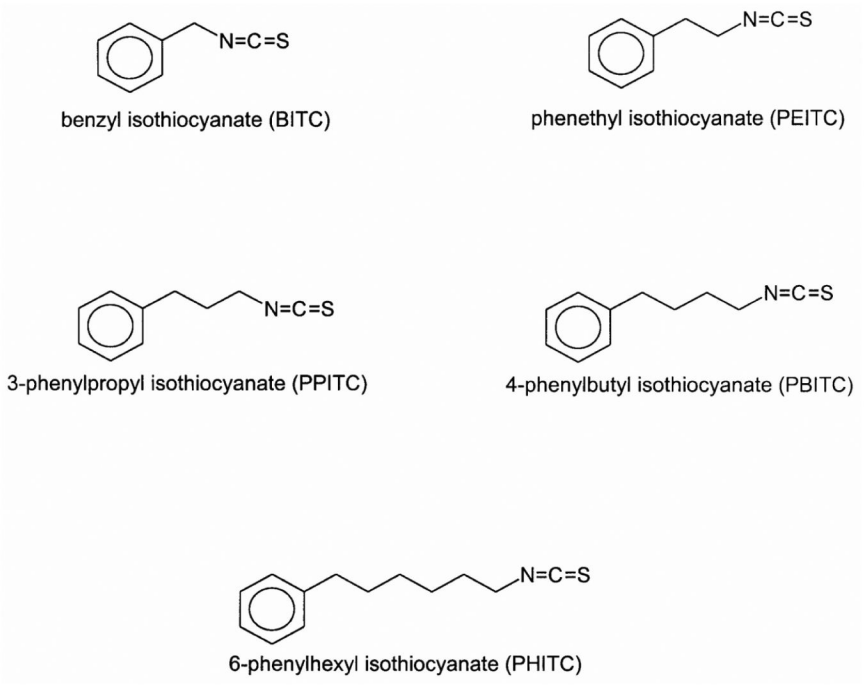

Figure 6.
Structures of isothiocyanates (from Stoner and Gupta, 2001).

Table 1

Agents that inhibit tumor initiation in the rat esophagus

Agents	Proposed mechanism of action	References
Ellagic acid	DNA adducts ↓ Phase II enzymes ↑	Mandal and Stoner, 1990 Mandal et al., 1988
Diallyl sulfide	P450 ↓ Phase II enzymes ↑	Wargovich et al., 1988 Brady et al., 1988
Theaflavins	?	Morse et al., 1997
(-)-epigallocatechin 3-gallate (EGCG)	Cyclin D1 ↓ COX-2 ↓ PGE2 ↓	Li et al., 2002 de Boer JG et al., 2004 Morse et al., 1997
Curcumin	P450 ↓	Mori et al., 2006 Ushida et al., 2000
Phenylethyl-isothiocyanate (PEITC)	P450 ↓	Stoner et al., 1991 Wilkinson et al., 1995
Phenylpropyl-isothiocyanate (PPITC)	P450 ↓	Wilkinson et al., 1995 Stoner and Morse, 1997
Phenylbutyl-isothiocyanate(PBITC)	P450 ↓	Stoner et al., 1995

Table 2

Agents that inhibit tumor progression in the rat esophagus

Agents	Proposed Mechanism of action	References
Green tea, black tea (decaffeinated)	?	Wang et al., 1995
PBIT*	iNOS inhibitor	Chen et al., 2004
L-748706	COX-2 inhibitor	Stoner et al, 2005
Resveratrol	PGE_2 signaling	Li et al., 2002
JTE-522	COX-2 inhibitor	Li et al., 2001
Irinotecan hydrochloride	Cell proliferation ↓	Fujiwara et al., 2004

* PBIT: *S,S*'-1,4-phenylene-*bis*(1,2-ethanediyl)*bis*-isothiourea

Table 3

Freeze-dried berries as effective agents against NMBA-induced esophageal tumorigenesis.

Agents	Proposed Mechanism of action	References
Inhibition of initiation		
Freeze-dried black raspberry	DNA adducts ↓ Glutathione S-transferase ↑	Kresty et al., 2001 Reen et al., 2006
Freeze-dried strawberry	DNA adducts ↓	Carlton et al., 2001
Inhibition of progression		
Freeze-dried black raspberry	Cell proliferation ↓ COX-2 ↓ iNOS ↓ VEGF ↓ c-Jun ↓ PCNA ↓	Chen et al., 2006 Chen et al., 2006 Kresty et al., 2001
Freeze-dried strawberry	Cell proliferation ↓	Carlton et al., 2001 Stoner et al., 1999
Brown rice and barn	Cell proliferation ↓	Kuno et al., 2004

Table 4

Agents tested in clinical trials for inhibitory effects against human esophageal SCC.

Agents	Activity			Mechanism	References
	No Effect	Inhibition	Enhancement		
Daily supplementation with vitamins and minerals	+				Rao et al., 1994
Calcium	+				Wang et al., 1993
Antitumor-B, 4-ECPR,Riboflavin*	+	+		?	Lin et al., 1990
		+		?	
Celecoxib + Selenomethionine	+	+		?	Limburg et al., 2005
Green tea	+				Wang et al., 2002

*4-ECPR: 4-ethoxycarbophenylretinamide