Inhibition of 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis in hamsters by tea and curcumin

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Tea is one of the most popular beverages consumed in the world. Curcumin, the major yellow pigment in turmeric, has been widely used as a spice, food preservative, coloring agent and an additive in cosmetic and drug preparations. Curcumin, alone and in combination with tea, significantly decreased the number of visible tumors by 35.1 or 39.6%, the tumor volume by 41.6 or 20.9%, and the SCC incidence from 92.3% (24/26) to 65.4% (16/24) and the squamous cell carcinoma (SCC) incidence from 76.9% (20/26) to 42.3% (11/26). The combination of tea and curcumin treatment, or no treatment for 18 weeks. The combination of both tea and curcumin significantly decreased the oral visible tumor incidence from 92.3% (24/26) to 69.2% (18/26) and the squamous cell carcinoma (SCC) incidence from 76.9% (20/26) to 42.3% (11/26). The combination of tea and curcumin also decreased the number of visible tumors by 35.1 or 39.6%, the tumor volume by 41.6 or 61.3% and the number of SCC by 53.3 or 51.3%, respectively. Green tea also decreased the number of dysplastic lesions. Curcumin also significantly decreased the SCC incidence. Tea and curcumin, singly or in combination, decreased the proliferation index in hyperplasia, dysplasia and papillomas. Only the combination treatment decreased the proliferation index in SCC. Tea alone and in combination with curcumin significantly increased the apoptotic index in dysplasia and SCC. Curcumin, alone and in combination with tea, significantly inhibited the angiogenesis in papilloma and SCC. The results suggested that green tea and curcumin had inhibitory effects against oral carcinogenesis at the post-initiation stage and such inhibition may be related to the suppression of cell proliferation, induction of apoptosis and inhibition of angiogenesis.

Introduction
Oral cancer is a common neoplasm worldwide, particularly in the developing countries such as India, Sri Lanka, Vietnam, the Philippines and Brazil, where it constitutes up to 25% of all kinds of cancers (1). In recent decades, oral cancer incidence and mortality rates have been increasing in the USA, Japan, Germany and Scotland, especially among young males (2–4). In the United States, there are ~43,000 new cases annually, resulting in ~11,600 deaths (5). The survival of patients with oral cancer has not improved significantly despite recent advances in radiotherapy and chemotherapy. Some of the patients cured by primary treatment develop a second cancer within a few years (6). Oral cancer usually develops from hyperplasia through dysplasia to carcinoma in the manner of ‘field cancerization’ due to carcinogen exposure (7). Oral leukoplasia is the most common premalignant lesion of oral cancer, and up to 20% of the patients with leukoplakia develop invasive carcinoma (8). Among many risk factors, tobacco and alcohol are the major ones in the development of oral carcinogenesis, being involved in >75% of oral cancers in the USA, France and Italy (9–11). There is increasing evidence for an association between a high consumption of fruits and vegetables and reduced risk of oral cancer, suggesting that natural products offer a protective effect against oral cancer (12,13).

The development of oral cancer is a multi-step process requiring initiation, promotion and progression. It can serve as a good model to investigate multi-step carcinogenesis. Due to its easy accessibility, the hamster buccal pouch is an excellent model system for the induction of oral squamous cell carcinoma (SCC) by chemical carcinogens and is useful for testing chemopreventive agents. Application of 7,12-dimethylbenz[a]anthracene (DMBA) to the cheek pouch of the Syrian golden hamster produces SCC that is histologically similar to human oral SCC. Carcinoma is preceded by a sequence of hyperplasia-papilloma/dysplasia-carcinoma, similar to human leukoplakia (14,15).

Tea (Camellia sinensis) is one of the most popular beverages consumed worldwide. Many studies have demonstrated that green tea, black tea and tea constituents have inhibitory effects on experimental tumorigenesis in a number of target organs such as skin, lung, esophagus, liver, small intestine, pancreas and bladder (16,17). We have detected high levels of tea catechins in human saliva after tea consumption, suggesting that the oral cavity could be a good target for tea to exert its chemopreventive effect against carcinogenesis (18). Oral administration of 1.5% green tea as the sole source of drink, starting from 2 weeks before initiation of the DMBA treatment to the end of the experiment, has had a significant inhibitory effect on oral carcinogenesis (19). It would be interesting to determine whether tea has an inhibitory effect on oral carcinogenesis at the post-initiation stage when the premalignant lesion has already developed.

Curcumin, the major yellow pigment in turmeric and curry, has been widely used as a spice, food preservative, coloring agent and an additive in cosmetic and drug preparations. Curcumin possesses antioxidant, anti-inflammatory and anti-

Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; SCC, squamous cell carcinoma; BrdU, bromodeoxyuridine; MVD, microvessels density.

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carcinogenic properties (20). It has been demonstrated that topical application of curcumin inhibits DMBA-initiated and 12-O-tetradecanoylphorbol-13-acetate-promoted skin tumors, and that dietary administration of curcumin suppresses the development of forestomach and colon tumors (21,22). Feeding of 500 p.p.m. curcumin during the initiation and post-initiation stages inhibited 4-nitroquinoline-1-oxide-induced tongue carcinogenesis (23). Curcumin has also been shown to exert significant growth inhibitory effects on oral precancerous and carcinoma cell lines, and the effect is synergistic with (-)-epigallocatechin-3-gallate, the most abundant polyphenol in tea (24).

The purpose of the present study was to investigate the effects of green tea and curcumin on DMBA-induced oral carcinogenesis in hamster cheek pouch at the post-initiation stage. The post-initiation model mimics the human cases with oral leukoplaikia or those with prior exposure to carcinogens, such as former and current smokers. We also aimed to understand chemopreventive mechanisms of green tea and curcumin by measuring cell proliferation, angiogenesis and apoptosis.

Materials and methods

Treatment of animals

All the experiments were conducted at Rutgers University under Protocol no. 91-024. Male Syrian golden hamsters (6 weeks old) weighing 60–80 g were purchased from Harlan (Indianapolis, IN). The animals were housed, four per cage in a room with controlled temperature and humidity with 12 h light:dark cycles. All animals were given AIN-93M diet (Research Diets, New Brunswick, NJ) and tap water ad libitum. After one week of acclimatization, the left pouch of 120 hamsters was topically treated with 100 µl of 0.5% DMBA (in mineral oil) (Sigma Chemical Company, St. Louis, MO) with a paintbrush three times/week for 6 weeks. Ten hamsters were used as the negative control (Group A). Eight animals were killed 2 days after the last DMBA treatment. The remaining hamsters were randomly divided into four groups (28 animals per group) and received no treatment (Group B), green tea (Group C), curcumin (Group D), or the combination of green tea and curcumin (Group E) for 18 weeks. Green tea solids, containing 15.1, 8.7, 4.3, 3.8% and 5.4% of (-)-epigallocatechin-3-gallate, (-)-epigallocatechin, (-)-epicatechin-3-gallate, (-)-epicatechin and caffeine, respectively (Thomas J.Lipton Tea Company, Englewood Cliffs, NJ) was made fresh to a 0.6% solution in tap water on Mondays, Wednesdays and Fridays; the solution was given to the hamsters as the sole source of drinking fluid. Curcumin (Turmeric type 97 containing 77% curcumin, 17% demethoxycurcumin and 3% bisdemethoxy-curcumin from Kalsec, Kalamazoo, MI) was dissolved in mineral oil, and 100 µl of the solution (10 µmol) was applied topically on the left buccal pouch three times/week. The body weights were monitored once every other week. The experiment was terminated at week 24. Hamsters were injected with bromodeoxyuridine (BrdU) (Sigma Chemical Company) at 50 mg/kg body weight 2 h before being killed by CO₂ asphyxiation.

Pathological and histopathological examinations

The whole cheek pouch was excised, flattened on the transparency plate and fixed in 10% PBS buffered formalin. The number of visible tumors in the oral cavity was counted; the length, width and height of each tumor were measured with a caliper. The tumor volume was calculated by the formula: volume = 4/3πr³ (where r was the average radius of the three diameter measurements in mm).

Formalin-fixed pouches were cut into 4–6 pieces of approximately equal width, Swiss-rolled, processed and then embedded in paraffin. Thirty sections (5 µm) were cut from each sample and the 1st, 15th and 30th slides were H&E stained for histopathological analysis. Basal cell hyperplasia, dysplasia, squamous cell carcinoma and papillomas were diagnosed with established criteria (25,26). The hyperplasia of oral epithelium was indicated by increased number of basal cells. The dysplasia was characterized by irregular epithelial stratification, increased number of mitotic figures, increased nuclear-to-cytoplasmic ratio and loss of polarity of basal cells. Papilloma was diagnosed by the invasion of underlying tissues, including those originating from papilloma and those from apparently normal epithelium. The numbers of oral lesions in the 1st, 15th and 30th slides were recorded (a large tumor that appeared in more than one slide was counted as one).

Apoptosis

Apoptotic cells were evaluated on H&E stained slides with the following morphological features: cell shrinkage, homogenous basophilic and condensed nuclei, nuclear fragmentation, marked eosinophilic condensation of the cytoplasm and sharply delineated cell borders surrounded with a clear halo (27). More than three noncontiguous, randomly selected fields (at least 2000 cells in each lesion) were counted under high-magnification (400×). The apoptotic index (percentage) was calculated as the number of apoptotic cells divided by the total number of epithelial cells counted.

Cell proliferation

To assess the proliferation of the squamous epithelium of the oral mucosa, tissue sections (25th, 16th and 29th) were immunohistochemically stained for BrdU. The avidin–biotin–peroxidase method was used with a rat monoclonal antibody (Serotec, Raleigh, NC) at the concentration of 5 µg/ml. More than three noncontiguous, randomly selected fields (at least 2000 cells) in each lesion were counted under 400x magnification. The proliferative index (percentage) was calculated as the number of positively stained cells divided by total number of epithelial cells counted.

Angiogenesis

Microvessels were detected by immunohistochemical staining for factor VIII/von Willebrand factor, a marker for vascular endothelial cells. After pretreatment with 0.25% trypsin for 10 min, tissue sections were immunostained with a rabbit anti-factor VIII antibody (1:800; DAKO Corporation, Carpenteria, CA) using the avidin–biotin–peroxidase method. In non-lesioned area and preinvasive lesions including hyperplasia, dysplasia and benign papillomas, the blood vessels in submucosa were counted. In carcinoma, peritumoral vessels were counted. The microvessels in three noncontiguous, randomly selected fields (200×) of each lesion were counted. The microvessel density (MVD) was expressed as the mean number of microvessels per mm².

Statistical analysis

The tumor incidence of different groups was compared by the χ² test. One way ANOVA followed by Dunn’s multiple test was used to compare the number of visible tumors, the number of various oral lesions. BrdU-labeling index, apoptotic index and MVD among these groups using the SAS computer software. The tumor volume was analyzed with signed rank test using the computer software Statview. Differences with calculated P values <0.05 were regarded as significant.

Results

General observations

Topical application of 0.5% DMBA to the left pouch of hamsters significantly decreased the body weight by 6.2% at week 6 compared with the non-treated group (Group A, Figure 1) (P < 0.05). This may be caused by the lower diet intake due to DMBA-induced inflammation, but all animals looked healthy. From weeks 8–20, the body weights were not significantly different among the different groups. After week 20, the body weights in Groups B–E were lower than in Group
A, possibly due to tumor development. Body weights were not significantly different among the four DMBA-treated groups during the period of weeks 6–22. However, at week 24, the body weights of Groups C and E were significantly lower than that of Group B (P < 0.01). This body weight lowering effect of green tea was also observed in previous studies (28). It may result from decreased nutrient absorption or increased energy expenditure due to caffeine.

Inhibition of tea and curcumin against DMBA-induced oral carcinogenesis

At week 6, all DMBA-treated animals had a visibly roughened granular surface on the mucosa with varying degrees of erythema and occasional white plaque-like lesion. Six of the eight animals analyzed developed dysplasia (75%) and all eight animals had hyperplasia (100%). The average numbers of hyperplasia and dysplasia per animal were 3.5 ± 0.7 and 1.5 ± 1.0, respectively. No SCC was observed in these animals.

At week 24, the combination of tea and curcumin treatment (Group E) significantly decreased the visible oral tumor incidence to 69.2% (18/26) from 92.3% (24/26) of the positive control (Group B) (P < 0.01). Although the tumor incidence of Groups C and D was less than that of Group B, the difference was not statistically significant (P > 0.05) (Table I).

Inhibition of angiogenesis by tea and curcumin

In DMBA-induced oral lesions, the neovasculatures primarily concentrated in the stromal areas and spread along stromal ridges on the periphery of epithelial lesions (Figure 5). A few microvessels were observed within the epithelium. The MVD of DMBA-induced various oral lesions were significantly higher than that of the non-lesioned area (P < 0.01). In Group B, the MVD in the areas of papilloma or SCC was significantly higher than those of hyperplasia and dysplasia and the MVD of SCC was significantly higher than that of papilloma (Figure 3C). There was no significant difference between MVD of hyperplasia and dysplasia. Compared with Group B, curcumin (Group D) and the combination (Group E) significantly decreased MVD in papilloma and SCC. However, the effect of green tea (Group C) on MVD was not significant in all the lesions (Figure 3C).

Discussion

In this study, topical application of DMBA to hamster cheek pouch for 6 weeks produced hyperplasia (100%) and dysplasia (75%), and the lesion progressed to SCC in 77% of the animals in 18 weeks without further treatment with a carcinogen or tumor promoter. This post-initiation model mimics the human case with oral leukoplakia or the former and current smokers, and therefore provides a good opportunity for studying chemopreventive agents.

The results indicated that tea and curcumin, alone and in combination, given after DMBA treatment effectively inhibited oral carcinogenesis. Decrease of tumor incidence, multiplicity and volume suggests that both green tea and curcumin retarded the progression of existing precancerous lesions and the growth of tumors in the oral mucosa. At the concentrations used, curcumin appears to have a stronger inhibitory effect on the progression from dysplasia to SCC than tea, since it decreased the incidence of SCC as well as the number of SCC. The combination of green tea and curcumin was more effective than either agent used alone. Higher concentrations of these agents are expected to be more efficacious; the dose–response relationship needs to be studied.

The results of the present study are in agreement with most previous studies concerning the inhibitory activity of green tea and curcumin against tumorigenesis in the skin, colon,
Table I. Inhibitory effect of green tea and curcumin on DMBA-induced oral carcinogenesis in hamster cheek pouch

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>Visible tumors</th>
<th>Microscopic lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tumor incidence (%)</td>
<td>No. of tumors</td>
</tr>
<tr>
<td>A</td>
<td>Negative control</td>
<td>10</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B</td>
<td>Positive control</td>
<td>26</td>
<td>92.3</td>
<td>2.42 ± 1.58b</td>
</tr>
<tr>
<td>C</td>
<td>0.6% green tea 10 μmol curcumin</td>
<td>27</td>
<td>81.4</td>
<td>1.57 ± 1.04c</td>
</tr>
<tr>
<td>D</td>
<td>10 μmol curcumin</td>
<td>26</td>
<td>76.9</td>
<td>1.46 ± 1.04c</td>
</tr>
<tr>
<td>E</td>
<td>0.6% green tea + 10 μmol curcumin</td>
<td>26</td>
<td>69.2a</td>
<td>1.15 ± 1.02c</td>
</tr>
</tbody>
</table>

The animals were given 0.6% green tea as drinking fluid (Group C), or 10 μmol curcumin by topical application (Group D), or the combination of green tea and curcumin (Group E) for 12 weeks after topical application of 0.5% DMBA to the left oral pouch of hamsters three times/week for 6 weeks. During the study period, two animals each in Groups B, D, E and one animal in Group C died. Tumor volume (mm³) was calculated by the formula: volume = 4/3πr³ (r represents the average radius of three diameter measurements in mm). The different oral lesions were expressed as the number of lesions per animal (mean ± SD) or incidence (%) in each group.

a Statistically different from Group B, P < 0.05, based on χ² test.
b,c Values with different superscripts in each column are significantly different, P < 0.05, based on ANOVA test followed by Dunn's multiple test.

a Statistically different from Group B, P < 0.05, based on Wilcoxon signed rank test.
Tea and curcumin inhibit oral carcinogenesis

Fig. 2. DMBA-induced oral lesions at week 24 (H&E staining). (a) Normal epithelium; (b) hyperplasia; (c) dysplasia; (d) papillomas; (e) papillary SCC; (f) SCC. Arrows indicate apoptotic cells which show cell shrinkage, nuclear condensation and apoptotic bodies.

Fig. 3. Effects of tea and curcumin on apoptosis, cell proliferation and angiogenesis. (A) Apoptotic index was calculated as the total number of apoptotic cells divided by total number of epithelial cells in each lesion evaluated (microscope setting ×400). (B) The proliferative index (%) was calculated as the total number of positively stained nuclei divided by total number of epithelial cells in each lesion evaluated (×400). (C) The microvessels density (MVD) was expressed as the mean number of microvessels/mm² (×200). Bars with different superscripts in each lesion are significantly different, P < 0.05 based on ANOVA test followed by Dunn’s multiple test.

Fig. 4. Cell proliferation in DMBA-induced oral lesions at week 24 (BrdU immunostaining and hematoxylin counterstaining). (a) Normal epithelium, ~5% BrdU-labeling index was detected; (b) basal cell hyperplasia (~9%); (c) dysplasia (~17%); (d) papillomas (~20%); (e) papillary SCC (~30%); (f) SCC (~30%).

Fig. 5. Angiogenesis in DMBA-induced various oral lesions (factor VIII immunostaining and hematoxylin counterstaining). (a) Normal epithelium; (b) basal cell hyperplasia; (c) dysplasia; (d) papillomas; (e) papillary SCC; (f) SCC.

esophagus, lung and other organs (28–30). To our knowledge, this is the first report showing an inhibitory effect of tea and curcumin on oral carcinogenesis at the post-initiation stage when the premalignant lesions have already been developed. Using the same hamster model, we previously showed that green tea was effective when given throughout the entire experimental period (19). Tanaka et al. (23) reported that feeding of 500 p.p.m. curcumin during the initiation and post-initiation stages inhibited 4-nitroquinoline-1-oxide-induced rat tongue carcinogenesis. Dietary and topical administration of turmeric (or curcumin) throughout the entire experiment period inhibited methylacetoxymethyl-nitrosamine-induced and DMBA-induced visible oral tumor yield and burden in hamster cheek pouches (31,32). Topical application of tea polyphenols (5 and 10 mg) and curcumin (0.6 and 6%) to the cheek pouch of hamsters at the initiation stage significantly inhibited the benzo[a]pyrene-induced cell proliferation (33). In vitro studies also demonstrated inhibitory effects of tea and curcumin on human oral premalignant and malignant epithelial cells, and a synergistic effect was observed when used in combination (24).

DMBA treatment produces carcinogen–DNA adducts which may induce G→A transition or A→T transversion (34). Such mutations have been observed frequently at exons 5–8 of the p53 gene, and at codon 61 of the Ki–ras gene in DMBA-induced hamster oral carcinomas (35,36). Mutation of the p53
gene may disrupt the balance between cell proliferation and apoptosis, and enhance angiogenesis which contributes to tumor development (37). The mutation of the ras gene may activate multiple signaling pathways leading to cell proliferation (38). In addition, topical multiple applications with DMBA induced inflammation and DNA oxidative damage in skin, which may play an important role in the promotion and progression of carcinogenesis (39). As a potent inflammatory mediator, leukotriene B4, formed through 5-lipoxygenation of arachidonic acid (40), was found much higher in DMBA-treated hamster oral tissue and human oral carcinoma tissue (41). All these factors can contribute to cell hyperproliferation, decreased apoptosis and angiogenesis in the progression from normal mucosa, through hyperplasia and dysplasia, to carcinoma.

In the present study, tea and curcumin, alone and combination, inhibited the cell proliferation in hyperplasia, dysplasia and papillomas, but only combination decreased the cell proliferation in SCC. On the other hand, we also observed an increased apoptosis in dysplasia and SCC, but not in hyperplasia and papillomas in hamsters treated with tea alone or tea and curcumin. This indicated that the dysplastic and cancerous cells are more susceptible to the pro-apoptotic effect of tea, a favorable property of a chemopreventive agent. In our studies, curcumin was not shown to increase apoptotic index. These observations are in general agreement with the previously observed anti-proliferative, pro-apoptotic and anti-promotion effects of tea polyphenols and curcumin (42–50). Caffeine in green tea can also promote UV- and radiation-induced apoptosis (51–53). We observed that the formation of microvessels was inhibited in papillomas and SCC by curcumin, suggesting curcumin may inhibit the further growth and progression of tumors. The results are consistent with the report by Arbiser et al. that curcumin had direct anti-angiogenic activity in vivo and in vitro (54). Although green tea and (−)-epigallocatechin-3-gallate have been shown to inhibit angiogenesis in other models (55,56), this inhibitory effect on angiogenesis in all lesions was not observed in the present model. Many studies have demonstrated that tea and curcumin can inhibit the activation of transcription factors such as AP-1 and NF-κB, enzyme activities of certain protein kinases, cyclooxygenase, lipooxygenase and ornithine decarboxylase, as well as the interaction of certain receptors with their ligands (42,43,57–62). Further research is required to investigate whether these mechanisms contribute towards the chemopreventive effect on oral carcinogenesis.

In conclusion, our results demonstrated that green tea and curcumin inhibited oral carcinogenesis at the post-initiation stage. Green tea and curcumin could be delivered to the oral mucosa at rather high concentrations. These agents may be explored as chemopreventive agents for humans at high risk of oral cancer such as those with leukoplakia and erythroplakia in former smokers. Proliferation, apoptosis and angiogenesis may be used as surrogate biomarkers for chemoprevention studies in the future.

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References


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