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

Ning Li¹ ², Xiaoxin Chen¹, Jie Liao¹, Guangyu Yang¹, Su Wang¹, Youssef Josephson¹, Chi Han², Junshi Chen², Mou-Tuan Huang¹ and Chung S. Yang¹

¹Laboratory for Cancer Research, College of Pharmacy, Rutgers University, 164 Frelinghuysen Road, Piscataway, New Jersey 08854, U.S.A.; and ²Institute of Nutrition and Food Hygiene, Chinese Academy of Preventive Medicine, 29 Nanwei Road, Beijing, 100050 China.

Summary

We studied the effects of tea and curcumin on DMBA-induced oral carcinogenesis in hamsters. DMBA solution (0.5% in mineral oil, 0.1 ml) was applied topically to the left cheek pouch of male Syrian golden hamsters 3 times per week for 6 weeks. Two days after the last treatment of DMBA, the animals received 0.6% green tea as drinking fluid, or 10 µmol curcumin applied topically 3 times per week, or the combination of green tea and curcumin treatment, or no treatment for 18 weeks. The combination of 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 and the tumor volume by 52.4% and 69.8%, as well as the numbers of SCC, dysplastic lesions, and papillomas by 62.0%, 37.5% and 48.7%, respectively. Green tea or curcumin treatment decreased the number of visible tumor by 35.1% or 39.6%, the tumor volume by 41.6% or 61.3% and the number of SCC by 53.3% and 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 bromodeoxyuridine (BrdU)-labeling index in hyperplasia, dysplasia, and papillomas. Only the combination treatment decreased the BrdU-labeling 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 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.

Keywords: Tea, curcumin, oral cancer, chemoprevention

Introduction

Oral cancer is a common neoplasm worldwide, particularly in the developing countries such as India, Srilanka, Vietnam, the Philippines and Brazil, where it constitutes up to 25% of all kinds of cancers. The survival of patients with oral cancer has not improved significantly despite recent advance in radiotherapy and chemotherapy. Some of the patients cured by primary treatment will develop a second cancer within a few years. Oral leukoplakia is the most common premalignant lesion of oral cancer, and up to 20% of the patients with leukoplakia will develop invasive carcinoma.

Hamster buccal pouch is an excellent model system for experimental induction of oral tumors by chemical carcinogens, and is useful for testing chemopreventive and therapeutic agents. Application of DMBA to the cheek pouch of the Syrian golden hamster produces squamous cell carcinoma that are histologically similar to those seen in human cases. Similar molecular changes were also described in the literature.

Tea is one of the most popular beverages consumed worldwide. Curcumin, the major yellow pigment in turmeric and curry, has been used widely as a spice, food preservative, yellow food coloring agent and additive in cosmetic and drug preparations. Many studies have demonstrated that tea and curcumin had significant inhibitory effects on tumorigenesis in a number of target organs but their effects on oral cancer were not well studied. Our research group detected high levels of tea catechins in human saliva after tea consumption, and this suggested a possible chemopreventive effect of tea on oral cancer. It has been shown that tea and curcumin exert significant synergistic growth inhibitory effects on oral precancerous and carcinoma cell lines.
The purpose of the present study was to investigate the effects of green tea and curcumin, alone or in combination, on oral carcinogenesis induced by DMBA in hamster cheek pouch and explore the chemopreventive mechanisms of green tea and curcumin by measuring cell proliferation, angiogenesis and apoptosis.

**Materials and Methods**

The left pouch of 120 Male Syrian golden hamsters was topically treated with 100 μl 0.5% DMBA (in mineral oil) three times per week for 6 weeks, ten hamsters were used as negative control (Group A). Eight animals were sacrificed two 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), 0.6% green tea solution as the sole source of drinking fluid (Group C), 10 μmol curcumin applied topically 3 times per week (Group D), or the combination of green tea and curcumin (Group E) for 18 weeks. The body weights were monitored once every other week. The experiment was terminated at week 24. Hamsters were injected with bromodeoxyuridine (BrdU) at 50 mg/kg body weight 2 hr before being sacrificed by CO₂ asphyxiation. The whole cheek pouch were excised and fixed in 10% PBS buffered formalin. The visible numbers of tumors in oral cavity were counted and the tumor volume was calculated by the formula: \[ \text{volume} = \frac{4}{3} \pi r^3 \] (r represents as average radius of three diameter measurements of tumor in mm).

**Histopathological Examination**

Formalin-fixed pouches were cut into 4-6 pieces of approximately equal width, swiss-rolled, processed and then embedded in paraffin. Thirty sections (5μm) of each sample were cut and the 1st, 15th and 30th slide were routinely H&E stained for histopathological analysis. Basal cell hyperplasia, dysplasia, squamous cell carcinoma and papillomas were diagnosed with established criteria (8). The numbers of oral lesions in the 1st, 15th and 30th slide were recorded (the same lesions appeared in more than one slide was counted as one lesion).

**Apoptotic index, Brdu-labeling index and the microvessel density (MVD)**

Apoptotic cells were evaluated on H&E stained slides with the morphological features. BrdU labeling cells and microvessels (factor VIII/von Willebrand factor) were detected by immunohistochemical staining with the avidin-bioti-peroxidase method. Apoptotic cell, Brdu-labeled cells and microvessels were counted in more than three noncontiguous, randomly selected fields of hyperlasia, dysplasia, papilloma and SCC lesion, respectively. The apoptotic index and proliferative index (percentage) were expressed as the number of positive cells per 100 cells counted. The microvessel density (MVD) was expressed as the mean number of microvessels per mm².

**Results and discussion**

**General observation**

Body weights did not have significant difference among the four DMBA treated groups from Weeks 6 to 22. However, at Week 24, the body weights of Groups C and E were significantly lower than that of Group B. This may be possibly due to the body weight lowering effect of green tea we observed previously in other animal models.

**Inhibition of tea and curcumin on DMBA-induced oral carcinogenesis**

At Week 6, six of 8 animals developed dysplasia (75%) and all 8 animals developed 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. This post-initiation model provides a good opportunity for us to develop chemopreventive strategies for humans at a high risk for oral cancer, especially leukoplakia and erythroplakia.
At week 24, the combination of tea and curcumin (Group E) significantly decreased the oral visible tumor incidence to 69.2% (18/26) from 92.3% (24/26, Group B). As compared with Group B, the average number of tumor in Groups C, D and E was significantly decreased by 35%, 39% and 52%, respectively, and the tumor volume was reduced by 41.6%, 61.3% and 69.8%, respectively (Table 1).

Histologically, the left buccal pouches of Group B-E animals presented areas of hyperplasia and dysplasia as well as papillomas and SCC. The combination of tea and curcumin significantly decreased the oral SCC incidence to 42.3% (11/26, Group E) from 76.9% (20/26, Group B) and curcumin alone also significantly decreased the SCC incidence to 50.0% (13/26, Group D). As compared with that of Group B, the average number of SCC per animal in Group C, D and E decreased by 53%, 51% and 62%, respectively. The average number of papillomas per animal in Group E was also decreased by 48.7%. In addition, the number of dysplastic lesion per animal in Groups C and E was significantly decreased by 31.5% and 37.5%, respectively, however, the number of hyperplasia was not different among four groups.

The results indicated that tea and curcumin, alone and in combination, given after DMBA treatment effectively inhibited oral carcinogenesis. 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, esophagus, lung and other organs. To our knowledge, this is the first report showing inhibitory effect of tea and curcumin on oral carcinogenesis at the post-initiation stage when the premalignant lesions have already been developed.

Induction of apoptosis and inhibition of cell proliferation and angiogenesis by tea and curcumin on DMBA-induced oral carcinogenesis

As compared with Group B, tea (Group C) and the combination (Group E) significantly increased the apoptotic index in dysplasia and SCC, 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; however, curcumin (Group D) had no effect on apoptosis in all the lesions (Fig. 1A). All the treatments with tea and curcumin inhibited cell proliferation significantly in hyperplasia, dysplasia and papillomas, but only the combination decreased cell proliferation in SCC (Fig. 1B). These observations are in general agreement with the previously observed anti-proliferative, pro-apoptotic and anti-promotion effects of tea polyphenols and curcumin.

As compared with Group B, curcumin (Group D) and the combination (Group E) significantly inhibited MVD in papilloma and SCC; however, green tea (Group C) had no effect on MVD in all the lesions (Fig. 1C), suggesting curcumin may inhibit the further growth and progression of tumors. The results is consistent with the report by Arbiser et al that curcumin had direct anti-angiogenic activity in vivo and in vitro. Although green tea and (-)-epigallocatechin-3-gallate have been shown to inhibit angiogenesis in other models, this inhibitory effect on angiogenesis in all lesions was not observed in the present model.

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.
### Table 1. 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 observations</th>
<th>Microscopic observations</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tumor incidence (%)</td>
<td>No. of tumors</td>
<td>Tumor volume (mm^3)</td>
<td>No. of hyperplasia</td>
<td>No. of dysplasia</td>
</tr>
<tr>
<td>A</td>
<td>Negative control</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.80±2.57</td>
<td>1.84±1.02</td>
</tr>
<tr>
<td>B</td>
<td>Positive control</td>
<td>26</td>
<td>92.3</td>
<td>2.42±1.55(^b)</td>
<td>99.71±107.90</td>
<td>6.63±2.39</td>
<td>1.26±0.92</td>
</tr>
<tr>
<td>C</td>
<td>0.6% green tea</td>
<td>27</td>
<td>81.4</td>
<td>1.57±1.04(^c)</td>
<td>42.51±37.05(^d)</td>
<td>6.19±2.32</td>
<td>1.69±1.35(^b,c)</td>
</tr>
<tr>
<td>D</td>
<td>10 μmol curcumin</td>
<td>26</td>
<td>76.9</td>
<td>1.46±1.04(^c)</td>
<td>38.49±46.39(^d)</td>
<td>6.11±3.11</td>
<td>1.15±0.76</td>
</tr>
<tr>
<td>E</td>
<td>0.6% green tea+10 μmol curcumin</td>
<td>26</td>
<td>69.2(^a)</td>
<td>1.15±1.02(^c)</td>
<td>30.11±41.03(^d)</td>
<td>6.11±3.11</td>
<td>1.15±0.76</td>
</tr>
</tbody>
</table>

\(^a\) Statistically different from Group B, p<0.05, based on Chi-square 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

\(^d\) Statistically different from Group B, p<0.05, based on Wilcoxon signed rank test
Figure 1. 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 x 400). B. The proliferative index (percentage) was calculated as the total number of positively stained nuclei divided by total number of epithelial cells in each lesion evaluated (x 400). C. The microvessels density (MVD) was expressed as the mean number of microvessels per mm$^2$ (x 200). Bars with different superscripts in each lesion are significantly different, p<0.05 based on ANOVA test followed by Dunn’s multiple test.