Effect of curcumin and ferulic acid on modulation of expression pattern of p53 and bcl-2 proteins in 7,12-dimethylbenz[a]anthracene-induced hamster buccal pouch carcinogenesis

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The modulating effect of curcumin and ferulic acid was investigated on expression pattern of apoptosis regulatory p53 and bcl-2 proteins in oral squamous cell carcinoma (OSCC). The OSCC was induced in the buccal pouch of golden Syrian hamster by painting with 0.5% 7,12-dimethylbenz[a]anthracene (DMBA) three-times a week for 14 weeks. The expression pattern of p53 and bcl-2 proteins was analyzed by immunohistochemical staining. We noticed 100% tumor formation in hamsters painted with DMBA alone for 14 weeks. Over expression of p53 and bcl-2 proteins was observed in the buccal mucosa of tumor-bearing hamsters. Oral administration of curcumin (80 mg/kg body wt) and ferulic acid (40 mg/kg body wt) to DMBA painted hamsters on days alternate to DMBA painting for 14 weeks completely inhibited tumor formation and down-regulated the expression pattern of p53 and bcl-2 proteins. Our results thus demonstrated the protective role of curcumin and ferulic acid on DMBA-induced abnormal expression of p53 and bcl-2 proteins in the buccal mucosa of golden Syrian hamsters.

Keywords: bcl-2, Curcumin, DMBA, Ferulic acid, Oral cancer, p53, SCC, Tumor

Oral cancer, one of the most disfiguring types of cancer constitutes the fifth most frequent cancer worldwide and accounts for 40-50% of all cancers in India. Such high incidence is due to tobacco chewing, smoking, betel quid chewing and alcohol consumption. Oral squamous cell carcinoma (OSCC), the most common cancer of the oral cavity accounts for 90% of all oral cancers. Despite significant advancement in oral cancer treatment strategy, it remains as a major cause of morbidity in human populations. The overall 5-year survival rate of oral cancer patients in advanced oral carcinoma is still at 50% only. 7,12-Dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis is a well-suited model for studying the biochemical, histological and molecular pathogenesis of oral cancer. OSCC in the hamsters has marked similarities to human oral carcinoma, both morphologically and histologically.

Malignant transformation is due to abnormalities in genes, which control cell cycle. Identification of mutated gene or its protein product during carcinogenesis can provide valuable information relating to tumor progression and outcome. Studies have demonstrated abnormalities in molecular regulators of cell proliferation and apoptosis in cancer of the oral cavity and oesophagus. p53 and bcl-2 can serve as molecular markers of carcinogenesis, including oral cancer, since genetic alterations in these genes have been shown to drive neoplastic development. The human p53 gene is located on the short arm of the 17th chromosome and encodes a protein composed of 393 amino acids. Its mutation or loss of function of p53 has been implicated in the pathogenesis of several cancers including oral carcinoma. Overexpression of mutant p53 protein has been shown in epithelial dysplasia and oral cancer. p53 protein plays a crucial role in the G1 phase of the cell cycle, wherein it arrests the cell cycle progression to S phase from G1 phase and thereby providing time for DNA repair. If the repair fails, p53 may trigger cell suicide by apoptosis.

The bcl-2 oncogenes, a member of family of genes encoding for protein which regulate apoptosis is first identified at the site of reciprocal translocation of the chromosome 18 in follicular carcinoma. This membrane-associated protein is present in endoplasmic reticulum, nuclear and outer mitochondrial membrane. Bcl-2, an inhibitor of...
apoptosis helps tumor cells to avoid programmed cell death\textsuperscript{19,20}. Overexpression of bcl-2 has been demonstrated in various malignant tumors, including oral carcinoma\textsuperscript{21-23}.

Antitumor agents exert their cytotoxic potential in tumor cells through apoptotic mechanism. Curcumin [1, 7-bis(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3,5-dione], the active principle of \textit{Curcuma longa} Linn. (Turmeric) has been shown to have potent anti-inflammatory, antioxidant and anti-carcinogenic properties, and induce apoptosis in a wide variety of cancer cells\textsuperscript{24-26}. It is also known to inhibit carcinogen-DNA adduct formation and thereby the tumor progress in experimental carcinogenesis\textsuperscript{27}.

Ferulic acid (3-methoxy, 4-hydroxy cinnamic acid), found in rice, wheat, barley, fruits and vegetables arises from the metabolism of phenylalanine and tyrosine through Shikimate pathway. The anti-carcinogenic potential of ferulic acid in skin, colon and tongue carcinogenesis has been reported\textsuperscript{28-30}. Ferulic acid may have proapoptotic effects in cancer cells and thereby leading their destruction\textsuperscript{31}. In the present study, modulatory effect of curcumin and ferulic acid on expression pattern of apoptosis regulatory proteins p53 and bcl-2 has been investigated in DMBA-induced hamster buccal pouch carcinoma, as it may provide valuable information relating to p53 and bcl-2 apoptotic potential during DMBA-induced oral carcinogenesis.

**Materials and Methods**

**Animals**

Male golden Syrian hamsters, 8-10 weeks old, weighing 80-120 g were procured from National Institute of Nutrition, Hyderabad, India and maintained in Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. The animals were housed in polypropylene cages and provided standard pellet diet (Agro Corporation Private Ltd., Bangalore, India) and water ad libitum. The standard pellet diet was composed of 21% protein, 5% lipids, 4% crude fiber, 8% ash, 1% calcium, 0.6% phosphorus, 3.4% glucose, 2% vitamin, and 55% nitrogen-free extract (carbohydrates). The animals were maintained under controlled conditions of temperature (27 ± 2°C) and humidity (55 ± 5%) with a 12 h light/dark cycle as per the principles and guidelines of the Ethical Committee for Animal Care of Annamalai University in accordance with Indian National Law on animal care and use.

7,12-Dimethylbenz[a]anthracene (DMBA), curcumin and ferulic acid were obtained from Sigma-Aldrich Chemical Pvt. Ltd., Bangalore, India.

**Experimental design**

The Institutional Animal Ethics Committee (Reg. no. 160/1999/CPCSEA), Annamalai University, Annamalainagar, Tamil Nadu, India approved the experimental design. A total number of 60 hamsters were randomized into six groups of 10 hamsters each.

Group 1 hamsters served as control and were painted with liquid paraffin (vehicle) alone thrice a week for 14 weeks on their left buccal pouches, while groups 2, 3 and 4 were painted with 0.5% DMBA in liquid paraffin using number 4 painting brush, which leaves approximately 0.4 mg DMBA on hamster buccal pouch in each application\textsuperscript{4,5}. Group 2 received no other treatment. Groups 3 and 4 were orally administered with curcumin (80 mg/kg body wt) and ferulic acid (40 mg/kg body wt), starting 1 week before the exposure to the carcinogen and continued on days alternate to DMBA painting until sacrifice. Groups 5 and 6 received oral administration of curcumin (80 mg/kg body wt) and ferulic acid (40 mg/kg body wt) alone, respectively throughout the experimental period. The experiment was terminated at the end of 14 weeks and all the animals were sacrificed by cervical dislocation. For histopathological examination, buccal mucosa tissues from control and experimental animals in each group were fixed in 10% formalin and routinely processed and embedded with paraffin and 2-3 µm sections were cut in a rotary microtome and stained with haematoxylin and eosin.

**Immunohistochemical analysis**

Paraffin embedded tissue sections were dewaxed and rehydrated through graded ethanol to distilled water. Endogenous peroxidase was blocked by incubation with 3% H\textsubscript{2}O\textsubscript{2} in methanol for 10 min. The antigen retrieval was achieved by adding citrate buffer solution (pH 6.0) and keeping in microwave for 10 min, followed by washing with Tris-buffered saline (pH 7.6). The tissue sections were then incubated with the universal proteinaceous blocking reagent power Block\textsuperscript{TM} for 15 min at room temperature to block non-specific binding, and further with the respective primary antibody (DAKO p53-DO-7 and DAKO bcl-2/100) overnight at 4°C. The bound primary antibodies were detected by incubation with their corresponding secondary antibodies, conjugated with horseradish peroxidase for 30 min at room temperature. After rinsing with
Tris-buffered saline, the antigen-antibody complex was detected using 3,3'-diaminobenzidine (Sigma, USA), the substrate of horseradish peroxidase. When acceptable colour intensity was reached, the slides were washed, counter stained with haematoxylin and covered with a mounting medium.

Results

Tumor incidence, tumor volume and burden as well as histological features noticed in buccal mucosa of control and experimental hamsters in each group are given in Table 1. We observed 100% tumor incidence with OSCC formation in all the hamsters, whose buccal pouches were painted with DMBA alone for 14 weeks. The mean tumor volume and burden of tumor-bearing hamsters (Group 2) were 321.6 mm$^3$ and 1029.1 mm$^3$, respectively. We also observed severe hyperkeratosis, hyperplasia, dysplasia and well-differentiated squamous cell carcinoma (SCC) in the buccal pouches of DMBA-alone painted hamsters.

Oral administration of curcumin (80 mg/kg body wt; Group 3) and ferulic acid (40 mg/kg body wt; Group 4) to DMBA painted hamsters on days alternate to DMBA painting for 14 weeks completely prevented OSCC progress. Although well-differentiated SCC was not developed in the buccal pouches of DMBA + curcumin (Group 3) and DMBA + ferulic acid (Group 4) treated hamsters, hyperplasia, hyperkeratosis and dysplasia were observed. Control hamsters painted with liquid paraffin alone (Group 1) and hamsters treated with curcumin alone (Group 5) and ferulic acid alone (Group 6) showed well-defined and intact epithelial layers without OSCC.

The p53 index and intensity of bcl-2 expression scoring in the buccal mucosa of control and experimental hamsters in each group are given in Table 2. The analysis showed a positive staining for p53 and bcl-2 in tumor tissues, which was more significant, as compared to normal tissues. Positive staining for p53 and bcl-2 were considered, when more than 10% of the tumor cells showed strong nuclear and cytoplasmic staining, respectively. We observed 80 and 85% positive staining for p53 and bcl-2, respectively in tumor tissues of group 2.

Increased nuclear expression of p53 and cytoplasmic expression of bcl-2 was also observed in the buccal

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 (Control)</th>
<th>Group 2 (DMBA alone)</th>
<th>Group 3 (DMBA + curcumin)</th>
<th>Group 4 (DMBA + ferulic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor incidence (OSCC)</td>
<td>-</td>
<td>100%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total number of tumors/hamsters</td>
<td>-</td>
<td>32/10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tumor vol. (mm$^3$)/hamsters</td>
<td>-</td>
<td>321.6 ± 30.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tumor burden (mm$^3$)/hamsters</td>
<td>-</td>
<td>1029.1 ± 98.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Histological features</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperkeratosis</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>-</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SCC</td>
<td>-</td>
<td>Well-differentiated</td>
<td>-</td>
<td>-</td>
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<tr>
<th>Parameters</th>
<th>Mutant p53</th>
<th>bcl-2</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>WS 0 0 0</td>
<td>WS 8 2 0</td>
</tr>
<tr>
<td>DMBA alone</td>
<td>1 3 1 6</td>
<td>0 2 8 1</td>
</tr>
<tr>
<td>DMBA + curcumin</td>
<td>7 2 1 5</td>
<td>4 5 1 1</td>
</tr>
<tr>
<td>DMBA + ferulic acid</td>
<td>6 3 1 4</td>
<td>7 3 0 0</td>
</tr>
<tr>
<td>Curcumin alone</td>
<td>3 0 0 0</td>
<td></td>
</tr>
<tr>
<td>Ferulic acid alone</td>
<td>0 0 0 0</td>
<td></td>
</tr>
</tbody>
</table>

0, Negative (<5%), WS, weak staining (5-25%), MS, moderate staining (25-50%), and SS, strong staining (>50%) of cells
The negative staining for p53 was noticed in the buccal mucosa of control animals. In normal epithelium, immunostaining for bcl-2 protein was identified in basal keratinocytes and dendritic cells adjacent to the basement membrane. We also graded p53 and bcl-2 expression according to the number of positively stained cells per 100 counted cells. The percentage positive cells were scored according to the method reported earlier. Oral administration of curcumin and ferulic acid to DMBA-painted hamsters of group 2 and 4, respectively significantly downregulated the expression of p53 and bcl-2 proteins. The hamsters treated with curcumin and ferulic acid alone (groups 5 and 6) showed no significant difference in the expression pattern of p53 and bcl-2 proteins, as compared to control group (Figs 1 and 2).

Fig. 1—Microphotographs (A-F) depicting immunoexpression of p53 in control and experimental hamsters in each group [A: Control, group 1 (expression not detectable, 0); B: DMBA alone, group 2 (overexpressed, SS); C: DMBA + curcumin, group 3 (downregulated, MS); D: DMBA + ferulic acid, group 4 (downregulated, MS); E: curcumin alone, group 5 (normal expression only in basal and immediate suprabasal cells, WS); and F: ferulic acid alone, group 6 (expression not detectable, 0). 0, negative (<5%), WS, weak staining (5-25%), MS, moderate staining (25-50%) and SS, strong staining (>50%) of cells [Immunohistostaining, 100x]

Fig. 2—Microphotographs (A-F) depicting immunoexpression of bcl-2 in control and experimental hamsters in each group [A: Control, group 1 (weak staining, WS); B: DMBA alone, group 2 (overexpression, SS); C: DMBA + curcumin, group 3 (downregulated, MS); D: DMBA + ferulic acid, group 4 (downregulated, MS); E: curcumin alone, group 5 (weak staining, WS); F: ferulic acid alone, group 6 (weak staining, WS). 0, negative (<5%), WS, weak staining (5-25%), MS, moderate staining (25-50%) and SS, strong staining (>50%) of cells [Immunohistostaining, 100x]
Discussion

In the present study, we observed 100% tumor formation in hamsters painted with DMBA alone. The tumors developed in the buccal mucosa were histopathologically confirmed as well-differentiated SCC. Oral administration of curcumin and ferulic acid completely prevented tumor progress and significantly reduced histopathological abnormalities in DMBA-painted hamsters, thus indicating potent suppressing effect of curcumin and ferulic acid on cell proliferation during DMBA-induced oral carcinogenesis.

Studies on immunohistochemical expression of p53 and bcl-2 in tumors can help to understand tumor biology and in predicting tumor prognosis. p53 and bcl-2 are involved in DNA repair and cell death. Altered expression of p53 promotes transformation, whereas bcl-2 blocks or delays apoptosis and enhances proliferation. Accumulation of mutant p53 protein in tumor cells is associated with more aggressive clinical phenotype. p53, the guardian of the genome prevents propagation of genetically damaged cells. It is also reported to repair DNA damage caused by physical, chemical and biological agents in the oral cavity prior to DNA replication.

p53 protein immunoreactivity increases with progression from normal mucosa to invasive carcinoma. Altered expression of p53 gene is reported in the majority of human malignant tumors. The positive staining of p53 gene in tumor cells is reportedly indicative of p53 mutation. DMBA-induced mutations have been demonstrated in p53 gene of hamsters, which share 75% homology with human p53 gene. It has been suggested that detection of mutant p53 protein in tumors may help in the treatment of oral cancer. Overexpression of p53 in the buccal mucosa of tumor bearing animals suggests accumulation of mutant p53 protein during DMBA-induced oral carcinogenesis.

Both p53 and bcl-2 are operative in the regulation of apoptosis and interact with each other in the assessment of tumor aggressiveness both in human and animal models of carcinogenesis. It has been suggested that mutant p53 can act as a substitute for bcl-2 function by inhibiting apoptosis. Overexpression of p53 and bcl-2 genes may substitute each other in the development of oral carcinoma. Overexpression of bcl-2 may occur secondary to p53 mutation and decreased programmed cell death. Altered expression of bcl-2 has been demonstrated in several cancers including oral carcinoma. Our results are in line with these findings.

Altered expression pattern of p53 and bcl-2 observed in buccal mucosa of tumor bearing animals was due to abnormal cell proliferation and evasion of apoptosis during DMBA-induced oral carcinogenesis. Oral administration of curcumin and ferulic acid to DMBA-painted hamsters significantly prevented the altered expression of p53 and bcl-2 during DMBA-induced oral carcinogenesis. Earlier studies have highlighted the antiproliferative, anticarcinogenic and apoptotic potential of curcumin and ferulic acid in experimental carcinogenesis. The modulating effect of curcumin and ferulic acid on p53 and bcl-2 expression was probably due to their apoptotic potential and significant role in the inhibition of abnormal cell proliferation occurring in DMBA-induced oral carcinogenesis. Thus, the present study demonstrated the modulatory effect of curcumin and ferulic acid on expression pattern of p53 and bcl-2 proteins in DMBA-induced oral carcinogenesis.

Acknowledgements

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