Chemopreventive Potential of Coumarin in 7,12-dimethylbenz[a]anthracene Induced Hamster Buccal Pouch Carcinogenesis

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Abstract

The aim of the present study was to investigate the chemopreventive effect of coumarin against 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch carcinogenesis by monitoring tumor incidence and histopathological changes as well as by analyzing the status of biochemical markers (lipid peroxidation, enzymatic and non-enzymatic antioxidants, phase I and phase II detoxification enzymes). Oral squamous cell carcinomas were induced in the buccal pouch of Syrian golden hamsters by painting with 0.5% DMBA in liquid paraffin three times a week for 14 weeks. We noted 100% tumor formation with marked abnormalities in the biomarkers status in hamsters treated with DMBA alone. Oral administration of coumarin at a dose of 100 mg/kg body weight (bw) to DMBA treated hamsters completely prevented the tumor formation as well as restored the status of biochemical variables. The results of the present study thus suggest that the chemopreventive effect of coumarin is probably due to its anti-lipid peroxidative potential and modulating effect on carcinogen detoxification agents in favor of the excretion of ultimate carcinogenic metabolites of DMBA during DMBA-induced hamster buccal pouch carcinogenesis.

Keywords: Antioxidants - chemoprevention - coumarin - detoxification agents - DMBA - oral cancer.

Introduction

Oral cancer, a cancer that has a poor prognosis, is the fifth most frequent cancer worldwide and affects more than 500,000 new cases each year (Warnakulasuriya, 2010). Majority of oral cancers (90%) arise in the squamous epithelium of the oral cavity and are usually preceded by leukoplakia and erythroplakia. While oral squamous cell carcinoma is the most common global cancer, this form of cancer comprising approximately one third of all cancers in Central and South East Asian countries (Warnakulasuriya, 2009). Epidemiological studies reported that highest incidence of oral cancers in India are due to wide spread habits of tobacco chewing, smoking, betel quid chewing with and without tobacco and alcohol consumption (Muwonge et al., 2008). Despite recent improvement in the diagnosis and treatment strategy of oral cancer, the prognosis and five-year survival rate of oral cancer has not changed dramatically in the past few decades (Warnakulasuriya, 2009).

Well established experimental animal models serve pivotal role to assess the chemopreventive efficacy of natural products and their bioactive components as well as synthetic chemicals. 7,12-dimethylbenz[a]anthracene (DMBA), a potent organ specific pro-carcinogen, mediates carcinogenesis by causing DNA damage and inducing chronic inflammation. Also excessive generation of reactive oxygen species (ROS) that occur during metabolic activation of DMBA causes oxidative DNA damage, contributing to neoplastic transformation (Silvan et al., 2011). DMBA induced oral carcinogenesis in hamsters is preceded by a sequence of precancerous lesions, which are quite similar to that of human oral cancers. Histopathologically, morphologically and at molecular level, DMBA induced hamster buccal pouch carcinogenesis is thus commonly employed to study the chemopreventive potential of natural products and synthetic agents (Manoharan et al., 2010).

Liver is constantly exposed to several toxic substances and thus plays putative role in the detoxification of toxic substances including carcinogenic and mutagenic entities. Measurement of detoxification agents in liver could thus help to assess the chemopreventive potential of the natural products. Phase I enzymes (cytochromes P450 and b5) are involved in the metabolic activation of pro-carcinogens into their active metabolites, where as phase II enzymes [glutathione reductase (GR), glutathione-S-transferase (GST) and DT-diaphorase)] and reduced glutathione (GSH) are involved in the detoxification of activated metabolites of the carcinogens. A positive association between genetic polymorphisms of drug metabolizing enzymes and susceptibility to oral cancer has been shown (Chandra et al., 2006). Extensive studies demonstrated impaired activities of drug metabolizing enzymes in the liver and buccal mucosa of hamsters treated with DMBA (Bhuvaneswari et al., 2005; Chandra et al., 2006).

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ROS are continuously generated in vivo due either to physiological metabolism or pathological alterations. ROS can cause damage to lipids, proteins and nucleic acids if they are excessively generated in the system. ROS destruct membrane lipids particularly PUFA and initiate a chain reaction known as lipid peroxidation, which has been implicated in the pathogenesis of several disorders including cancer (Reuter et al., 2010). The deleterious effects of ROS are counteracted by an array of sophisticated enzymatic [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)] and non-enzymatic [GSH and vitamin E] antioxidants. Altered status of lipid peroxidation and antioxidants were reported in several cancers including oral cancer (Manoharan et al., 2012).

Chemoprevention, a promising and appealing approach in experimental oncology, deals with the prevention of cancer by using non-cytotoxic natural products or synthetic compounds (Malik et al., 2006). Chemopreventive agents suppress, inhibit, halt or reverse the process of carcinogenesis either by inhibiting the metabolic activation of carcinogens or by enhancing the detoxification of ultimate carcinogenic metabolites. Also, they suppress tumor formation by inhibiting the excessive generation of ROS and by improving the antioxidant defense mechanism. Coumarin, a member of the benzopyrone family of compounds, is found throughout the plant kingdom and at high levels in Cinnamon bark oil, Cassia leaf oil and Lavender oil. Coumarin is also present in considerable amounts in some of the human diets. It possesses an array of pharmacological effects including anti-thrombotic, vasodialatory, anti-mutagenic and antioxidant properties (Lacy and O’Kennedy, 2004). It has been reported that coumarin inhibited DMBA-induced mammary tumor formation in rats and benzo(a)pyrene induced neoplasia in the forestomach of mice (Feuer et al., 1976; Wattenberg et al., 1979). Von Angerer et al. (1994) reported anti-neoplastic and anti-metastatic activities of coumarin in transplanted prostate tumors of the rat. Coumarin reduced the incidence of spontaneous tumors in MTV-H-ras transgenic mice (Tseng, 1991). Previous studies from our laboratory demonstrated the protective effect of coumarin against DMBA-induced genotoxicity and DMBA-induced cell surface glycoconjogates abnormalities during DMBA-induced hamster buccal pouch carcinogenesis (Baskaran et al., 2011a; 2011b). There was however no experimental studies on the chemopreventive effect of coumarin against DMBA-induced hamster buccal pouch carcinogenesis. The present study was thus designed to focus the chemopreventive effect of coumarin in DMBA-induced hamster buccal pouch carcinogenesis.

**Materials and Methods**

**Chemicals**

DMBA, coumarin and biochemicals such as reduced glutathione, reduced nicotinamide adenine dinucleotide (NADH), 1,1’,3,3’-tetramethoxypropane, were obtained from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. All chemicals and solvents used were AR grade.

**Animals**

Male golden Syrian hamsters, 8-10 weeks old, weighing 80-120 g were used for the study. The animals were obtained from National Institute of Nutrition, Hyderabad and maintained in Central animal house, Rajah Muthiah Institute of Health Science, Annamalai University, Annamalainagar, India. The hamsters were housed in polypropylene cages at room temperatures (27±2°C) with relative humidity 55±5%, in an experimental room. In Annamalainagar, the LD (light: dark) cycle is almost 12:12 h. The local institutional animal ethics committee (Registration Number 160/1999/ CPCSEA), Annamalai University, Annamalainagar, India approved the experimental design (Proposal No.731, dated 02.09.2010). The animals were maintained as per the principles and guidelines of the ethical committee for animal care of Annamalai University in accordance with the Indian National Law on animal care and use. The animals were provided with standard pellet diet (Amrut Laboratory Animal Feed, Mysore Feeds Limited, Bangalore, India) and water ad libitum.

**Induction of oral squamous cell carcinoma**

Tumors were induced in each hamster’s buccal pouch with topical application of 0.5% DMBA in liquid paraffin three times a week for 14 weeks (Shklar, 1982). The total number of tumors in the hamsters’ buccal pouch was determined macroscopically at the time of sacrifice of animals.

**Dose dependent study**

Different doses of coumarin (50, 100 and 150 mg/kg bw) were assessed to find out the effective chemopreventive dose in DMBA-induced hamster buccal pouch carcinogenesis. A dose of 100 mg/kg bw coumarin has shown potent chemopreventive potential in DMBA treated hamsters as compared to rest of the doses. Due to these reasons, the dose of 100 mg/kg bw was chosen.

**Experimental design for chemoprevention study**

A total of 40 hamsters were randomized into four groups of ten hamsters in each. Group I hamsters served as control and were painted with liquid paraffin three times a week for 14 weeks on their left buccal pouches. Groups II and III hamsters were painted with 0.5% DMBA in liquid paraffin three times a week for 14 weeks on their left buccal pouches. Group II hamsters received no other treatment. Group III hamsters were orally given coumarin at a dose of 100 mg/kg bw/day, starting one week before exposure to the carcinogen and continued on days alternate to DMBA painting, until the sacrifice of the hamsters. Group IV hamsters received oral administration of coumarin (100 mg/kg bw) alone throughout the experimental period. The experiment was terminated at the end of 16th week and all hamsters were sacrificed by cervical dislocation. Biochemical studies were conducted on plasma, liver and buccal mucosa of control and experimental hamsters in each group. For histopathological examination, buccal mucosal tissues were fixed in 10% formalin and routinely processed and embedded with paraffin, 2-3 μm sections were cut in a
rotary microtome and stained with haematoxylin and eosin.

Biochemical estimations

Blood samples were collected into heparinized tubes. Plasma was separated by centrifugation at 1000×g for 15 min. Tissue samples from hamsters were washed with ice cold saline and dried between folds of filter paper, weighed and homogenized using appropriate buffer in an all glass homogenizer with Teflon pestle and used for biochemical estimations.

The levels of cytochrome P₄₅₀ and b₅ in both the liver and buccal mucosa were determined according to the method of Omura and Sato (1964). Cytochrome P₄₅₀ was measured by the formation of pigment on reaction between reduced cytochrome P₄₅₀ and carbon monoxide. The pigment was read with an absorbance maximum at 450 nm. The difference spectrum between reduced and oxidised cytochrome was used as an index to measure the level of cytochrome b₅. GR activity in liver tissue homogenate was assayed using the method employed by Carlberg and Mannervik (1985). The enzyme activity was assayed by measuring the formation of reduced glutathione when the oxidized glutathione (GSSG) is reduced by reduced nicotinamide adenine dinucleotide phosphate (NADPH). The activity of GST in liver and buccal mucosa tissue homogenate was assayed using the method employed by Habig et al. (1974). GST activity was measured by incubating the tissue homogenate with the substrate 1-chloro-2,4-dinitrobenzene (CDNB). The absorbance was followed for 5 min at 540 nm after the reaction was started by the addition of reduced glutathione. The activity of DT-diaphorase in the liver was estimated according to the method of Ernster (1967) based on the utilization of GSH by the enzyme. The color developed was read at 520 nm. One unit of enzyme is taken as the amount of enzyme required to give 50% inhibition of nitroblue tetrazolium (NBT) reduction. The activity of CAT in plasma and buccal mucosa was assayed using the method described by Sinha (1972) based on the utilization of H₂O₂ by the enzyme. The color developed was read at 620 nm. One unit of the enzyme is expressed as micromoles of H₂O₂ utilized per minute. The activity of GPx in plasma and buccal mucosa was determined using the method employed by Rotruck et al. (1973) based on the utilization of GSH by the enzyme. One unit of the enzyme is expressed as micromoles of GSH utilized per minute.

Protein determination

The protein content was determined by the method of Lowry et al. (1951). The peptide bonds (-CONH-) in polypeptide chain react with copper sulphate in an alkaline medium to give a blue colored complex. In addition, tyrosine and tryptophan residues of proteins cause reduction of the phosphomolybdate and phosphotungstate components of the Folin-Ciocalteau reagent to give bluish products read at 640 nm, which contribute towards enhancing the sensitivity of this method.

Statistical analysis

The values are expressed as mean±SD. The statistical comparisons were performed by one way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT), using SPSS version 12.0 for windows (SPSS Inc. Chicago; http://www.spss.com). The values are considered statistically significant if the p value was less than 0.05.

Results

The tumor incidence, tumor volume and tumor burden of control and experimental hamsters in each group are shown in Table 1. In hamsters treated with DMBA alone, 100% tumor formation with mean tumor volume (389.68 mm³) and tumor burden (1324.91 mm³) was observed. Oral administration of coumarin (100 mg/kg bw) completely prevented the tumor incidence in hamsters treated with DMBA. No tumor was observed in control hamsters treated with liquid paraffin alone as well as
counarin alone administered hamsters.

Figure 1 shows the histopathological features of control and experimental hamsters in each group. A myriad of histopathological changes (severe hyperkeratosis, hyperplasia, dysplasia and well-differentiated squamous cell carcinoma of the epithelium), was observed in hamsters treated with DMBA alone (Figure 1b). A mild to moderate preneoplastic lesions (hyperplasia and dysplasia) were noticed in DMBA+counarin treated hamsters (Figure 1c). Hamsters administrated with coumarin alone showed well defined and intact epithelial layers similar to that of the control hamsters (Figure 1a and d).

Figure 2 shows the activities of phase I (cytochromes P<sub>450</sub> and b<sub>5</sub>) and phase II (GR, GST and DT-diaphorase) detoxification enzymes and glutathione content (GSH) in the liver of control and experimental hamsters in each group. The activities of phase I enzymes were increased (p<0.05) whereas phase II enzymes and GSH content were significantly decreased (p<0.05) in hamsters treated with DMBA alone as compared to control hamsters. Oral administration of coumarin to hamsters treated with DMBA significantly restored (p<0.05) the activities of the detoxification enzymes and GSH to near normal range. Coumarin alone treated hamsters showed no significant difference in the status of detoxification enzymes and GSH content as compared to control hamsters.

Figure 3 shows the status of phase I (cytochromes P<sub>450</sub> and b<sub>5</sub>) and phase II (GST) detoxification enzymes and the content of GSH, GSSG, and GSH/GSSG ratio in the buccal mucosa of control and experimental hamsters in each group. The status of phase I (cytochrome P<sub>450</sub> and b<sub>5</sub>) and phase II (GST) detoxification enzymes and GSH and GSH/GSSG ratio were significantly increased (p<0.05) whereas GSSG content was decreased (p<0.05) in tumor-bearing hamsters as compared to control hamsters. Oral administration of coumarin to hamsters treated with DMBA significantly brought back (p<0.05) the status of phase I and phase II detoxification enzymes and glutathione content to near normal range. Hamsters treated with coumarin alone showed no significant difference in the status of phase I and phase II detoxification enzymes, GSH, GSSG, and GSH/GSSG ratio as compared to control hamsters.

Figure 4 shows the status of TBARS, enzymatic (SOD, CAT, GPx) and non-enzymatic (GSH, Vitamin E) antioxidants in the plasma of control and experimental hamsters in each group. The concentration of TBARS was increased (p<0.05), whereas the activities of enzymatic antioxidants (SOD, CAT, GPx) and the levels of non-enzymatic antioxidants (GSH and Vitamin E) were significantly decreased (p<0.05) in tumor bearing hamsters as compared to control hamsters. Oral administration of coumarin to hamsters treated with DMBA significantly brought back (p<0.05) the concentration of TBARS and antioxidants to near normal range. Hamsters treated with coumarin alone showed no significant difference in TBARS and antioxidants status as compared to control

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**Table 1. Incidence of Oral Neoplasm and Histopathological in the Control and Experimental Animals in Each Group (n=10)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group VI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control DMBA alone</td>
<td>DMBA+Counarin</td>
<td>Counarin alone</td>
<td></td>
</tr>
<tr>
<td>Tumour incidence (oral squamous cell carcinoma)</td>
<td>0%</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Total number of tumours/animals</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tumor multiplicity</td>
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<td>3.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tumor frequency</td>
<td>0</td>
<td>34</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tumour volume (mm&lt;sup&gt;3&lt;/sup&gt;/animals)</td>
<td>389.68±17.92</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tumour burden (mm&lt;sup&gt;3&lt;/sup&gt;/animals)</td>
<td>1324.91±64.38</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

*Values are expressed as mean±SD for 10 hamsters in each group. Tumor volume was measured using the formula, \( V = \frac{4}{3} \pi \times D_1 \times D_2 \times D_3 \), where \( D_1 \), \( D_2 \) and \( D_3 \) are the three diameters (mm) of the tumor. Tumor frequency = number of average tumors per animal. Tumor frequency = number of tumors per group.
Chemopreventive Potential of Coumarin in DMBA Induced Hamster Buccal Carcinogenesis

Figure 3. Status of Phase I and Phase II Enzymes and Glutathione Content in the Buccal Mucosa of Control and Experimental Hamsters in Each Group. Values are expressed as mean±SD for 10 hamsters in each group. Values that do not share a common superscript between groups differ significantly at p<0.05. (DMRT). X- Micromoles of cytochrome P450; Y- micromoles of cytochrome b5; A-micromoles of 1-chloro 2,4 dinitrobenzene (CDNB)/ reduced glutathione conjugate formed per minute.

Figure 4. Status of TBARS and Antioxidants in the Plasma of Control and Experimental Hamsters in Each Group. Values are expressed as mean±SD for 10 hamsters in each group. Values that do not share a common superscript between groups differ significantly at p<0.05. (DMRT). A- The amount of enzymes required to inhibit 50% nitroblue-tetrazolium (NBT) reduction. B- Micromoles of H2O2 utilized/second. C- Micromoles of glutathione utilized/min.

Figure 5. Status of TBARS and antioxidants in the buccal mucosa of control and experimental hamsters in each group. Values are expressed as mean±SD for 10 hamsters in each group. Values that do not share a common superscript between groups differ significantly at p<0.05. (DMRT). A- The amount of enzymes required to inhibit 50% nitroblue-tetrazolium (NBT) reduction. B- Micromoles of H2O2 utilized/second. C- Micromoles of glutathione utilized/min.

Discussion

In the present study, the chemopreventive potential of coumarin was assessed by monitoring the tumor incidence, tumor volume and tumor burden during DMBA-induced hamster buccal pouch carcinogenesis. The status of lipid peroxidation, antioxidants and phase I and II detoxification agents was used as biochemical end points to assess the chemopreventive potential of coumarin in DMBA-induced hamster buccal pouch carcinogenesis. We observed 100% tumor formation with severe hyperplasia, keratosis, and dysplasia in hamsters treated with DMBA alone. The oral pathologist confirmed the tumors histopathologically as well differentiated squamous cell carcinoma. An interesting observation of the present study was no tumor formation in the buccal mucosa of hamsters treated with DMBA+coumarin. We, however, noticed precancerous lesions such as hyperplasia and dysplasia in DMBA and coumarin treated hamsters. Control hamsters and hamsters treated with coumarin alone showed well defined intact epithelial layers. The present study suggests that coumarin has the potential to suppress or inhibit tumor formation during DMBA-induced hamster buccal pouch carcinogenesis. Khan et al. (2004) reported that the ring-opened products of lactone ring serve as nucleophiles to scavenge the reactive ultimate carcinogenic metabolites during carcinogenesis. They also reported that coumarin reduced chemical induced oxidative stress in the kidney of Wistar rats. The chemopreventive potential of coumarin relies on its aromatic ring fused to a condensed lactone ring.

Phase I and II drug metabolizing enzymes have pivotal role in the metabolism of carcinogens and other drugs (Alias et al., 2009; Vinothkumar and Manoharan, 2011). An imbalance in the activities of these enzymes results in carcinogenesis. Increase in phase I enzymes accompanied by decrease in phase II enzymes in the liver of hamsters treated with DMBA confirms the accumulation of the ultimate carcinogenic metabolite of DMBA, dihydrodiol epoxide as well as impaired activities of these enzymes in the liver (Manoharan et al., 2010). Enhanced activities of phase I and phase II enzymes in the buccal mucosa might be due to repeated DMBA exposure. Oral administration of coumarin at a dose of 100 mg/kg bw to hamsters treated with DMBA restored the status of phase I and II enzymes,
which suggest that coumarin either blocked the metabolic activation of DMBA by neutralizing the carcinogenic electrophiles or stimulated the activities of phase II enzymes to enhance the excretion of the carcinogenic metabolites.

In recent years free radical research has been utilized to focus the chemopreventive potential of the test compound since ROS play putative role in signal transduction pathways and phagocytosis (physiological concentrations) as well as in the pathogenesis of several cancers if they are excessively generated (Wittgen and van Kempen, 2007). A poor antioxidant defense mechanism in the cells makes them more prone to the cytotoxic effects of carcinogens and ROS. Oxidative stress thus results in the cell due to imbalance in the status of pro-oxidants and antioxidants. Over production of ROS and impaired activities of antioxidants has been reported in the plasma of oral cancer patients and oral tumor bearing animals (Baskar et al., 2004; Anusu and Manoharan, 2011). Our results support these findings.

Vitamin E and glutathione, well known non-enzymatic antioxidants, play crucial role in scavenging several ROS and in the inhibition of tumor formation. Tumor cells utilize these antioxidants from circulation for their rapid growth and to escape from the toxic effects of ROS. Lowered activities of SOD, CAT and GPx were reported in DMBA-induced oral carcinogenesis (Anusu and Manoharan, 2011). Increased plasma TBARS observed in hamsters treated with DMBA alone (tumor bearing hamsters) could be due to poor antioxidant defense mechanism occurring during DMBA-induced oral carcinogenesis. Low levels of ROS promote cell proliferation whereas high levels of ROS induce cell apoptosis or necrosis. Low content of PUFA, a substrate for lipid peroxidation, has been reported in oral carcinogenesis (Krishnakumar et al., 2009). Low levels of TBARS were reported in highly proliferating tumors such as oral cancer (Kowsalya et al., 2011). Decreased levels of TBARS in the buccal mucosa of hamsters treated with DMBA alone (tumor bearing hamsters) could be due to low PUFA content or due to abnormal cell proliferation occurring in oral carcinogenesis. Reduced GSH and GPx have putative role in the regulation of cell proliferation. A positive association between GSH content and cell proliferation has been reported (Manoharan et al., 2012). An increase in GSH/GSSG ratio has been shown in oral carcinogenesis (Silvan et al., 2011). Lowered activities of SOD and CAT were reported in both human and experimental oral carcinogenesis (Manoharan et al., 2005; Balakrishnan et al., 2008). Our results corroborate these observations. Oral administration of coumarin restored the status of lipid peroxidation and antioxidants in the circulation and buccal mucosa of hamsters treated with DMBA, which suggest that coumarin showed pronounced anti-lipid peroxidative property as well as modulating effect on detoxification cascade in favor of the excretion of carcinogenic metabolites during DMBA-induced hamster buccal pouch carcinogenesis. Although there was no tumor formation in hamsters treated with DMBA+coumarin, moderate hyperplasia and dysplasia were noticed. The present study will be extended to confirm whether coumarin inhibited the tumor formation or just delayed the tumor formation in hamsters treated with DMBA.

Acknowledgements

Financial support from University Grants Commission (UGC), New Delhi to Mr. N. Baskaran in the form of UGC-RGNF-SRF is gratefully acknowledged.

References


