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Research Article

Pro-apoptotic potential of ferulic acid during 7,12-dimethylbenz(a)anthracene induced hamster buccal pouch carcinogenesis

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ABSTRACT

The present study has investigated the pro-apoptotic potential of ferulic acid by analysing the activities of caspase-3 and 9 as well as by investigating the expression pattern of pro-apoptotic protein, Bax, during 7,12-dimethylbenz(a)anthracene (DMBA)-induced hamster buccal pouch carcinogenesis. Oral tumors were developed in the buccal pouch of hamsters by painting with 0.5% DMBA three times a week for 14 weeks. We noticed 100% tumor formation, histopathologically confirmed as well differentiated squamous cell carcinoma, with marked abnormalities in the activities of caspase-3 and 9 and Bax expression pattern in hamsters treated with DMBA alone. Oral administration of ferulic acid at a dose of 40mg/kg bw to hamsters treated with DMBA, not only completely prevented the formation of oral tumors but also restored the activities of caspase 3 and 9 and up regulated the expression of Bax. The present study thus suggests that the pro-apoptotic potential of ferulic acid might have inhibited tumor formation during DMBA-induced hamster buccal pouch carcinogenesis.

Keywords: Oral cancer; ferulic acid; Bax; caspases.

INTRODUCTION

Apoptosis plays pivotal role in the removal of unwanted damaged cells from the body. Under normal situations, apoptosis is essential to regulate the balance between cell proliferation and cell death. Apoptosis causes cell death by inducing blebbing, cytoplasmic membrane shrinkage, dissipation of mitochondrial membrane potential, nuclear condensation and DNA fragmentation (Hengartner, 2000 & Xu, et al., 2010). Impairment in apoptosis disrupts the maintenance of tissue homeostasis and can lead to several diseases including cancer. Evasion of apoptosis to various toxic stimuli is one of the characteristic features of neoplastic cells. Several chemopreventive agents exhibited antitumor efficacy through apoptotic induction (Kamesaki, 1998).

Bax, a proapoptotic protein of Bcl-2 family, is present predominantly in the cytosol. Bax, is responsible for transferring apoptotic signals to the mitochondria (Pluta, et al., 2010). Lowered Bax expression is associated with poor prognosis in oral squamous cell carcinoma. Imbalance in Bcl-2 /Bax ratio has been recognized as a common phenomenon cancer (Simonart & Van Vooren, 2002). Extensive studies have pointed out that the expression of bax was reduced in preneoplastic and neoplastic lesions (Loro, et al., 1999; Manoharan, et al., 2011). Tumor tissues with higher Bcl-2 or lower Bax expression escape from apoptosis, which enhance the tumor progression and aggressiveness (Gao, et al., 2012). Natural products or synthetic agents that induce apoptosis have thus great potential to emerge as novel chemotherapeutic agents.

Caspases, intracellular cysteine proteases, are important components of apoptotic cascade (Porter & Janicke, 1999). Caspases exist in cells as inactive proenzymes, which are activated in response to pro-apoptotic stimuli (Li & Yuan, 2008; Logue & Martin, 2008). In apoptosis two types of caspases are involved, which include initiator caspases and effector caspases. While the initiator caspases (2, 8, 9 and 10) are involved in initiating caspase activation cascade, effector caspases (3, 6 and 7) are responsible for demolition of the cells during apoptosis (Sanders & Parker, 2002; Zimmermann, et al., 2001). Caspase-9 is an initiator caspase for mitochondrian specific apoptotic pathway. Upon activation, caspase-9 stimulates the effector caspase-3 to complete the process of apoptosis. Caspase-3, synthesized as a latent pro-enzyme, is involved in the degradation of the cell in the final stages of apoptosis. Inactivation or reduced expression of caspase-3 and 9 has been implicated in the pathogenesis of cancer.

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Ferulic acid (4-hydroxy- 3-methoxy cinnamic acid) a polyphenol, is present abundantly in bananas, citrus fruits, bamboo shoots, egg plants and cabbage (Zhao & Moghadasian, 2008). Ferulic acid possesses diverse biochemical and pharmacological effects, which include antioxidant, anti-inflammatory, anti-genotoxic, anticancer, neuroprotective and hepatoprotective properties (Anselmi, et al., 2004; Joshi, et al., 2006; Rukkumani, et al., 2004). Previous studies from our laboratory demonstrated the chemopreventive potential of ferulic acid against DMBA- induced oral, skin and mammary carcinogenesis (Balakrishnan, et al., 2008; Alias, et al., 2009; Baskaran, et al., 2010). The present study focuses the modulating effect of ferulic acid on the activities of caspase 3 and 9 and the expression pattern of Bax during DMBA-induced hamsters buccal pouch carcinogenesis.

MATERIALS AND METHODS

Animals

Male golden Syrian hamsters, aged 8–10 weeks, weighing 80–120 g, were purchased from the National Institute of Nutrition, Hyderabad, India and were maintained in the Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. The animals were housed in polypropylene cages and provided with a standard pellet diet (Agro Corporation Pvt. Ltd., Bangalore, India) and water *ad libitum*. The animals were maintained under controlled conditions of temperature (27±2°C) and humidity (55±5%) with a 12 h light/dark cycle.

Chemicals

The carcinogen, 7,12-dimethylbenz(a)anthracene and ferulic acid was obtained from Sigma-Aldrich Chemical Pvt. Ltd., Bangalore, India. Bax, primary antibodies were purchased from Dako, Carprinteria, CA, USA. Power Block^{TM} reagent and secondary antibody conjugated with horseradish peroxidase were purchased from BioGenex, San Ramon, CA, USA. The caspase 3 and -9 colorimetric assay kits were purchased from Biovision, Mountain View, CA, USA.

Experimental design

The institutional animal ethics committee (Register number 160/1999/CPCSEA), Annamalai University, Annamalainagar, India, approved the experimental design. A total number of 40 hamsters were categorized into four groups of ten hamsters in each. Group I hamsters served as vehicle control and were painted with liquid paraffin alone 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 III hamsters were orally administered with ferulic acid at a dose of 40mg/kg bw/day, starting one week before exposure to the carcinogen and continued on days alternate to DMBA painting, until the completion of the experiment. Group IV hamsters were orally administered with ferulic acid alone at a dose of 40mg/kg bw/day throughout the experimental period. The experiment was terminated at the end of 16th week and all hamsters were sacrificed by cervical dislocation.

Immunohistochemical staining

Paraffin embedded tissue sections were dewaxed and rehydrated through grade ethanol to distilled water. Endogenous peroxidase was blocked by incubation with 3% H_2O_2 in methanol for 10 minutes. The antigen retrieval was achieved by microwave in citrate buffer solution (2.1 g citric acid/L D.H_2O; 0.37g EDTA/L D.H_2O; 0.2g Trypsin) (pH 6.0) for 10 minutes, followed by washing step with Tris-buffered saline (8g NaCl; 0.605g Tris) (pH 7.6). The tissue section was then incubated with power BlockTM reagent (BioGenex, San Ramon, CA, USA), universal proteinaceous blocking reagent, for 15 minutes at room temperature to block non-specific binding sites. The tissue sections were then incubated with the primary antibody (Bax - Dako, Carprinteria, CA, USA) overnight at 4°C. The bound primary antibody was detected by incubation with the secondary antibody conjugated with horseradish peroxidase (BioGenex, San Ramon, CA, USA) for 30 minutes at room temperature. After rinsing with Tris-buffered saline, the antigen-antibody complex was detected using 3,3´-diamminobenzidine, the substrate of horseradish peroxidase. When acceptable color intensity was reached, the slides were washed, counter stained with hematoxylin and covered with a mounting medium. Each slide was microscopically analyzed and enumerated the percentage of the positively stained cells semi-quantitatively. The percentage of positive cells was scored according to the method of Nakagawa et al. (1994) as follows: 3+ = strong staining, more than 50% of cells were stained; 2+ = moderate staining, between 20 and 50% of cells were stained; 1+ = week staining, between 1 and 20% of cells were stained; 0 = negative, less than 1% of cell staining.

Estimation of caspase 3 and 9 activities by enzyme linked immunosorbent assay [ELISA]

The activities of caspase-3 and 9 were assayed in the buccal mucosa using ELISA kits for caspase-3 and 9 according to the manufacturer's instructions. The caspase-3 and 9 assays are based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD – pNA and LEHD–pNA respectively at 405nm in a microtiter plate reader.

Statistical analysis

Values are expressed as mean ± standard deviation (SD). Statistical comparisons were performed by one-way analysis of variance followed by Duncan's Multiple Range Test. The results were considered statistically significant if the *p* values were less than 0.05.

Table 1: Incidence of oral neoplasm in control and experimental hamsters in each group (n=10)

Parameter	Group I Control	Group II DMBA alone	Group III DMBA + Ferulic acid	Group VI Ferulic acid alone
Tumor incidence (oral squamous cell carcinoma)	0	100%	0	0
Total number of tumors/animals	0	33/10	0	0
Tumor volume (mm^3)/ animals	0	312.45 $\pm$ 28.72	0	0
Tumor burden (mm^3)/ animals	0	1031.08 $\pm$ 94.77	0	0

Tumor volume was measured using the formula, $v = (4/3)\pi\ [D_1/2][D_2/2][D_3/2]$ where D1, D2 and D3 are the three diameters (mm^3) of the tumor. Tumor burden was calculated by multiplying tumor volume and the number of tumors/animal.

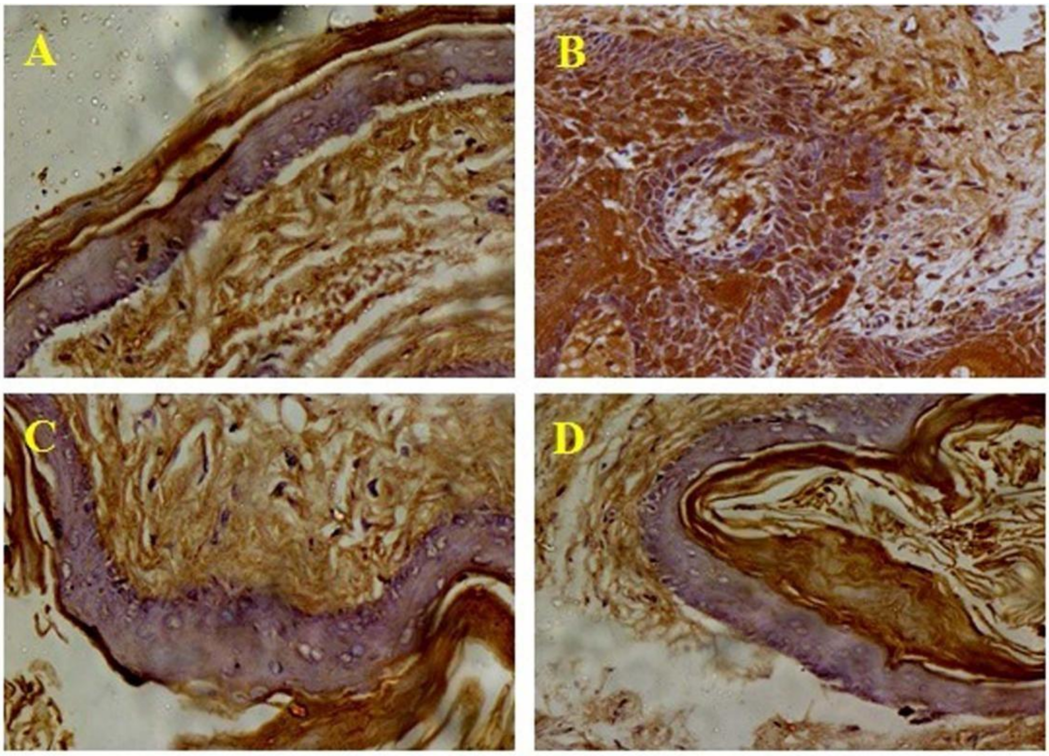

Figure 1: Immunoexpression pattern of Bax protein observed in the buccal mucosa of control and experimental hamsters in each group

A and **D** - Control and ferulic acid alone (nuclear expression positive); **B** - DMBA alone (nuclear expression negative); **C**- DMBA + ferulic acid (nuclear and cytoplasmic expression positive [⟶]).

RESULTS

The tumor incidence, tumor volume and tumor burden of control and experimental hamsters are shown in Table 1. We observed 100% tumor formation with mean tumor volume (312.45 mm^3) and tumor burden (1031.08 mm^3) in hamsters treated with DMBA alone. The tumor was histopathologically confirmed as well differentiated squamous cell carcinoma. Oral administration of ferulic acid at a dose of 40mg/kg bw significantly prevented the tumor incidence, tumor volume and burden in hamsters treated with DMBA. Tumors were not formed in control hamsters painted with liquid paraffin alone as well as hamsters administered with ferulic acid alone.

The immunoexpression pattern of Bax and the score of positively stained cells in control and experimental hamsters in each group are depicted in figure 1 and Table 2 respectively. Decreased expression of Bax was noticed in hamsters treated with DMBA alone. Oral administration of ferulic acid at a dose of 40mg/kg bw to hamsters treated with DMBA significantly restored the expression pattern of Bax. Hamsters treated with ferulic acid alone revealed expression similar to that of control hamsters.

The activities of caspase 3 and 9 in the buccal mucosa of control and experimental hamsters in each group is shown in figure 2. The activities of caspase 3 and 9 were decreased in hamsters treated with DMBA alone. Oral administration of ferulic acid to hamsters treated with DMBA brought back the activities of caspase 3 and 9 to near normal range. No significant difference was noticed in the status of above markers in control hamsters and hamsters administered with ferulic acid alone.

DISCUSSION

Apoptotic (pro and anti-apoptotic) proteins could serve as promising molecular markers to assess the progression and aggressiveness of malignant tumors. Deregulation of apoptotic proteins has been associated with tumor development and progression (Danial & Korsmeyer, 2004). Bcl-2 family consists of anti-apoptotic and pro-apoptotic proteins. They are located in the outer mitochondrial membrane, nuclear envelope and endoplasmic reticulum of cells. Bax/Bcl-2 ratio could be used as a marker to evaluate the outcome of an apoptotic stimulus. Bax/Bcl-2 ratio could also be used to assess the response of tumor cells to chemotherapy. Previous studies from our laboratory demonstrated increase in p53 and Bcl-2 expression in oral tumor bearing hamsters as well as decreased expression of p53 and Bcl-2 in hamsters treated with DMBA + ferulic acid (Balakrishnan, et al., 2010).

Table 2: The score of positively stained cells of Bax in control and experimental hamsters in each group

Groups / Markers	Bax			
	0	1+	2+	3+
Control	2	3	4	1
DMBA	8	2	0	0
DMBA + Ferulic acid	1	2	2	5
Ferulic acid alone	2	4	2	2

Values are given as number of hamsters (n = 10). The percentage positive cells were scored as: 3+ = strong staining, more than 50% of cells were stained, 2+ = moderate staining, between 20 and 50% of cells were stained 1+ = week staining, between 1 and 20% of cells were stained, 0 = negative, less than 1% of cell staining.

Caspases play putative role in the initiation and execution of the apoptotic process. It has been reported that impaired activation of initiator caspases (caspases 2,8,9 and 10) and executioner caspases (caspases 3,6 and 7) in tumor cells make them resistance to apoptotic death (Logue & Martin, 2008). Several studies pointed out those anticancer drugs induce apoptosis through activation of caspase cascade (Kaufmann & Earnshaw, 2000; Zimmermann, et al., 2001). Caspase-3 is one of the key components of apoptosis and its apoptotic role is executed by initiator caspases 8 and 9 (Cohen, 1997). Low expression of caspase 3 was shown in gastric and breast carcinoma (Park, et al., 2005). Activation of caspases-3, the key and essential member of caspases family, plays crucial role in the DNA fragmentation process and other morphological changes associated with apoptosis. A large number of in vitro studies suggested that natural products and synthetic agents induce caspase-3 dependent apoptosis in cancer cells (Preston, 2001; Liu, et al., 2010).

Caspase 9 plays an important role in apoptosis and impaired caspase-9 activation has profound consequences including morphological deformation of the brain. Multicellular organisms need caspase-9 to remove damaged cells to escape from the proliferative diseases (Hakem, et al., 1998). Insufficient caspase-9 activation in the cell contributes to chemotherapeutic drug resistance in various cancer models (Kuida, et al., 1998; Liu, et al., 2002). It has been reported that polymorphisms in the coding regions of caspase-9 gene predispose to various cancers such as lung, bladder and colorectal cancers (Park, et al., 2006; Gangwar, et al., 2009; Theodoropoulos, et al., 2011). While Bcl-2 is involved in the inhibition of caspase cascade, Bax promotes the activation of caspase cascade.

Low expression of Bax accompanied by decreased activities of caspase 3 and 9 in the buccal mucosa of hamsters treated with DMBA alone suggests that the buccal mucosa was protected from apoptosis and render more susceptible to tumor progression. Oral administration of ferulic acid at a dose of 40mg/ kg bw to hamsters treated with DMBA restored the expression of Bax and the activities of caspases. The results of the present study suggest that ferulic acid revealed significant apoptotic potential during DMBA-induced hamster buccal pouch carcinogenesis.

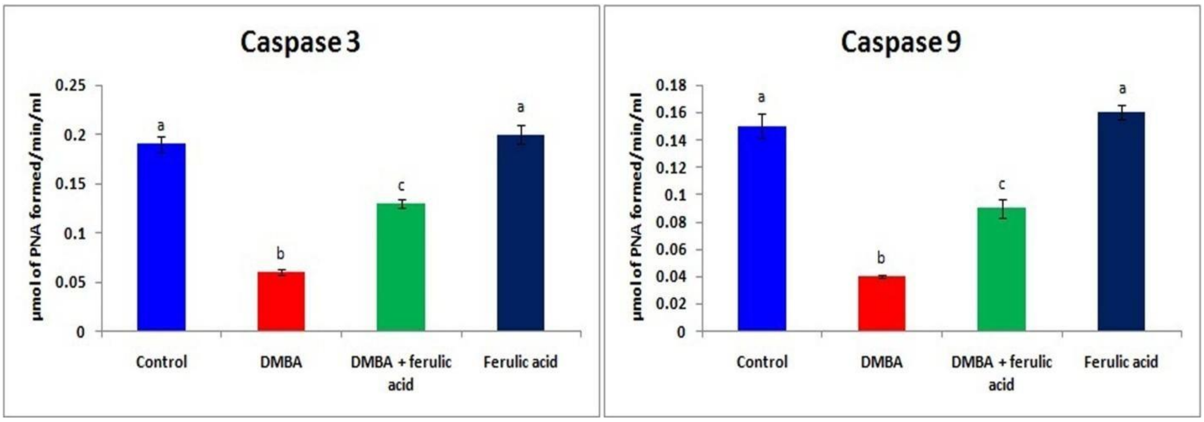

Figure 2: Caspase 3 and 9 activities in control and experimental hamsters in each group

Values are expressed as mean ± SD for 10 hamsters in each group. Values that do not share a common superscript letter between groups differ significantly at $p < 0.05$. (Analysis of variance followed by DMRT).

The present study thus concludes that ferulic acid might have inhibited oral tumor formation in hamsters treated with DMBA through apoptotic induction, as evidenced by increased activities of caspases and increased expression of Bax in DMBA + ferulic acid treated hamsters.

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