Abstract- The present study designed to investigate the protective effects of [6]-shogaol on 7,12-Dimethylbenz (a)anthracene (DMBA) induced buccal pouch carcinogenesis in male golden Syrian hamsters. Oral squamous cell carcinoma was developed in left buccal pouch of hamster by painting with 0.5% of DMBA, three times in a week. We observed an altered status of apoptotic associated gene expression (P53, bcl-2, Caspase-3 and TNF-α) were observed in the DMBA alone painted hamsters as compared to control hamsters. Oral administration of [6]-shogaol at a dose of 20 mg/kg b.wt to DMBA treated animals on alternative days for 14 weeks significantly reduced the neoplastic changes and improved the status of apoptosis associated gene expression. These observations confirmed that [6]-shogaol not only suppress the neoplastic changes but also effectively enhance the apoptotic associated gene expression in DMBA treated animals.

Keywords- [6]-shogaol, Apoptosis, DMBA, Hamster.

INTRODUCTION

APOPTOSIS has become a major research area in the biomedical sciences. It is also termed programmed cell death, plays diverse roles in embryogenesis and normal homeostasis, as well as in tumorigenesis [1]. The apoptotic processes are executed by a number of factors, which have inhibitory or stimulatory effects [2]. p53, tumor suppressor gene, product is a multifunctional molecule that influences the cell cycle, DNA repair and apoptosis by regulating transcription and interacting directly with other proteins [3].

 Proteins of the bcl-2 family together with caspases have among others been identified as essential components of the intracellular apoptotic signaling pathways [4]. Caspases are the heart of the apoptotic machinery. Several caspases have been shown to be key executioners of apoptosis, among the family of caspase; Caspase-3, a key factor in apoptosis execution and the activation of caspase-3 is crucial for mitochondrial dependent and independent apoptotic pathways [5]. TNF-α has been described as a powerful anti-cancer effectors and cytokine produced by immune cells such as macrophages and lymphocytes. It mediates tumor cell killing primarily by apoptosis [6].

 Many medicinal plants based chemopreventive agents are rich in antioxidant phytochemicals, recognized to exert their anti-carcinogenic effects by inhibiting cell proliferation, inducing cell differentiation and apoptosis [7]. The pungent phenolic constituents derived from ginger are believed to possess many interesting pharmacological and physiological activities. [6]-shogaol [1-4-hydroxy-3-methoxyphenyl-4-decen-3-one] is one of the major biologically active compounds found in the rhizome of Zingiber officinale [8]. The available literature also documented that the [6]-shogaol shows it anti-hepatotoxic effect against galactosamine induced cytotoxicity in primary cultured rat hepatocytes and protects against LPS induced inflammation in macrophage cells [9]. Pan et al., (2008) reported that [6]-shogaol inhibits the growth of human cancer cells and induces apoptosis in COLO 205 cells through modulation of mitochondrial functions regulated by reactive oxygen species [10]. However, no scientific reports were available on literature about apoptosis associated genes of p53, bcl-2, caspase-3 and TNF-α gene regulation of [6]-shogaol on DMBA induced carcinogenesis in male golden Syrian hamsters. The present study is designed to screen the protective role of [6]-shogaol assessed by histological examinations and immunohistochemical techniques on DMBA induced carcinogenesis.

Protective Effects of [6]-Shogaol on Histological and Immunohistochemical Gene Expression in DMBA Induced Hamster Buccal Pouch Carcinogenesis

Suresh Kathiresan¹*, Arokia Vijayaanand Mariadoss¹, Rajasekar Muthusamy¹, and Sivakumar Kathiresan²

Suresh Kathiresan¹ is with the Department of Biochemistry and Biotechnology, Annamalai University, Annamalai Nagar-608 002, Tamilnadu, India. (Tel.: +91-4144-239141(Extn.*209);Telefax:+91-4144-238080;e-mail:suraj_cks@yahoo.co.in
Arokia Vijayaanand Mariadoss¹ is with the Department of Biochemistry and Biotechnology, Annamalai University, Annamalai Nagar-608 002, Tamilnadu, India. (e-mail:mavijaibt@gmail.com)
Rajasekar Muthusamy¹ is with the Department of Biochemistry and Biotechnology, Annamalai University, Annamalai Nagar-608 002, Tamilnadu, India. (e-mail:generajasekar@gmail.com)
Sivakumar Kathiresan² is with the Department of Botany, Annamalai University, Annamalai Nagar-608 002, Tamilnadu, India. (e-mail:kshivam69@gmail.com)
II. MATERIALS AND METHODS

A. Animals And Experimental Design

Male golden Syrian hamsters (80 ± 120 g), were obtained from National Institute of Nutrition, Hyderabad, India. The experiments were designed and conducted in accordance with the institutional guidelines. Hamsters were divided into 4 groups of 10 each. Group I, served as an untreated control. The Group II and Group III were painted with 0.5% DMBA in liquid paraffin three times a week for 14 weeks on the left buccal pouches using (No. 4 brush) to induce the buccal pouch carcinogenesis. Group II received no other treatment. Group III animals were orally administered with [6]-shogaol (20 mg/kg b.wt; dissolved in 0.5% DMSO) starting one week before the exposure to the carcinogen and continuing on alternate days of the DMBA painting until the animals were sacrificed.

B. Sample Collections

At the end of the experimental period, the hamsters were sacrificed by cervical decapitation under anesthetic conditions (Ketamin 30 mg/kg, i.p.). The control and experimental animal buccal tissues were immediately removed, washed using ice-cold phosphate buffer solution (pH 7.4), and then the buccal tissues were divided for assessment of histological and immunohistochemical studies.

C. Tumor Study

The left sides of the control and experimental animal’s buccal pouch was excised and flattened on a transparency. Visible tumors in the oral cavity were counted and the diameter of each tumor was measured with a vernier caliper.

D. Histopathological Evaluation

Histological slides were prepared by according to the method of Ramos Vara, (2005). The buccal pouches of the hamsters were fixed in 10% buffered formaldehyde and dehydrated in rising concentrations of ethanol and embedded in paraffin. 5 µm thickness of tissue sections were mounted on frosted glass slides and dried for overnight. The sections were then deparaffinized with xylene, rehydrated with alcohol and water. The rehydrated sections were stained using H&E and viewed under the microscope 40X magnifications (Olympus BX51 microscope, Tokyo, Japan).

E. Immunohistochemical Studies

Monoclonal anti-mouse p53 antibody, monoclonal anti-mouse bcl-2 antibody and monoclonal anti-mouse caspases-3 antibody (Sigma Aldrich Inc., Santa Cruz, CA) and the anti-mouse horseradish-peroxidase conjugated secondary antibodies were obtained from Biogenex, San Ramon, CA. To examine the expression patterns and inter correlation between apoptosis related molecular markers p53, bcl-2, caspase-3 and TNF-α were prepared by according to the method of Cotran et al., (1994) [11].

F. Statistical Analysis

The percentage of positive cells in immunohistochemical were scored according to the method of Nakagawa et al., [1994] as follows: +++=strong staining, more than 50% of cells were stained; ++=moderate staining, between 20 and 50% of cells were stained; +=week staining, between 1 and 20% of cells were stained; 0=negative, less than 1% of cell staining.

III. RESULTS

Fig. 1 and Table 1 show the tumor incidence, tumor volume and tumor burden of hamsters in all experimental groups along with the histopathological features. In Group II animals (DMBA alone treated), we have observed 100% tumor formation with mean volume (98.12 ± 9.43), tumor burden (1601±161.1). For histopathological confirmation; we noticed severe keratosis, hyperplasia, dysplasia and oral neoplasm. In Group III (DMBA+ [6]-shogaol) we noticed mild preneoplastic lesions like mild dysplasia, keratosis and hyperplasia which indicated the key role of [6]-shogaol in regaining the antioxidant status. The buccal pouch of Group I (Control) and Group IV ([6]-shogaol alone) animals resembles the same with no tumor formation and preneoplastic lesions.
Hamster buccal pouch carcinogenesis (HBP) is an excellent model to study oral carcinogenesis, for the reason, that the development of DMBA induced squamous cell carcinoma in HBP simulates many of the structural and biological alterations that occur in its human counterpart [13]. These evaluations are likely to be helpful in identifying intermediate biomarkers that would help in monitoring clinical trials or evaluating drug effect measurements. This study was designed to investigate the ability of [6]-shogaol to modulate expression of the apoptosis related proteins which are implicated in DMBA induced hamster buccal pouch carcinogenesis, including p53, bcl-2 caspase-3 and TNF-α.

Recent study have been documented that p53 most frequent genetic alteration observed in carcinomas. In particular, a high incidence of p53 mutation has been demonstrated in many experimental carcinogenesis including oral cancer [14]. In the present investigation, we noticed significant expression of mutant p53 in buccal tissues of DMBA treated hamsters which may be due to the significant molecular alterations at p53 gene. Moreover, p53 is implicated in the induction of two distinct apoptotic signaling pathways that lead to the activation of the caspases which mediate apoptosis. The extrinsic pathway involves engagement of particular death receptors that belong to the TNF receptor family and, leads to a cascade of activation of caspases, including caspase-3, which in turn induce apoptosis [15]. The finding of this study conclude that oral administration of [6]-shogaol decreased p53 level to improve apoptosis process.

There are various signaling pathways of apoptosis within an organism, of which the mitochondrial pathway is one of the most important. Bcl-2 family proteins are key regulatory factors of the mitochondrial pathway. Several studies have shown that overexpression of bcl-2 prevents the mitochondrial release of cytochrome c, thereby inhibiting the activation of caspases cascade and apoptosis [16]. Over expression of antiapoptotic proteins such as bcl-2 may contribute to tumor formation in DMBA-treated animals. We monitored well defined expression of bcl-2 gene in buccal tissues of DMBA treated hamsters which may be due to the enhanced inhibition of release of cytochrome C by bcl-2 gene. Oral administration of [6]-shogaol reduced the expression of bcl-2 in DMBA painted hamsters which may be due to the reduced release of cytochrome C from mitochondria and increasing the expression of apoptotic gene. The results of the present study validate our conviction that DMBA causes carcinogenesis via up regulation of p53, down regulation of bcl-2 levels and inducing mitochondrial damage. Further, [6]-shogaol at a dose of 20 mg/kg b.wt prevented the up regulation of Bax and maintained mitochondrial integrity hence, protected the DMBA induced carcinogenic process in experimental animals. Thus, these observations hold promise for further molecular target oriented studies.

Caspase is an important apoptosis signaling molecule that triggers a cascade of molecular interaction leading to apoptosis. Studies have shown that the extrinsic activation triggers the hallmark Caspase cascade of apoptotic pathway, in which caspase-3 plays a dominant role [17]. By inducing the release of mitochondrial cytochrome c, p53 may be able to

Fig. 3 and Table 2 show the immunohistochemical gene expression of p53, bcl-2, caspase-3 TNF-α in control and experimental animals of each group. The presence of cells with clear and unequivocal staining identified in p53 positive cells. Bcl-2 cells were identified as diffused golden yellow colour in cytoplasmic staining and caspase-3 were identified as red brown in color. The positive cells of TNF-α predominantly were located with brown yellow color in cytoplasm. We observed a significant and sequential raise in protein levels by p53, bcl-2, TNF-α and down regulation of caspase-3 gene expression were observed in DMBA alone treated hamsters as compared to control hamsters. Oral administration of [6]-shogaol at a dose of 20mg/kg b.wt effectively restored the expression pattern of p53, bcl-2, caspase-3 and TNF-α. No significant changes were observed in expression of p53, bcl-2, TNF-α, and caspase-3 in control and [6]-shogaol alone treated animals.

Table II

<table>
<thead>
<tr>
<th>Groups</th>
<th>p53</th>
<th>Bcl-2</th>
<th>Caspase-3</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13</td>
<td>0</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>DMBA</td>
<td>3</td>
<td>7</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>DMBA + [6]-shogaol</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>[6]-shogaol alone</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>9</td>
</tr>
</tbody>
</table>

Fig. 3 Immunohistochemical expression pattern of P53, bcl-2, Caspase-3 and TNF-α in buccal mucosa of control and experimental animals in each group


IV. DISCUSSION

Molecular and immunohistochemical markers have been found to be useful in characterizing the progression of human cancer. These markers include changes in several genes that encode the expression of cell proliferation regulators [12].
activate effector caspases, including caspase-3. Activation of caspase-3 subsequently leads to apoptotic cell death through cleavage of a broad spectrum of target proteins [18]. [6]-shogaol may induce apoptosis mediated by p53 through the down regulation of bcl-2 and up regulation of Bax. In this study we were seen a significant increase in caspase-3 expression in [6]-shogaol treated animals. This finding is similar to previous observations of lupeol mediated expression pattern of apoptotic markers in DMBA oral carcinogenesis [19]. Our analysis revealed that [6]-shogaol induces p53-mediated apoptosis in DMBA treated animals.

TNF-α is a multifunctional key cytokine involved in the modulation of numerous immune response, known to possess potent antitumor activity both in vivo and in vitro, mediator of a variety of cellular responses, including apoptotic or necrotic cell lysis and proliferation [20]. It triggers a number of signal transduction processes, which lead to either apoptosis or proliferation. A wide range of intracellular components are implicated in TNF-α-induced cell killing, including pertussis toxin-sensitive guanine nucleotide binding protein, phospholipase A2, phospholipase D activation, and DNA damage [21]. We found that the expression of TNF-α significantly increased in DMBA treated animals, due to the marked elevation of ROS during DMBA metabolism that probably leads to increase in the synthesis of TNF-α. Moon et al., (2008) reported that, Sulforaphan, a biologically active material”, J Pathol, vol. 176, pp. 361-372, Aug 1995. 

In conclusion, we have shown that oral administration of [6]-shogaol at a dose of 20 mg/kg b.wt significantly improve the apoptotic associated gene expression of p53, bcl-2, Caspase-3 and TNF-α. However, the findings of this study are inadequate for clinical trials, further are warranted to fulfill the mechanisms of [6]-shogaol against DMBA induced hamster buccal pouch carcinogenesis.

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