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ALTERED CYTOKERATIN EXPRESSION DURING CHEMOPREVENTION OF HAMSTER BUCCAL POUCH CARCINOGENESIS BY S-ALLYLCYSTEINE

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We examined the effect of S-allylcysteine (SAC), a water-soluble garlic constituent, on cytokeratin expression, a sensitive and specific marker for differentiation status during 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis in male Syrian hamsters. Hamsters were divided into four groups of six animals each. Animals in group 1 were painted with a 0.5% solution of DMBA in liquid paraffin on the right buccal pouches three times a week for 14 weeks. Group 2 animals were painted with DMBA as in group I, and in addition they received orally 200 mg/kg of SAC on days alternate to DMBA application. Group 3 animals received SAC as in group 2. Group 4 animals received neither DMBA nor SAC and served as the control. The hamsters were killed after an experimental period of 14 weeks. Cytokeratin expression was detected by Western blot analysis using monoclonal antibodies AE1 and AE3. In DMBA-induced HBP tumors, the decreased expression of high molecular weight cytokeratins of molecular mass between 55–70 kDa was observed. Administration of SAC (200 mg/kg) to animals painted with DMBA suppressed the incidence of DMBA-induced carcinomas and was associated with restoration of normal cytokeratin expression. The results of the present study suggest that inhibition of HBP tumorigenesis by SAC may be due to its regulatory effects on differentiation, tumor invasiveness, and its ability to migrate and form metastases.

Key words: *DMBA, chemoprevention, oral cancer, hamster buccal pouch, cytokeratins, garlic, S-allylcysteine*

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Abbreviations: DMBA – *7,12-dimethylbenz[a]-anthracene,* HBP – *hamster buccal pouch,* OSCC – *oral squamous cell carcinoma,* SAC – *S-allylcysteine,* SDS-PAGE – *sodium dodecylsulfate polyacrylamide gel electrophoresis*

INTRODUCTION

Squamous cell carcinoma of the oral cavity (OSCC), the fifth most common cancer worldwide, is the leading cause of mortality and morbidity in the Indian subcontinent. Despite advances in cancer detection and treatment, the mortality due to OSCC remains high and the five-year survival rate is among the lowest of the major cancers [22, 23].

Chemoprevention offers a practical approach to control the incidence of oral cancer. Monitoring of the alterations in cytokeratin expression has emerged as an excellent tool to analyze oral cancer development as well as chemoprevention [11]. Cytokeratins have a number of advantages for use as cellular markers as they are abundant, very stable and highly antigenic [9, 20]. Cytokeratins are members of the family of intermediate filaments and form a major component of mammalian epithelial cell cytoskeleton [21]. Cytokeratin expression is closely related to the differentiation state of the epithelial cell [13, 24]. Qualitative and spatial differences in cytokeratin expression have been observed between normal oral epithelium and OSCCs [13, 32].

The buccal pouch of the Syrian hamster is an excellent model to investigate oral cancer development and intervention by chemopreventive agents. Squamous cell carcinomas induced by the application of 7,12-dimethylbenz[a]anthracene (DMBA) to the hamster buccal pouch (HBP) are morphologically and histologically similar to human tumors [12]. In addition, hamster tumors express many metabolic and molecular markers that are expressed in human oral cancers [8]. Recently, we demonstrated a similar pattern of changes in the oxidant-antioxidant status of human oral tumors and DMBA-induced HBP tumors [7]. These findings have provided an expanded rationale to test chemopreventive agents in the HBP model.

A number of natural and synthetic agents have been tested for their chemopreventive efficacy in the HBP model. We have documented the chemopreventive potential of plant extracts including neem leaf and garlic during DMBA-induced HBP carcinogenesis [1, 2].

Garlic (*Allium sativum* Linn.) has been used as a spice and medicinal herb for centuries. S-allylcysteine (SAC), a minor constituent of regular crushed garlic is a non-toxic, water-soluble, organosulfur compound. It is considered as one of the important biologically active constituents of garlic. SAC has come under extensive study in the light of its anticancer effects both *in vitro* and *in vivo* [15, 19]. SAC has been reported to inhibit 1,2-dimethylhydrazine-induced colon cancer, and DMBA- and N-methyl-nitrosourea-induced mammary tumorigenesis [18, 26, 30]. The antiproliferative effects of SAC against human neuroblastoma and melanoma cell lines have also been demonstrated [31, 33].

In previous reports from this laboratory, we demonstrated a positive correlation between the chemopreventive efficacy of SAC against DMBA-induced HBP carcinogenesis and its modulatory effects on lipid peroxidation, antioxidant and detoxification systems [3–5]. Recently, we reported induction of apoptosis by SAC that may contribute to its chemopreventive properties [6]. We undertook the present study to investigate the effect of SAC on cytokeratin expression during DMBA-induced HBP carcinogenesis.

MATERIALS and METHODS

Animals

All the experiments were carried out on male Syrian hamsters aged 8–10 weeks, weighing 85–90 g, obtained from the Central Animal House, Annamalai University, India. The animals were housed six to a polypropylene cage and provided food and water *ad libitum*. The animals were maintained under controlled conditions of temperature and humidity with an alternating 12-hour light/dark cycle. All animals were fed standard pellet diet (Hindustan Lever Limited, India).

Chemicals

DMBA was purchased from Sigma Chemical Company, USA. S-allylcysteine (SAC) was kindly provided by Wakunaga Pharmaceutical Co. Ltd. (Hiroshima, Japan) and the purity was confirmed to be 99.9% by HPLC analysis. For experimental purposes, it was dissolved in distilled water.

Treatment schedule

The animals were randomized into experimental and control groups, and divided into four groups of six animals each. Animals in group 1 were painted with a 0.5% solution of DMBA in liquid paraffin on the right buccal pouches using a number 4 brush three times a week for 14 weeks. Each application leaves approximately 0.4 mg DMBA [2]. Group 2 animals were painted with DMBA as in group 1. In addition, the animals were administered SAC at a dose of 200 mg/kg orally on days alternate to DMBA application [30]. Animals in group 3 received only SAC as in group 2. Group 4 (untreated control) animals received neither DMBA nor SAC. The experiment was terminated at the end of 14 weeks and all animals were sacrificed by cervical dislocation after overnight fasting. At sacrifice, the buccal pouch was excised, grossly examined and a portion of the tissue was used for histopathological examination. The remaining tissues were stored at –70°C until used. Cytokeratin expression was studied in buccal pouch tumor tissues in animals that developed tumors and in normal pouch tissues in animals that did not develop tumors.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Approximately, 50 mg of each tissue sample was subjected to lysis in a sample buffer containing 62.5 mM Tris (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and bromophenol blue. The protein concentrations of the lysates were determined spectrophotometrically. Equivalent protein extracts (60 μg) from each sample were electrophoresed on 10% SDS-PAGE gels and electrophoretically transferred onto polyvinylidene difluoride membranes (Immobilion, Millipore, Bedfore, MA, USA). The blot was treated for 1 h with blocking solution containing 3% BSA in Tris-buffered saline (TBS) containing 150 mM NaCl and 50 mM Tris pH 7.4 at room temperature. The blot was rinsed once with TBS and incubated with 1:500 dilution of a mixture of monoclonal antibodies AE1 and AE3 (Progen, Germany) for 2 h. The blot was washed with TBS-T (0.1% Tween 20) four times, 10 min each. Cytokeratins were detected by incubating corresponding horseradish-conjugated secondary antibodies (1:4000 dilution) to AE1 and AE3 for 30–45 min at room temperature. After four 10-minute washes in TBS-T, the transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham, UK) according to the manufacturer's instructions.

RESULTS

Histopathological observations

Table 1 summarizes the incidence of oral preneoplastic lesions and neoplasms in different groups. Exophytic tumors induced by DMBA in the oral cavity of hamsters in group 1 were well-differentiated squamous cell carcinomas. The incidence of oral neoplasms in group 1 was 100%, whereas in group 2, only mild hyperplasia was observed. No malignant neoplasms or preneoplastic lesions were observed in animals in groups 3 and 4.

Effect of SAC on cytokeratin expression

The differences in patterns of cytokeratins reacting with a mixture of MAb AE1/MAb AE3 antibodies are shown in Figure 1. In the control (group 4) as well as in DMBA and SAC-treated groups (group 2), cytokeratin polypeptides of molecular mass 67 kDa (K1), 63 kDa (K3), 52 kDa (K13), 48 kDa (K16) and 40 kDa (K19) were found to be expressed. In DMBA and SAC-treated group (group 2),

Table 1. Tumor incidence and histopathological changes in control and experimental animals in each group (n = 6)

Group	Treatment	Keratosis	Hyperplasia	Dysplasia	Squamous cell carcinoma
1	DMBA	+ to ++ (100)	+ to ++ (100)	++ (100)	+++ 6/6 (100)
2	DMBA+ S-allylcysteine	+ (100)	+ (100)	–	0/0
3	S-allylcysteine	–	–	–	0/0
4	Control	–	–	–	0/0

+ = mild; ++ = severe; +++ = well differentiated. Percentage of animals with lesions is shown in parentheses

Table 2. Changes in cytokeratin expression in different experimental groups

Group	Pattern of cytokeratin expression						
	K1		K3	K4	K13	K16	K19
	73 KDa	67 KDa	63 KDa	59 KDa	52 KDa	48 KDa	40 KDa
DMBA	–	↓	↓	↑	↓	↓	↓
DMBA + SAC	–	↔	↔	↓	↔	↔	↔
SAC	↔	↔	↔	↔	↔	↔	↔
Control	↔	↔	↔	↔	↔	↔	↔

↓ = decreased expression; ↑ = increased expression; ↔ = normal expression; – = nil

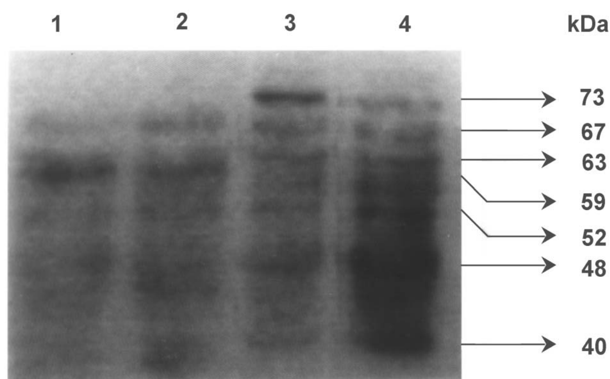

Fig. 1. Western blot analysis of proteins extracted from buccal pouch mucosa of hamster cheek pouch from control and experimental groups using a mixture of monoclonal antibodies AE1 and AE3. Lane 1: DMBA. Lane 2: DMBA and SAC. Lane 3: SAC. Lane 4: control

low expression of cytokeratins of molecular mass 59kDa (K4) was observed. The cytokeratin polypeptide pattern in DMBA-induced oral tumors (group 1) showed low expression at 67 kDa, 63 kDa, 52 kDa, 48 kDa, 40 kDa and high expression at 59 kDa. An additional high molecular weight cytokeratin, 73 kDa was expressed only in control group and in animals treated with SAC but not in animals treated with DMBA. Table 2 summarizes the changes in cytokeratin expression in different experimental groups.

DISCUSSION

Alterations in cytokeratin expression have been reported during DMBA-induced HBP carcinogenesis [11, 16, 29]. We observed decreased expression of K1, K3, K13, K16 and K19 and an increased expression of K4 in DMBA-induced oral tumors. Loss of the differentiation-associated cytokeratins K1 and K13 appears to be characteristic of malignant progression. Loss of both K1 and K13 is recognized to occur in mouse skin two-stage carcinogenesis system, DMBA-induced HBP carcinogenesis as well as in human tumors [11, 13, 16, 25, 32]. The decreased expression of K1, a 65–75 kDa high molecular weight cytokeratin is in agreement with previous reports of loss of high molecular weight cytokeratin polypeptides during DMBA-induced HBP carcinogenesis [11, 16, 27, 28]. Failure of the expression of high molecular weight cytokeratins reflects changes in differentiation of epithelial cells during oral cancer development.

Lin et al. [16] reported reduced expression of K16 during DMBA-induced HBP carcinogenesis. Down-regulation of K19 has been documented in oral cancer [10, 14]. Our findings substantiate these reports. Down-regulation of K19 has been reported to increase the invasive behavior and migratory capability of OSCCs [10, 17]. Dysregulated cytokeratin expression in DMBA-induced oral tumors observed in our study may indicate an invasive phenotype of the malignant cell population.

Here we report for the first time an increase in K4 expression during DMBA-induced HBP carcinogenesis. Presumably, this protein is produced in response to the hyperplastic stimulus. Whether the presence of K4 in the oral tumors is a simple consequence of hyperplasia or is a specific event during DMBA-induced carcinogenesis remains to be elucidated. An additional 73 kDa band was observed in pouch mucosa of control animals and in animals administered SAC alone. Further studies are needed to account for the appearance of this cytokeratin.

Administration of SAC to DMBA-painted animals reverted cytokeratin expression to normal pattern, rather than toward cancer expression. Regulation of K19 and normal cytokeratin expression by SAC suggests that they may have regulatory effects on tumor invasiveness, and its ability to migrate and form metastases. Schwartz et al. [28] used altered cytokeratin expression to monitor the chemopreventive potential of GSH and vitamin E. Both GSH and vitamin E increased high molecular weight cytokeratins and restored normal cytokeratin expression during DMBA-induced HBP carcinogenesis. Our results lend credence to the report that altered cytokeratin expression could be used as a marker to monitor tumor regression by chemopreventive agents. We suggest that one of the mechanisms of tumor inhibition by SAC is an influence on cellular differentiation. Further studies with specific monoclonal antibodies could provide valuable information on the effect of SAC on cytokeratin expression.

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