Original Article

Retinoic acid receptor-β mRNA expression during chemoprevention of hamster cheek pouch carcinogenesis by garlic

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The effect of aqueous garlic (*Allium sativum* Linn.) on retinoic acid receptor β (RARβ) mRNA expression was investigated in male Syrian hamsters during 12-dimethyl enz[a]anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis. RARβ mRNA expression was analysed by slot blotted hybridization with radiolabelled RAR-β probe. In DMBA-induced HBP tumours, decreased expression of RARβ mRNA was observed. Administration of garlic (250 mg/kg body weight) to animals painted with DMBA restored RARβ mRNA expression to normal pattern suggesting that this may be one of the mechanisms by which garlic exerts its chemopreventive effects.

Key Words: retinoic acid receptor-β, 7,12-dimethylbenz[a]anthracene (DMBA), chemoprevention, garlic, oral cancer

Introduction

Medicinal plants have received growing attention in recent years as potential chemopreventive agents. Garlic (*Allium sativum* Linn.) used as a spice and medicinal herb for centuries, exhibits a wide range of properties including immunomodulatory, hepatoprotective, antioxidant, antimutagenic and anticarcinogenic effects. The anticancer effects of garlic have been documented in both epidemiological and experimental studies.[1,2] In previous studies from this laboratory, we have shown a positive correlation between the chemopreventive potential of garlic and its inducing effects on antioxidants and detoxification systems during N-methyl-N'-nitro-N-nitrosoguanidine-induced rat stomach carcinogenesis and 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis.[3-5]

The buccal pouch of the Syrian hamster has been widely used as an ideal model for the analysis of oral cancer development and intervention by chemopreventive agents. Squamous cell carcinomas (SCCs) induced by the application of 7,12-dimethylbenz[a]anthracene (DMBA) to the hamster buccal pouch (HBP) have been found to be morphologically and histologically similar to human tumours. In addition, hamster tumours express many biochemical and molecular markers that are expressed in oral cancer.[6,7] Previously, we demonstrated similarities between human and hamster tumours with respect to the oxidant-antioxidant status.[8] We have documented the chemopreventive potential of garlic and S-allylcysteine, a water-soluble garlic constituent during DMBA-induced HBP carcinogenesis using lipid peroxidation and the status of the antioxidants glutathione (GSH) and the GSH-dependent enzymes glutathione peroxidase and glutathione S-transferase as biomarkers of chemopre-vention.[9,10] Recently, we demonstrated induction of cellular differentiation and apoptosis by garlic in the HBP model.[11,12]

Of late, the nuclear receptors for retinoids have assumed significance as key molecular targets for new chemopreventive agents.[13] The expression of retinoic acid receptor-β (RAR-β), the best studied member of the retinoic acid receptor family, is known to be decreased in a wide variety of malignancies, including human oral cancer.[14-18] RAR-β which functions as a tumour suppressor gene, mediating the anti-cancer effects of retinoids in different cancer cell types, has emerged as a novel biomarker for chemoprevention.[19] We therefore undertook the present study to investigate the effect of garlic on RAR-β mRNA expression during DMBA-induced HBP carcinogenesis.

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Materials and methods

Animals

All the experiments were carried out with male Syrian hamsters aged 8-10 weeks weighing 85-90g obtained from the Central Animal House, Annamalai University, India. The animals, housed six to a polypropylene cage, were provided food and water *ad libitum* and maintained under controlled conditions of temperature and humidity with an alternating 12-hours light/dark cycle. All animals were fed standard pellet diet (Hindustan Lever Limited, India). The animals used in the present study were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India and approved by the ethics committee of Annamalai University.

Preparation of garlic extract

An aqueous extract of garlic was prepared by homogenising 250mg of freshly peeled cloves in 10mL of double-distilled water to give a concentration of 25mg of dry cloves/mL.[20] The homogenate was centrifuged at 3000 rpm for 10 min to remove particulate matter and the supernatant was used for the experiment. The weight of the particulate matter after drying was found to be 4% of the total extract. The supernatant fraction at this stage contained 96% of the total extract corresponding to 240mg of dry garlic.

Treatment schedule

The animals were randomised into experimental and control groups and divided into four groups of six animals each. Animals in group 1 were painted with a 0.5 per cent solution of DMBA (Sigma Chemical Company, St Louis MO, USA) 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.[9] Group 2 animals painted with DMBA as in group 1, received in addition aqueous garlic extract at a dose of 250 mg/kg body weight orally on days alternate to the DMBA application. Animals in group 3 received only garlic as in group 2. Group 4 (untreated control) animals received neither DMBA nor garlic. The experiment was terminated at the end of 14 weeks and all animals were sacrificed by cervical dislocation after an overnight fast. At sacrifice, the buccal pouch was excised, grossly examined, and a portion of the tissue was used for histopathological examinations. The remaining tissues were immediately stored at -70°C until use.

Total RNA isolation and slot blotted hybridisation

Total RNA from tissues was isolated by the guanidinium thiocyanate-phenol-choloroform extraction method as described by Chomczymski and Sacchi.[21] Appropriate dilutions of total RNA were denaturated and applied to Hybond-N' (Amersham, UK) using a slot blotting device (BioRad). The membrane was washed, air-dried and hybri-dised to radiolabelled RAR-β probe (kindly provided by Prof. Pierre Chambon, IGBMC, Strasbourg, France). Fifty nano-grams of the isolated probe was labelled with {α-^{32}P} dNTP by the random primer method (DNA polymerase Klenow fragment, dNTPs and hexanucleotide primers). The labelled probe was separated from unincorporated nucleotides by gel filtration, measured for radioactivity with a beta scintillator, denatured by boiling for 5min and cooled on ice. The denatured probe (10^8 CPM DNA/mL hybridisation solution) was hybridised to prehybridised membrane bound RNA at 65°C in hybridisation solution overnight with constant shaking. The membrane was washed under stringent conditions and exposed to X-ray film at -70°C for 72 h. Densitometry was performed on a Microtek Scanmaker IISP flat bed scanner and quantitated with NIH image 1.52 software.

Statistical analysis

Statistical significance for densitometric analysis was carried out using analysis of variance followed by Duncan's multiple range test (DMRT). Treatment mean differences with $P<0.05$ were considered statistically significant.

Results

Table 1 summaries the incidence of HBP tumours and preneoplastic dysplasia in different groups. Exophytic tumours induced by DMBA in the buccal pouch of hamsters in group 1 were well-differentiated squamous cell carcinomas (Fig.1). The incidence of SSCs in group 1 was 100 per cent whereas in group 2, only mild hyperplasia was observed (Fig.2). No premalignant or malignant lesions were observed in groups 3 and 4. Administration of garlic to animals painted with DMBA restored RAR-β mRNA expression to normal pattern. A representative slot blot autoradiogram is shown in Fig. 3.

Discussion

This investigation is the first to analyse RAR-β mRNA expression during DMBA-induced HBP carcinogenesis. Low expression of RAR-β mRNA in HBP tumours observed in this study corroborates with similar findings in SCCs of the oral cavity, lung cancer, breast cancer as well as in several cancer cell lines.[15,16,22]

Table 1. Incidence of hyperplasia, dysplasia and squamous cell carcinomas in control and experimental hamsters (N=6)

Group	Treatment	Hyperplasia	Dysplasia	Squamous cell carcinoma
1	DMBA	+ to ++	++	6/6 (100)
2	DMBA+ garlic	+	-	0/6 (0)
3	Garlic	+	-	0/6 (0)
4	Control	-	-	0/6 (0)

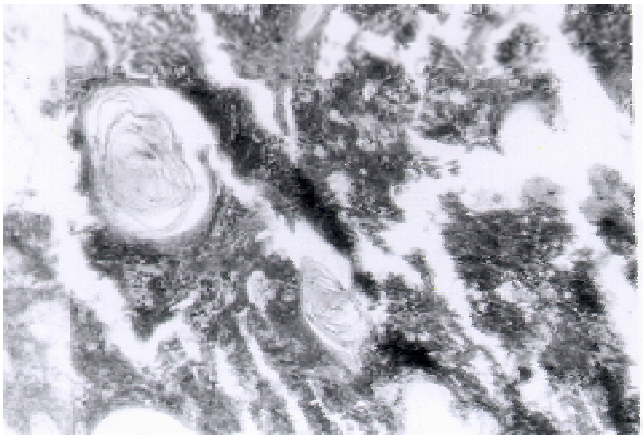

Figure 1. Well-differentiated squamous cell carcinoma in an animal painted with DMBA (Group 1) showing epithelial and keratin pearls (H and E x 10).

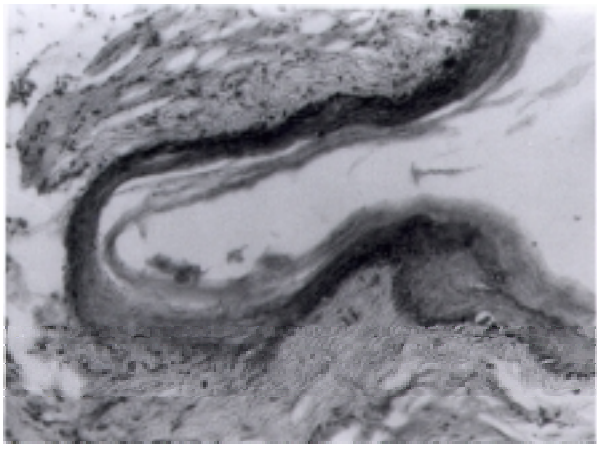

Figure 2. Epithelium showing hyperkeratinization and mild hyperplasia in a hamster treated with DMBA + garlic (group 2) (H and E x 10).

RAR-β has been suggested to regulate specific genes associated with suppression of carcinogenesis. Downregulation of RAR-β expression may lead to an increase in the activity of anti-activator protein (AP-1) associated with enhanced expression of the proto-oncogenes *c-fos* and *c-jun* resulting in hyperproliferation and expansion of malignant clones.[23,24] Decrease in RAR-β expression may be a key mechanism by which SCCs and other types of tumours escape the control of normal growth and differentiation. This would impair heterodimerization with other retinoid X receptors (RXRs). Defects in retinoid receptor expression such as RAR-β may result in an abrogate retinoid signalling pathway facilitating cancer development.[25]

An important finding in this study is the significant association between RAR-β expression and chemoprevention by garlic. Administration of aqueous garlic extract to DMBA-painted animals restored RAR-β mRNA expression. Transfection of RAR-β gene is recognised to decrease the tumorigenicity of human lung cancer cells as well as retinoic acid-dependent

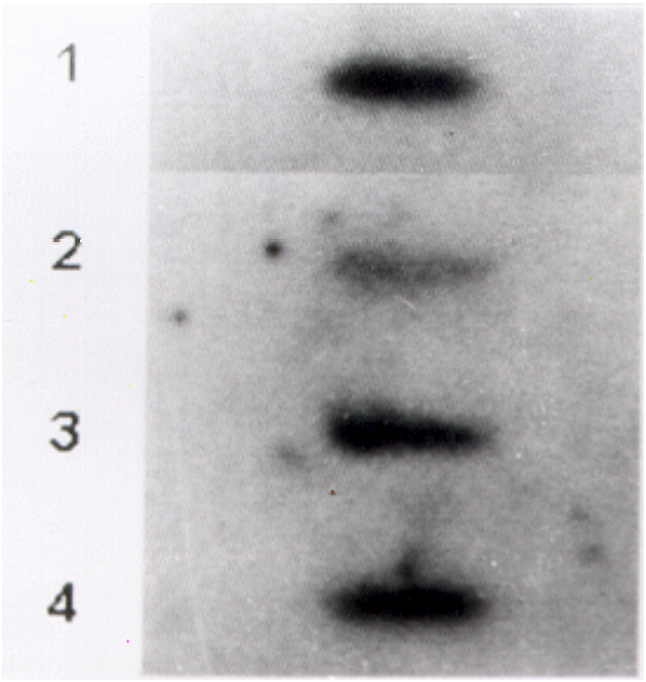

Figure 3. Slot blot analysis of RAR-β mRNA expression in buccal pouch mucosa of hamster cheek pouch from control and experimental groups. Lane 1: control; Lane 2: DMBA; Lane 3: DMBA+ garlic; Lane 4: garlic.

suppression of cell proliferation.[26] A number of studies have shown a correlation between RAR-β expression and induction of apoptosis.[27-29] Previously, we demonstrated the antiproliferative and apoptosis-inducing properties of garlic during DMBA-induced HBP carcinogenesis.[9,12] Restoration of RAR-β by garlic observed in the present study may inhibit cell proliferation and facilitate apoptosis.

The results of the present study taken together with our previous reports validate the chemopreventive efficacy of garlic. We feel that restoration of RAR-β mRNA expression may be one of the major mechanisms by which garlic exerts its chemopreventive effects. Further research on the effect of garlic on *c-fos* and *c-jun* during DMBA-induced HBP carcinogenesis may shed new light on the molecular mechanisms of inhibition of chemically induced oral carcinogenesis

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