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GGT Reduction in Beta Carotene-Inhibition of Hamster Buccal Pouch Carcinogenesis

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Abstract—*Levels of activity for gamma glutamyl transpeptidase (GGT) were studied in hamster buccal pouches developing DMBA-induced epidermoid carcinomas and in pouches in which carcinogenesis was inhibited by topical application of beta carotene. The beta carotene acted to inhibit tumor development when applied topically on days alternate to the application of* 0.25% *DMBA in heavy mineral oil thrice weekly for* 22 weeks. *Forty male young adult Syrian hamsters were divided into four equal groups. Group* 1 *had DMBA applied to left buccal pouches thrice weekly. Group* 2 *had DMBA applied as in Group* 1 *but also beta carotene thrice weekly on days alternate to the DMBA application. Group* 3 *animals were painted with only beta carotene and Group* 4 *animals were untreated controls. The left buccal pouches were dissected at autopsy and divided in half. One half was fixed in formalin, sectioned in paraffin and stained with hematoxylin–eosin for histologic study. The other half was prepared for the histochemical demonstration of GGT activity using epithelial whole mount preparations. GGT activity was found to be reduced in the left buccal pouches of those animals treated with both beta carotene and DMBA when compared to those animals treated with DMBA alone.*

INTRODUCTION

GAMMA glutamyl transpeptidase (GGT) (gamma glutamyltransferase) has been found to be an effective histochemical marker for the development of malignant tumors. It was first described as a marker for chemical carcinogenesis in rat liver by Fiala and associates [1–4] and others [5, 6]. A GGT increase was used to demonstrate the rapid development of premalignant liver cell populations following carcinogen use, leading to a clearer interpretation of the sequential development of liver cancer in experimental animals from clonal sites [7–10].

Fiala *et al.* were able to demonstrate that GGT activity could be found in significantly greater quantities in epithelial tumours such as carcinomas of prostate, mammary gland, or colon [11]. Even when the tissue of origin normally contained little or no GGT activity, such as tongue, urinary bladder, larynx, or esophagus, the carcinomas arising from these tissues were rich in GGT activity [11].

De Young and associates demonstrated an increase in GGT activity in normal to neoplastic mouse skin [12] and Solt was not only able to demonstrate GGT activity in hamster buccal pouch mucosa undergoing chemical carcinogenesis, but found that the GGT was localized to small foci, suggestive of clones of altered cells [13]. Solt noted the intraepithelial plaques of intense GGT activity in whole mounts of hamster buccal pouch epithelium after 4 weeks of topical DMBA treatment. The plaques disappeared when DMBA treatment was discontinued. The discrete nature of the plaques and their microscopic size suggested that they may have originated from a single carcinogen-altered cell. In further studies, Solt and Shklar found that the GGT-stained plaques underwent rapid lateral growth during the period of DMBA application, with the GGT-stained cell populations overcoming the inhibitory effect of DMBA on normal cell replication [14].

Since GGT activity is a common finding in hamster buccal pouch undergoing chemical carcinogenesis, and is absent in normal, untreated control buccal pouch epithelium, its relationship to carcinogenesis could receive further confirmation by the investigation of experimental tumors undergoing inhibitory influences. Hamster buccal pouch carcinogenesis has been shown to be inhibited by retinoids [15, 16], alpha tocopherol [17] and beta carotene [18]. An experiment was

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designed to study the degree of activity of GGT in hamster buccal pouch carcinogenesis inhibited by beta carotene as compared to control animals developing buccal pouch carcinomas.

MATERIALS AND METHODS

Forty male Syrian hamsters (*Mesocricetus auratus*) were divided into four equal groups of experimental animals.

Group 1 animals were painted on the left buccal pouch thrice weekly with a 0.25% solution of DMBA in heavy mineral oil using a No. 4 sable brush.

Group 2 animals were painted thrice weekly with DMBA as in Group 1. On alternate days the left buccal pouches were painted with a 0.25% solution of beta carotene (Sigma) dissolved in mineral oil. 250 μg/ml (25 mg/kg).

Group 3 animals were painted with beta carotene thrice weekly as in Group 2.

Group 4 animals were untreated controls.

Animals were killed 22 weeks after the start of the procedure. Tumors were counted, measured and the buccal pouches photographed. The buccal pouches were then excised and each pouch was divided in half for 70% alcohol fixation and for 1.0% glacial acetic acid fixation. Alcohol fixation was used for paraffin embedding and hematoxylin–eosin staining. Acetic acid fixation was used for epithelial whole mount preparations and GGT histochemical staining. All animals were autopsied and liver and spleen were removed for histologic study.

The whole mounts were prepared according to the technique used by Solt [13]. After remaining in an ice-cold aqueous solution of 1.0% acetic acid with constant stirring for 1 hr, the pouch tissues were rinsed and placed in a shallow pan of distilled water, and the epithelia detached from the underlying connective tissue with a wooden tongue depressor. The epithelial fragments were then placed, basal layer up, on glass slides, air dried and secured to the glass surface with glycerin gelatin. Localization of GGT within the whole mounts was achieved using the histochemical method of Rutenburg *et al.* [15].

A freshly prepared solution containing the substrate glutamyl-4-methoxy-2-naphthylamide, the diazonium coupling reagent Fast blue BB salt, and the acceptor glycylglycine was transferred by pipet onto the surface of each whole mount. The incubation was performed at room temperature for 20–30 min. After staining, cover slips were placed using glycerin as a mounting medium. These stained whole-mount preparations were held under refrigeration to retard crystallization of the reaction products.

In order to quantitate the number of GGT-stained lesions, the outline of each whole mount preparation was traced onto graph paper with sq mm divisions. The number of GGT-stained lesions was determined microscopically and expressed as a function of epithelial surface area. To obtain values for individual hamsters, the lesion counts and whole-mounts areas of both pouches were combined. The size of individual GGT-stained plaques were determined by measuring each lesion with a calibrated ocular micrometer. For this purpose, lesion size was defined as (length + width) ÷ 2, where length is the maximum dimension of the lesion measured along its long axis and is the maximum width of the lesion measured perpendicular to the long axis. For circular lesions, the size is equal to the diameter. Statistical analysis for significance was carried out using the 2-tailed Student's *t*-test for unpaired data. The *P* values were indicated. The chi-square test was used to compare the proportion of animals with tumor.

RESULTS

Gross observations

The Group 2 animals (DMBA + B Carotene) were found to have fewer tumors than the Group 1 animals (DMBA alone) and those tumors that did develop were smaller in size than those in the Group 1 animals. Nine of 10 animals in Group 1 had grossly visible, palpable tumors, while only 4 of 10 animals in Group 2 had gross tumors (Table 1, Graph 1).

Microscopic observations

All tumors in both Group 1 and Group 2 animals were epidermoid carcinomas. In both groups the carcinomas were of the well differentiated to moderately differentiated histologic types. In general, the carcinomas in the pouches of the Group 2 animals appeared to be less invasive and better differentiated, with less pleomorphism and increased formation of keratin.

Histochemical observations (GGT)

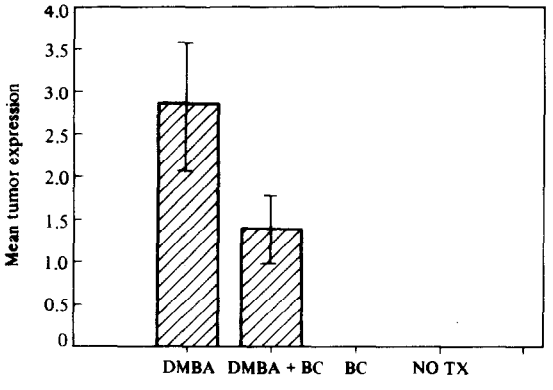
The left buccal pouch epithelial whole mounts of the Group 2 animals were found to have fewer foci of GGT activity than those of the Group 1 animals (Table 2, Graph II). In addition, the size of the GGT positive foci were significantly smaller in the Group 2 animals.

DISCUSSION

The results show a significant decrease in GGT activity in hamster buccal pouches undergoing chemical carcinogenesis when the tumor development is inhibited by beta carotene. The correlation between depressed tumor formation and

Table 1. *Summary of gross observations*

Group	Treatment	Mean tumor expression (Mean number × mean diameter in mm)	Animals with tumor/total in group
I	DMBA	2.88 ± 1.49	9/10
II	DMBA + beta carotene	1.40 ± 0.76	4/10
III	Beta carotene	0	0/10
IV	No treatment	0	0/10
		P value	
	Student's *t*-test Mean tumor expression	chi-square test Animals with tumor	
I vs II	≤ .001	≤ .001	

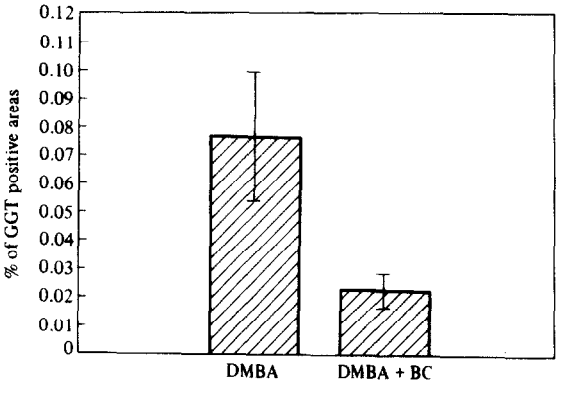


Graph 1

Table 2. GGT *positive foci in buccal pouch epithelium*

Group I: DMBA only
x = 0.077, S.D. = 0.045

Group II: DMBA and beta carotene
x = 0.023, S.D. 0.012



Graph 2

depressed GGT activity is clear, and would support the concept of Solt that GGT activity may be a marker for hamster buccal pouch carcinogenesis [13] as it appears to be in a variety of tissues.

Beta carotene has been shown previously to be associated with inhibition of carcinogenesis [20]. The significant reduction in tumor formation and the low level of GGT foci in the Group 2 animals (DMBA + beta carotene) may indicate a reduction in chemical agents considered to be related to GGT activity (e.g. lipid peroxidation products such as singlet oxygen or free radicals or gamma glutamyl compounds). Therefore, beta carotene may, though its intrinsic antioxidant capacity, decrease the need for GGT activity [21]. In addition, GGT is required for the conversion of LTE_4 (leukotriene) to its end product SRSA (slow reacting substance of anaphylaxis). SRSA can enhance blood flow to a tumor site by altering vascular permeability and capacity, which has been demonstrated in the hamster buccal pouch.

The physiological role of GGT has several interpretations. It appears to act as a glutathionase, catalyzing the transfer of the gamma-glutamyl group to numerous peptide and amino acid acceptors.

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