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Diurnal variation of γ-glutamyl transpeptidase activity during DMBA-induced hamster buccal pouch carcinogenesis

L-M Lin, YK Chen

Oral Pathology Department, School of Dentistry, Kaohsiung Medical College, Kaohsiung, Taiwan, ROC

OBJECTIVE: The aim of the present study is to assess the diurnal variation of γ-glutamyl transpeptidase (GGT) during 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch carcinogenesis.
MATERIALS AND METHODS: The right buccal pouches of 108 Syrian golden hamsters, divided into three experimental groups, were treated three times weekly with 0.5% DMBA in mineral oil over an 11-week treatment regimen. The left buccal pouches were untreated and served as the controls. Within each group, six animals were killed at 4-h intervals (04.00, 08.00, 12.00, 16.00, 20.00 and 24.00) for 24 h. GGT histochemical stain, according to the method of Ruthenberg and coworkers, was applied. The number of GGT-positive foci in the pouch mucosa was recorded at 3, 7 and 11 experimental weeks.
RESULT: Diurnal variation of GGT histochemical activity during DMBA-induced hamster buccal pouch carcinogenesis was substantiated in the present study.
CONCLUSION: This investigation highlights the importance of the diurnal variation in experimental oral carcinogenesis.

Keywords: diurnal variation; 7,12-dimethylbenz[a]anthracene (DMBA)-carcinogenesis; γ-glutamyl transpeptidase (GGT); hamster buccal pouch

Introduction

γ-glutamyl transpeptidase (GGT) (E.2.3.2.2.), a plasma membrane-bound enzyme, is one of the most important enzymes of glutathione (GSH) metabolism. It catalyzes the transfer of the γ-glutamyl group of GSH to amino acids or peptides, and participates in the regulation of amino acids across the cell membrane (Meister and Larsson 1989; Curthoys, 1990). GGT plays a role in multistage hepatocarcinogenesis, therefore being regarded as a (pre)neoplastic marker (Hendrich and Pitot, 1987). Furthermore, GGT activity has been detected histochemically in 7,12-dimethylbenz[a]anthracene DMBA-induced hamster buccal pouch carcinogenesis (Solt *et al*, 1987; Zhang *et al*, 1987) and in various human carcinomas (Fiala *et al*, 1980; Gerber and Thung, 1980; Levine *et al*, 1983; Calderon-Solt and Solt, 1985) including oral cancers (Mock *et al*, 1987).

GGT, GSH and glutamate are closely related in the γ-glutamyl cycle (Meister and Larsson, 1989; Curthoys, 1990). A distinct diurnal variation of GSH in rat tissues (Boor *et al*, 1979; Farooqui and Ahmed, 1984) has already been reported whereas glutamate phase shifts circadian rhythms in hamsters (Miejer *et al*, 1988) were also observed. However, to our knowledge, diurnal variation of GGT has not been adequately explored. This led us to investigate whether diurnal variation of GGT activity is also present.

Hamster buccal pouch is the most widely accepted experimental model of oral carcinogenesis (Salley, 1954; Lin *et al*, 1989; Lin and Chen, 1991). Diurnal variation in cell population kinetics of hamster buccal pouch mucosa during DMBA-induced carcinogenesis was reported by our laboratory (Lin and Goepp, 1986). The current study is designed to assess the possibility that GGT shows diurnal variation in DMBA-treated hamster buccal pouch mucosa. GGT histochemical activity was determined at 4-h intervals (04.00, 08.00, 12.00, 16.00, 20.00 and 24.00) for 24 h during DMBA-induced hamster buccal pouch carcinogenesis.

Materials and methods

Hamsters and carcinogen treatments

All chemicals, procured from Sigma Chemical Company (St Louis, Missouri, USA), were of the highest purity commercially available. The protocol of this study ensures humane practices. One hundred and eight non-inbred young (6 weeks old) male Syrian golden hamsters (purchased from National Taiwan University Breeding Laboratory), weighing about 100 g at the commencement of the experiment, were randomly divided into six groups. The animals were maintained under constant conditions (22°C, 12:12 h light/dark cycle) in an air-conditioned animal house. They were fed with standard laboratory pellets and tap water *ad libitum*. After allowing a week to acclimatize to the new surroundings, 0.5% DMBA in heavy mineral oil solution was applied three times weekly (09.00 on Monday, Wednesday and Friday) to the medial walls of the right buccal

Correspondence: Dr Li-Min Lin, Oral Pathology Department, School of Dentistry, Kaohsiung Medical College, 100, Shih-Chuan 1st Road, Kaohsiung, Taiwan, ROC
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Table 1 GGT-stained foci per pouch in each of the time periods: 04.00, 08.00, 12.00, 16.00, 20.00 and 24.00 in each killing period

Time periods	*Killing periods*		
	3-week	*7-week*	*11-week*
Daytime			
04.00	(1,1,0,2,3,2)	(3,4,3,4,5,3)	(5,6,5,5,6,5)
08.00	(1,0,0,3,4,2)	(5,4,4,4,5,6)	(7,6,6,9,6,9)
12.00	(1,1,1,2,2,1)	(2,2,3,2,3,4)	(5,4,4,4,5,5)
Subtotal	[27]	[66]	[102]
(mean ± s.d.)	(1.5 ± 1.1)[a]	(3.7 ± 1.1)	(5.7 ± 1.4)[b]
Night time			
16.00	(1,1,0,1,1,0)	(3,2,2,5,4,6)	(6,7,7,9,6,9)
20.00	(1,0,0,1,0,0)	(4,3,2,7,4,7)	(9,6,9,10,8,10)
24.00	(0,0,0,2,1,0)	(2,1,2,4,5,3)	(5,6,5,7,8,5)
Subtotal	[9]	[66]	[32]
(mean ± s.d.)	(0.5 ± 0.6)[a]	(3.7 ± 1.7)	(7.3 ± 1.7)[b]
Total[c]	{36}	{132}	{234}

The numbers of GGT-stained foci per pouch for each of six hamsters in each time period were shown in ()
[a]GGT activity of day periods was significantly greater than night periods ($P < 0.05$, Student's *t*-test)
[b]GGT activity of night periods was significantly greater than day periods ($P < 0.05$, Student's *t*-test)
[c]An elevation in the total number of GGT-stained foci during DMBA-induced hamster buccal pouch carcinogenesis was observed.

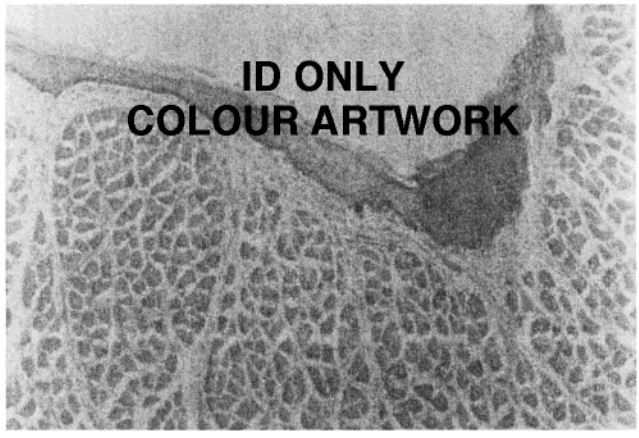

Figure 1 Two GGT-positive foci in a 7-week DMBA-treated pouch (×40)

pouches of all the animals with six strokes of a No. 4 camel-hair brush which had wiped against the side of the container containing the solution (Morris, 1961). The left pouches remained untreated and served as the controls. After 3 weeks painting (3 days following the last DMBA treatment), a group of 36 animals was withdrawn randomly from the painting schedule and killed six at a time by an overdose of diethyl ether in a gas jar for each time period: 04.00, 08.00, 12.00, 16.00, 20.00 and 24.00, respectively. The killed hamsters were fixed in a supine position with pins. Bilateral pouches were exposed by dissection, cut from their oral openings to their caudal ends along the middle of their lateral walls and examined grossly. Subsequently, both pouches were excised and stretched on card papers to prevent distortion of the specimens during processing. Then, similar procedures were carried out repeatedly after 7 and 11 weeks DMBA painting.

GGT histochemistry

Four fresh samples of approximately 1 cm × 1 cm in diameter were obtained from each DMBA-treated pouch. These fresh tissues were quick-frozen in liquid nitrogen. Five micron serial tissue sections were cryostat-cut perpendicular to the epithelial surface and mounted on glass slides. Between sample sections, there was at least a space of 250 μm in order to avoid the possibility that the two consecutive sections contain the same foci. Two sections for each sample were processed to demonstrate GGT activity histochemically according to the method described by Rutherburg *et al* (1969) by incubating in a mixture containing the substrate γ-L-glutamyl-4-methoxy-2-naphthylamide, the diazonium coupling reagent fast blue BB salt and the acceptor glycylglycine for 30 min at room temperature. After rinsing with 0.85% sodium chloride, the tissue sections were dipped in 0.2 M copper sulphate solution, rinsed again in 0.85% sodium chloride, and washed in distilled water. The sections were counter-stained with haematoxylin. Slides were cover-slipped with glycerin gelatin mounting medium. Sections of hamster kidney, rich in GGT activity, were used as positive controls of GGT histochemical staining. Representative contiguous sections from each sample were processed and stained with haematoxylin and eosin (H&E) staining. One sample was taken randomly from the control pouches, fixed by immersion in 10% neutral buffered formalin for 24 h at room temperature, routinely processed for paraffin embedding, and stained for H&E staining.

GGT slides were refrigerated to prevent formation of crystallization products, which distort histochemical assessment. GGT-positive foci in each section were identified with a light microscope and photographed to record histologic findings. The brick red reaction products typical of

Table 2 Diurnal variations of GGT-positive foci during each painting period of hamsters during DMBA-induced carcinogenesis

Killing periods	*Time periods*					
	04.00	*08.00*	*12.00*	*16.00*	*20.00*	*24.00*
3 weeks	1.50 ± 0.96	1.67 ± 1.49	1.33 ± 0.47	0.67 ± 0.47	0.33 ± 0.47	0.50 ± 0.76
7 weeks	3.67 ± 0.75	4.67 ± 0.75	2.67 ± 0.75	3.67 ± 1.49	4.50 ± 1.89	2.83 ± 1.34
11 weeks	5.33 ± 0.47	7.17 ± 1.34	4.50 ± 0.50	7.33 ± 1.25	8.67 ± 1.37	6.00 ± 1.16

this enzyme activity involving the pouch epithelial lining were depicted as positive foci. The numbers of positive foci, irrespective of the intensity in the reaction products, of the six time periods (04.00, 08.00, 12.00, 16.00, 20.00 and 24.00) over 24 h in the right pouches of each animal were enumerated. Consequently, the total number of GGT-positive foci for each animal was the summation of the number of positive foci from the eight representative tissue sections taken from the DMBA-treated pouch. Since GGT-positive foci are randomly distributed within the pouch mucosa, the number of GGT-positive foci obtained may serve as a relative index of the total number of stained-foci present in the pouch mucosa (Zhang and Mock, 1987).

Statistical method
When appropriate, the means of the number of GGT foci in each killing period were compared using Student's *t*-test with $P < 0.05$ as the level of significance.

Results

GGT histochemistry
The brick red reaction products typical of GGT activity were seen involving the DMBA-induced pouch mucosa (Figure 1). As shown in Table 1; an elevation in the number of GGT-positive foci during DMBA-induced hamster buccal pouch carcinogenesis was observed. In addition, the average numbers of GGT-stained foci in each of the time periods: 04.00, 08.00, 12.00, 16.00, 20.00 and 24.00 were increased from 3-week, 7-week and 11-week killing periods (Table 2). No GGT activity was demonstrated in the untreated control pouches. The kidney sections (positive controls) proved to be positive.

Diurnal variation of GGT
Examination of GGT-stained specimens during the day periods (04.00, 08.00, 12.00) and night periods (16.00, 20.00, 24.00) revealed that GGT histochemical activity during the day periods was significantly greater than night periods in the 3-week DMBA painting group ($P < 0.05$, Student's *t*-test). Then, passing through 7-week DMBA painting, a reverse relationship was observed with the enzyme activity in the night periods being statistically higher than day periods in the 11-week DMBA painting group ($P < 0.05$, Student's *t*-test) (Table 1).

Diurnal variations of the average numbers of GGT-positive foci were found in each killing period of hamsters during DMBA-induced carcinogenesis (Table 2). The pattern of diurnal variation of GGT histochemical activity appeared to be similar for the 7-weeks and 11-week DMBA painting groups. Furthermore, the amplitude of diurnal variation seemed to be increased by a longer period of DMBA treatment.

Discussion

GGT expression during DMBA-induced hamster buccal pouch carcinogenesis has already been documented by a number of authors (Solt *et al*, 1987; Zhang and Mock, 1987). However, to our knowledge, diurnal variation of GGT activity in DMBA-induced hamster buccal pouch carcinogenesis has not been previously demonstrated. The phenomena of diurnal variation of GGT histochemical activity during DMBA-induced hamster buccal pouch carcinogenesis have clearly been demonstrated in the current study. The higher degree of diurnal variation of GGT histochemical activity upon a longer period of DMBA treatment in the current investigation may be corroborated by our previous study (Lin and Goep, 1986).

The GGT-positive foci would surely be induced by DMBA during hamster pouch carcinogenesis. It may be suggested that under the influence of some and yet not completely understood diurnal variation associated factors (intrinsic and/or extrinsic), the GGT activity was either switched on or off during neoplastic formation; thus producing the phenomena of diurnal variation. These exact intrinsic and/or extrinsic biological parameters attributed to the phenomena of diurnal variation of GGT in DMBA-induced hamster buccal pouch mucosa remain to be explored. However, the influence of a photoperiod upon animals is noteworthy to be considered. The effects of a photoperiod on the growth of colon cancer in mice (Waldrop *et al*, 1989) and melanoma in hamsters (Stanberry *et al*, 1983) were reported. Shah *et al* (1984) also found that continuous daylight increased the number of tumours induced by DMBA. In the present study, a 12-h light/dark cycle was used. Different photoperiods, such as continuous 24-h lighting system, may be employed in future to investigate whether there is an effect upon tumour growth and the diurnal cycle of GGT activity.

In conclusion, over 100 animals were employed in this study, with a moderate number of hamsters in each time period. Nonetheless, the diurnal variation of GGT in DMBA-carcinogenesis in hamsters is substantiated in the present study. It constitutes a preliminary step towards the comprehension of the diurnal rhythm of those biological parameters that are measured in the process of carcinogenesis.

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