Expression of γ-Glutamyltranspeptidase Activity in Human Oral Squamous Cancers

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SUMMARY

The enzyme activity of γ-glutamyltranspeptidase(GGT) was determined in tissues from human oral cancers and normal controls. Biochemical assay revealed that differentiated cancers had higher GGT activity compared with poorly differentiated cancers. The analysis of GGT histochemical activity was the same as that of the biochemical assay, however, the distribution pattern of GGT staining was heterogeneous. Although not all of the differentiated cancers had GGT histochemical activity, frequently GGT activity was detected only in highly keratinized horn pearls of individually keratinized cells, but not in proliferative cells. The significance of GGT expression in squamous cancers is discussed.

Key words: GGT, Oral cancer, Differentiation

INTRODUCTION

GGT is a membrane-bound enzyme which catalizes the transfer of the γ-glutamyl group of glutathione to amino acids and peptides and is also involved in the regulation of amino acids across the cell membranes(18). Normal adult liver cells contain only small amounts of GGT activity, but in the fetal and neonatal rat liver GGT activity is substantially higher(2). In contrast to normal adult liver cells, precancerous lesion and cancer cells of liver seem to contain increased amounts of GGT as revealed in rats during hepatocarcinogenesis (2, 7, 12). Similarly GGT activity has been detected in human cancers of the liver(9), colon(8), breast(14), pancreas(25) and skin(4). Recently we have also reported that GGT has several advantages and serves as a good marker for the study of hamster oral carcinogenesis(16, 23) and for diagnosis of human head and neck cancers(3). Thus, it has been suggested that GGT is simply a marker of a fetal phenotype or cell dedifferentiation(24), and a cursory inspection would suggest that GGT in a marker of cell proliferation. However, recent reports on GGT expression by in vivo and in

vitro systems suggest that GGT is a marker of cell differentiation, or aging rather than cell dedifferentiation or proliferation(20, 21). Hence, the present study was undertaken to examine both biochemically and histochemically, whether correlations exist between elevated GGT activity and degree of cell differentiation in human oral squamous cancers of varying degrees of differentiation.

MATERIALS AND METHODS

Specimens

Biochemical and histochemical assays were made in tissues from 19 human oral squamous cancers, control oral mucosal tissues from 5 patients with other oral diseases and fresh kidney tissue from a human autopsy. These specimens were cut into 2 pieces to apply GGT assays. For the biochemical assay, subepithelial tissues were carefully removed from the specimens to avoid contamination, so far as possible. These specimens were frozen at −80℃ until used.

Biochemical assay

The enzyme source was extracted by a modification of a previously reported method(25). Specimens were homogenized in ice-cold 10 mM Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. The homogenate was centrifuged at 105,000 ×g for 60 min to pellet the microsomal membrane filter fraction. The pellet was incubated in 10 nM Tris-HCl buffer (pH 8.0) containing 0.5% Triton X-100 at 4 ℃ for 120 min. The solution was further treated with bromelain (1 mg per 10 mg protein) at 37℃ for 60 min and centrifuged at 105,000 ×g for 60 min. GGT was assayed in the supernatant according to the previously published procedure(17). Tissue supernatants of 100 μl were incubated with 500 μl substrate mixture at 37℃ for 5 min. The final reaction mixture contained 2.5 mM L-γ-glutamyl-p-nitroanilide, 50 nM glycylglycine and 0.01 M Tris-HCl buffer (pH 8.5). The reaction was terminated by an addition of 1.5 M acetic acid. Enzyme activity (μmol of p-nitroaniline liberated per mg of protein per min) was calculated using a molar extinction coefficient of p-nitroaniline at 410 nm of 8800 M^{-1}. cm^{-1}. Protein was determined according to the method of Lowly *et al.*(15).

Histochemical assay

Serial frozen sections of these tissues were cut at 6 μm. The initial sections were fixed in 10% formalin solution and stained with hematoxilin-eosin for tissue diagnosis and correlation with GGT activity. The remaining sections were fixed in cold acetone for 60 min and incubated according to the method described by Rutenberg *et al.*(22). Sections were immersed at room temperature for 30 min in a freshly prepared solution containing 1 ml substrate solution of 2.5 mg γ-glutamyl

-4-methoxy-2-nahthylamide per m*l*, 5.0m*l* Tris buffer (0.1 M) pH 7.4, 14 m*l* 0.85% NaCl, 10 mg glycylglycine and 10 mg Fast Blue BBN. After rinsing with a solution of 0.85% NaCl, 0.1 M $CuSO_4$ and 0.85% NaCl, the sections were mounted in glycerol. Some sections were counterstained with hematoxilin.

RESULTS

Histology

Of the 19 oral squamous cancers examined in the present study, histologically, 7 cancers were well differentiated, 9 moderately differentiated, 3 poorly differentiated(Tables 1, 2).

GGT biochemical activity

Table 1 shows the specific biochemical activity of GGT in tissue homogenates. The mean level of GGT activity for oral mucosal tissue as control was 0.015 with a standard deviation of 0.007 and a range of 0.008-0.026. GGT activity in kidney

Table 1 *GGT biochemical activity in human oral cancers and controls*

Tissue	Total	GGT specific activity* (Range)
Normal oral mucosa	5	0.015±0.007(0.008-0.026)
Kidney	1	2.554
Squamous cancer		
Well differentiated	7	0.219±0.074(0.094-0.311)
Moderately differentiated	9	0.197±0.088(0.031-0.351)
Poorly differentiated	3	0.031±0.012(0.019-0.045)

* μmol/mg protein/min
Mean±SD

Table 2 *GGT histochemical activity in human oral cancers and controls*

Tissue	Total	GGT histochemical activity		
		GGT-positive		GGT-negative
		Whole*	Patchy**	
Normal oral epithelium	5	0	0	5
Squamous cancer				
Well differentiated	7	4	3	0
Moderately differentiated	9	3	5	1
Poorly differentiated	3	0	0	0

* Uniform staining for all of the cancer cell nests
** Patchy staining for cancer cell nests

examined simultaneously as control was markedly higher as compared with that of normal oral mucosal tissue with an increase of about 170 fold intensity. Well differentiated cancers had a mean level of 0.219 ± 0.074 which was higher, but not so significantly different, compared with that of moderately differentiated cancers showing a mean activity of 0.197 ± 0.088. In contrast to these cancers, GGT activity in the poorly differentiated cancers was reduced to lower levels of 0.031 ± 0.012.

GGT histochemical activity

The results of GGT histochemical staining are shown in Table 2. No GGT activity was noted in any of the normal oral epithelia of 5 mucosal tissues. However, GGT activity was observed in the secretory portions of subepithelially located minor salivary glands, but not in the ductal portions. As previously de-

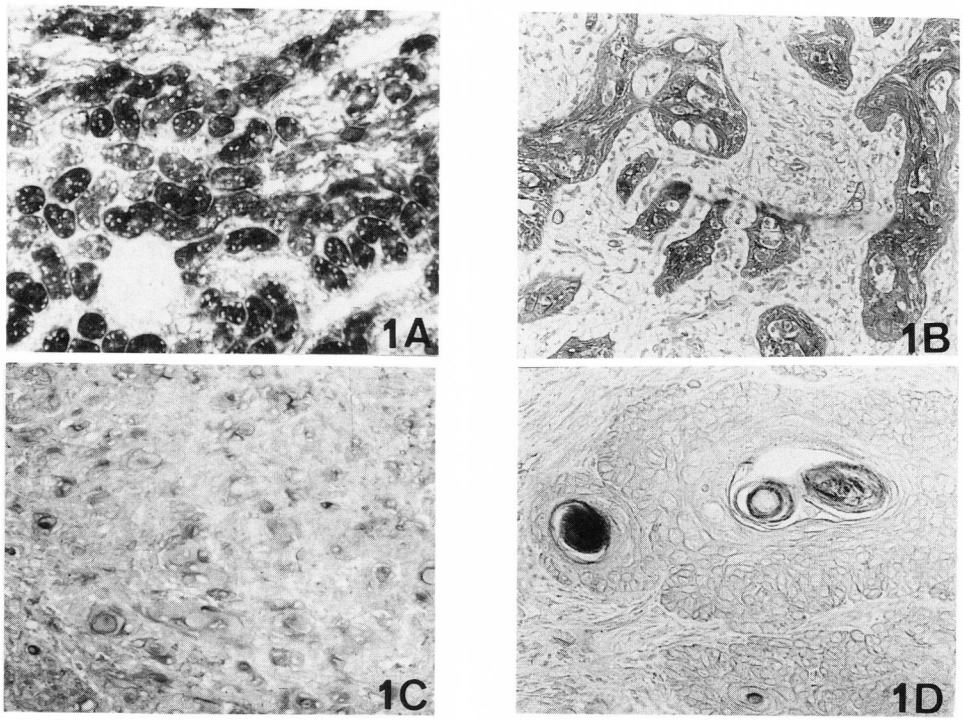

Fig. 1 GGT enzyme histochemistry without counterstain. A, GGT-positive tubules in kidney. × 85. B, Whole GGT-positive cancer cell nests in moderately differentiated squamous cancer. × 170. C, GGT-positive individually keratinized cancer cells in moderately differentiated squamous cancer. No enzyme activity was detected in the proliferative cancer cells. × 190. D, GGT-positive horn pearls in well differentiated squamous cancer. × 109.

scribed(11), tubules in kidney showed markedly intense GGT activity (Fig. 1A). However, the staining pattern was heterogeneous in oral cancers. Of 19 cancers examined, 7 well and 8 moderately differentiated cancers were GGT-positive, while 3 poorly differentiated cancers showed no positive reaction for GGT. In 7 of 15 GGT-positive cancers, whole cancer cell nests were uniformly stained with intense reaction (Fig. 1B), whereas, in 8 cancers variation in the localization and intensity of GGT activity was observed: frequently, GGT activity was heterogeneously positive with both positive and negative cancer cell nests and was restricted to keratinized horn pearls and individually keratinized cells (Figs. 1C, 1D). Occasionally, even in the keratinized portions no GGT activity was found. Tissues incubated without substrates were always invariably negative for GGT staining.

DISCUSSION

Attempts to use GGT as a marker in carcinogenesis have been extended to other organ systems than rat liver(5, 6). However, the exact significance of the elevated GGT activity in precancerous lesions and cancers is unknown. Recent reports have shown that GGT expression was not necessarily linked to cell proliferation, but rather related to cell differentiation(21, 22). These reports led us to investigate the correlation between GGT activity and cell differentiation in squamous cancers of different histologic type.

In the present study, GGT biochemical and histochemical analysis for human oral cancers has provided evidence for a difference of the expression of GGT activity between cancers with different degrees of differentiation. GGT histochemical activity was observed only in differentiated cancers, but not in poorly differentiated cancers and normal oral mucosal controls. Biochemically, significantly higher GGT activdty was also measured in differentiated cancers as compared to poorly differentiated cancers and normal controls. Groscurth *et al.*(10) found biochemical levels of GGT activity to be higher in well differentiated rather than in poorly differentiated human lung squamous cancer cell lines transplanted to nude mice. These suggest that there might be some relationship between GGT activity and tumor differentiation. However, histochemically GGT activity was not present in all the differentiated cancers. Moreover, the GGT-positive cancers had a heterogeneous pattern of staining with GGT-positive and negative cancer cell nests. Furthermore, in the GGT-positive cancers, GGT activity was frequently localized in such restricted portions of cancer cell nests as in keratinized horn pearls and individually keratinized cells. Occasionally, even keratinized portions had no GGT activity. This heterogeneous pattern of GGT activity in squamous cancers is not consistent with the possible correlation between GGT expression and tumor differentiation.

It has been suggested that the transport of γ-glutamyl amino acids is dependent on intracellular glutathion levels(1). As nothing is known of the role of glutathion in squamous cancer, special consideration must be given to it. Laishes *et al.*(13) reasoned that staining artifact was an unlikely cause for heterogeneous phenotypic expression. In the present study, it was clear that there was no staining artifact because kidney tissue used simultaneously as control showed uniformly intense staining of GGT activity on each examination, and there was no demonstrable activity in keratinized layers of normal oral epithelium. Some authors have speculated that GGT activity in benign tissue might be a reflection of normal γ -glutamyl amino acid transport relating to secretion, whereas in precancerous lesions and cancers it might be a reflection of impairment in intracellular glutathion metabolism(19) and that GGT might be an enzyme that provides a selective advantage to a cell undergoing stress during malignant transformation, resulting in depletion of glutathione(11). Zang and Mock(26) speculated that the expression of GGT activity may be switched off with malignant transformation, and while newly occurring proliferative cells may stop expressing the activity, already GGT-positive cells may be differentiated. This would explain the heterogeneous staining of GGT activity which was frequently expressed only in keratinized portions of cancer cell nests. Additional data are required to determine, whether GGT in squamous cancers is a special marker that is related to the mechanism of keratinization, whether malignant transformation of kerationcyte requires the metabolic changes of glutathione to be expressed as a fetal phenotype, and or whether the expression of GGT activity is modulated during malignant transformation.

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