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Sequential expression of placental glutathione S-transferase (GST-P) during DMBA-induced hamster buccal pouch squamous cell carcinogenesis

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The aim of the present study was to investigate the sequential expression of placental glutathione S-transferase (GST-P) during 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch squamous cell carcinogenesis. Both immunohistochemical and immunoblot analyses were employed to detect the epithelial GST-P in hamster buccal pouch mucosa over a 15-week treatment regimen. No GST-P positivity was demonstrated in the pouches of the control group. GST-P positive cells were first noted as early as 1 week after DMBA applications. A gradual increase in both the mean number and size of GST-P-positive foci was noted in the first 12 experimental weeks, but a plateau level was approached thereafter. The early GST-P-positive-area were located in the basal layer, or occasionally in the middle layer, of DMBA-treated hamster buccal pouch mucosa. Later, the stained sites became enlarged and were scattered randomly in different layers or in the whole thickness of the dysplastic and non-dysplastic epithelium. The keratin layer was only occasionally involved during the first 12 weeks of DMBA treatment but positive staining was more noticeable in the final stage of the experiment. Both exophytic (8–12 weeks) and invasive (13–15 weeks) squamous cell carcinomas showed GST-P positivity, in both cytoplasmic and nuclear components. Immunoblot analysis revealed no band in the crude tissue extracts of the control pouches whereas GST-P polypeptide of molecular weight approximately 26 kD was demonstrated in DMBA-treated pouches over the whole 15-week treatment regimen. Results of the present work indicate that GST-P is a stable and persistent label for almost all of the carcinogen-altered cells during DMBA-induced hamster buccal pouch carcinogenesis. Immunohistochemically detectable GST-P may be a potential marker throughout oral chemical carcinogenesis.

Key words: DMBA-carcinogenesis; hamster; oral; placental glutathione S-transferase; tumor marker

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Cytosolic glutathione S-transferases (GSTs: EC 2.5.1.18) are a multigene family of detoxification and metabolizing enzymes, which have been linked with the susceptibility of tissues to chemical carcinogens and resistance of tumors to chemotherapy (1). They catalyze the conjugation of potentially mutagenic electrophilic compounds to the reduced tripeptide glutathione (1). GSTs have been studied most extensively in rat hepatic tissues. At least twelve distinguishable forms of rat GST isoenzymes have been identified (2), among which the placental form (GST-P) was isolated and purified from rat placenta by SATO *et al.* (3) and KITAHARA *et al.* (4). This rat GST-P was

shown to contain two dimers of molecular weight 26 kD (5). Human placental GST (GST π), which corresponds to rat GST-P, has also been isolated (6). A large degree of interspecies homogeneity has been demonstrated by immunological criteria (5).

Rat GST-P was found to be present in relatively high concentrations in preneoplastic and neoplastic lesions during rat chemical hepatocarcinogenesis compared to normal rat hepatocytes, therefore being regarded as a (pre)neoplastic marker (3–5). A similar enhancement of GST-P isoenzyme has also been observed in preneoplastic and neoplastic lesions in rat lung (7), and in hamster liver and pancreas (8). Furthermore, GST-P expression has been demonstrated in hamster buccal pouch mucosa treated by 7,12-dimethylbenz[a]anthracene (DMBA), but was absent from normal pouch keratinocytes (9). Recently, GST π was found to be overexpressed immunohistochemically in oral premalignant and malignant epithelial lesions, and may be a potential tumor marker in oral carcinogenesis (10, 11). In addition, HIRATA *et al.* (12), using enzyme immunoassay, demonstrated that GST π was useful in early diagnosis, prediction of disease extent and outcome of patients with oral cancers. These findings (10–12) have thus led to the importance of establishing whether the placental GST-positive cells in oral premalignant lesions will eventually be transformed to overtly malignant cells. Consequently, it is critical to investigate the sequential changes occurring in placental GST isoenzyme during oral carcinogenesis. However, such kinetics of placental GST-positive cells may not be readily derived from individual human biopsies. With the use of the hamster buccal pouch model, first introduced by SALLEY (13) and further developed by MORRIS (14), it is possible to follow the changes of epithelial GST-P expression during a period of oral chemical carcinogenesis.

Immunohistochemical methods have been used in most previous studies concerning the expression of placental GST in oral mucosa (9–11, 15). Other methods of detecting this isoenzyme have been described, including high performance liquid chromatography in ovarian tumors (16), immunoblot analysis in breast tumors (17), and Western blot and Northern hybridization in breast tumors (18). All methods of GST isoenzyme detection may be subject to a degree of experimental error. It is therefore more appropriate to use multiple investigative techniques to detect placental GST expression (18). Review of the literature concerning the kinetics of placental GST in hamster buccal pouch mucosa revealed only one such related study (15). However, in that report (15) no information is available about the use of immunoblot analysis to detect the oral epithelial GST-P isoenzyme. Therefore, in the present study both immunohistochemical and immunoblot analyses were employed to detect epithelial GST-P in hamster buccal pouch mucosa.

Material and methods

Hamsters and carcinogen treatment

Eighty non-inbred male adult Syrian golden hamsters (purchased from the National Taiwan University Breeding Laboratory), aged approximately 6 weeks and weighing 120 g at the commencement of the experiment, were randomly divided into 16 groups (15 experimental groups and one control group), each containing five animals. The animals were maintained under constant conditions (22°C, 12 h light/dark cycle) and fed with water as well as laboratory chow *ad libitum*. After allowing a week to adjust to the new surroundings, 0.5% DMBA in heavy mineral oil solution was applied thrice weekly with a No. 4 camel-hair brush to both the buccal pouches of all the animals in the experimental groups, whereas pouches of the five animals in the control groups were treated with mineral oil alone. At the end of each week (three days following the last DMBA treatment), one group of five animals was omitted randomly from the painting schedule and was killed by a lethal dose of diethyl ether. Pouches were inverted and examined grossly; the number of tumor growths was counted and the diameter of the tumors was measured. The mucosa was excised. Finally, all the remaining animals in the experimental groups, as well as the five animals in the control group, were killed at the end of the experiment.

The experimental procotol for obtaining samples from the pouches is illustrated in Fig. 1. From each pouch, four samples (A1–D1) of approximately 1 cm in length were fixed in 10% neutral buffered formalin solution, dehydrated in ascending alcohols, cleared in xylene and embedded in paraffin. Sections were then cut at 4 μm in thickness, stained with hematoxylin and eosin (H & E) or immunostained with GST-P antisera, and examined by light microscopy. Four other samples (A2–D2) adjacent to those used for immunohistochemistry were frozen at −80°C for GST-P isoenzyme extraction.

Immunohistochemistry for GST-P isoenzyme

Serial sections were cut from each sample. One of the sections was prepared for GST-P immunohistochemistry while another was used for H & E staining. There were therefore four slides from each pouch and eight slides from each

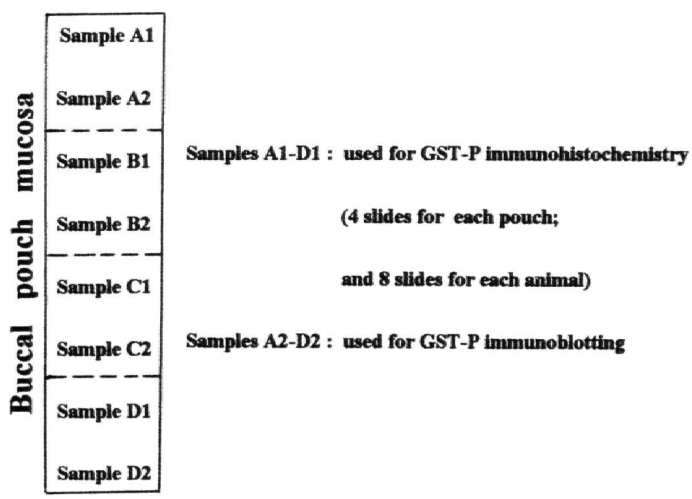

Fig. 1. Illustration of the experimental protocol for obtaining samples from the control as well as DMBA-treated pouches.

animal for GST-P staining. Immunohistochemical staining of GST-P isoenzyme was performed by the standard avidin-biotin peroxidase complex (ABC) technique according to HSU *et al.* (19). Polyclonal antibodies against rat GST-P were raised in rabbits (20) and obtained from Biotrin International Lab., Dublin, Ireland [Cat no. BIO23 Yp(r)]. The antisera have no cross-reactivity with GSTα or GSTμ enzymes but strong cross-reaction with human and mouse π enzymes (information from the manufacturer). Also, it has been established that hamster GST-P is indistinguishable from rat GST-P immunohistochemically (8). Paraffin sections were passed through xylene and a series of graded alcohols (99% to 70%) and then treated sequentially with 3% H_2O_2 (Sigma), normal goat serum (Dakopatts, 1:20), anti-GST-P (1:200) rabbit polyclonal antibodies, and then with biotin-bound anti-rabbit IgG antibody (Vectastain ABC kit, PK4001, 1:400) and avidin-biotin-peroxidase complex (Vectastain ABC kit, PK4001). The binding sites of peroxidase were determined using diaminobenzenes (DAB) as the substrate. Sections were then lightly counterstained with Meyer's hematoxylin before mounting. The GST-P-positive foci in each histological section were counted under a light microscope. The number of GST-P-positive foci for each animal was the sum of the numbers of stained foci from eight representative sections from both DMBA-treated pouches. The size of individual GST-P-stained foci was determined by measuring the positive foci using a calibrated ocular micrometer. Negative controls were included by replacing the immune sera with preimmune rabbit sera.

Extraction of GST-P isoenzyme

The GST-P isoenzymes were extracted by the method of PETERS *et al.* (21), with some modifications. All procedures were performed at 4°C, unless otherwise stated. Tissues (~100 mg) were homogenized in ~5 volumes of ice-cold 20 mM tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 1.4 mM dithiothreitol with 10 strokes in glass/glass tissue grinders. The homogenates were centrifuged at 10,000×*g* at 4°C for 10 min and the cytosolic fractions were obtained after recentrifugation at 150,000×*g* for 1 h. Supernatants were frozen in liquid nitrogen and stored at −80°C until used for gel electrophoresis and the subsequent immunoblot analysis.

Table 1. Involvement of GST-P positivity in different layers of pouch mucosa in different killing periods during DMBA-induced hamster cheek pouch carcinogenesis

	Control	Weeks 1–4	Weeks 5–12	Weeks 13–15
Keratin layer	−	−	−	+
Middle layer	− (−)	+ (+)	+ (+)	+ (+)
Basal layer	− (−)	+ (+)	+ (+)	+ (+)

−: negative staining; +: positive staining; (−): negative nuclear staining; (+): positive nuclear staining.

Immunoblotting for GST-P isoenzyme

Immunoblot analysis was used to identify the GST-P proteins, separated by SDS-PAGE electrophoresis (22) (12.5% w/v acrylamide), by the method described by TOWBIN *et al.* (23).

After SDS-PAGE electrophoresis, the unstained gel was immersed in the transfer buffer (20 mM tris-HCl, 150 mM glycine, 20% methanol) for 30 min. The proteins were transferred to nitrocellulose membranes (Biotrace, pore size 0.45 μm) in a Bio Trans Semidry electrophoretic transfer unit (Gelman Science Inc.) with a constant current (0.8 A/cm of gel) for 2 h at 4°C. The nitrocellulose paper was first incubated in 1% Tween-20 in tris-buffered saline (TBS) for 1 h and washed twice with TBS containing 0.05% Tween-20.

Immediately following transfer, the nitrocellulose sheet was incubated in TBS for 1 h with 5% bovine serum albumin (BSA). The nitrocellulose sheet was then incubated in diluted 1:200 rabbit anti-rat GST-P (Biotrin International Lab., Dublin, Ireland) for 2 h at room temperature, washed, and then reacted with TBS-diluted 1:500 goat anti-rabbit IgG peroxidase conjugate (Sigma) for 2 h at room temperature. The nitrocellulose sheet was washed in freshly prepared substrate solution containing 0.05% DAB and 0.005% hydrogen peroxide in 50 mM TBS (pH 7.4) for 5 min.

Results

Gross

Gross examination of all the pouches of the control group revealed no obvious changes. The week one and week two DMBA-treated pouches demonstrated erythema and erosion, which were then followed by repair in week three. By the fourth week there was thickened mucosa with a rough surface and whitish granular appearance. Subsequently, papillomatous tumor growths first developed in weeks eight to twelve. Invasive endophytic squamous cell carcinomas were observed in the final stage (weeks 13 to 15) of the experiment. Furthermore, a gradual increase in both the mean number and size of tumors was noted in each killing period.

Histopathology

Microscopically, no obvious changes were observed in the control pouches, while the one-week and two-weeks DMBA-treated pouches showed an acute inflammatory reaction with engorged blood vessels and severe mucos-

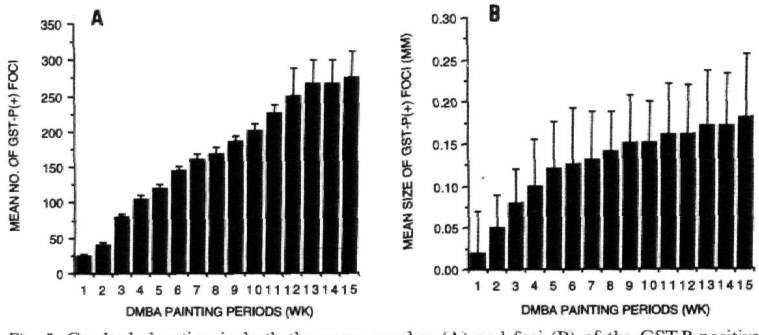

Fig. 2. Gradual elevation in both the mean number (A) and foci (B) of the GST-P-positive foci in the first 12 experimental weeks was noted but a plateau level was approached in the final stage of the experiment (13–15 weeks).

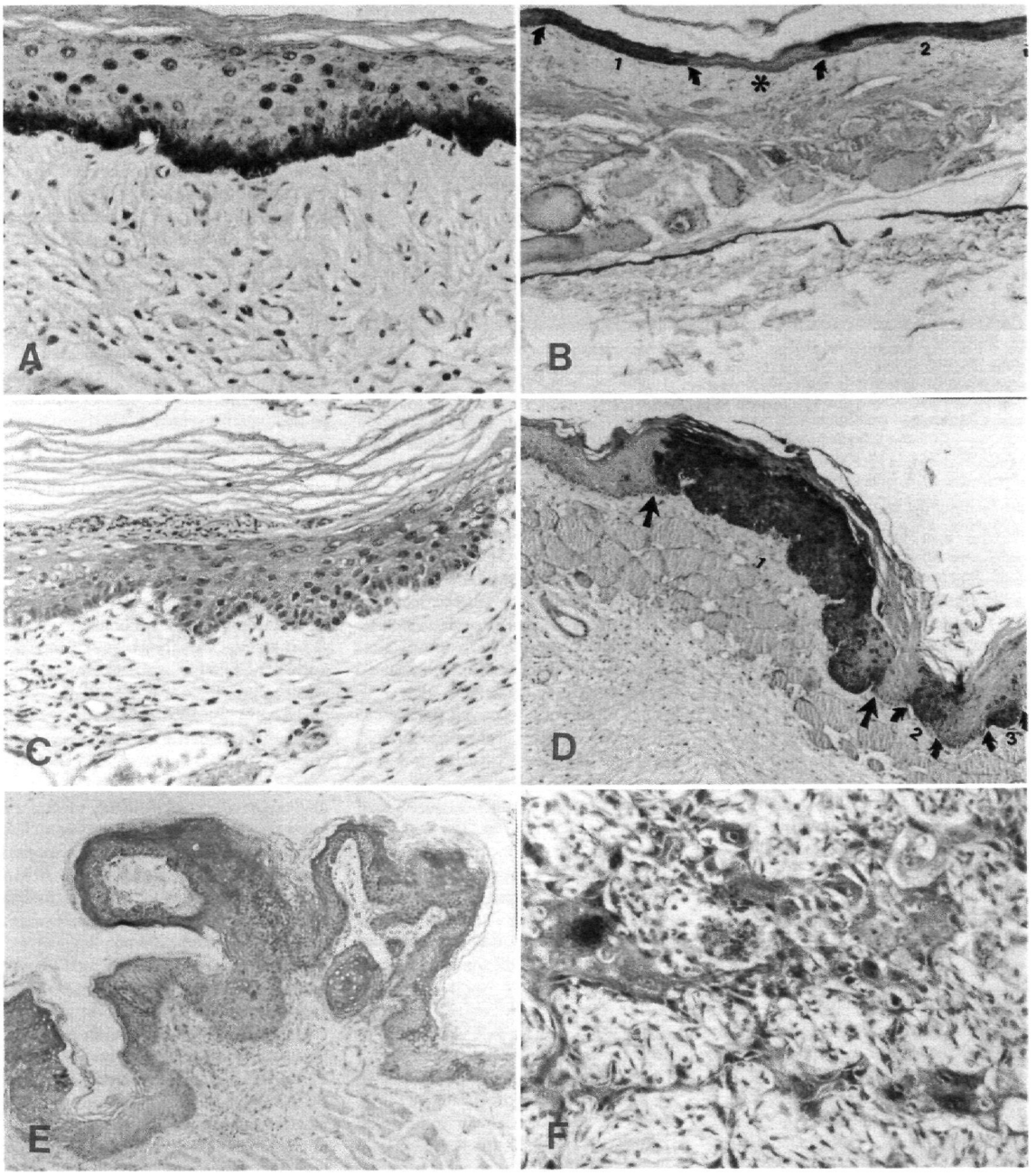

Fig. 3. A. Early GST-P positivity comprised stained cells in the basal layer or multiple single cells in the middle layer of a 3-week DMBA-treated pouch (×100). *B.* Two GST-P-positive foci, indicated by the curved arrows and labelled respectively as 1 and 2, in a pouch of 4-week DMBA applications. These GST-P-positive sites were morphologically indistinguishable from the negative area (asterisk). Focus 1 involved the entire thickness of the mucosa but spared the keratin layer. The left portion of focus 2 involved only the middle layer, whereas the right part occupied the whole thickness, with the exception of the keratin layer, of the pouch mucosa (×40). *C.* A GST-P-positive focus in an area of mild dysplasia from a pouch of 6-week DMBA applications (×100). Both cytoplasmic and nuclear staining are observed. *D.* A large GST-P-stained area, designated as 1, in an area of moderate dysplasia in a pouch of 9-week DMBA applications. Two smaller GST-P-positive foci, labelled respectively as 2 and 3, are seen in the same pouch. Focus 2 involves the whole thickness, while focus 3 involves only the lower portion of the pouch mucosa (×40). *E.* GST-P staining in an exophytic squamous cell carcinoma of a pouch after 12-week DMBA applications (×40). *F.* GST-P staining in an invasive squamous cell carcinoma in a pouch after 15-week DMBA applications (×100).

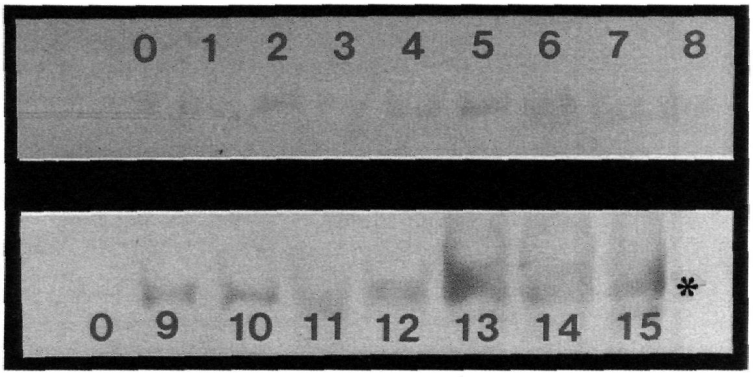

Fig. 4. GST-P polypeptide was found to be of molecular weight approximately 26 kD (asterisk) in the DMBA-treated pouches for the whole 15 week treatment regimen (lanes 1 to 15). No band could be detected in the control pouches (lane 0). Note the relatively weaker GST-P staining in pouches of hamsters treated with DMBA for less than 9 weeks as compared to those of more than 9 weeks.

itis. Following the stage of repair in weeks 3 to 5, there was hyperkeratosis, acanthosis and lymphocytic infiltration. Mild dysplastic changes were first seen in the pouches at week 6. The severity of dysplasia increased in the following experimental periods (weeks 6 to 8). The first histologic evidence of overt squamous cell carcinoma was noted in week 8 pouches. Then, between weeks 8 to 12, the carcinomas increased in size and were accompanied by areas of ulceration. Invasion of tumor cells into the underlying fibrous connective tissue as well as muscle tissue was noted in the final stage of the experimental period (weeks 13 to 15). These malignant cells demonstrated hyperchromatism, pleomorphism, disorientation and abnormal mitosis.

GST-P immunohistochemistry

No GST-P positivity was found in any of the pouches of the control group (Table 1). GST-P-positive cells were first noted in the pouch mucosa after 1 week of three DMBA applications (Table 1). A gradual increase in both the mean number and size of the GST-P-positive foci was observed in the first twelve experimental weeks but a plateau level was approached in the final stage of the experiment (13–15 weeks) (Fig. 2). In addition, the number of positively stained foci was elevated coincident with the increase in the severity of epithelial dysplasia (Figs. 2, 3C–3D). The early GST-P-positive staining was chiefly located in the basal layer or, occasionally, in the middle layer of DMBA-induced hamster buccal pouch mucosa (Fig. 3A). Later, the positive sites were enlarged and were scattered randomly in different layers or throughout the whole thickness of the non-dysplastic (Fig. 3B) and dysplastic (Figs. 3C, 3D) mucosa. In the non-dysplastic pouch mucosa, GST-P-positive sites were morphologically indistinguishable from the negative areas (Fig. 3B). GST-P staining was only occasionally seen to involve the keratin layer in the first 12 weeks of DMBA treatment but was more noticeable in the final stage of the experiment (13–15 weeks). Both exophytic (weeks 8–12; Fig. 3E) and invasive squamous cell carcinomas (weeks 13–15; Fig. 3F) showed GST-P positivity in cytoplasmic and nuclear locations (Figs. 3A–3D). The control stainings after replacing the immune sera with preimmune rabbit sera proved negative.

GST-P immunoblotting

No band could be detected in any of the samples from control pouches (Fig. 4). GST-P polypeptide was found to be of molecular weight approximately 26 kD in DMBA-treated pouches for the whole 15 weeks of the experimental period. In addition, relatively weaker GST-P immunoblot reactivity was obtained in pouches from hamsters treated with DMBA for less than 9 weeks, as compared to those of more than 9 weeks (Fig. 4).

Discussion

GST-P staining persisted for the whole treatment period in the present experiment, including invasive squamous cell carcinomas (weeks 13 to 15), though both the mean number and size of the positive foci approached a plateau level. This is thought to be due to a saturation effect' because nearly all of the epithelial cells in the mucosa will have responded to the carcinogenicity of DMBA. Furthermore, in contrast to the first 12 weeks of the DMBA treatment period (when GST-P staining only occasionally involved the keratin layer), GST-P positivity was noticed in keratin after a longer period of DMBA applications (>12 weeks). These positive staining areas may be associated with the fact that some GST-P-active epithelial cells have been shed towards the surface of the pouch mucosa in the later stages of oral chemical carcinogenesis. In addition, the results described in the present study also suggest that GST-P activity is enhanced in relation to the severity of epithelial dysplasia.

The initiated hamster keratinocytes are indistinguishable morphologically from one another as well as from the surrounding normal keratinocytes in H&E-stained sections. Therefore, the search for reliable markers that can detect the putative initiated cells in hamster buccal pouch mucosa is of interest. Judging from the results of the present study, GST-P is a potential candidate for such a marker, as it may serve as a means to allow recognition of the altered preneoplastic cell population. The participation of GST-P in the initiation phase has also been supported in two other immunohistochemical studies conducted respectively in the hamster pancreas (24) and rat liver (25).

However, in order to confirm the validity of GST-P as a useful tumor marker, the central question of the relationship between GST-P-positive cells and rapid focal lesion development induced by DMBA must be answered. It should be emphasized that it is not known whether all or only a certain proportion of the GST-P-positive single cells are, in fact, the sites of development into foci and conversion finally to overt epithelial neoplasms. Only a small portion of GST-P-positive hepatic cells were found to develop into neoplastic foci in rat chemical hepatocarcinogenesis (26). In an attempt to clarify whether the GST-P-stained epithelial cells in the early stage of DMBA-induced hamster buccal pouch carcinogenesis are indeed the precursors of progressive lesions, a prospective *in vitro* experiment to follow the fate of GST-P-positive foci in cul-

tured pouch keratinocytes treated with DMBA is presently under investigation in our laboratory.

Because the ultimate reactive carcinogenic form of DMBA is electrophilic, GST-P is induced to eliminate the genotoxic DMBA-adducts from the keratinocytes in hamster buccal pouch mucosa. GST-P may contribute to carcinogen inactivation in the initiation phase of oral carcinogenesis. The appearance of this detoxification enzyme, GST-P, could potentially increase the capacity of pouch keratinocytes to withstand the burden of carcinogens in the initiation phase, thus reducing the risk of cancer development. However, MacCaughan *et al.* (27) suggested that the role of GST-P in models of chemical carcinogenesis may be its permissive effect on cell cycle activity and downregulation of apoptosis, thus allowing expansion of a population of initiated cells. GST-P may alternatively confer an anticarcinogenic defense process to normal pouch keratinocytes on exposure to carcinogens and provide protection against the development of cancer. However, the same isoenzyme, GST-P, induced in the early period of oral chemical carcinogenesis may, at least partially, render the putative atypical keratinocytes more susceptible to clonal expansion (27).

The c-Ha-*ras* and c-N-*ras* oncogenes have been found to be involved early in the transformation process in rat chemical hepatocarcinogenesis (28, 29). In addition, the promoter region of the GST-P gene in rat (30, 31) and the GST π gene in human (33) contains a consensus sequence for a *ras*-responsive element. The increased epithelial GST-P expression in the early period of hamster buccal pouch carcinogenesis may therefore be the result of deregulation of the c-*ras* gene family. Further examination of the molecular changes should provide a more precise role for GST-P in relation to the initiation phase of oral chemical carcinogenesis.

Nuclear GST-P staining in the present report may indicate that GST-P is involved in DMBA-induced hamster buccal pouch carcinogenesis by, for instance, acting as the 'carrier protein' to eliminate the genotoxic substances from cytosol (33). In addition, positive nuclear staining may indicate that GST-P is accumulated in the nuclei of the pouch keratinocytes under the stimulus of DMBA during the process of chemical carcinogenesis. Nuclear staining of human placental GST isoenzyme has also been recognized in (pre)malignant lesions of human oral mucosa (10, 11) and cutaneous tissues (34, 35). Such nuclear staining has been regarded as critical in malignant transformation and linked to progression in transitional cell carcinoma of the bladder (36).

As far as we know, this report may include the first immunoblot identification of epithelial GST-P in DMBA-induced hamster buccal pouch carcinogenesis. The results obtained using immunoblot analysis are consistent with the immunohistochemical findings. Similar to rat tissues (5), GST-P polypeptide of molecular weight approximately 26 kD has been demonstrated in DMBA-treated pouches for the whole 15-week treatment regimen. The relatively weaker GST-P immunoblot staining in pouches from hamsters treated with DMBA for less than 9 weeks may be due to the reduced number and size of the GST-P foci when compared to those of the later experimental periods. In view of the immunoblot and immunohistochemical analyses, the latter surely has the advantage of permitting the ready assessment of GST-P at the cellular level, which may not be detected with immunoblotting. However, immunoblotting does remain a valuable adjunct to immunohistochemistry in the detection of GST-P isoenzymes in fresh tissues from DMBA-treated hamster buccal pouch mucosa.

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