Short Communication

Placental glutathione S-transferase isoenzyme expression during promotion of two-stage hamster cheek-pouch carcinogenesis

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Abstract

Glutathione S-transferases (GSTs) are the products of a multigene family. A well-established function of GSTs is to metabolize carcinogens by catalysing the conjugation of electrophilic substrates to glutathione. Whether placental GST (GST-P) is expressed during the promotion of two-stage hamster buccal-pouch mucosa (HBPM) carcinogenesis was investigated here, using 7,12-dimethylbenz[a]anthracene (DMBA) as the initiator and 12-O-tetradecanoylphorbol-13-acetate (TPA) as the promoter. Cytoplasmic and nuclear staining for GST-P was seen in pouches treated with DMBA for 4 or 16 weeks, as well as in those treated with DMBA for 4 weeks and then TPA for 12 weeks. No GST-P positivity was seen in any pouches treated with only TPA or with mineral oil for either 4 or 16 weeks. The average number of GST-P-stained foci in the groups treated with DMBA for 16 weeks (246 ± 96; mean $\pm$ SD) or DMBA for 4 weeks followed by TPA for 12 weeks (186 ± 67) was significantly higher than in pouches treated with only DMBA for 4 weeks (97 ± 24). These results demonstrate that TPA alone is not sufficient for GST-P expression in hamster buccal pouch mucosa. However, after being initiated with DMBA, then promoted with TPA, GST-P activity is induced in hamster buccal pouch mucosa during squamous-cell carcinogenesis. This underpins the suggestion that GST-P may play an important part during the promotion stage of oral carcinogenesis. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Glutathione S-transferase; Promotion; DMBA-carcinogenesis

Glutathione S-transferases, the products of a multi-gene family (Sato, 1989), are divided into four classes, α, μ, π (Mannervik et al., 1985) and θ (Meyer et al., 1991). A well-established function of these enzymes is their ability to metabolize carcinogens by catalysing the conjugation of potentially mutagenic electrophilic substrates to glutathione, thereby enhancing the elimination of the deleterious compounds as water-soluble molecules (Mannervik, 1985).

The hamster buccal-pouch mucosa provides is one of the most widely accepted experimental models of oral carcinogenesis (Gimenez-Conti and Slaga, 1993). In spite of anatomical and histological differences between pouch mucosa and human buccal tissue, carcinogenesis protocols induce premalignant changes and

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Table 1
The experiments and the average numbers of GST-P-stained foci per hamster. 10 hamsters are included in each group; DMBA, 7,12-dimethylbenz[a]anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate; MO, mineral oil; $^*p < 0.05$ when compared respectively with A-3 and D-1; $^{**}p > 0.05$ when compared with each other

Group[a]	Treatment protocol	Mean no. of GST-P-stained foci/hamster
A-1	DMBA[b] $\xrightarrow{\text{4 wk}}$ Killed	97±24*
A-2	DMBA $\xrightarrow{\text{4 wk}}$ No treatment $\xrightarrow{\text{12 wk}}$	108±40*
A-3	DMBA $\xrightarrow{\text{16 wk}}$	246±96**
B-1	TPA[c] $\xrightarrow{\text{4 wk}}$ Killed	0±0
B-2	TPA $\xrightarrow{\text{4 wk}}$ No treatment $\xrightarrow{\text{12 wk}}$	0±0
B-3	TPA $\xrightarrow{\text{16 wk}}$	0±0
C-1	MO[d] $\xrightarrow{\text{4 wk}}$ Killed $\xrightarrow{\text{12 wk}}$	0±0
C-2	MO $\xrightarrow{\text{4 wk}}$ No treatment $\xrightarrow{\text{12 wk}}$	0±0
C-3	MO $\xrightarrow{\text{16 wk}}$	0±0
D-1	DMBA $\xrightarrow{\text{4 wk}}$ TPA $\xrightarrow{\text{12 wk}}$	186±67**
D-2	TPA $\xrightarrow{\text{12 wk}}$ DMBA $\xrightarrow{\text{4 wk}}$	89±23*

carcinomas that are similar to the development of pre-malignancy and malignancy in human oral cancer. Hyperkeratosis, dysplasia, and exophytic and endophytic squamous-cell carcinomas are usually observed in the cheek-pouch model. Previous studies (Zhang, 1994; Chen and Lin, 1996, 1998) have shown enhanced amounts of GST-P in buccal pouches treated with DMBA, suggesting that this enzyme may be involved in the promotion stage of oral carcinogenesis. However, as DMBA is an initiator as well as a promo-
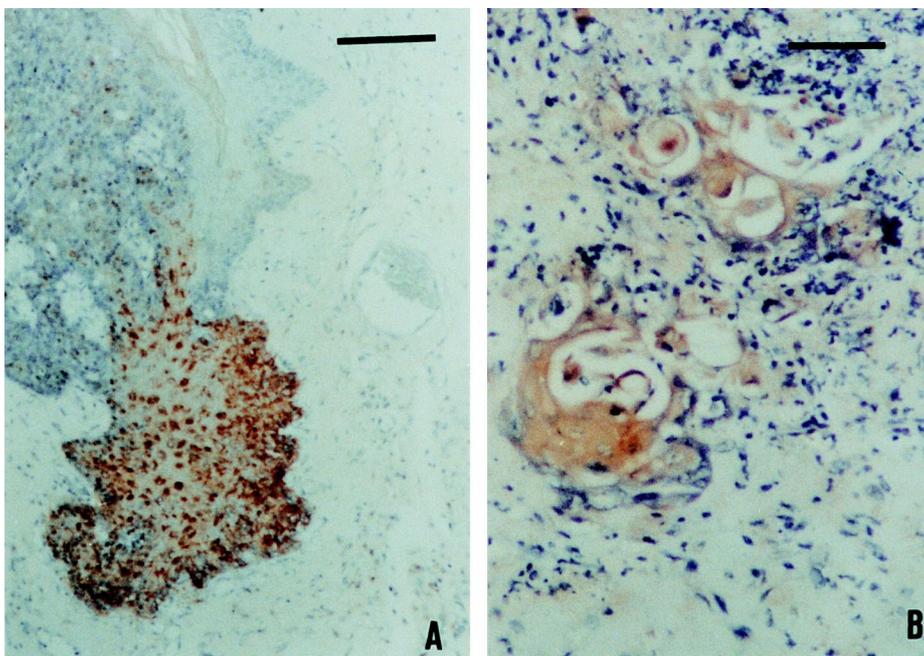
Fig. 1. (A) Representative section of pouch mucosa treated with DMBA for 4 weeks and TPA for 12 weeks. Clusters of GST-P-positive foci are seen in a region of epithelial hyperplasia. (B) Multiple GST-P-positive foci in a section of pouch mucosa treated with DMBA for 16 weeks (avidin–biotin peroxidase complex stain). (A) ×120; bar = 20 μm; (B) ×320; bar = 40 μm.

ter, the involvement of GST-P in the synergism of oral carcinogenesis is not certain. The present study was designed to test whether GST-P is expressed in the promotion stage during two-stage carcinogenesis in hamster cheek pouch, using DMBA as the initiator and TPA as the promoter (Lin et al., 1997).

The DMBA, TPA, and mineral oil (Sigma Bio Sciences, St Louis, Missouri) were of the highest purity commercially available. A 0.5% DMBA solution in mineral oil, stored at room temperature, was used (Gimenez-Conti and Slaga, 1993; Morris, 1961). TPA (40 μg/ml) was stored at 4°C until use (Lin et al., 1997).

Outbred young (6-week-old; 110 animals) male Syrian golden hamsters (*Mesocricatus auratus*) (purchased from the National Science Council Animal Breeding Centre, ROC), weighing about 100 g at the beginning of the experiment, were randomly divided into four main groups. Each main group was then further divided into two to three subgroups (Table 1). The animals were housed under constant conditions (22°C, 12-h light/dark cycle), and fed with tap water and standard Purina laboratory chow *ad libitum*.

The treatment regimen is shown in Table 1. The protocol ensured humane practices. After allowing the animals 1 week of acclimatization to the new surroundings, both pouches of each animal were painted with different combinations of DMBA, TPA and mineral oil solutions at 9 a.m. on Monday, Wednesday, and Friday of each week, using a No.4 sable-hair brush. Approximately 0.2 ml of each solution was applied topically to the medial walls of both pouches at each painting.

At the end of 16 weeks (3 days after the last treatment), all animals, with the exception of groups A-1, B-1, C-1 (killed at 4 weeks), were killed by a lethal dose of diethyl ether at the same time of the day (9 a.m.) to avoid the influence of diurnal variation. The hamsters were then fixed in a supine position with pins. Their pouches were exposed by dissection, cut from the oral opening to the caudal end along the middle of the lateral wall, and examined grossly. Both pouches were then excised and placed on cardboard to prevent distortion.

Six specimens of approximately equal length were taken from each pouch. They were fixed in 10% neutral-buffered formalin solution, dehydrated in ascending alcohols, cleared in xylene, and embedded in paraffin for light microscopy. Two serial sections of each specimen were then cut at 4-μm-thickness. One of the sections was prepared for GST-P immunohistochemistry while another was used for haematoxylin–

eosin staining. Therefore, six slides from each pouch and twelve from each animal were used for GST-P immunostaining.

Immunohistochemical staining of GST-P isoenzyme was by the avidin–biotin peroxidase complex technique (Hsu et al., 1981), as previously described (Chen and Lin, 1996, 1998). Polyclonal antibodies against rat GST-P raised in rabbits (Rushmore et al., 1988) were obtained from Biotrin International Lab., Dublin, Ireland [Cat. no. BIO23 Yp(r)]. The antisera did not cross-react with α- or μ-isoenzymes, but showed strong cross-reactivity with human and mouse π-isoenzymes (information from manufacturer). The possible patterns of GST-P positivity, randomly located in a pouch mucosa, may vary from a single cell to a cluster of a various numbers of cells without the interruption of unstained cells (Chen and Lin, 1998). The total number of GST-P-positive foci for each animal was the sum of the stained foci in the 12 representative slides obtained from both treated pouches. Negative controls were included by substituting the antiserum with preimmune rabbit serum. The significance of the difference between the average numbers of GST-P-stained foci in the various groups was assessed by student *t*-test.

The mean numbers of GST-P-positive foci in each group are shown in Table 1. The groups treated with DMBA for 4 weeks (A-1), DMBA for 4 weeks followed by no treatment (A-2) or TPA for 12 weeks followed by DMBA for 4 weeks (D-2) had similar mean numbers of GST-P positive foci per animal; these numbers were significantly lower than those in the group treated with DMBA for 16 weeks (A-3) and the group treated with DMBA for 4 weeks followed by TPA for 12 weeks (D-1) ($p < 0.05$). The mean numbers of GST-P-stained foci did not vary significantly between groups A-3 and D-1 ($p > 0.05$).

GST-P positivity in group D-1 (DMBA 4 weeks, then TPA 12 weeks) comprised clusters of stained cells distributed randomly throughout the whole layer of the mucosa with the exception of the keratin; both cytoplasmic and nuclear staining was observed (Fig. 1A). Staining in cytoplasm and nuclei was also seen in pouches of groups A-1, A-2, and D-2, and usually involved many single, positive cells located randomly throughout the mucosa. Multiple GST-P-positive foci were noted in group A-3 (DMBA 16 weeks) (Fig.1B). No GST-P positivity was seen in any pouches treated with only TPA (groups B-1, B-2 and B-3) or mineral oil (groups C-1, C-2 and C-3). The control sections were negative.

The induction of GST-P during two-stage carcinogenesis has not, to the best of our knowledge, been demonstrated previously in hamster buccal-pouch mucosa. Our results show that TPA alone is not sufficient for GST-P expression in the buccal pouch.

However, after cells have been initiated with DMBA, TPA is able to fix the DMBA-induced damage to keratinocytes, producing the proliferation of a clone of cells arising from a damaged cell. GST-P activity is then induced during the progressive squamous-cell carcinogenesis, implying a definite role for this enzyme during the promotion stage of oral carcinogenesis. Therefore, this study is an important extension of previous work (Zhang, 1994; Chen and Lin, 1996, 1998), because it further clarifies the hypothesis that GST-P chemically induced in the hamster cheek pouch is a potential marker of the progressive squamous-cell carcinogenesis. These results agree with those observed in hepatocytes cells treated with TPA; TPA activation alone was insufficient for GST-P expression in rat liver (Sakai et al., 1988). Furthermore, these data are compatible with our previous studies (Chen and Lin, 1995, 1997) of π-class glutathione S-transferase in oral premalignant and malignant lesions in humans.

The induction of GST-P by chemical carcinogens could help the pouch keratinocytes to resist the burden of carcinogen. However, the role of GST-P in models of chemical carcinogenesis may be its permissive effect on cell-cycle activity and downregulation of apoptosis, allowing proliferation of the initiated cells (Macaughan et al., 1994). Thus, GST-P may provide protection against the effect of carcinogens but the same enzyme also renders the initiated pouch keratinocytes more vulnerable to clonal expansion (Macaughan et al., 1994).

The alteration of gene expression by TPA is conceivably related to protein kinase C (Nishizuka, 1984), which might remain inactive in the absence of initiation by a carcinogen, but be activated upon initiation with DMBA. TPA promotion then proceeds as a result of a signal-transduction cascade triggered by protein kinase, and GST-P is expressed.

It is worth noting that the rat *GST-P* gene contains two TPA-responsive elements (Sakai et al., 1988; Okuda et al., 1989). The responsive element in the *GST-P* gene is homologous to the sequence that binds to activator protein 1, which is activated by TPA (Sakai et al., 1988). One of the causes of GST-P expression during hepatocarcinogenesis is the activation of a protein kinase-mediated signal pathway followed by activation of activator protein 1 in an early stage of this process (Sakai et al., 1988). By analogy, the GST-P expression in oral carcinogenesis may be similarly responsive.

In conclusion, multiple factors may be involved in GST-P expression during the promotion of oral carcinogenesis in hamsters, and further study of these factors is required to elucidate the exact mechanisms involved. Understanding the role of GST-P in this stage of carcinogenesis may be valuable in comparing the potency of various promoters (Lin et al., 1996): the

higher the GST-P activity in the promotion period, the greater the potency of the promoters.

Acknowledgements

References

Chen, Y.K., Lin, L.M., 1995. Immunohistochemical demonstration of epithelial glutathione S-transferase isoenzymes in normal, benign, premalignant and malignant human oral mucosa. J. Oral Pathol. Med 24, 316–321.

Chen, Y.K., Lin, L.M., 1996. Sequential expression of placental glutathione S-transferase (GST-P) during DMBA-induced hamster buccal pouch squamous cell carcinogenesis. J. Oral Pathol. Med 25, 388–394.

Chen, Y.K., Lin, L.M., 1997. Evaluation of glutathione S-transferase activity in human buccal epithelial dysplasias and squamous cell carcinomas. Int. J. Oral Maxillofac. Surg 27, 205–209.

Chen, Y.K., Lin, L.M., 1998. Placental glutathione S-transferase isoenzyme expression in polycyclic aromatic hydrocarbon-induced hamster buccal pouch mucosa. Eur. J. Cancer [B] Oral Oncol 34, 180–185.

Gimenez-Conti, I.B., Slaga, T.J., 1993. The hamster cheek pouch carcinogenesis model. J. Cell. Biochem 17F (Suppl.), 83–90.

Hsu, S.M., Raine, L., Fanger, H., 1981. Use of avidin–biotin–peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem 29, 577–580.

Lin, L.M., Chen, Y.K., Lai, D.R., Huang, Y.L., 1996. Minimal arecaidine concentrations showing a promotion effect during DMBA-induced hamster cheek pouch carcinogenesis. J. Oral Pathol. Med 25, 65–68.

Lin, L.M., Chen, Y.K., Lai, D.R., Huang, Y.L., Chen, H.R., 1997. The cancer-promoting effects of 12-O-tetradecanoylphorbol-13-acetate and collagenase in hamster buccal pouch carcinogenesis. Chin. Dent. J 16, 148–155.

Macaughan, F.M., Brown, A.L., Harrison, D.J., 1994. The effect of inhibition of glutathione S-transferase P on the growth of the Jurkat human T cell line. J. Pathol 172, 357–362.

Mannervik, B., Alin, P., Gutherberg, C., et al., 1985. Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. Proc. Natl. Acad. Sci. USA 82, 7202–7206.

Mannervik, B., 1985. The isoenzymes of glutathione transferase. Adv. Enzymol. Rel. Areas Mol. Bio 57, 357–417.

Meyer, D.H., Coles, B., Pemble, S.E., et al., 1991. Theta, a new class of GSH transferases purified from rat and man. Biochem. J 274, 409–414.

Morris, A.L., 1961. Factors influencing experimental carcinogenesis in the hamster cheek pouch. J. Dent. Res 40, 3–15.

Nishizuka, Y., 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature 308, 693–698.

Okuda, A., Imagawa, M., Maeda, Y.M., Sakai, M., Muramatsu, M., 1989. Structural and functional analysis of an enhancer GPE1 having a phorbol 12-O-tetradecanoate 13-acetate responsive element-like sequence found in the rat glutathione transferase P gene. J. Bio. Chem 264, 16919–16926.

Rushmore, T.H., Harris, L., Nagai, M., et al., 1988. Purification and characterization of P-52 (glutathione S-transferase-P or 7-7) from normal liver and putative preneoplastic liver nodules. Cancer Res 48, 2805–2812.

Sato, K., 1989. Glutathione transferases as markers of preneoplasia and neoplasia. Adv. Cancer Res 52, 205–255.

Sakai, M., Okuda, A., Muramatsu, M., 1988. Multiple regulatory elements and phorbol 12-tetradecanoate 13-acetate responsiveness of the rat placental glutathione transferase gene. Biochem 85, 9456–9460.

Zhang, L., 1994. The value of placental glutathione S-transferase and γ-glutamyl transpeptidase as markers of altered foci during hamster pouch carcinogenesis. Carcinogenesis 15, 105–109.