The mRNA expression of placental glutathione S-transferase isoenzyme in hamster buccal-pouch carcinomas using reverse transcription–polymerase chain reaction

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Abstract

Placental glutathione S-transferase (GST-P) may facilitate cell proliferation and inhibit apoptosis, hence allowing for the expansion of a population of initiated tumor cells. The enhanced expression of GST-P at the protein level has been reported previously in chemically induced oral carcinomas in hamster buccal-pouch mucosa but the expression of GST-P at the mRNA level has not yet been demonstrated. The purpose of the present study was to assess the GST-P mRNA expression in 7,12-dimethyl-benz[a]anthracene (DMBA)-induced hamster buccal-pouch carcinomas using a reverse transcription–polymerase chain reaction (RT–PCR). Thirty-five outbred, young (6 weeks old), male, Syrian golden hamsters (*Mesocricatus auratus*) were randomly divided into one experimental group (15 animals), and two control groups (10 animals each). Bilateral pouches of a group of 15 animals of the experimental group were painted with a 0.5% DMBA solution three times a week for 12 weeks while each animal of one of the control groups was similarly treated with mineral oil. Another control group of 10 animals was untreated throughout the experiment. Areas of dysplasia and squamous-cell carcinomas with a 100% tumor incidence developed in all of the DMBA-treated buccal pouches. The mineral oil-treated and untreated pouches revealed no obvious changes. Placental glutathione S-transferase mRNA was demonstrated to be present amongst all the 12-week DMBA-treated hamster buccal-pouch mucosa animals, but not for the untreated animals or the animals for which the buccal pouch was treated with mineral oil. Multiple potential regulatory pathways including gene amplification, enhanced mRNA stability, chromosomal translocation/gene rearrangement, and hypomethylation of the promoter region can contribute to the overexpression of GST-P mRNA in DMBA-induced hamster buccal-pouch carcinomas. Further study is necessary to completely understand which candidate mechanism(s) will contribute principally to the increased GST-P mRNA expression in oral experimental carcinogenesis. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a family of phase II detoxification enzymes that can be divided into at least five classes, α, μ, π, θ and ξ [1,2]. The main function of such GSTs is to catalyze the conjugation of glutathione (GSH) to an electrophilic site of a broad range of potentially toxic and carcinogenic compounds, thereby making such compounds less biologically active and enabling their excretion [1].

It has been proposed that π-class GST might be a useful marker for the detection of preneoplastic and neoplastic cells in humans, and an increased π-class GST expression has been found in a wide variety of human tumors [3–9]. Recently, an enhanced expression of placental GST (GST-P) has been demonstrated in both 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal-pouch carcinomas [10–15] and 4-nitroquinoline 1-oxide (NQO)-induced rat lingual carcinomas [16,17]. Furthermore, enzyme activity [18–20], immunohistochemical [16,21,22] and immunoblot analyses [19] have shown that π-class GST expression is elevated within human oral premalignant and malignant lesions compared with normal tissues or benign lesions.

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The hamster buccal-pouch mucosa provides one of the most widely accepted experimental models for oral carcinogenesis [23]. In spite of anatomical and histological differences between pouch mucosa and human buccal tissue, carcinogenesis protocols induce premalignant changes and carcinomas in pouch mucosa that are similar to the development of premalignancy and malignancy in human oral cancer. To the best of our knowledge, mRNA expression of GST-P isoenzyme has not been demonstrated in oral experimentally induced carcinogenesis. The purpose of the present study is to investigate the mRNA expression of GST-P isoenzyme in DMBA-induced hamster buccal-pouch carcinomas using a reverse transcription–polymerase chain reaction (RT–PCR).

2. Materials and methods

2.1. Hamsters and their specific treatment

Thirty-five outbred, young (6 weeks old), male, Syrian golden hamsters (*Mesocricatus auratus*; purchased from National Science Council Animal Breeding Centre, ROC), weighing about 100 g each at the beginning of the experiment, were randomly divided into one experimental group (15 animals), and two control groups (10 animals each). 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 protocol ensured humane practices as regards the handling of the test animals. Subsequent to allowing the animals 1 week of acclimatization to their new surroundings, both pouches of the experimental group of 15 animals were painted with a 0.5% DMBA solution at 09.00 a.m. on Monday, Wednesday and Friday of each week, using a No. 4 sable-hair brush. Both pouches of each animal of one of the control groups of 10 animals were similarly treated with mineral oil. Approximately 0.2 ml of the respective solution was applied topically to the medial walls of both pouches at each painting. Another control group of 10 animals was untreated throughout the experiment.

At the end of 12 weeks of such treatment (3 days following the last treatment), in order to avoid the influence of diurnal variation [24], all of the animals from each group were simultaneously killed at 09.00 a.m. using a lethal dose of diethyl ether. The animals' pouches were exposed by dissection, and cut from their oral opening to their caudal ends along the middle of their lateral walls and examined grossly. Both pouches were then excised. A portion of the pouch tissue was immediately frozen in liquid nitrogen for subsequent RNA extraction. Another portion was fixed in 10% neutral buffered formalin solution, dehydrated in ascending alcohols, cleared in xylene, and embedded in paraffin for light microscopy.

2.2. Extraction of RNA

Total RNA was extracted by homogenizing the hamster buccal-pouch tissue specimens in guanidium isothiocyanate followed by ultracentrifugation in caesium chloride, as described previously [25]. The RNA concentration was determined from the optical density at a wavelength of 260 nm (by using an OD_{260} unit equivalent to 40 μg/ml of RNA). In brief, the pouch tissue (∼250 mg) was added to 500 μl of denaturing solution (4 M guanidium thiocyanate, 25 mM sodium citrate [pH = 7.0], 0.5% sodium N-sarosine) with 50 μl of 2 M NaOAc [pH = 4.0], 500 μl of water-saturated phenol, and 100 μl of chloroform:isoamyl alcohol (1:1, v/v). The homogenized pouch tissue plus solution was then vortexed thoroughly. The RNA was subsequently precipitated with an equal volume of isopropanol. The pellet was rinsed twice with 1 ml of 75% ethanol and dried in air. The RNA was re-suspended in 100 μl of diethlpyrocarbonate (DEPC)-treated water at a final concentration of 1 μg/μl and stored at −80°C until further use.

2.3. RT reaction (cDNA synthesis)

Isolated total RNA (1 μg) was reverse-transcribed to cDNA in a reaction mixture (with a final volume of 20 μl) containing 4 μl of $MgCl_2$ (5 mM), 2 μl of 10× reverse transcription buffer (10 mM Tris–HCl, [pH = 9.0], 50 mM KCl, 0.1% Triton® X-100), 2 μl of deoxynucleotide triphosphate (dNTP) mixture (1 mM each), 0.5 μl of recombinant RNasin® ribonuclease inhibitor (1 μ/μl), 15 units of avian-myeloblastosis-virus (AMV) reverse transcriptase (High Conc.; 15 μ/μg), 0.5 μg of oligo(dT)$_{15}$ primer (Promega, catalogue No. A3500, WI, USA). The reaction mixture was incubated for 15 min at 42°C. The AMV reverse transcriptase was inactivated by heating for 5 min at 99°C and then incubated at 0–5°C for 5 min to prevent it from binding to the cDNA to form RNA–cDNA hybrids.

2.4. PCR amplification

All oligonucleotide primers were purchased from Genset Corp. (La Jolla (CA), USA). The primer pairs were chosen from the published cDNA sequences of mouse GST-P [26, GenBank accession no. X53451], and human β-actin [27, GenBank accession no. X-00351]. Primers for PCR reactions were as follows: GST-P: sense 5′-TCA TCT ACA CCA ACT ATG AG-3′ 9 (nt 328–347), and antisense 5′-GCC ACA TAG GCA GAG AGC AG-3′ (nt 534–553). These primers for GST-P encompass two introns and result in a 226-bp cDNA product, β-actin: sense 5′-AAC CGC GAG AAG ATG ACC CAG ATC ATG TTT-3′ (nt 628–657), and antisense 5′-AGC AGC CGT GGC CAT CTC TTG CTC

GAA GTC-3′ (nt 1048–1077). The expected size of the PCR product from primers of β-actin is 350-bp.

The 20 μl first-strand cDNA synthesis reaction product obtained from the reverse transcriptase (RT) reaction was diluted to 100 μl with nuclease-free water. The PCR amplification reaction mixture (with a final volume of 100 μl) contained 20 μl of diluted, first-strand cDNA reaction product ($<$10 ng/μl), 2 μl of cDNA reaction dNTPs (200 μM each), 4 μl of $MgCl_2$ (2 mM), 8 μl of 10× reverse transcription buffer (10 mM Tris–HCl, pH = 9.0, 50 mM KCl, 0.1% Triton® X-100), 50 pmol of upstream primer, 50 pmol of downstream primer, and 2.5 units of *Taq* DNA polymerase (Promega, catalogue No. M7660, WI, USA).

The PCRs were carried out on a DNA thermal cycler (TaKaRa MP, Tokyo, Japan). Thermocycling conditions included denaturing at 94 °C for 1 min (one cycle), then denaturing at 94 °C (60 s), annealing at 55 °C (60 s) for GST-P or at 60 °C (60 s) for β-actin, and extending at 72 °C (60 s) for 30 cycles and a final extension at 72 °C for 7 min. The β-actin primers were utilized as positive controls. Negative controls without RNA and without reverse transcriptase were also performed. Amplification products were analyzed by electrophoresis in a 2% agarose gel along with the DNA molecular weight marker (Boehringer, Mannheim, Germany) containing ethidium bromide. The PCR products were visualized as bands with a UV transilluminator. Photographs were taken with a Polaroid DS-300 camera. The PCR products were then sequenced to confirm their identities using T7 Sequenase version 2.0 kit (Amersham International, Little Chalfont, UK).

3. Results

3.1. Gross observations and histopathology

Two of the 15 animals of the DMBA-treated group unfortunately died during the experiment. Gross and histopathological changes in the 12-week DMBA-treated pouches were similar to those described in our previous study [12]. Areas of dysplasia and squamous cell carcinomas with a 100% tumor incidence were apparent in all of the 12-week DMBA-treated pouches. The mineral oil-treated and untreated pouches revealed no obvious changes.

3.2. RT–PCR

A band corresponding to approximately 226-bp was observed for all the hamster buccal-pouch tissue specimens treated with DMBA for 12 weeks (Fig. 1). Upon direct sequencing, the 226-bp band was confirmed to be part of the *GST*-P gene. No such bands were noticed for the untreated animals, the mineral oil-treated tissues
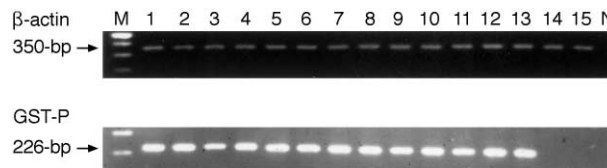

Fig. 1. A band corresponding to approximately 226-bp was observed for all the hamster buccal-pouch tissue specimens treated with DMBA for 12 weeks (lanes 1–13). No bands were noticed for untreated (lane 14), mineral oil-treated (lane 15), and the negative control (lane N) samples. All samples except the negative control sample revealed bands of β-actin (350-bp). Lane M is the DNA molecular-weight marker.

and the negative-control samples (Fig. 1). All samples except the negative-control samples revealed bands of β-actin (350-bp).

4. Discussion

Under the influences of high doses of DMBA, GST-P has been reported to have been over-expressed in hamster buccal-pouch mucosa [10–15]. The induction of GST-P may facilitate cell proliferation and inhibit apoptosis, hence allowing the clonal expansion of a population of initiated keratinocytes of pouch mucosa [28]. The expression of GST-P mRNA in chemically induced oral carcinomas has not, to the best of our knowledge, been demonstrated previously in hamster buccal-pouch mucosa. Using RT-PCR, GST-P mRNA was demonstrated to be present in the buccal-pouch mucosa from all the 12-week DMBA-treated hamsters but not so for the untreated or mineral oil-treated pouch tissues in the current study. The overexpression of mRNA of π-class GST, however, has already been found to be present in squamous cell carcinomas of human cutaneous [29], head and neck [30], and esophageal [31] tissues.

Rat GST-P has been considered to constitute one of the best marker enzymes for chemically induced hepatocellular carcinoma [32]. The same enzyme has also been indicated as a useful marker in both chemically induced hamster buccal-pouch [10–15] and rat lingual [16,17] carcinomas. Being assessed at the mRNA level in the present study, we further demonstrate that GST-P is also a valuable marker at the mRNA level in DMBA-induced hamster buccal-pouch carcinomas.

The finding of overexpression of GST-P mRNA in DMBA-induced hamster buccal-pouch carcinomas compared with the untreated and mineral oil-treated counterparts in the current study correlates well with the previous studies indicating the finding of the over-expression of GST-P at protein level under similar experimental conditions [10–15] using immunohisto-chemical and immunoblot techniques. This suggests that *GST*-P gene expression may be regulated at the

level of mRNA, although the molecular mechanisms that regulate the increased GST-P mRNA expression in DMBA-induced hamster buccal-pouch carcinomas are still not completely understood.

One of the possible mechanisms to achieve over-expression of GST-P mRNA is through gene amplification [29]. It has been claimed that gene amplification does not seem to play a key role in mRNA over-expression of GST isoenzyme because amplification was only present in two of 10 head and neck squamous-cell carcinomas manifesting GST-π mRNA overexpression [29]. Southern blot analysis, however, will have been conducted in the subsequent study. Therefore, whether or not gene amplification accounts significantly for the GST-P mRNA overexpression in DMBA-induced hamster buccal-pouch carcinomas remains to be verified.

Changes in mRNA turnover rate are recognized as a major control point in the regulation of gene expression [33,34]. Increased mRNA stability has been observed in human breast-carcinoma cell lines [33] and human bladder-carcinoma cell line [34]. Therefore, these data [33,34] suggest that the stabilization of GST-P mRNA can act as one of the mechanisms governing the mRNA overexpression. Furthermore, chromosomal translocation/gene rearrangement of *GST*-P gene may also result in mRNA overexpression [29]. Further studies incorporating digestion using restriction enzymes such as *Eco*RI or other restriction enzymes may be necessary to unequivocally identify or exclude chromosomal translocation/gene rearrangement results in GST-P mRNA overexpression in DMBA-induced hamster buccal-pouch carcinomas.

Changes to the level of CpG-site methylation appear to play a role in gene activation/suppression during carcinogenesis. In general, there is an inverse relationship between promoter CpG-site methylation and the potential for transcription [35]. Recently, hypomethylation of the GST-P promoter region in GST-P positive liver neoplasms has been reported [35], indicating that methylation of the promoter CpG site is at least one possible mechanism that could potentially lead to transcriptional activation of GST-P in hepatocarcinogenesis. Thus, by analogy, elevated GST-P mRNA expression in DMBA-induced hamster buccal-pouch carcinomas could be due to the enhanced promoter activity and increased transcription resulting from hypomethylation of hamster GST-P promoter region, perhaps as a consequence of exposure to high levels of DMBA carcinogen.

Clearly, evidence is apparent suggesting that multiple candidate mechanisms may contribute to modulating the expression of GST-P mRNA in DMBA-induced hamster buccal-pouch carcinomas. It may be a difficult issue to resolve, however, this being an important issue to address in further studies in order to fully elucidate the potential regulatory pathways that control the increased expression of GST-P mRNA in oral experimental carcinogenesis.

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