Glutathione transferases in rat hepatoma cells

Effects of ascites cells on the isoenzyme pattern in liver and induction of glutathione transferases in the tumour cells

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Rat hepatoma cells grown intraperitoneally as an ascites tumour were analysed with respect to their contents of cytosolic glutathione transferases. In contrast with normal liver tissue, the hepatoma cells were dominated by the class Pi glutathione transferase 7-7. All the major hepatic enzyme forms were down-regulated to almost undetectable concentrations. Livers of rats bearing ascites-hepatoma cells expressed low, but significant, amounts of protein which, by electrophoretic and immunochemical properties, appeared identical with transferase 7-7. This enzyme is not detectable in normal hepatocytes. Treatment of rats with *trans*-stilbene oxide induced the expression of transferase 7-7 in the livers of normal rats as well as in hepatoma-cell-bearing animals. In addition, a 2-fold induction of transferase 7-7 was measured in the hepatoma ascites cells. No significant elevation of any other enzyme forms in the hepatoma cells was noted.

INTRODUCTION

Glutathione transferases (GSTs; EC.2.5.1.18) play an important role in detoxication by catalysing the conjugation of GSH with a large number of electrophilic compounds, including some carcinogens [1]. Liver cytosol is the richest source of these enzymes in all the species studied so far. Human liver [2–7] and mouse liver [8] contain multiple forms of GST. However, rat liver, the most extensively studied organ, expresses at least 13 forms of enzyme (for a detailed review, see [9]). The cytosolic mammalian enzymes have been classified into three groups on the basis of similarities in structural and kinetic properties [10,11]. All cytosolic forms of GST are dimeric proteins composed of identical or non-identical subunits [12]. This structural feature is the basis for a systematic nomenclature based on designation of the subunits by Arabic numerals [13]. In the rat, eight subunits have been characterized and named [14,15]. Rat subunit 7, which has been identified in kidney [16], lung [17] and placenta [18], is present in liver only in trace amounts, i.e. in the bile-duct epithelium [19–21].

In contrast, GST 7-7 occurs in high concentration in chemically induced pre-neoplastic rat hepatocyte nodules [22–24] and in rat primary hepatomas [25]. This enzyme appears to be abundant also in many cancer cells and may serve as a marker for (pre)neoplastic tissues [21]. It would seem important to correlate the expression of this class Pi GST to particular stages in the development of cancer [26,27]. In the present study the composition of GST in a rat hepatoma cell line was investigated as a representative of rat tumour cells. A preliminary account of some of the present results has already been presented [28].

MATERIALS AND METHODS

Immunoblotting [29] was carried out as described by Berzins *et al.* [30]. Other materials and methods were described previously [31].

Tumour cells

Rat Zajdela hepatoma cells induced by dimethylaminoazobenzene (cf. [32]) were kindly provided by Dr. B. D. Nelson of our Department. Frozen cell suspension [stored at -80°C in 10% (v/v) glycerol] was thawed and injected intraperitoneally in a dose of 3×10^6 cells in 0.5 ml of 0.9% (w/v) NaCl into male Sprague–Dawley rats weighing 150 g. The animals were kept in a 12 h-day/12 h-night cycle and had free access to food. After 12–15 days, cells were harvested from the intraperitoneal cavity by use of syringe and, after 1:1 dilution with 0.9% (w/v) NaCl, were used for injection into new rats. A volume of 0.5 ml of the diluted suspension was sufficient to produce 3–5 g (wet weight) of cells in 30–40 ml of cell suspension in each rat after 1 week. The animals were killed by cervical dislocation. The cell suspension was taken out by a syringe with a 2 mm-thick injection needle. Afterwards, the abdominal cavity was rinsed with 10 ml of 0.9% NaCl in order that the remaining cells might be collected.

Tumour-cell cytosol

Fresh cell suspension was chilled on ice and centrifuged for 5 min at 500 rev/min. The supernatant fraction was removed by aspiration and the cell pellet was suspended in 4 vol. of ice-cold 0.9% (w/v) NaCl solution. The cells were washed repeatedly with 0.9% NaCl solution

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; DTE, dithioerythritol; f.p.l.c. fast protein liquid chromatography; GST, glutathione transferase; PAGE, polyacrylamide gel electrophoresis; tSBO, *trans*-stilbene oxide; IC_{50} , concentration causing 50% inhibition.

followed by centrifugation until the sediment was free from blood.

The pellet was then suspended (25 %, w/v) in ice-cold 50 mM-sodium phosphate, pH 7.4, containing 0.9% NaCl, 1 mM-EDTA and 0.2 mM-DTE, and sonicated for 20 s (Branson Sonifier model S125). The sonicated material was centrifuged at 20000 *g* for 30 min. The supernatant fraction was further centrifuged at 105000 *g* for 1 h in order to obtain the cytosol fraction.

Induction with *trans*-stilbene oxide (tSBO)

Rats (150 g) were injected daily with 60 mg of tSBO dissolved in 1 ml of corn oil [33] for 4 days. The control animals received the same dose of corn oil without tSBO. In animals inoculated with hepatoma cells, administration of tSBO was initiated 48 h after the injection of hepatoma cells. Cell cytosol was prepared as described above.

Purification of GSTs

Hepatoma cell cytosol (1 vol.) was run through 5–6 vol. of Sephadex G-25 (coarse grade) equilibrated with 10 mM-Tris/HCl, pH 7.8, containing 1 mM-EDTA and 0.2 mM-DTE (buffer A). GST-containing fractions were pooled and loaded on to an affinity column containing *S*-hexylglutathione linked to epoxy-activated Sepharose 6B [34] equilibrated with buffer A. The column was washed with 0.2 M-NaCl in buffer A to get rid of non-specifically bound proteins. The bound transferases were eluted with 5 mM-*S*-hexylglutathione solution in buffer A containing 0.2 M-NaCl. The affinity pool was desalted on 15–20 vol. of Sephadex G-50 equilibrated with buffer A without EDTA and subsequently concentrated to 0.5 mg of protein/ml by ultrafiltration on an Amicon PM 10 filter. The above purification steps were all carried out at 4 °C.

The concentrate was adjusted to pH 9 with triethylamine and applied on a Mono P HR 5/20 column equilibrated with 25 mM-triethylamine/HCl, pH 10.5, for chromatofocusing at 22 °C. GSTs were eluted with Pharmalyte (pH 10.5–8) diluted 1:170 (v/v) with deionized water, followed by a second elution mixture composed of 0.75 ml of Pharmalyte (pH 10.5–8) and 3.75 ml of Polybuffer 96 adjusted to pH 7.5 and diluted to 150 ml with water. The activity still bound to Mono P was eluted with 2 M-NaCl.

The isoenzyme eluted at pH 8.7 in chromatofocusing was further purified by ion-exchange chromatography on Mono S HR 5/5 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 15 mM-sodium acetate, pH 5.15. The activity was eluted with a linear concentration gradient of 0–0.1 M-NaCl in the same buffer.

Purification of GSTs from liver

Livers were thoroughly washed with 0.25 M-sucrose containing 1 mM-EDTA and 0.2 mM-DTE and homogenized in the same medium. Preparation of the cytosol fraction and all further steps of enzyme purification were performed as described previously [31].

RESULTS

Isoenzyme pattern in hepatoma cells

The cytosol fraction of rat hepatoma cells displayed a GST activity with 1-chloro-2,4-dinitrobenzene (CDNB)

of 0.25–0.3 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein⁻¹. After affinity chromatography, 85–90 % of the activity applied was recovered. Fast protein liquid chromatofocusing following the affinity chromatography resulted in elution of GST activity at pH 9.55 and 8.7. The procedures were identical with those used for isolation of GSTs from normal rat liver [31], which makes direct comparison of the chromatographic profiles possible (Fig. 1). Analysis by SDS/PAGE indicated that the first, minor, peak, obtained after chromatofocusing of the material from hepatoma cells has an apparent molecular mass of 25 kDa. All the fractions in the second, major, peak and its tail show the presence of protein subunits of two different apparent molecular masses, namely 24 and 26.5 kDa. Ouchterlony double-diffusion analyses of the separated fractions were performed with antibodies raised against rat GSTs 1-1, 2-2, 3-3, 4-4 and 7-7. The first peak (after concentration) showed precipitin lines only with antibodies against rat GST 1-1. All the fractions of the second peak gave precipitin lines with antibodies against the class Pi enzymes, rat GST 7-7 or human placental GST π [35], as well as with the class Mu enzyme rat GST 4-4. Further purification on the cation-exchanger Mono S resulted in a homogeneous protein, with an apparent subunit molecular mass of 24 kDa. This purified protein reacted only with antibodies against human GST π and rat 7-7 (GST-P) [18]. The pooled material from the Mono S column had an activity with ethacrynic acid which was 15 % of that with CDBN. The IC_{50} values of various compounds, with CDBN as electrophilic substrate, are given in Table 1. The purity and homodimeric character of this enzyme was further confirmed by plotting fractional velocity against the logarithm of the inhibitor concentration, which gave the characteristic slope of –58 at 50 % inhibition (cf. [36]). All available data indicate that the major enzyme in the rat hepatoma cells is identical with GST 7-7.

Acidic and neutral GSTs remaining bound to the Mono P column at pH 7.5 were eluted with 2 M-NaCl. This pool accounts for 4–5 % of the total activity eluted.

Hepatoma cells exposed to tSBO

The cytosol fraction of hepatoma cells grown in animals treated with tSBO had a specific GST activity with CDBN of 0.45–0.50 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein⁻¹ (Table 2). The treated animals did not show any morphological abnormalities. The protein profile after chromatofocusing on Mono P (not shown) displayed the same pattern as that for the hepatoma cells of animals not treated with tSBO. There was no significant change in the qualitative behaviour of the different peaks in further purification on Mono S, on SDS/PAGE, in Ouchterlony immunodiffusion or in inhibition studies.

Rat liver

Control animals weighing 150–165 g, after a period of 7 days, grew to a weight of 210–235 g. The weight of the livers were in the range of 10.0–11.5 g, and the cytosol fraction had a specific GST activity of 1.1 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein⁻¹. The protein profile after chromatofocusing in the pH range 10.5–7.5 accurately reproduced the pattern previously published [31].

The weight of livers from matching animals bearing hepatoma ascites cells were in the range of 7–8 g. The specific cytosolic GST activity ranged from 0.66 to

Fig 1. Elution pattern after chromatofocusing of GSTs in rat ascites hepatoma cells

Affinity-purified material from the cytosol fraction was separated on a Mono P chromatofocusing column of the f.p.l.c. system. The arrow indicates the change in elution buffer. ●, Activity determined with CDNB; △, activity measured with ethacrynic acid as second substrate; —, A_{280} ; ----, pH. The inset shows a corresponding profile for rat liver cytosol (symbols mean the same as in the main Figure).

Table 1. Characteristics of cytosolic GSTs in rat hepatoma cells

The properties of the GSTs from hepatoma ascites cells are compared with those of corresponding rat GSTs previously characterized.

Property	Transferase...	I*	I-1†	II‡	7-7†
Subunit molecular mass (kDa)		25	25	24	24
pH of elution (chromatofocusing)		9.55	> 9	8.7	8.7
Reactions with antibodies against rat GST:					
1-1		+	+	—	—
2-2		—	—	—	—
3-3		—	—	—	—
4-4		—	—	—	—
7-7		—	—	+	+
Specific activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) with:					
CDNB		55	50	21	24
Ethacrynic acid		0.05	0.08	3.2	3.8
Cumene hydroperoxide		3.5	3.1	0.08	0.05
IC_{50} (μM)					
Cibacron Blue		0.5	0.6	0.3	0.3
Haematin		0.03	0.05	4	4
Triphenyltin chloride		0.5	0.5	10	10
<i>S</i> -(<i>p</i> -Bromobenzyl)glutathione		50	50	1	1

* The enzyme contained in the first peak obtained by chromatofocusing (Fig. 1).

† Data from [16].

‡ The enzyme contained in the major component of second peak obtained by chromatofocusing (Fig. 1), further purified by ion-exchange chromatography on a Mono S f.p.l.c. column.

Table 2. Comparison of cytosolic GST activities in rat liver and hepatoma ascites cells

Source	Specific activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)*
Hepatoma cells	0.25
Hepatoma cells, induced†	0.5
Liver, normal	1.1
Liver, induced†	3.2
Liver, animal with hepatoma ascites cells	0.7
Liver, animal with hepatoma ascites cells, induced†	2.0

* Activity measured with CDNB is expressed per mg of cytosolic protein.

† 'Induced' refers to material obtained from rats treated with tSBO (cf. the Materials and methods section).

Fig 2. Analysis of immunoreactive class Pi GST polypeptide in the cytosols of ascites hepatoma cells and rat liver

Equal amounts of protein of each cytosol fraction were subjected to SDS/PAGE. The proteins were transferred on to nitrocellulose paper by the method described in [30]. Antiserum against human GST π (which cross-reacts with rat GST 7-7) was used to detect GST 7-7. Immuno-complexes were radiolabelled with ^{125}I and revealed by autoradiography. Lane 1, ascites cells; lane 2, ascites cells exposed to tSBO; lane 3, control liver; lane 4, liver from animals treated with tSBO; lane 5, liver from ascites-cell-bearing animals; lane 6, liver from ascites-cell-bearing animals treated with tSBO.

$0.8 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. The activity profile obtained by chromatofocusing of the affinity-purified GSTs was similar to that obtained for control animals (cf. inset to Fig. 1), with the exception that the fourth peak, which corresponds to GST 3-3, was approx. 2-fold lower in comparison with the other peaks in the livers of the rats carrying hepatoma cells (results not shown). Further, the fifth peak contained a protein with subunits of apparent molecular mass 24 kDa in addition to its normal constituent, GST 3-4. This protein, which was identified as GST 7-7 (Figs. 2 and 3), was not evident in livers of untreated rats [31]. The different fractions of both the fifth and the sixth peaks in the protein contained GST 7-7, as evidenced by positive reactions with corresponding antibodies. A detailed study of this isoenzyme was not made, since the considerably higher concentrations of GSTs 3-4 and 3-? [31] in peaks V and VI made the isolation of this minor component difficult.

Fig 3. Analyses by SDS/PAGE of different GST-containing fractions

Purified fractions were separated by SDS/PAGE [37]. The protein bands were stained with Coomassie Brilliant Blue G. Lane 1, GST 3-4 fraction containing subunit of low molecular mass purified from livers of ascites-hepatoma-cell-bearing animals by chromatofocusing; lane 2, purified GST 1-2 (reference); lane 3, purified GSTs from ascites hepatoma cells; major peak eluted from Mono P after further purification on Mono S; lane 4, affinity-purified material from ascites hepatoma cells; lane 5, affinity-purified material from liver of ascites-hepatoma-cell-bearing animals treated with tSBO; lane 6, affinity-purified material from normal liver.

Livers from animals with hepatoma cells showed a 2–3-fold increase in cytosolic GST activity after induction with tSBO. Analysis by chromatofocusing demonstrated that subunits 1 and 3 were present at higher levels (results not shown). Qualitatively there is no significant difference from the isoenzyme pattern of normal liver, except for the appearance of GST 7-7 (cf. Fig. 3, lanes 5 and 6). In a separate experiment, the effect of tSBO on normal liver (in rats not bearing hepatoma cells) was examined. A similar increase of subunits 1 and 3 in addition to an elevated concentration of subunit 7 was noted (see Fig. 2). A comparison of the cytosolic specific activities of the various preparations is made in Table 2.

DISCUSSION

GSTs in hepatoma cells

Affinity chromatography of the cytosol from hepatoma cells results in the recovery of 85–90 % of GST activity as compared with 75 % in liver, indicating that none of the major enzyme forms normally found in hepatocytes were lost in the purification procedure. In the hepatoma cells subunit 2 could be detected neither by chromatofocusing nor by SDS/PAGE or Ouchterlony immunodiffusion experiments. However, when the affinity pool was subjected to SDS/PAGE followed by immunoblotting, trace amounts of subunit 2 were indicated (results not shown).

Chromatofocusing in the pH range 9–7.5 results in the separation of GSTs 3-3, 3-4, 3-? and 4-4 in normal liver [31]. The corresponding chromatography of the hepa-

toma cytosol fraction gave rise only to one major peak, which was eluted at pH 8.7. The appearance of a very small peak in the hepatoma protein profile at the position of GST 3-3 in normal liver chromatograms suggests the presence of subunit 3. The protein eluted at pH 8.7 accounted for 85–90% of the total activity recovered from the Mono P column. The results of the present investigation (cf. Table 1) indicate strongly that this enzyme is identical with GST 7-7.

Induction with tSBO resulted in a 100% increase in the cytosolic specific GST activity in the hepatoma cells (Table 2). Comparison of protein profiles after chromatofocusing indicates that this is fully accounted for by an elevation of the concentration of GST 7-7. Immunoblot analysis confirmed the increase in the concentration of subunit 7 (see Fig. 3, lanes 1 and 2).

GSTs in livers from tumour-cell-bearing animals

The livers of rats bearing hepatoma ascites cells showed decreased cytosolic specific GST activities, which in part might be explained by the decreased amount of GST 3-3 (cf. Table 2). Whether this low level is due to undernourishment of the liver or a result of some direct physiological link is not clear. Livers from ascites-cell-bearing rats also contain a small amount of an isoenzyme composed of subunit 7 (or a very similar subunit) in the 3-4 and 3-? fractions (Fig. 4, lane 1).

After treatment with tSBO the livers of normal rats and animals bearing hepatoma cells show increased amounts of subunits 1, 3 and 7. The induction by tSBO of GST 7-7 in normal livers was previously unnoticed because of differences in the procedures for separation and identification of the various enzyme forms [38,39]. In normal rats, the amounts of hepatic GST 7-7 after tSBO treatment is much higher than the amounts in livers from rats bearing hepatoma cells in the absence of tSBO treatment (Fig. 3, lanes 4 and 5).

Regulation of GST expression

It is well known that GSTs are inducible and that the effect is specific for certain subunits (cf. [9]). The present study shows a dramatic alteration in the isoenzyme pattern in hepatoma cells as compared with that of the liver tissue. GST 7-7 is the single dominant form in the tumour cells, whereas it is not detectable in the normal hepatocyte. In pre-neoplastic nodules, GST 7-7 is also expressed at a high level [22–24], but several additional enzyme forms are also present. Other GSTs also occur in high concentration in solid hepatoma tumour tissue [25] in contrast with the results obtained with ascites cells in the present work. In the hepatoma ascites cells, all the normal major hepatic GSTs are down-regulated to an almost undetectable level. Even after induction with tSBO, all basic enzyme forms except GST 7-7 remained at a low level.

An important additional finding in the present investigation was that GST 7-7 is induced to a small, but significant, level in the livers of rats bearing ascites hepatoma cells (cf. Fig. 4, lane 1). This result suggests that a humoral factor is transmitting an effect from the tumour cells in the peritoneal cavity to the liver. Contamination of the liver with ascites cells was excluded by careful dissection and rinsing of the liver.

Class Pi GSTs, such as GST 7-7 in the rat, have been found to be present at high levels in tumours also in mouse [40], hamster [41] and man [7,42–46]. The signi-

ficance of this expression in neoplastic tissues and its relationship to the stepwise development of cancer [26,27] is not known. However, it may be significant that the rat transferase 7-7 gene [47] contains a base sequence, TGACTCAG, approx. 30 nucleotides upstream of the TATA box in the 5' non-coding region, which is similar to, or identical with, *cis* elements recognized by a phorbol-ester-modulated *trans*-acting protein AP-1 [48]. Binding of AP-1 to this sequence appears to stimulate transcription of genes for collagenase, stromelysin and metallothionein II_A, which, directly or indirectly, may be linked to tumour promotion.

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