Selective expression of glutathione transferase isoenzymes in chemically induced preneoplastic rat heptocyte nodules

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Isoenzymes of glutathione transferase were shown to occur at selectively altered levels in rat hepatocyte nodules produced by 2-acetylaminofluorene treatment. Changes were measured by different substrates, antibodies raised against purified glutathione transferases, and by purification of the major isoenzymes. Isoenzymes composed of subunits 1, 2 and 3, expressed in normal liver tissue, all occurred at increased concentrations in nodules, whereas the level of transferase 4-4 was decreased. The most conspicuous change was the appearance of glutathione transferase 7-7 (or transferase P), the concentration of which in negligible in normal liver.

Glutathione transferase *Hepatocyte nodule* *Isoenzyme induction* *Preneoplastic marker* *Immunoprecipitation*

1. INTRODUCTION

The development of hepatocyte nodules is a model widely used to study carcinogenesis in liver. Several methods are available to induce formation of such nodules and all the resulting model systems show similar biochemical characteristics (review [1]). The hepatocyte nodules are known origins of liver cancer. Farber has recently suggested that these alterations of the liver tissue arise as a physiological response to environmental perturbation [1]. Furthermore, it was proposed that they have survival value because they afford increased resistance by more effective excretion and detoxication of certain carcinogens. The hepatocyte nodules are also less susceptible to the cytotoxic effects of hepatotoxins, such as aflatoxin B_1, 2-acetylaminofluorene and diethylnitrosamine, and they are able to grow under conditions that severely inhibit cell proliferation of the surrounding tissue. In order to understand the biological significance of the chemically-induced hepatocyte nodules, it is of great importance to elucidate the pattern of the enzymes involved in the metabolism of carcinogens and other cytotoxic compounds.

Glutathione transferases (EC 2.5.1.18) catalyze conjugation of electrophilic compounds, including carcinogens and mutagens, with glutathione [2]. They also display binding capacity for different classes of hydrophobic substances [3]. The isoenzyme pattern of glutathione transferase has been most extensively studied in rat liver but the enzymes are widely distributed in animal tissues [4]. The nomenclature of the dimeric rat isoenzymes has recently been revised and is now based on their respective subunit composition [5]. Here we describe the purification, identification, and quantitation of glutathione transferase isoenzymes in hepatocyte nodules, in which the concentrations of these enzymes are known to be elevated [6–8]. In particular, the nature of the polypeptide of apparent M_r 21000 that is characteristic of the preneoplastic tissue [9] has been investigated.

2. EXPERIMENTAL

2.1. *Materials*

Male Wistar rats weighing about 100–130 g

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were used (Möllegaard Breeding Centre, Ejby, Denmark). The rats were kept under a 12 h light and dark cycle at constant temperature and humidity. Nodules were prepared by intermittent feeding of 2-acetylaminofluorene according to a protocol described by Epstein et al. [10] and modified for Wistar rats by Eriksson et al. [11]. Nodules were harvested between 25 and 34 weeks after the start of the regimen, and at least 2 weeks after release from the last period of 2-acetylaminofluorene feeding. Before harvesting the rats were starved for 48 h to reduce intracellular glycogen content. Rats were anesthetized with hexobarbital and livers were perfused with ice-cold 0.25 M sucrose before the nodules were scooped out. The cytosolic glutathione transferase isoenzymes were isolated as described [12] using an *S*-hexylglutathione–Sepharose 6B affinity matrix and chromatofocusing on a Mono P column of the FPLC system (Pharmacia, Uppsala). Anti-transferase 5-5 antibodies were a generous gift from W.B. Jakoby, National Institutes of Health, Bethesda, MD; all other antibodies were available in the laboratory.

2.2. *Methods*

Enzyme activities were determined spectrophotometrically at 30°C. The assay conditions were as cited for 1,2-dichloro-4-nitrobenzene (DCNB) [13], 1-chloro-2,4-dinitrobenzene (CDNB), *trans*-4-phenyl-3-buten-2-one (t-PBO), ethacrynic acid, and bromosulfophthalein (BSP) [14].

Protein concentration was determined by the method of Lowry et al. [15] after trichloroacetic acid precipitation. SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli [16], and single radial immunodiffusion by the method of Mancini et al. [17].

3. RESULTS

3.1. *Determination of glutathione transferase levels in the cytosol fraction*

Cytosolic fractions of liver nodules from 4 different preparations were found to have a glutathione transferase activity of 4.2–6.9 μmol/min per mg protein with CDNB as the electrophilic substrate (table 1). These values are 2.5–4.0-times higher than those in the liver cytosol of untreated animals. The induction of activity with ethacrynic acid and BSP was very similar in magnitude to that measured with CDNB, whereas the activity with t-PBO was increased maximally 2-fold.

Table 1

Comparison of specific glutathione transferase activities with different substrates in the cytosol fraction of normal rat liver and hepatocyte nodules

Preparation	Specific activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)			
	CDNB	BSP	t-PBO	Ethacrynic acid
Normal liver	1.7	0.014	0.010	0.042
Nodules I[ab]	5.5	0.066	0.018	0.19
Nodules II	4.2	0.050	0.017	0.19
Nodules III[b]	5.2	0.048	0.021	0.22
Nodules IV	6.9	0.078	0.021	0.21

[a] Preparation I contained pooled material from several rats. The remaining preparations were from individual animals

[b] The preparation did not give a peak corresponding to glutathione transferase 4-4 upon chromatofocusing (cf. fig.1)

Table 2

Quantitation of glutathione transferase isoenzymes in the cytosol fraction of normal rat liver and hepatocyte nodules by immunoprecipitation

Preparation	Relative cytosolic content of proteins reacting with antibodies directed to transferase				
	Rat 1-1	Rat 2-2	Rat 3-3	Rat 4-4	Human π
Normal liver	(1)	(1)	(1)	(1)	n.d.
Nodules I	2.3	2.6	3.4	0.6	1.5
Nodules II	3.0	2.9	2.7	0.6	1.0
Nodules III	2.8	3.2	3.0	0.6	1.1
Nodules IV	4.6	3.3	3.4	0.8	1.1

The values for nodules are expressed relative to those in normal liver, except for those obtained with antibodies against human transferase π. The latter values are estimated as per cent of total cytosolic protein. The values for normal liver of proteins reacting with the anti-rat antibodies each corresponds to approx. 2% of the cytosolic protein; n.d., not detectable

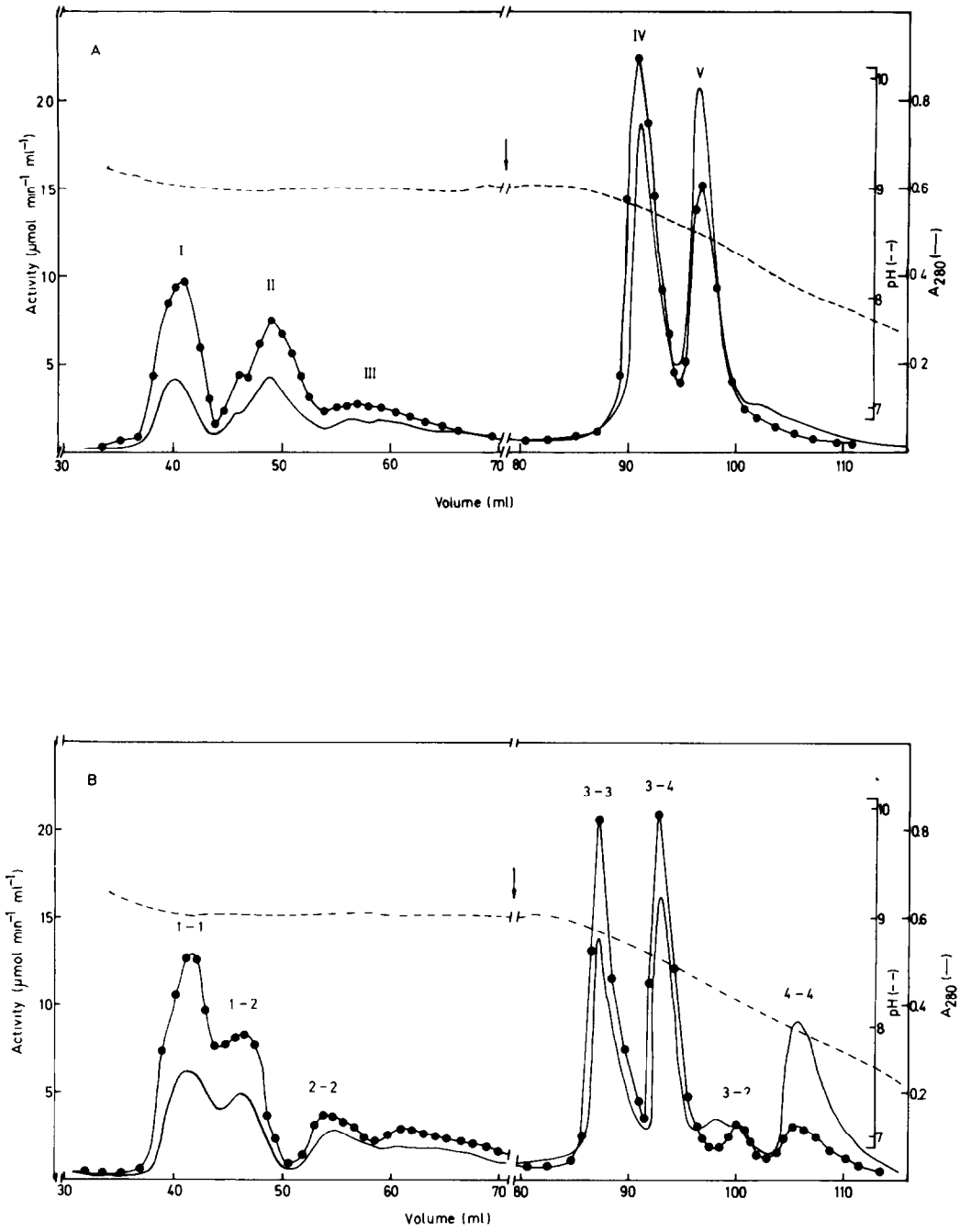

Fig.1. Separation of glutathione transferase isoenzymes by chromatofocusing. The samples analyzed contained glutathione transferases isolated by affinity chromatography of the cytosol fractions of hepatocyte nodules (A) and normal rat liver tissue (B). The preparations correspond to the first two lines in tables 1 and 2. Enzyme activity (●) was measured with CDNB as electrophilic substrate; A_{280} (——) and pH (– – –) were monitored continuously during elution [12].

Quantitative immunoprecipitation [17] demonstrated that the specific content of glutathione transferase isoenzymes precipitable with antibodies to rat glutathione transferases 1-1, 2-2 and 3-3 increased 2.3–4.6-fold, whereas the value for transferase 4-4 decreased (table 2). It was recently found in our laboratory (C. Guthenberg et al., unpublished) that antibodies raised against human glutathione transferase π from placenta cross-react with rat glutathione transferase 7-7 isolated from rat kidney or lung [18,19]. By using these antibodies, significant levels of immunoprecipitable material in the nodule cytosol fraction were demonstrated, whereas normal liver did not contain detectable amounts.

3.2. *Isolation of basic glutathione transferase isoenzymes*

Hepatocyte nodules (6 g) were homogenized in 0.25 M ice-cold sucrose (20% w/v). All the steps up to chromatofocusing were performed at 4°C. The homogenate was centrifuged at 105000 × *g* for 2 h and the supernatant fraction (18 ml) was collected. The pooled material was passed over a Sephadex G-25 column (4 × 20 cm), equilibrated with 10 mM Tris-HCl, 0.2 mM dithiothreitol (DTE), pH 7.8 (buffer A). The resulting active fraction (55 ml) was applied onto an *S*-hexylglutathione-Sepharose 6B column (2 × 17 cm), packed in buffer A. The affinity matrix was washed with about 5 bed vols of buffer A fortified with 0.2 M NaCl. The adsorbed glutathione transferases were eluted with 5 mM *S*-hexylglutathione in the salt-fortified buffer A (100 ml). After desalting on Sephadex G-25 packed in buffer A, the sample was concentrated by ultrafiltration to 14 ml. Four ml were used for chromatofocusing, performed as described by Ålin et al. [12].

The separation of the isoenzymes from hepatocyte nodules by use of chromatofocusing in the range between pH 10.6 and 7.6 gave 4 distinct and one broad peak of activity with CDNB as substrate (fig.1A). The peaks were designated I–V in the order of elution as the pH gradient decreased. For comparison, the isoenzymes of normal liver cytosol from male Wistar rats were separated under identical conditions (fig.1B). Peaks I, II, III and IV were identified with glutathione transferases 1-1, 1-2, 2-2, and 3-3, respectively, by their position in the elution profile, apparent subunit M_r, and reactivity with specific antibodies [12]. The specific activity of the peak fractions also supported the identification. Peak V contained two polypeptides with apparent subunit M_r 26500 and 24000, respectively, occurring in unequal amounts. The pooled material from this peak was applied to a cation exchange column (Mono S of the FPLC system) equilibrated with 5 mM sodium phosphate buffer, 0.2 mM DTE, pH 5.8, and eluted with a linear gradient of 0–0.1 M NaCl in the phosphate buffer. This separation gave as the major component a homogeneous isoenzyme having an apparent subunit M_r of 24000. This value is based on revised M_r values for the glutathione transferases [5] and corresponds to the value of 21000 earlier reported as characteristic for a polypeptide in nodules [9]. This M_r value is lower than those of the known basic glutathione transferases in normal liver cytosol. The non-typical hepatic isoenzyme lacked reactivity with antibodies raised against rat glutathione transferases 1-1, 2-2, 3-3, 4-4, and 5-5 in Ouchterlony double diffusion analysis. When tested with antibodies against the human glutathione transferases α-ϵ, μ and π, a positive precipitin reaction was obtained only with anti-transferase π antibodies.

4. DISCUSSION

The cytosolic glutathione transferase activity with CDNB in hepatocyte nodules is considerably higher (2.5–4-times) than in normal liver [6–8], and the same is true for the activities measured with BSP and ethacrynic acid (table 1). The increased activity with BSP may be ascribed specifically to an increased concentration of glutathione transferase subunit 3, since this is the only known subunit significantly active with this substrate [4,12,20]. The 2-fold elevated activity with t-PBO would similarly suggest an increased concentration of subunit 4 [4,12,20], but this interpretation is contradicted by a near 2-fold decrease in protein immunoprecipitable with anti-transferase 4-4 antibodies (table 2). The remaining explanation appears to be that an as yet unidentified isoenzymes is responsible for the increased activity with t-PBO. The activities with CDNB and ethacrynic acid are less discriminatory among

subunits, but it may be stated that none of the glutathione transferases of normal rat liver has a sufficiently high specific activity with ethacrynic acid [4,12,20] to explain the 5-fold increase in cytosolic activity (table 1). Evidently, the latter activity to a significant extent has to be ascribed to glutathione transferase 7-7 [18,19], demonstrated by its cross-reaction with antibodies to human transferase π (table 2).

The 2.3–4.6-fold increase in the levels of proteins reacting with antibodies to transferases 1-1, 2-2, and 3-3 (table 2) is in good agreement with the 3–5-fold increase of mRNAs for the corresponding protein subunits [21].

In the affinity chromatography, only 2% of the CDNB activity passed unretained through the gel, which is less than found for normal liver (3–5%). It is known that transferase 5-5 is not adsorbed to the affinity gel [22]. The present findings suggest that the level of transferase 5-5 is decreased in hepatocyte nodules. In fact, no precipitin reaction with anti-transferase 5-5 antibodies could be detected in the Ouchterlony double diffusion analysis of different nodule preparations.

Four of the components separated by chromatofocusing (fig.1) were identified with transferase isoenzymes 1-1, 1-2, 2-2, and 3-3, respectively. Peak V contained an isoenzyme not detected in normal liver cytosol. This isoenzyme was coeluted with transferase 3-4 in the chromatofocusing, but the two components can subsequently be separated by ion-exchange chromatography. The most characteristic properties of this isoenzyme are low apparent subunit M_r, relatively high activity with ethacrynic acid, and a positive precipitin reaction with antibodies raised against human placental transferase π. An isoenzyme which by all available criteria appears identical with the non-typical hepatic glutathione transferase in the nodules has been purified from lung, kidney and placenta [18,19,23]. This enzyme has been named rat glutathione transferase 7-7 [18] or transferase P [24]. Sato et al. have reported that the rat placental glutathione transferase has immunochemical identity with an isoenzyme present in nodules [8,23,24].

By comparing different nodule preparations we found that some do not contain a component corresponding to transferase 4-4 (cf. fig.1) although all nodules are produced by the same method. To some extent the subunits of transferase 4-4 might occur in the hybrid form transferase 3-4, owing to the increased levels of subunit 3. However, the quantitative immunoprecipitation (table 2) shows that the total level of subunit 4 does decrease. In preparations expressing transferase 4-4, the amount of isoenzyme 7-7 is significantly lower than in those that lack transferase 4-4. Whether this inverse relationship between transferases 4-4 and 7-7 reflects a particular stage in the evolution of the nodules is not known at present. It has been reported that all known basic transferase isoenzymes are decreased in poorly differentiated rat hepatoma cells [8], and it may be that transferase 4-4 is the most sensitive isoenzyme in the liver.

In conclusion, rat glutathione transferase 7-7 corresponds to the characteristic polypeptide [9] and to transferase P [8,23,24] earlier characterized as a good marker for early stages of cancer development in rat liver.

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REFERENCES

[1] Farber, E. (1984) Biochim. Biophys. Acta 738, 171–180.
[2] Chasseaud, L.F. (1979) Adv. Cancer Res. 29, 175–274.
[3] Smith, G.J. and Litwack, G. (1980) Rev. Biochem. Toxicol. 2, 1–47.
[4] Mannervik, B. (1985) Adv. Enzymol. 57, 357–417.
[5] Jakoby, W.B., Ketterer, B. and Mannervik, B. (1984) Biochem. Pharmacol. 33, 2539–2540.
[6] Kitahara, A., Satoh, K. and Sato, K. (1983) Biochem. Biophys. Res. Commun. 112, 20–28.
[7] Åström, A., DePierre, J.W. and Eriksson, L.C. (1983) Carcinogenesis 4, 577–581.
[8] Kitahara, A., Satoh, K., Nishimura, K., Ishikawa, T., Ruike, K., Sato, K., Tsuda, H. and Ito, N. (1984) Cancer Res. 44, 2698–2703.

[9] Eriksson, L.C., Sharma, R.N., Roomi, M.W., Ho, R.K., Farber, E. and Murray, R.K. (1983) Biochem. Biophys. Res. Commun. 117, 740–745.
[10] Epstein, S., Ito, N., Merkow, L. and Farber, E. (1967) Cancer Res. 27, 1702–1711.
[11] Eriksson, L.C., Torndal, U.-B. and Andersson, G. (1983) Cancer Res. 43, 3335–3347.
[12] Ålin, P., Jensson, H., Guthenberg, C., Danielson, U.H., Tahir, M.K. and Mannervik, B. (1985) Anal. Biochem. 146, 313–320.
[13] Booth, J., Boyland, E. and Sims, P. (1961) Biochem. J. 79, 516–524.
[14] Habig, W.H., Pabst, M.J. and Jakoby, W.B. (1974) J. Biol. Chem. 249, 7130–7139.
[15] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Mancini, G., Carbonara, A.O. and Heremans, J.F. (1965) Immunochemistry 2, 235–254.
[18] Guthenberg, C., Jensson, H., Nyström, L., Österlund, E., Tahir, M.K. and Mannervik, B. (1985) Biochem. J., in press.
[19] Robertson, I.G.C., Jensson, H., Guthenberg, C., Tahir, M.K., Jernström, B. and Mannervik, B. (1985) Biochem. Biophys. Res. Commun. 127, 80–86.
[20] Mannervik, B. and Jensson, H. (1982) J. Biol. Chem. 257, 9909–9912.
[21] Pickett, C.B., Williams, J.B., Lu, A.Y.H. and Cameron, R.G. (1984) Proc. Natl. Acad. Sci. USA 81, 5091–5095.
[22] Meyer, D.J., Christodoulides, L.G., Tan, K.H. and Ketterer, B. (1984) FEBS Lett. 173, 327–330.
[23] Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. and Sato, K. (1985) Proc. Natl. Acad. Sci. USA, in press.
[24] Sato, K., Kitahara, A., Satoh, K., Ishikawa, T., Tatematsu, M. and Ito, N. (1984) Gann 75, 199–202.