Anomalous elevation of glutathione S-transferase P-form (GST-P) in the elementary process of epigenetic initiation of chemical hepatocarcinogenesis in rats

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The molecular mechanism of the specific expression of glutathione S-transferase P-form (GST-P) in the rat hepatic preneoplastic foci and ‘GST-P-positive’ single cells requires elucidation. Immunochemical and stereological analyses revealed that the enzyme level in preneoplastic foci was 150–250-fold (6.7 ± 2.4 mg/g liver and 0.29 ± 0.1 mM subunits) higher than in normal cells. GST-P content in the single cells was higher than in preneoplastic foci, as determined by densitometry. In addition, the single cells were larger in cell diameter and area, corresponding to 2–3-fold increase in cell volume, relative to normal cells, but showed a significant shrinkage of their nuclei. Prior to the induction of single cells in the liver by diethylnitrosamine (DEN), microsomes were severely damaged as reflected by the low yield (~60% that of untreated controls) after 2 h of DEN injection. Considering that GST-P is mainly a binding protein for GSH conjugates of endogenous carcinogens, together with our findings of morphological expansion, low viability of single cells and microsomal damage, our results suggest anomalous elevation of the ligand counterparts to lethal levels in preneoplastic cells, especially in single cells. We propose that the epigenetic mechanism rather than the genetic mechanism could account for GST-P induction in hepatocytes.

Introduction

The molecular and cellular processes of initiation of chemical hepatocarcinogenesis of experimental animals require elucidation (1–3). Isozymic alteration of phase II detoxification enzymes, such as epoxide hydrolase, glutathione S-transferase (GST) and UDP-glucuronosyltransferase, is a biochemical feature of preneoplasia and neoplasia (4–6). Although several aspects of these changes remain to be characterized, these markers may be useful for elucidating the mechanism of initiation. Of the preneoplastic marker enzymes, rat GST P-form (GST-P) is markedly and specifically inducible in preneoplastic foci (6–8). Therefore, this enzyme has been used in both the basic analysis of carcinogenesis in experimental animals and in screening for carcinogens and carcinogenic modifiers (8,9).

Of particular interest, single cells heavily positive for GST-P were detected in the rat liver as early as 2–3 days after i.p. administration of diethylnitrosamine (DEN) (10,11). Similar cell populations were detected in mouse and hamster livers and their preneoplastic characteristics were identified in Nrf2 transactivator gene knockout mice (12–14). However, the physiological significance of the specific expression of GST-P in preneoplastic cell populations, why it is inducible by so many carcinogens, whether or not the GST-P-positive single cell populations are precursors of preneoplastic foci and what is the genetic change(s), if any, are matters of great interest. GST-P belongs to the pi class of GST, a family of multifunctional enzymes that catalyze GSH conjugation of a wide variety of exogenous and endogenous compounds, as well as acting as a binding protein of organic anions and carcinogens (15,16). Despite extensive studies, the substrate specificity of GST-P remains unclear as it shows low and broad enzymatic activities against various compounds except for some (16–18). As reported earlier, however, the physical organic chemistry approach indicated that GST-P acts mainly as a binding protein of GSH conjugates of endogenous carcinogens such as acrolein and 4-hydroxyalkenals (18).

In order to address these problems, we reassessed in the present study the GST-P protein in hepatic preneoplastic foci using the stereological analysis of Campbell et al. (19). Our results showed anomalously high GST-P contents in preneoplastic foci and especially in single cells. The findings, together with other previous data (11,18), strongly suggest the accumulation of endogenous carcinogens up to lethal levels in small cell populations, giving rise to a ‘state of emergency’. Based on the critical event in hepatocytes prior to initiation together with various other data including those of Grass-Kraupp et al. (20), we postulate a specific induction of GST-P in preneoplastic cells is an epigenetic mechanism rather than a genetic one. Although the genetic initiation mechanism has been generally accepted (1–3,21), Bannasch et al. (22,23) theoretically discussed epigenetic events as a plausible cause of the evolution of preneoplastic hepatocarcinogenesis, and Prehn (24) raised a question on whether gene mutations are ‘cause or effect’ in malignant neoplasia.

Materials and methods

Chemicals
DEN was purchased from Wako Pure Chemical Industries (Osaka, Japan). Biotin-labeled goat anti-rabbit immunoglobulin IgG and avidin-biotin-peroxidase complex (Vectorstain ABC kit, PK 4001) were obtained from Vector Laboratories (Burlingame, CA). Enzyme substrates and other chemicals were obtained from commercial sources and were of high reagent grade.

Animals
Male Sprague–Dawley rats, aged 5 weeks and weighing 130–150 g, were obtained from CLEA, Japan (Tokyo, Japan) and maintained in the Institute for Animal Experiments of Hirosaki University. All animal experiments were conducted according to the Guidelines for Animal Experimentation of Hirosaki University.

Preparation of livers of rats bearing GST-P-positive cell populations
Single cells and mini-foci positive for GST-P were induced in the rat liver using the method described previously by Solt-Farber (25,26). In brief, a bolus dose of 200 mg/kg DEN was injected i.p. into male Sprague–Dawley rats. Single cells and preneoplastic foci were examined 5 days later (10,11).

Abbreviations: DEN, diethylnitrosamine; GST-P, glutathione S-transferase P-form; single cells, GST-P-positive single hepatocytes; NNM, N-nitrosomorpholine.
Preneoplastic foci were examined 5 weeks after administration of DEN (Figure 1).

**Immunohistochemical staining**

Liver sections (6 µm thick) were stained immunohistochemically with polyclonal antibody against rat GST-P using the avidin-biotin-peroxidase complex method as described previously (7). The area of GST-P-positive preneoplastic foci was measured with NIH image analysis software v.1.61, and the volume of these foci was estimated according to the stereological analysis of Campbell et al. (19). The correction factor for distortion of paraffin sections on extension to glass slides at 50°C was 0.90 ± 0.03 (mean ± SD). Measurement of hepatocyte size

Single cells and preneoplastic foci were stained under the same conditions with GST-P antibody. Stained GST-P-positive single cells were photographed under 100-fold magnification, followed by scanning the colored images on slides by a color scanner (Epson E-2000). The nuclear and cellular diameters were measured by the above software with an appropriate external reference. GST-P staining intensity of preneoplastic foci was compared with that of single cells by densitometry using the above software. Statistical significance of differences was analyzed using Student’s t-test.

**Preparation of cytosolic and microsomal fractions and enzyme assays**

Rats were administered DEN i.p. at a bolus dose of 200 mg/kg. After appropriate time intervals from 0 to 12 h, the animals were killed. For preparation of cytosols, livers were homogenized with 5 vol of 50 mM Tris–HCl pH 7.4, 6.25 mM DTT, 192 mM KCl and 5 mM EDTA in a Potter–Elvehjem homogenizer on ice, centrifuged at 5000 g for 10 min followed by centrifugation at 105,000 g for 40 min. Cytosolic glycogen phosphorylase and glucose 6-phosphate dehydrogenase (G6PDH) were assayed as reported previously (26). GST was assayed with 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid as substrates (27). Microsomal fractions were prepared from the livers of DEN-treated animals according to method described by Omura et al. (28) with slight modifications. Livers were cut into small pieces with scissors and homogenized with 10 vol of 0.25 M sucrose and 0.2% deoxycholate for ATPase assay (29) or in 0.15 M KCl and 50 mM Tris–maleate pH 6.6, for glucose 6-phosphatase (G6Pase) assay (26).

**Results**

**Stereological estimation of GST-P protein content in preneoplastic foci**

As GST-P was specifically localized immunohistochemically in preneoplastic foci (Figure 2a), the actual concentration of the marker enzyme in the areas of mini-foci was estimated using the stereological analysis of Campbell et al. (19). As seen in Figure 3, GST-P protein content in liver homogenate (mg/g liver) correlated linearly with the volume of preneoplastic foci (r = 0.84). The linear relationship agreed well with the specific localization of GST-P in microscopic foci. GST-P content in the normal rat liver was 34.3 ± 6.8% (n = 6), while it was higher (131 ± 46 mg/g liver) in preneoplastic liver (n = 20) (6). The mean volume of preneoplastic foci estimated from the GST-P-positive mini-foci area was 19.5 ± 6.8% (n = 20). The protein content of the microscopically positive cell population was thus calculated to be [(131 ± 46)/195] = 6.7 ± 2.4 mg/g liver, corresponding to 0.29 ± 0.10 mM concentration of the enzyme subunit of $M_r$ 23,307 (30). The GST-P content in preneoplastic foci can also be estimated from extrapolation to the volume of these foci at 100% in the plot, which was identical to the estimated value.

**Semi-quantitative estimation of GST-P protein content in single cells**

The GST-P protein content of the single-cell population was estimated semi-quantitatively from the relative immunohistochemical staining of these cells and that of preneoplastic foci, which were stained under the same conditions (Figure 2). Using an arbitrary scale based on the densitometric staining intensity, we found that the staining intensity of single-cell population was 1.23-fold higher than that of preneoplastic foci (Table I and Figure 4). Thus, the GST-P content of the single-cell population was 8.2 ± 2.9 mg/g (0.35 ± 0.12 mM subunit). For example, the estimated GST-P content of the single-cell population shown in Figure 2c was as high as 10.2 mg/g (0.43 mM subunit). These results indicate that the amount of
Table I  Nuclear and cellular size of rat hepatocyte populations.

<table>
<thead>
<tr>
<th></th>
<th>GST-P staining&lt;sup&gt;a&lt;/sup&gt;</th>
<th>n</th>
<th>Diameter (µm)</th>
<th>Area (µm&lt;sup&gt;2&lt;/sup&gt;)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nucleus</td>
<td>Cell</td>
</tr>
<tr>
<td>Normal cells</td>
<td>3 ± 2</td>
<td>30</td>
<td>8.6 ± 1.0</td>
<td>19.6 ± 3.0</td>
</tr>
<tr>
<td>Single cells</td>
<td>103 ± 20</td>
<td>19</td>
<td>8.6 ± 1.6</td>
<td>23.9 ± 4.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Foci cells</td>
<td>84 ± 21</td>
<td>35</td>
<td>7.4 ± 1.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21.2 ± 2.7&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>n</sup>, Number of samples. Diameter represents the average value of horizontal and vertical dimensions. Values are mean ± SD. Numbers in parentheses are percentage values relative to normal cells.

<sup>a</sup>Relative densitometric staining intensity of GST-P.

<sup>b</sup>P < 0.05 and <sup>c</sup>P < 0.005, compared with normal cells.

Fig. 4. GST-P contents in normal hepatocytes, single cells and preneoplastic foci. GST-P contents were estimated as described in the text. Values in parentheses represent the number of samples. Data are mean ± SD. *P < 0.05, compared with foci and nodules.

GST-P expressed in the single-cell population was markedly higher than in the preneoplastic foci.

Enlargement of single-cell populations

Examination of GST-P-positive single cells showed that these cells were very large. In the next step, we measured the size of these cells and compared it with that of preneoplastic and normal cells (Table I). As all single GST-P-positive cells examined were mononuclear cells, binuclear cells of normal and preneoplastic foci cells were excluded for such analysis. To measure the cell diameter and size, 19–35 dissected cells and nuclei were selected at random. On average, the mean cell diameter and area of single cells were 1.22- and 1.56-fold greater than those of normal cells, respectively (Table I). One- and two-dimensional increments corresponded to a respective 1.82- and 1.95-fold increase in the three-dimensional volume of single cells. The diameter and area of the single cell shown in Figure 2c were, for example, 1.45- and 1.89-fold larger than those of the normal cells, corresponding to 3.0- and 2.6-fold increase in the cell volume. The size of hepatocytes was, thus, in the order of GST-P-positive single cells greater than preneoplastic foci cells which were greater than normal hepatocytes, i.e. the single cells were much larger in size and more hyperplastic than preneoplastic foci as seen in Figure 5. In contrast to the prominent expansion of the cytoplasmic portion, the nuclear area was apparently in the order of normal hepatocytes greater than GST-P-positive single cells greater than preneoplastic foci cells, suggesting shrinkage of nuclei of GST-P-positive cells (Table I). The enlargement of the cytoplasmic fraction and shrinkage of the nuclear compartment of GST-P-positive single cells and foci cells may reflect severe cell injury. Ploidy analysis of single cells including cytofluorometry should be performed in future studies.

Microsomal damage of rat liver by DEN prior to induction of single cells

We also analyzed the serial effects of DEN on the microsomal and cytosolic components of rat liver as a function of time from 0 to 12 h. As seen in the time course studies shown in Figure 6a, DEN treatment had no effect on the cytosolic enzyme activities of glycogen phosphorylase, G6PDH and GST. Thus, judging from the appearance of ‘no leakage of cytosolic enzymes from hepatocytes’, the result indicated that the carcinogen was weakly necrogenic. The enzymatic activities of glycogen phosphorylase, G6PDH and GST activity with CDNB of untreated rat livers were 11.9 ± 0.7, 2.0 ± 0.12 and 159 ± 11 U/g of liver, respectively, when
assayed under appropriate conditions. In contrast, the yield of microsomal fractions decreased progressively and significantly up to 2 h followed by a recovery to the control levels (Figure 6b). The microsomal yields after 2 h were 58.7% for protein and 56.5 and 48.4% for G6Pase and ATPase activities, respectively. Microsomal protein, and G6Pase and ATPase activities of control livers were 23.7 ± 0.01 and 0.6 ± 0.002 and 0.07 ± 0.01 U/g of liver, respectively. These results clearly indicate that the microsomal components are highly susceptible to the carcinogenic action of DEN.

Discussion

Based on the experimental fact that GST-P is prominent in its immunohistochemical sensitivity for detection of preneoplastic cell populations among preneoplastic marker enzymes (7–9,31,32), an approach was made in the present study to evaluate the protein expressed in ‘GST-P-positive’ cell populations. The quantitative anomaly observed for a biochemical component might be an important clue to solve various basic problems of rat chemical carcinogenesis.

Anomalous elevation of GST-P in preneoplastic foci and single cells

Immunohistochemical and stereological analyses revealed that GST-P expression in preneoplastic foci and nodules was 150–250-fold (6.7 ± 2.4 mg/g liver and 0.29 ± 0.10 mM subunits) higher than in normal cells. The amount is much higher than the 30–50-fold increase at homogenate level as reported originally (6). The degree of elevation and the absolute concentration of GST-P are too high for an enzyme catalyst, and also high for a binding protein (15,16). As examined, the H-site hydrophobicity of GST-P was the lowest among the seven major GSTs of alpha, mu and pi classes, whereas the G-site binding affinity of GST-P (affinity for GSH) was the highest, being 5–8 and 10–20-fold higher than those of mu and alpha species, respectively. The physical chemistry data suggest that GST-P acts mainly as a binding protein for GSH conjugates of endogenous carcinogens such as acrolein and 4-hydroxyalkenals that are still active or activated all the more (18,33). In addition to the quantitative data, our results showed that the size of single cells was increased 200–300% compared with normal cells and was also much larger than those of foci. In contrast to the anomalous increase in the volume of the cytoplasm, however, the nuclei of single cells tended to diminish in size, reflecting severe cell injury. The anomalous rise of GST-P in single cells, morphological expansion and the functional characteristics are indicative of the generation and intracellular accumulation of a lethal level of endogenous ligands in certain hepatocytes, giving rise to single ‘GST-P-positive’ cells in a ‘state of emergency’. The biochemical anomaly, cell kinetics and various other data are not contradictory to consider that GST-P-positive single hepatocytes are inducible through epigenetic mechanism rather than genetic one as discussed in the following section.

Induction of GST-P-positive single cells in the elementary process of epigenetic initiation

The genetic mechanism for induction of GST-P-positive single hepatocytes can be postulated according to Farber (1,34) and Pitot et al. (3,35). In this mechanism, shown schematically in Figure 7a, carcinogens modify DNA bases and the lesion is then fixed via cell division followed by activation of initial oncogene(s) and induction of GST-P-positive single cells. The mutation process is, however, unknown in principle, as it is latent and minor (1,3). In contrast, in the epigenetic initiation mechanism (Figure 7b), carcinogens injure/worsen membranous GSX excretion machinery of hepatocytes resulting in the accumulation of endogenous carcinogens to lethal levels. GST-P is then induced to counteract the cytotoxic effects of the endogenous carcinogens giving rise to ‘GST-P-positive’
single cells. Accordingly, whereas genetic initiation is a latent and static process, epigenetic mechanism is a radical and dynamic one.

As reported earlier (11), i.p. injection of DEN in rats resulted in the transient induction of single cells, which reached a peak level (12 400 ± 4200 GST-P-positive cells/cm³ or 124 000/10 g of liver) after 5 days, but >80% of cells were lost rapidly thereafter. Accordingly, the viability of DEN-induced single cells was suggested to be very low (<20%) due to extracellular and intracellular injury by carcinogens and resultant autointoxication. It should, however, be considered that the single cells are, at least, actively synthesizing high concentrations of GST-P protein suggesting that they are living cells being in struggle for survival. The higher the expression of GST-P to defend the stress of autointoxication, the more advantageous they may be to avoid apoptotic or necrotic death. It is conceivable, however, that all single cells are destined to death. This means that GST-P induction is a ‘signal of death’ apart from its physiological role, which is totally inapplicable to ‘resistant’ foci. Therefore, only a proportion of single cells may proliferate as estimated by Pitot et al. (35); ~1% of single cells develop into altered hepatic foci. How do the smaller cells in foci develop from the larger single cells? Although the exact mechanism is not clear at present, intracellular stress on cell division may be relieved significantly. Namely, the enlarged single cells may give rise to smaller daughter cells as a result of sharing ‘pains’. It should be stressed here that induction of the single cell is not an intrinsic phenomenon following the administration of necrogenic dose of DEN (200 mg/kg), as these cells are also inducible at non-necrogenic or subcarcinogenic dose of the carcinogen (10, 20 and 40 mg/kg) (10,11,36).

Recently, Gras-Kraupp et al. (20) have made detailed kinetic examination on positive cell populations induced in the rat liver after administration of N-nitrosomorpholine (NNM) through gastric gavage. According to their stochastic growth model analysis, single cells were induced at a rate of 12 000 per day/liver until a maximal number of 176 000 was reached (0–14 day, expansion), thereafter their number diminished to 134 000 (14–28 day, regression) and then remained constant (28–51 day, stabilization). Several experimental facts could not, however, be explained by genetic mechanism. First, no more than ~1% of single cells were estimated to replicate within 36 h of their formation suggesting that DNA replication and cell division were not associated with their formation. Secondly, the GST-P-positive single cells were noted to be suffering from genotoxic and cytotoxic effects of NNM even on day 107.5 post-NNM. Thirdly, the static mechanism could not account for the high birth and death rates of single cells at various points. Therefore, they explained ‘Since the molecular mechanisms of induction of the GST-P-positive phenotype are not known, the appearance of GST-P-positive hepatocytes could be due to epigenetic rather than genetic events’.

Biochemical and pathological changes in plasma membrane components in preneoplastic and neoplastic cell populations are well documented (1–3,36–38). Although macroscopic, hepatocyte membranous components were found in our study to be fairly susceptible to the carcinogenic action of DEN judging from the low yield of microsomal preparations after 2 h of DEN injection, which preceded the induction of single cells in the rat liver. In this regard, it is of interest that Wu et al. (39) demonstrated that the functional defect of ATPase 7B, a membranous polypeptide but not an oncogenic product, was responsible for spontaneous carcinogenesis of LEC rats. The mutant animals were also noted to be susceptible to DEN carcinogenesis (40). It should be noted that GST-P was gradually but strongly inducible in primary cultures of normal rat hepatocytes in the absence of any carcinogen up to 1 or 2 weeks (41,42). As GST-P could be a measure of intracellular stress, the result indicates gradual accumulation of endogenous carcinogens in normal hepatocytes in vitro, that might be, as a matter of course, unrelated to genetic change(s). Accordingly, one possible mechanism for the marked induction of GST-P in certain hepatocytes by carcinogens is impairment of membranous GSH conjugate excretion machinery such as MRP protein (43,44).

No significant oncogenic changes have been detected in preneoplastic cells of experimental animals except for some occasional activation of c-myc or H-ras (45,46). We, therefore, promote the concept of an epigenetic mechanism, in which genetic change is not necessary for transformation of hepatocytes into GST-P-positive cells, whereby a strong driving force arises intracellularly on/prior to initiation (Figure 7b).

As questioned by Levin et al. (5), preneoplastic antigen, epoxide hydrolase, is inducible by 2-AAF, ethionine and DEN, but the marker enzyme does not play a role in the metabolism of these carcinogens. It is also difficult at present to explain why DEN is such a strong inducer of GST-P when this carcinogen is unrelated to GSH conjugation (47). As the physiological meaning(s) of tumor marker expression remains largely unclear, isozymic change appears to be an intrinsic feature of preneoplastic cells, being independent of physiological conditions. Based on the present findings, however, the physiological meaning of the isozymic change of early markers, phase II marker enzymes including GST-P, primarily to detoxicate endogenous lipid peroxidation end products that accumulate intracellularly, rather than to detoxicate exogenous carcinogens. In other words, isozymic alteration of the early marker enzymes represents a biochemical response to defend against strong intracellular stress. Whereas early marker expression is characteristic of initiation, late marker expression is closely related with promotion and progression in the multistage hepatocarcinogenesis. As demonstrated by Hacker et al. (48) using laser-microdissected specimens of individual preneoplastic liver foci for pyruvate kinase M2, late marker enzymes, most of which are glycolytic isoenzymes, may play principal roles in the energy metabolism and proliferation of malignant cells (1–3,8).

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