Inhibition of Early-phase Exogenous and Endogenous Liver Carcinogenesis in Transgenic Rats Harboring a Rat Glutathione S-Transferase Placental Form Gene

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Hepatocarcinogenesis initiated with N-nitrosodiethylamine (DEN) and that initiated by feeding of a choline-deficient, L-amino acid-defined (CDAA) diet were compared in transgenic male Wistar rats harboring a rat glutathione S-transferase placental form (GST-P) gene (GST-P-Tg rats) and non-transgenic (N-Tg) rats. Eight-week-old GST-P-Tg and N-Tg rats were administered DEN intraperitoneally at 100 mg/kg body weight, subjected to a selection procedure with 2-acetylaminofluorene and CCl4, and killed at the end of weeks 5 and 12. Other groups were fed the CDAA diet for 12 weeks and killed. Five weeks after the DEN treatment, numbers and sizes of γ-glutamyltransferase (GGT)- or GST-P-positive lesions and 8-hydroxyguanine (8-OHG) levels in the livers were significantly less in GST-P-Tg rats than in N-Tg rats. The lesion numbers were unchanged between the ends of weeks 5 and 12 in GST-P-Tg rats, but decreased in N-Tg rats. The lesion sizes were increased in GST-P-Tg rats, but unchanged in N-Tg rats. While the proliferating cell nuclear antigen labeling indices (PCNA L.I.) in and surrounding the lesions were decreased, more prominently in GST-P-Tg rats than in N-Tg rats, the 8-OHG levels were also decreased but similarly in both cases. After 12 weeks on the CDAA diet, the lesion incidences, numbers and sizes, 8-OHG levels, PCNA L.I. in and surrounding the lesions, and liver injury were significantly less in GST-P-Tg rats than in N-Tg rats. These results indicate that insertion of a rat GST-P transgene alters the early phase of exogenous and endogenous rat hepatocarcinogenesis, presumably due to enhanced detoxification by GST-P expressed both transiently during the initiation and chronically in the altered hepatocyte populations.

Key words: Glutathione S-transferase placental form — N-Nitrosodiethylamine — Choline-deficient, L-amino acid-defined diet — Transgenic rat — Rat liver carcinogenesis

The glutathione S-transferases (GSTs; EC 2.5.1.18) are a family of multifunctional, phase II detoxification enzymes catalyzing the conjugation of the reduced form of glutathione with electrophilic metabolites of xenobiotics.1,2 Among the isozymes of the GSTs, GST placental form (GST-P) is present in small quantities in various rat tissues, but only in trace amounts in normal liver.3,4 It is lacking in fetal liver and not increased in regenerating liver,5 but has attracted particular attention since it is highly expressed in putative preneoplastic, focal lesions as well as in hepatocellular carcinomas induced by most chemical carcinogens.3,4,5,6 GST-P has been shown to be a very useful marker for quantitation of lesions in assessment of both initiation and promotion.5,6 However, the biological roles and the mechanisms underlying GST-P expression within the preneoplastic focal lesions are still largely obscure.

To cast light on this problem, we have cloned the rat GST-P gene7,8 and identified a strong enhancer, GST-P enhancer I, having 2 activator protein 1 site-like sequences at −2.5 kb of the 5′ flanking region.11–14 Using transgenic rats, we have revealed that the GST-P gene is trans-activated by GST-P enhancer I playing an essential role as a cis element, with at least partial mediation of activator protein 1, in the induction of GST-P at the transcriptional level in the livers of rats treated with N-nitrosodiethylamine (DEN).9–11 or lead.16

With regard to biological roles, the expression of GST-P within putative preneoplastic, focal lesions has been considered to be a part of the “resistant phenotype” of initiated hepatocytes, with a decrease in activation along with an increase in detoxification of the carcinogen.16,17 This phenotype is believed to facilitate clonal expansion during the promotion phase.18 It still remains largely unknown, however, what is the precise role of GST-P in the early phase of rat liver carcinogenesis. The aim of the present study was to explore this question in detail in transgenic rats using two different rat liver carcinogenesis models; one featuring initiation with the exogenous carcinogen, DEN, and the other, chronic feeding of a cho-
line-deficient, L-amino acid-defined (CDAA) diet. While hepatocellular carcinomas are induced through the development of putative preneoplastic, focal lesions in both models, DEN forms DNA adducts but does not produce cirrhosis, whereas the CDAA diet, not containing any known carcinogen, causes continuous liver injury that is reflected in intrahepatocellular fat accumulation, hepatocyte death, connective tissue deposition and frank cirrhosis.

MATERIALS AND METHODS

Animals  Wistar rats were originally obtained from Charles River Japan, Inc., Atsugi. To produce transgenic rats harboring a GST-P gene, a plasmid, pEB 64, was prepared by inserting a 6.4-kb fragment between −2.9 kb (EcoRI) and +3.5 kb (BamHI) of GST-P genomic DNA into the SalI site of pUC 8. The pEB 64 was linearized by SalI digestion and microinjected into male pronuclei of fertilized eggs of rats, and the eggs were transferred to oviducts of pseudopregnant rats. Male transgenic animals were selected by Southern blot analysis of the high-molecular DNA extracted from the surgically removed tails of newborn rats, which are hereafter referred to as GST-P-Tg rats. Non-transgenic (non-treated), age-matched male rats served as controls (N-Tg rats). The working of the transgene was confirmed by western blot analysis of GST-P protein levels. In total, 57 GST-P-Tg and 57 N-Tg rats were used in the present study at an age of 8 weeks.

Animal treatment  GST-P-Tg rats were divided into groups T-1, T-2, T-3, T-4, T-5, T-6, T-7, T-8, T-9 and T-10 consisting of 6, 5, 5, 5, 8, 5, 6, 5, 7 and 5 animals, respectively. N-Tg rats were similarly divided into groups N-1 to N-10. For the DEN experiments (see Tables I and II), groups T-1, T-5, N-1 and N-5 received a single intra-peritoneal administration of DEN (Wako Pure Chemicals Industries, Ltd., Kyoto) at the dose of 100 mg/kg body weight and were subjected to the selection procedure. The GGT activity was histochemically demonstrated by 2-acetylaminofluorene (Wako) and carbon tetrachloride (Wako) were administered at a dietary concentration of 0.02% from the end of week 2 to that of week 4 and as a single dose of 1 ml/kg body weight by gavage at the end of week 3, respectively. Groups T-2, T-6, N-2 and N-6 received DEN but were not subjected to the selection procedure. Groups T-3, T-7, N-3 and N-7 received the vehicle (0.9% sodium chloride solution) and were subjected to the selection procedure. Groups T-4, T-8, N-4 and N-8 received the vehicle, but were not subjected to the selection procedure. Rats in groups T-1 to T-4 and N-1 to N-4 were killed at the end of week 5, whereas those in groups T-5 to T-8 and N-5 to N-8 were killed 12 weeks after the beginning of the experiments. For the experiments featuring the CDAA diet (see Table III), groups T-9 and N-9 were chronically fed the CDAA diet (Dyets Inc., Bethlehem, PA), while groups T-10 and N-10 were maintained on the control choline-supplemented, L-amino acid-defined (CSAA) diet (Dyets). The composition of these diets was described previously. All rats were killed 12 weeks after the commencement.

Animals were killed under light ether anesthesia by exsanguination from the abdominal aorta after collection of blood to prepare serum samples, and livers were excised and weighed. Five-millimeter-thick slices were immediately taken from the left lateral, median and right lateral lobes of the liver, fixed in ice-cold acetone and processed for embedding in paraffin. Five serial 4-μm-thick sections were prepared from each fixed liver slice. Two were used for histological examination after routine hematoxylin-eosin and Azan-Mallory stainings, and the other 3 were employed for histochemical and immunohistochemical assessments. The remaining liver portions were immediately frozen under liquid nitrogen and stored at −80°C until use for biochemical analyses.

During the experimental period, the animals were weighed weekly. The diets were changed twice a week, food consumption was monitored, and fresh water was always available.

Quantitative analysis of the development of putative preneoplastic, focal lesions  The development of the focal lesions were quantitatively analyzed, by measuring their γ-glutamyltransferase (GGT; EC 2.3.2.2)- and GST-P-positivities, as an end-point marker for carcinogenicity. The GGT activity was histochemically demonstrated by the method of Rutenberg et al. GST-P was detected immunohistochemically by the avidin-biotin complex method as previously described, using a rabbit anti-rat-GST-P polyclonal antibody (Medical and Biological Laboratories Co., Nagoya) and a Vectastain Elite ABC kit (rabbit IgG, Vector Laboratories, Inc., Burlingame, CA). The numbers and sizes of the lesions were determined with an image analyzing system as described elsewhere and the three-dimensional correction procedure of Campbell et al. Only foci consisting of more than 10 altered hepatocytes were included in the assessment since these are considered to be less reversible than single-positive cells or foci of a few positive cells.

Proliferative activity of hepatocytes within GGT-positive lesions and in the background parenchyma  The proliferative activity of hepatocytes was assessed by means of immunohistochemical demonstration of proliferating cell nuclear antigen (PCNA) using the enhanced polymer one-step staining system (EPOS system; Dako A/S, Copenhagen, Denmark). The PCNA labeling indices (PCNA LI) both inside the GGT-positive lesions (when several large lesions were detected) and in the surrounding (non-tumoral) areas, identified by reference to
serial specimens stained for GGT, were calculated as percentages of labeled cells among approximately 3000 (altered) hepatocytes, counted at the light microscope level.

**Determination of the 8-hydroxyguanine (8-OHG) levels in liver DNA** The levels of 8-OHG were assessed as a parameter of oxidative DNA damage. The 8-hydroxydeoxyguanosine (8-OHdG) levels in the nucleoside samples were measured by electrochemical detection after high-performance liquid chromatographic separation as detailed previously, using liver portions weighing approximately 200 mg (groups T-9 and N-9) or 100 mg (the other groups) and a Sepagene kit (Sanko Jun-Yaku Co., Ltd., Kyoto) for DNA extraction. Data were expressed as numbers of 8-OHdGs per 10^5 deoxyguanosines (dGs) in deoxynucleosides by calibration against curves from runs of standard samples containing known amounts of authentic 8-hydroxy-2′-dG (Wako) and 2′-dG (Sigma).

**Other biochemical determinations** Liver triglyceride (TG) and hydroxyproline (HP) contents were individually measured in the samples of groups T-9, T-10, N-9 and N-10. TG was extracted from 500 mg of wet tissue by the method of Folch using liver portions weighing approximately 200 mg (groups T-9 and N-9) or 100 mg (the other groups) and a Sepagene kit (Sanko Jun-Yaku Co., Ltd., Kyoto) for DNA extraction. Data were expressed as numbers of 8-OHdGs per 10^5 deoxyguanosines (dGs) in deoxynucleosides by calibration against curves from runs of standard samples containing known amounts of authentic 8-hydroxy-2′-dG (Wako) and 2′-dG (Sigma).

**Statistics** Statistical analyses were carried out using a personal computer, basically as previously described. The Student-Newman-Keuls multiple comparisons test was employed to assess the statistical significance of intergroup differences of means for multiple groups after one-way analysis of variance to determine the variations among group means, followed by Bartlett’s test to determine the homogeneity of variance. For assessment of intergroup differences of means for pairs of 2 particular groups and the incidence data, the Mann-Whitney non-parametric test and Fisher’s exact test were performed, respectively. The intergroup difference was considered significant when the P value was less than 0.05.

**RESULTS**

**GST-P protein in non-treated GST-P-Tg rats** Southern blot analyses revealed that each GST-P-Tg rat had 33 copies of the transgene. No particular histological changes were noted in the livers of non-treated GST-P-Tg rats at 20 weeks of age (group T-8), and no expression of GST-P protein was immunohistochemically detected in the hepatocyte lobuli of such rats. This was confirmed by western blot analyses showing that the amounts of protein in the livers and kidneys of GST-P-Tg rats were in the same range as those of N-Tg rats. Nevertheless, the transgene was working, since the amounts of GST-P protein in the livers and kidneys were significantly increased by 2.22- and 2.10-fold in GST-P-Tg rats, respectively, while no significant increments were obtained in N-Tg rats, when 10-week-old animals were treated with lead at 3 intraperitoneal doses (every 24 h) of 100 mmol/kg body weight and left for another 24 h in a preliminary western blot study (data for this paragraph not shown).

**Liver carcinogenesis initiated with DEN, 5-week study** (Table I) All animals survived until the scheduled termination. Final body weights were slightly higher in group T-4 than in group T-1, while values for groups N-2 to N-4 were slightly higher than the N-1 value. No differences were found between matched groups of GST-P-Tg and N-Tg rats. There were no intergroup differences in relative liver weights (data for this paragraph not shown).

The number of GGT-positive lesions in group T-1 was significantly less than that in group N-1. Similarly, the average size of the lesions in group T-1 was significantly less than that in group N-1. Similar results were obtained for GST-P-positive lesions, and GST-P protein was expressed solely inside the putative neoplastic lesions, both in the transgenic and non-transgenic cases, as previously described (data not shown). There were no differences in terms of PCNA LI between the matched groups of GST-P-Tg and N-Tg rats. The 8-OHG levels in groups T-1 and T-2 were lower than those in groups N-1 and N-2, respectively. The ALT activity in group T-1 was lower than that in group N-1.

**Liver carcinogenesis initiated with DEN, 12-week study** (Table II) All animals remained in good condition until the end of the experiment. There were no intergroup differences in final body or relative liver weights. There were no particular histological changes in any of the groups other than the development of altered hepatocyte focal lesions (data for this paragraph not shown).

No differences were seen in terms of GGT-positive lesion numbers between matched groups of GST-P-Tg and N-Tg rats. Comparing the data in Table II with those in Table I, however, the number of lesions in group T-5 was in the same range as the group T-1 value, whereas that in group N-5 was significantly less than the group N-1 value. There were again no significant differences in terms of the lesion sizes between matched groups of GST-P-Tg and N-Tg rats. The size of the lesions in group T-5 was significantly larger than the group T-1 value, whereas that in group N-5 was in the same range as the group N-1 value (compare Tables I and II). Similar results were obtained for GST-P-positive lesions (data not shown). There were significant changes in hepatocyte proliferative...
Table I. Incidences, Numbers and Sizes of GGT-positive Lesions, PCNA L.I. and 8-OHG Levels in Liver, and Serum ALT Activities in GST-P-Tg and N-Tg Rats Given DEN and Then Killed at the End of Week 5

| Group | Rats | Treatment(s) | Effective number of rats | Incidence (%) | Number/cm³ | Average volume (mm³) | Within GGT-positive lesions | Surrounded area | 8-OHG (8-OHG/10⁵ dG) | ALT (unit/liter) |
|-------|------|-------------|--------------------------|---------------|-------------|-------------------|---------------------------|----------------|---------------------|----------------|
| T-1   | GST-P-Tg | DEN+selection | 6 | 100 | 170±50⁰ | 0.066±0.049⁰ | 30.2±2.0 | 30.3±1.8 | 20.71±6.91⁰ | 32±10⁰ |
| T-2   | GST-P-Tg | DEN         | 5 | 100 | 9±13¹ | 0.002±0.003 | 19.1±6.3³ | 17.4±3.0³ | 2.48±0.49⁸ | 29±5 |
| T-3   | GST-P-Tg vehicle+selection | 5 | 0² | 0² | — | 12.3±2.1¹ | 2.75±0.99³ | 33±4 |
| T-4   | GST-P-Tg vehicle | 5 | 0² | 0² | — | 3.9±0.2³ | 0.55±0.15⁰ | 28±2 |
| N-1   | N-Tg | DEN+selection | 6 | 100 | 356±109 | 0.207±0.146 | 28.6±2.9 | 27.0±3.0 | 26.73±9.13 | 54±14 |
| N-2   | N-Tg | DEN | 5 | 100 | 34±11¹ | 0.007±0.002⁰ | 15.1±2.5¹ | 15.1±1.3⁰ | 13.82±4.7² | 39±7³ |
| N-3   | N-Tg vehicle+selection | 5 | 0² | 0² | — | 14.2±3.4 | 2.56±1.5² | 33±9³ |
| N-4   | N-Tg vehicle | 5 | 0² | 0² | — | 3.9±0.2³ | 0.47±0.20³ | 31±6³ |

a) The values other than the incidence data are means±SD.
b) Significantly different from the group N-1 value.
c) Significantly different from the group T-1 value.
d) Significantly different from the group N-2 value.
e) Significantly different from the group T-2 value.
f) Significantly different from the group N-3 value.
g) Significantly different from the group T-3 value.

Table II. Incidences, Numbers and Sizes of GGT-positive Lesions, PCNA L.I. and 8-OHG Levels in Liver, and Serum ALT Activities in GST-P-Tg and N-Tg Rats Administered DEN and Killed at the End of Week 12

| Group | Rats | Treatment(s) | Effective number of rats | Incidence (%) | Number/cm³ | Average volume (mm³) | Within GGT-positive lesions | Surrounded area | 8-OHG (8-OHG/10⁵ dG) | ALT (unit/liter) |
|-------|------|-------------|--------------------------|---------------|-------------|-------------------|---------------------------|----------------|---------------------|----------------|
| T-5   | GST-P-Tg | DEN+selection | 8 | 100 | 150±79⁰ | 0.225±0.105 | 15.2±1.4² | 11.7±1.3⁰ | 4.77±2.8⁴ | 49±14 |
| T-6   | GST-P-Tg | DEN | 5 | 100 | 17±12² | 0.005±0.003⁶ | 12.3±0.8 | 12.3±1.5³ | 0.88±0.6⁴ | 50±15 |
| T-7   | GST-P-Tg vehicle+selection | 6 | 17² | 2.4⁶ | 0.010±0.025⁰ | 10.4±1.3³ | 0.55±0.2³ | 38±4 |
| T-8   | GST-P-Tg vehicle | 5 | 0² | 0² | — | 2.9±0.3³ | 0.57±0.2¹ | 33±5 |
| N-5   | N-Tg | DEN+selection | 8 | 100 | 186±64 | 0.332±0.202 | 23.1±2.3³ | 21.1±1.4 | 6.01±3.3 | 46±5 |
| N-6   | N-Tg | DEN | 5 | 100 | 26±13² | 0.018±0.024³ | 16.5±1.7³ | 16.5±1.2³ | 0.86±0.5¹ | 45±4 |
| N-7   | N-Tg vehicle+selection | 6 | 0² | 0² | — | 15.6±1.6³ | 0.58±0.25¹ | 43±4 |
| N-8   | N-Tg vehicle | 5 | 0² | 0² | — | 2.7±0.3³ | 0.60±0.22³ | 46±10 |

a) The values other than the incidence data are means±SD.
b) Significantly different from the value in the surrounding area.
c) Significantly different from the group N-5 value.
d) Significantly different from the group T-5 value.
e) Significantly different from the group N-6 value.
f) Significantly different from the group T-6 value.
g) Significantly different from the group N-7 value.
h) Significantly different from the group T-7 value.

activity between GST-P-Tg and N-Tg rats; i.e., PCNA L.I. of groups T-5, T-6 and T-7 were significantly lower than those of groups N-5, N-6 and N-7, respectively, in the surrounding area. Similarly, the PCNA L.I. of group T-5 was significantly lower than that of group N-5 also inside the GGT-positive lesions. Furthermore, both within the lesions and in the surrounding areas, the values of groups T-5 and N-5 were significantly less than those of
groups T-1 and N-1, respectively, this decrease being more prominent in GST-P-Tg rats (compare Tables I and II). There were no significant differences in terms of the 8-OHG levels or serum ALT activities between matched groups of GST-P-Tg and N-Tg rats. 

Liver carcinogenesis due to 12 weeks feeding of the CDAA diet (Table III) No rats died during the course of the experiment. There were no intergroup differences in final body weights. Relative liver weight in group T-10 was significantly less than that in group T-9, and that in group N-10 was significantly less than that in N-9. No significant differences were found between matched groups of GST-P-Tg and N-Tg rats. Histologically, in the livers of N-Tg rats fed the CDAA diet for 12 weeks (group N-9), severe intrahepatocellular fat accumulation and numerous dead hepatocytes were diffusely seen, with the development of borderline cirrhosis, in good accordance with our earlier findings on male Fischer 344 rats.24, 25, 39, 42) In contrast, cirrhotic changes were limited in GST-P-Tg rats (group T-9) with far fewer dead hepatocytes, but no apparent alteration in intrahepatocellular fat accumulation (data for this paragraph not shown).

In the GST-P-Tg rats, GST-P-positive lesions were found in only 1 rat of group T-9 (14% incidence). In contrast, all rats of group N-9 had such lesions. The incidence, number and size of the lesions in group T-9 were all significantly less than those in group N-9. Similar results were obtained for the GST-P-positive lesions, with only 2 rats positive in group T-9 (29% incidence) (data not shown). There were no significant differences in terms of PCNA L.I. between matched groups of GST-P-Tg and N-Tg rats. The 8-OHG level in group T-9 was significantly lower than that in group N-9. Liver TG and HP contents of GST-P-Tg, but not N-Tg rats as described above, could, in turn, be inhibited, resulting in a reduction of the number of initiated cells and the magnitude of the alterations occurring in the initiated cells, which may then be respectively reflected in the decrease of the numbers and sizes of GST- and GST-P-positive lesions 5 weeks after DEN administration in GST-P-Tg rats. The reduced 8-OHG formation and the absence of serum ALT activity increment in GST-P-Tg rats at the end of week 5 may be supportive of the above ideas, because these changes occur as results of the metabolic activation of DEN. On the other hand, once the initiating process is accomplished, the altered hepatocytes express the new phenotype.18, 19) As one of the enzymes involved, GST-P is

**Table III. Incidences, Numbers and Sizes of GGT-positive Lesions, PCNA L.I., 8-OHG Levels, TG Contents and HP Contents in Liver, and Serum ALT Activities in GST-P-Tg and N-Tg Rats Fed the CDAA Diet and Killed at the End of Week 12**

| Group | Rats | Diet | Effective number of rats | GGT-positive lesions | PCNA L.I. | 8-OHG (8-OHdG/10^5 dG) | TG (mg/g wet liver) | HP (mg/g dry liver) | ALT (unit/liter) |
|-------|------|------|------------------------|---------------------|--------|------------------|------------------|-------------------|------------------|
| T-9   | GST-P-Tg | CDAA | 7 | 14%b | 0.008±0.00330 | 18.6 | 9.14±1.3630 | 0.62±0.3030 | 0.86±0.1730 | 35±5 |
| T-10  | GST-P-Tg | CSAA | 5 | 0 | 9.14±1.3630 | 0.62±0.3030 | 0.86±0.1730 | 35±5 |
| N-9   | N-Tg | CDAA | 7 | 100% | 0.610±0.048 | 33.4±0.80 | 27.3±3.4 | 14.95±2.51 | 332±13 | 2.29±0.15 | 72±8 |
| N-10  | N-Tg | CSAA | 5 | 0 | 2.4±0.60 | 0.62±0.3030 | 0.86±0.1730 | 35±5 |

- a) The values other than the incidence data are means±SD.
- b) Significantly different from the group N-9 value.
- c) Significantly different from the group T-9 value.
- d) Significantly different from the value in the surrounding area.
Hepatocarcinogenesis in GST-P-transgenic Rats

Phenotypically expressed to act as a phase II enzyme conferring resistance against carcinogens in the vast majority of these altered hepatocytes, and the putative preneoplastic, focal lesions are formed by clonal expansion. In GST-P-Tg rats, GST-P was expressed solely in the focal lesions as shown previously and herein. While over 90–95% of such lesions become remodeled or redifferentiated to normal-appearing liver with reversion of the phenotype, the minority persist with a spontaneous proliferation rate that is almost balanced by cell loss so that they grow very slowly. In this context, the comparison of the data at the end of week 5 with that at the end of week 12 may indicate that the focal lesions that had progressed to a more advanced stage in GST-P-Tg rats than in N-Tg rats, since the lesions induced in the transgenic animals were more persistent than those in the non-transgenic animals, with an increase in size, and because the PCNA L.I. in the lesions, as well as in the surrounding areas, were decreased more prominently in GST-P-Tg rats than in N-Tg rats.

The inhibitory effects of insertion of GST-P transgenes were also prominent on rat liver carcinogenesis by chronic feeding of the CDAA diet. In this case, oxidative injury to subcellular components and regeneration, continuous as long as the diet is fed, play major roles in injury to subcellular components and regeneration, continuous as long as the diet is fed, play major roles in cell proliferation with chronic exposure to the CDAA diet in GST-P-Tg rats. Since cell proliferation is a prerequisite for the initial stage of acquiring the phenotypical changes, GST-P appears to provide such protection.

In conclusion, the insertion of GST-P transgenes alters the early phase of rat liver carcinogenesis, presumably by providing enhanced detoxification and protection against damage to cells and DNA leading to carcinogenesis. The present results suggest that, while GST-P itself always has the same functions, the enzyme at first acts in favor of the normal hepatocytes during the initiation stage and then acts in favor of altered hepatocyte populations thereafter. However, further analyses will still be required to clarify the findings obtained in the present study. For instance, it would be essential to measure directly the GST-P protein level, enzyme activity, the levels of major ethylated DNA adducts, the 8-OHG level and the magnitude of liver toxicity in the initiation phase under the present experimental protocols, especially the one featuring initiation with DEN.

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