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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 CCl_4, 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.[3–5] It is lacking in fetal liver and not increased in regenerating liver,[3] 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.[3,4,6,7] GST-P has been shown to be a very useful marker for quantitation of lesions in assessment of both initiation and promotion.[8,9] 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* gene[10,11] and identified a strong enhancer, GST-P enhancer I, having 2 activator protein 1 binding 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)[3,15,16] or lead.[17]

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.[18,19] This phenotype is believed to facilitate clonal expansion during the promotion phase.[20] 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-

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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,[21, 22] 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.[23–26]

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 (*Eco*RI) and +3.5 kb (*Bam*HI) of *GST-P* genomic DNA into the *Sal*I site of pUC 8.[12] The pEB 64 was linearized by *Sal*I digestion and microinjected into male pronuclei of fertilized eggs of rats, and the eggs were transferred to oviducts of pseudopregnant rats.[27] Male transgenic animals were selected by Southern blot analysis of the high-molecular DNA extracted from the surgically removed tails of newborn rats,[17] 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 intraperitoneal 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 of Cayama *et al.*[28] in which 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.[26] 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 livers, 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.*[29] GST-P was detected immunohistochemically by the avidin-biotin complex method as previously described,[30] 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[31] and the three-dimensional correction procedure of Campbell *et al.*[32] 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).[33, 34] The PCNA labeling indices (PCNA L.I.) 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,[35] 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[36] and determined by the method of Spayd *et al.*[37] using a Triglyceride E-TESTWako kit (Wako). The HP determination was performed according to Kivirikko *et al.*,[38] as detailed elsewhere.[39] The alanine aminotransferase (ALT; EC 2.6.1.2) activity in serum samples was individually measured with an ultraviolet-assay method[40] using an automatic Monarch Chemistry System analyzer (Instrumentation Laboratory, Inc., Lexington, MA).

Statistics Statistical analyses were carried out using a personal computer, basically as previously described.[41] 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 in 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 preneoplastic lesions, both in the transgenic and non-transgenic cases, as previously described[15] (data not shown). There were no differences in terms of PCNA L.I. 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, while 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	GGT-positive lesions			PCNA L.I.		8-OHG (8-OHdG/10^5 dG)	ALT (unit/liter)
				Incidence (%)	Number/cm^3	Average volume (mm^3)	Within GGT-positive lesions	Surrounding area		
T-1	GST-P-Tg	DEN+selection	6	100	170±50[a, b]	0.066±0.049[b]	30.2±2.0	30.3±1.8	20.71±6.91[b]	32±10[b]
T-2	GST-P-Tg	DEN	5	100	9±13[c]	0.002±0.003	19.1±6.3[c]	17.4±3.0[c]	2.48±0.49[c, d]	29±3
T-3	GST-P-Tg	vehicle+selection	5	0[c, e]	0[c]	—	—	12.3±2.1[c]	2.75±0.99[c]	33±4
T-4	GST-P-Tg	vehicle	5	0[c, e]	0[c]	—	—	3.9±0.2[c, e, g]	0.55±0.15[c]	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±31[b]	0.007±0.002[b]	15.1±2.5[b]	15.1±1.3[b]	13.82±4.22[b]	39±7[b]
N-3	N-Tg	vehicle+selection	5	0[b, d]	0[b]	—	—	14.2±3.4	2.56±1.52[b]	33±9[b]
N-4	N-Tg	vehicle	5	0[b, d]	0[b]	—	—	3.9±0.2[b, d, f]	0.47±0.20[b, d]	31±6[b]

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	GGT-positive lesions			PCNA L.I.		8-OHG (8-OHdG/10^5dG)	ALT (unit/liter)
				Incidence (%)	Number/cm^3	Average volume (mm^3)	Within GGT-positive lesions	Surrounding area		
T-5	GST-P-Tg	DEN+selection	8	100	150±79[a]	0.225±0.105	15.2±1.4[b, c]	11.7±1.3[c]	4.77±2.84	49±14
T-6	GST-P-Tg	DEN	5	100	17±12[d]	0.005±0.003[d]	12.3±0.8	12.3±1.5[e]	0.88±0.64[d]	50±15
T-7	GST-P-Tg	vehicle+selection	6	17[d, f]	2±4[d]	0.010±0.025[d]	—	10.4±1.3[g]	0.55±0.23[d]	38±4
T-8	GST-P-Tg	vehicle	5	0[d, f]	0[d]	—	—	2.9±0.3[d, f, h]	0.57±0.21[d]	33±5
N-5	N-Tg	DEN+selection	8	100	186±64	0.332±0.202	23.1±2.3[b]	21.1±1.4	6.01±3.31	46±9
N-6	N-Tg	DEN	5	100	26±13[c]	0.018±0.024[c]	16.5±1.7[c]	16.5±1.2[c]	0.86±0.51[c]	45±4
N-7	N-Tg	vehicle+selection	6	0[c, e]	0[c]	—	—	15.6±1.6[c]	0.58±0.25[c]	43±4
N-8	N-Tg	vehicle	5	0[c, e]	0[c]	—	—	2.7±0.3[c, e, g]	0.60±0.22[c]	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, GGT-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 lower 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 less than that in group N-9. Liver TG and HP contents and serum ALT activity were all significantly less in group T-9 than in group N-9, essentially in line with the above-mentioned histological findings.

DISCUSSION

The present results clearly indicate that the early phase of liver carcinogenesis due to exogenously administered DEN is inhibited in GST-P-Tg rats as compared with N-Tg rats. The metabolic activation of DEN below 100 mg/kg body weight is completed within 3–24 h,[43] affording major ethylated DNA adducts (O^4-ethylthymidine, 7-ethylguanine and O^6-ethylguanine)[44, 45] and 8-OHG.[41] Then, at least a single round of cell proliferation is required for the altered phenotype to appear in initiated hepatocytes.[20, 28] We have shown that GST-P mRNA is over-expressed on a whole-liver basis as a part of the immediate detoxifying reactions within 5 h after DEN administration, reaching a peak level at 12 h and then declining.[46] This phenomenon should be strictly distinguished from the persistent expression in putative preneoplastic, focal lesions.[3, 4, 6, 7] Since GST-P protein was induced within 24 h after acute lead intoxication in the livers of GST-P-Tg, but not N-Tg rats as described above, insertion of rat *GST-P* genes could provide an additional amount of GST-P protein, also shortly after DEN exposure, to enhance the detoxification. If so, DEN activation 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 GGT- 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)
				Incidence (%)	Number/cm^3	Average volume (mm^3)	Within GGT-positive lesions	Surrounding area				
T-9	GST-P-Tg	CDAA	7	14[b]	2±7[a, b]	0.008±0.003[b]	18.6	22.7±7.0[b]	9.14±1.36[b]	281±33[b]	1.23±0.43[b]	37±5[b]
T-10	GST-P-Tg	CSAA	5	0	0	—	—	2.4±0.3[c]	0.62±0.30[c]	44±12[c]	0.86±0.17[c]	35±2
N-9	N-Tg	CDAA	7	100	16±8	0.610±0.048	33.4±0.8[d]	27.3±3.4	14.95±2.51	332±13	2.29±0.15	72±8
N-10	N-Tg	CSAA	5	0[b]	0[b]	—	—	2.4±0.6[b]	0.62±0.30[b]	53±7[b]	0.96±0.03[b]	30±4[b]

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.

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.[3, 4, 6, 7] In GST-P-Tg rats, GST-P was expressed solely in the focal lesions as shown previously[15] and herein. While over 90–95% of such lesions become remodeled or redifferentiated to normal-appearing liver with reversion of the phenotype,[47–51] the minority persist with a spontaneous proliferation rate that is almost balanced by cell loss[52] so that they grow very slowly.[52, 53] 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 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 initiation and subsequent growth of the initiated cells.[24–26, 31, 39, 42, 54] Under these circumstances, therefore, the enhanced antioxidative effects of GST-P[55, 56] owing to the expression of the inserted transgene may be of prime significance. Using recombinant human GST isozymes, Berhane *et al*. recently demonstrated that GST-P1-1 (a human analogue of GST-P) is associated with protection against oxidative damage to nucleic acids, whereas GST-A1-1 (a human analogue of GST-A) is linked to protection against lipid peroxidation.[57] If rat GSTs act similarly to human GSTs, enhanced expression of GST-P by transgene insertion might be expected to efficiently protect liver DNA from oxidative stress. This would explain the lack of liver damage and 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,[20, 28] GST-P appears to provide such protection.

In conclusion, the insertion of rat *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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REFERENCES

1) Chasseaud, L. F. The role of glutathione and glutathione *S*-transferase in the metabolism of chemical carcinogens and other electrophilic agents. *Adv. Cancer Res.*, **29**, 175–274 (1979).
2) Mannervik, B. and Danielson, U. H. Glutathione transferases—structure and catalytic activity. *CRC Crit. Rev. Biochem. Mol. Biol.*, **23**, 283–337 (1988).
3) Sugioka, Y., Fujii-Kuriyama, Y., Kitagawa, T. and Muramatsu, M. Changes in polypeptide pattern of rat liver cells during chemical hepatocarcinogenesis. *Cancer Res.*, **45**, 365–378 (1985).
4) Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. and Sato, K. Purification, induction and distribution of placental glutathione transferase: a new marker enzyme for preneoplastic cells in the rat chemical carcinogenesis. *Proc. Natl. Acad. Sci. USA*, **82**, 3964–3968 (1985).
5) Sato, K. Glutathione *S*-transferases and hepatocarcinogenesis. *Jpn. J. Cancer Res.* (*Gann*), **79**, 556–572 (1988).
6) Ito, N., Tatematsu, M., Hasegawa, R. and Tsuda, H. Medium-term bioassay system for detection of carcinogens and modifiers of hepatocarcinogenesis utilizing the GST-P positive liver cell focus as an endpoint marker. *Toxicol. Pathol.*, **17**, 630–641 (1989).
7) Ito, N., Hasegawa, R., Imaida, K., Hirose, M., Shirai, T., Tamano, S. and Hagiwara, A. Medium-term rat liver bioassay for rapid detection of hepatocarcinogenic sub-

stances. *J. Toxicol. Pathol.*, **10**, 1–11 (1997).
8) Tatematsu, M., Mera, Y., Ito, N., Satoh, K. and Sato, K. Relative merits of immunohistochemical demonstrations of placental, A, B and C forms of glutathione *S*-transferase and histochemical demonstration of γ-glutamyl transferase as markers of altered foci during liver carcinogenesis in rats. *Carcinogenesis*, **6**, 1621–1626 (1985).
9) Hendrich, S., Campbell, H. A. and Pitot, H. C. Quantitative stereological evaluation of four histochemical markers of altered foci in multistage hepatocarcinogenesis in the rat. *Carcinogenesis*, **8**, 1245–1250 (1987).
10) Sugioka, Y., Fujii-Kuriyama, Y., Kitagawa, T. and Muramatsu, M. Cloning and the nucleotide sequence of rat glutathione *S*-transferase P cDNA. *Nucleic Acid Res.*, **13**, 6049–6057 (1985).
11) Okuda, A., Sakai, M. and Muramatsu, M. The structure of the rat glutathione *S*-transferase P gene and related pseudogenes. *J. Biol. Chem.*, **262**, 3858–3863 (1987).
12) Sakai, M., Okuda, A. and Muramatsu, M. Multiple regulatory elements and phorbol 12-*O*-tetradecanoate 13-acetate responsiveness of the rat placental glutathione transferase gene. *Proc. Natl. Acad. Sci. USA*, **85**, 9456–9460 (1988).
13) Okuda, A., Imagawa, M., Maeda, Y., Sakai, M. and Muramatsu, M. Structure and functional analysis of an enhancer GPEI having a phorbol 12-*O*-tetradecanoate 13-acetate responsive element-like sequence found in the rat glutathione *S*-transferase P gene. *J. Biol. Chem.*, **264**, 16919–16926 (1989).
14) Okuda, A., Imagawa, M., Sakai, M. and Muramatsu, M. Functional cooperativity between two TPA responsive elements in undifferentiated F9 embryonic stem cells. *EMBO J.*, **9**, 1131–1135 (1990).
15) Morimura, S., Suzuki, T., Hochi, I., Yuki, A., Nomura, K., Kitagawa, T., Nagatsu, I., Imagawa, M. and Muramatsu, M. Trans-activation of glutathione transferase P gene during chemical hepatocarcinogenesis of the rat. *Proc. Natl. Acad. Sci. USA*, **90**, 2065–2068 (1993).
16) Suzuki, T., Imagawa, M., Hirabayashi, M., Yuki, A., Hisatake, K., Nomura, K., Kitagawa, T. and Muramatsu, M. Identification of an enhancer responsible for tumor marker gene expression by means of transgenic rats. *Cancer Res.*, **55**, 2651–2655 (1995).
17) Suzuki, T., Morimura, S., Diccianni, M. B., Yamada, R., Hochi, S., Hirabayashi, M., Yuki, A., Nomura, K., Kitagawa, T., Imagawa, M. and Muramatsu, M. Activation of glutathione transferase P gene by lead requires glutathione transferase P enhancer I. *J. Biol. Chem.*, **271**, 1626–1632 (1996).
18) Farber, E. The biochemistry of preneoplastic liver: a common metabolic pattern in hepatocyte nodules. *Can. J. Biochem. Cell Biol.*, **62**, 486–494 (1984).
19) Roomi, M. W., Ho, R. K., Sarma, D. S. R. and Farber, E. A common biochemical pattern in preneoplastic hepatocyte nodules generated in four different models in the rat. *Cancer Res.*, **45**, 564–571 (1985).
20) Farber, E. The step-by-step development of epithelial cancer: from phenotype to genotype. *Adv. Cancer Res.*, **70**, 21–48 (1996).
21) Pegg, A. E. Formation and metabolism of alkylated nucleosides; possible role in carcinogenesis by nitroso-compounds and alkylating agents. *Adv. Cancer Res.*, **25**, 195–269 (1977).
22) Saffhill, R., Margison, G. P. and O'Connor, P. J. Mechanisms of carcinogenesis induced by alkylating agents. *Biochim. Biophys. Acta*, **823**, 111–145 (1985).
23) Nakae, D., Yoshiji, H., Mizumoto, Y., Horiguchi, K., Shiraiwa, K., Tamura, K., Denda, A. and Konishi, Y. High incidence of hepatocellular carcinomas induced by a choline deficient L-amino acid defined diet in rats. *Cancer Res.*, **52**, 5042–5045 (1992).
24) Denda, A., Tang, Q., Endoh, T., Tsujiuchi, T., Horiguchi, K., Noguchi, O., Mizumoto, Y., Nakae, D. and Konishi, Y. Prevention by acetylsalicylic acid of liver cirrhosis and carcinogenesis as well as generation of 8-hydroxydeoxyguanosine and thiobarbituric acid-reactive substances caused by a choline-deficient, L-amino acid-defined diet in rats. *Carcinogenesis*, **15**, 1279–1283 (1994).
25) Endoh, T., Tang, Q., Denda, A., Noguchi, O., Kobayashi, E., Tamura, K., Horiguchi, K., Ogasawara, H., Tsujiuchi, T., Nakae, D., Sugimura, M. and Konishi, Y. Inhibition by acetylsalicylic acid, a cyclooxygenase inhibitor, and *p*-bromophenacyl bromide, a phospholipase A_2 inhibitor, of both cirrhosis and enzyme-altered nodules caused by a choline-deficient, L-amino acid-defined diet in rats. *Carcinogenesis*, **17**, 467–475 (1996).
26) Nakae, D., Yoshiji, H., Maruyama, H., Kinugasa, T., Denda, A. and Konishi, Y. Production of both 8-hydroxydeoxyguanosine in liver DNA and γ-glutamyltransferase-positive hepatocellular lesions in rats given a choline-deficient, L-amino acid-defined diet. *Jpn. J. Cancer Res.*, **81**, 1081–1084 (1990).
27) Hochi, S., Ninomiya, T., Honma, M. and Yuki, A. Successful production of transgenic rats. *Anim. Biotechnol.*, **1**, 175–184 (1990).
28) Cayama, E., Tsuda, H., Sarma, D. S. R. and Farber, E. Initiation of chemical carcinogenesis requires cell proliferation. *Nature*, **275**, 60–61 (1978).
29) Rutenberg, A. M., Kim, H., Fishbein, J., Hauker, J. S., Wasserkrug, H. C. and Seligman, R. Histochemical and ultrastructural demonstration of γ-glutamyltranspeptidase activity. *J. Histochem. Cytochem.*, **17**, 517–526 (1968).
30) Obara, T., Makino, T., Ura, H., Yokose, Y., Kinugasa, T., Moore, M. A., Sato, K. and Konishi, Y. Comparative histochemical investigation of the glutathione *S*-tranferase placental form and γ-glutamyltranspeptidase during *N*-nitrosobis(2-hydroxypropyl)amine-induced pancreatic carcinogenesis in hamster. *Carcinogenesis*, **7**, 801–805 (1986).
31) Mizumoto, Y., Nakae, D., Yoshiji, H., Andoh, N., Horiguchi, K., Endoh, T., Kobayashi, E., Tsujiuchi, T., Shimoji, N., Denda, A., Tsujii, T., Nagao, M., Wakabayashi, K. and Konishi, Y. Inhibitory effects of 2-*O*-octadecylascorbic acid and other vitamin C and E derivatives on the induction of enzyme-altered putative preneoplastic lesions in the livers of rats fed a choline-deficient, L-amino

acid-defined diet. *Carcinogenesis*, **15**, 241–246 (1994).
32) Campbell, H. A., Pitot, H. C., Potter, V. R. and Laishes, B. A. Application of quantitative stereology to the evaluation of enzyme-altered foci in rat liver. *Cancer Res.*, **42**, 465–472 (1982).
33) Tsutsumi, Y., Serizawa, A. and Kawai, K. Enhanced polymer one-step staining (EPOS) for proliferating cell nuclear antigen (PCNA) and Ki-67 antigen: application to intraoperative frozen diagnosis. *Pathol. Int.*, **45**, 108–115 (1995).
34) van der Loos, C. M., Maruko, T. and Becker, A. E. The use of enhanced polymer one-step staining reagents for immunoenzyme double-staining. *Histochem. J.*, **28**, 709–714 (1996).
35) Nakae, D., Mizumoto, Y., Kobayashi, E., Noguchi, O. and Konishi, Y. Improved genomic/nuclear DNA extraction for 8-hydroxydeoxyguanosine analysis of small amounts of rat liver tissue. *Cancer Lett.*, **97**, 233–239 (1995).
36) Folch, J. L. A simple method for isolation and purification of total lipids from animal tissues. *J. Biol. Chem.*, **226**, 497–509 (1957).
37) Spayd, R. W., Bruschi, B. A., Dappen, G. M., Eikenberry, J. N., Esders, T. W., Figueras, J., Goodhue, C. T., LaRossa, D. D., Nelson, R. W., Rand, R. N. and Wu, T. W. Multilayer film elements for clinical analysis: applications to representative chemical determinations. *Clin. Chem.*, **24**, 1343–1350 (1978).
38) Kivirikko, K. I., Lailinen, O. and Prockop, D. J. Modification of a specific assay for hydroxyproline in urine. *Anal. Biochem.*, **19**, 249–255 (1967).
39) Tamura, K., Nakae, D., Horiguchi, K., Akai, H., Kobayashi, Y., Andoh, N., Satoh, H., Denda, A., Tsujiuchi, T., Yoshiji, H. and Konishi, Y. Inhibition by *N*-(4-hydroxyphenyl)retinamide and all-*trans*-retinoic acid of exogenous and endogenous development of putative preneoplastic, glutathione *S*-transferase placental form-positive lesions in the livers of rats. *Carcinogenesis*, **18**, 2133–2141 (1997).
40) Bergmeyer, H. U., Scheibe, P. and Wahlefeld, A. W. Optimization of methods for aspartate aminotransferase and alanine aminotransferase. *Clin. Chem.*, **24**, 58–73 (1978).
41) Nakae, D., Kobayashi, Y., Akai, H., Andoh, N., Satoh, H., Ohashi, K., Tsutsumi, M. and Konishi, Y. Involvement of 8-hydroxyguanine formation in the initiation of rat liver carcinogenesis by low dose levels of *N*-nitrosodiethylamine. *Cancer Res.*, **57**, 1281–1287 (1997).
42) Denda, A., Endoh, T., Kitayama, W., Tang, Q., Noguchi, O., Kobayashi, Y., Akai, H., Okajima, E., Tsujiuchi, T., Tsutsumi, M., Nakae, D. and Konishi, Y. Inhibition by piroxicam of oxidative DNA damage, liver cirrhosis and development of enzyme-altered nodules by a choline-deficient, L-amino acid-defined diet in rats. *Carcinogenesis*, **18**, 1921–1930 (1997).
43) Scherer, E., Steward, A. P. and Emmelot, P. Kinetics of formation of O^6-ethylguanine in, and its removal from liver DNA of rats receiving diethylnitrosamine. *Chem.-Biol. Interact.*, **19**, 1–11 (1977).
44) Scherer, E., Timmer, A. P. and Emmelot, P. Formation by diethylnitrosamine and persistence of O^4-ethylthymidine in rat liver DNA *in vivo*. *Cancer Lett.*, **10**, 1–6 (1980).
45) Williams, G. M., Iatropoulos, M. J., Wang, C. X., Ali, N., Riverson, A., Peterson, L. A., Schultz, C. and Gebhardt, R. Diethylnitrosamine exposure-responses for DNA damage, centrilobular cytotoxicity, cell proliferation and carcinogenesis in rat liver exhibit some non-linearities. *Carcinogenesis*, **17**, 2253–2258 (1996).
46) Suzuki, T., Imagawa, M., Yamada, R., Yokoyama, K., Kondo, S., Itakura, K. and Muramatsu, M. Acute changes in liver gene expression in the *N*-nitrosodiethylamine-treated rat. *Carcinogenesis*, **15**, 1759–1761 (1994).
47) Kitagawa, T. Histochemical analysis of hyperplastic lesions and hepatomas of the liver of rats fed 2-fluorenylacetamide. *Gann*, **62**, 207–216 (1971).
48) Kitagawa, T. and Pitot, H. C. The regulation of serine dehydratase and glucose-6-phosphatase in hyperplastic nodules of rat liver during diethylnitrosamine and *N*-2-fluorenylacetamide feeding. *Cancer Res.*, **35**, 1075–1084 (1975).
49) Kitagawa, T. Sequential phenotypic changes in hyperplastic areas during hepatocarcinogenesis in the rat. *Cancer Res.*, **36**, 2534–2539 (1976).
50) Enomoto, K. and Farber, E. Kinetics of phenotypic maturation of remodeling of hyperplastic nodules during liver carcinogenesis. *Cancer Res.*, **42**, 2330–2335 (1982).
51) Tatematsu, M., Nagamine, T. and Farber, E. Redifferentiation as a basis for remodeling of carcinogen-induced hepatocyte nodules to normal appearing liver. *Cancer Res.*, **43**, 5049–5058 (1983).
52) Rotstein, J., Sarma, D. S. R. and Farber, E. Cell cycle kinetics of rat hepatocytes in early putative preneoplastic lesions in hepatocarcinogenesis. *Cancer Res.*, **44**, 2913–2917 (1984).
53) Farber, E. Clonal adaptation during carcinogenesis. *Biochem. Pharmacol.*, **39**, 1837–1846 (1990).
54) Nakae, D., Mizumoto, Y., Yoshiji, H., Andoh, N., Horiguchi, K., Shiraiwa, K., Kobayashi, E., Endoh, T., Shimoji, N., Tamura, K., Tsujiuchi, T., Denda, A. and Konishi, Y. Different roles of 8-hydroxyguanine formation and 2-thiobarbituric acid-reacting substance generation in the early phase of liver carcinogenesis induced by a choline-deficient, L-amino acid-defined diet in rats. *Jpn. J. Cancer Res.*, **85**, 499–505 (1994).
55) Meyer, D. J., Beale, D., Tan, K. H., Coles, B. and Ketterer, B. Glutathione transferases in primary rat hepatomas: the isolation of a form with GSH peroxidase activity. *FEBS Lett.*, **184**, 139–143 (1985).
56) Robertson, I. G. C., Jensson, H., Mannervik, B. and Jernström, B. Glutathione transferases in rat lung: the presence of transferase 7-7, highly efficient in the conjugation of glutathione with the carcinogenic (+)-7β,8α-dihydroxy-9α,10α-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. *Carcinogenesis*, **7**, 295–299 (1986).
57) Berhane, K., Widersten, M., Engström, Å., Kowarichm, J. W. and Mannervik, B. Detoxication of base propenals and other α, β-unsaturated aldehyde products of radical reactions and lipid peroxidation by human glutathione transferases. *Proc. Natl. Acad. Sci. USA*, **91**, 1480–1484 (1994).