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π-Class Glutathione-S-Transferase-Positive Hepatocytes in Aging B6C3F1 Mice Undergo Apoptosis Induced by Dietary Restriction

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Liver sections from aging* ad libitum*-fed and diet-restricted B6C3F1 male mice were evaluated immunohistochemically for π-class glutathione S-transferase (GST-II). GST-II immunostaining of hepatocytes was diffuse and occurred in periportal regions of hepatic acinus, whereas perivenous areas were weakly stained or were stain-free. Expression of GST-II was significantly diminished in diet-restricted mice in all age groups and was associated with a marked decrease in liver tumor development. As most spontaneous liver tumors were GST-II positive, it can be speculated that they developed from GST-II-positive initiated hepatocytes. To determine whether dietary restriction induces apoptosis in GST-II-positive hepatocytes, 24-month-old* ad libitum*-fed mice were introduced to 40% diet restriction. After 1 week of diet restriction, a decrease in GST-II expression was associated with a threefold increase in the frequency of apoptotic bodies as detected by terminal deoxynucleotidyl transferase-mediated d-UTP nick end labeling of DNA fragments. A two-step immunohistochemical procedure revealed that approximately 70% of apoptotic bodies were GST-II positive. These results suggest that spontaneous, potentially preneoplastic hepatocytes in tumor-prone B6C3F1 mice are eliminated by apoptosis with dietary restriction. (Am J Pathol 1996, 149:1585–1591)

It has been unequivocally demonstrated that dietary restriction (DR) reduces the incidence of spontaneous and chemically induced tumors in rodents and increases their life span relative to *ad libitum*-fed (AL) counterparts.[1–3] The mechanistic basis for this phenomenon remains unclear. Experimental studies have suggested that apoptotic cell death provides a protective mechanism by removing senescent, DNA-damaged, or diseased cells that could interfere with normal function or lead to neoplastic transformation.[4] Recently, evidence has been presented to indicate that DR eliminates preneoplastic cells through apoptosis.[5,6]

Hepatocellular preneoplastic foci and tumors in F344 rats are immunoreactive for π-class glutathione S-transferase (GST-P) and appear to arise from single GST-P-positive hepatocytes, which occur spontaneously as a function of age.[7] It has been suggested that the single GST-P-positive hepatocytes that occur after initiating doses of carcinogens or develop spontaneously in the livers of aging rats reflect the extent of initiation.[7–10] Unlike the rat, hepatocytes in the adult male mouse constitutively express an exceptionally high level of glutathione S-transferase II (GST-II), which is immunologically related to rat GST-P and is regulated by testosterone.[11] The physiological relevance of sex-specific expression of GST-II in mouse is not known; however, inbred male mice have a much higher frequency of spontaneous liver tumors than female mice.[12,13]

In the present study, using immunohistochemical techniques, we show the following: 1) in tumor-prone B6C3F1 male mice, GST-II expression in the liver is significantly decreased with DR and this decrease

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correlates with the reduced incidence of spontaneous liver tumors; 2) most murine spontaneous liver tumors are GST-II positive, regardless of strain, sex, or diet; 3) the proportion of GST-II-positive spontaneous liver tumors increases with age; and 4) GST-II-positive hepatocytes in aging B6C3F1 male mice can undergo apoptosis induced by DR.

Materials and Methods

Animals and Experimental Procedures

Male and female B6C3F1 and C57BL/6 mice were housed under specific-pathogen-free conditions with a 12-hour light/dark schedule and maintained as a part of the Project on Caloric Restriction at the National Center for Toxicological Research (Jefferson, AR) as described previously.[14] At 4 weeks of age, the mice were singly allocated to polycarbonate cages and fed *ad libitum* the NIH-31 open-formula diet (Purina Mills, Richmond, IN). At 14 weeks of age, a step-wise procedure was initiated in the animals allocated to the restricted diet to obtain the desired level of restriction (40%) over a 2-week period. The restricted diet (the same NIH-31 open-formula diet) was fortified with approximately 1.67 times the amount of vitamins as the *ad libitum* diet to insure that the DR mice consumed the same level of micronutrients as the AL animals. At 12, 18, 24, 30, and 36 months of age, 15 mice from each group were randomly selected and sacrificed between the hours of 10:00 and noon. All animals were examined for gross and microscopic pathological lesions.

To evaluate changes in apoptotic activity and GST-II expression with acute DR, the same step-wise procedure was initiated in a separate group (n = 15) of 24-month-old AL B6C3F1 male mice. Liver samples were taken 1 week and 3 weeks after reaching 40% DR.

Immunohistochemistry

Formalin-fixed, paraffin-embedded liver sections were processed for immunohistochemical demonstration of GST-II by the biotin-extravidin-peroxidase detection system (Sigma Chemical Co., St. Louis, MO). Primary antibodies (rabbit polyclonal anti-human GST-P, Dako Corp., Carpinteria, CA) were used at 1:200 dilution. The specificity of the Dako antibodies is similar to that reported for other polyclonal antibodies to GST-P.[15–17] Nonspecific staining was blocked with normal goat serum (Sigma). Liver sections were incubated in biotinylated goat anti-rabbit antibodies and extravidin-conjugated horseradish peroxidase. Staining was developed with diaminobenzidine substrate, and sections were counterstained with hematoxylin and analyzed using a binocular microscope (Nikon Biophot, Yokohama, Japan). For negative control, normal rabbit serum (Sigma) or phosphate-buffered saline (PBS) replaced the primary antibodies. Positive control for GST-P consisted of diethylnitrosamine-(DEN)-initiated rat liver foci. The percentage of spontaneous liver tumors that were GST-II positive was determined in aging AL and DR B6C3F1 and C57BL/6 mice of both sexes.

To facilitate identification of apoptotic bodies (ABs), terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP nick end labeling (TUNEL) of DNA fragments was used (Oncor ApopTag Detection System, Gaithersburg, MD). Briefly, permeabilized liver sections were enzymatically labeled with digoxigenin-nucleotide via TdT and subsequently exposed to horseradish-peroxidase-conjugated anti-digoxigenin antibody. Staining was developed in diaminobenzidine and sections were counterstained with methyl green. The slides were viewed with a 60× objective on a binocular microscope (Nikon Biophot) and the image was captured using a color video camera (World Video, Boyertown, PA). An Optimas image analysis system (Optimas Corp., Seattle, WA) was used to quantify the percent incidence of ABs in 600 microscopic images (~15,000 hepatocytes) per animal.

For detection of GST-II-positive ABs, the TUNEL procedure was followed by GST-II labeling using the biotin-extravidin-alkaline phosphatase detection system (Sigma). Staining was developed with New Fuchsin substrate (Biogenex, San Ramon, CA). Normal rabbit serum replaced anti-GST-P antibodies in negative controls. The DEN-treated rat liver sections served as positive controls. The percent incidence of GST-II-positive (alkaline-phosphatase-stained) ABs was obtained by dividing the total number of GST-II-positive ABs by the total number of ABs counted per liver section and expressed per 100 cells. To avoid the possible systemic influence of distant tumors on apoptotic rate, the ABs were counted in livers obtained from animals confirmed to be free of apparent neoplasms by gross necropsy and microscopic examination of major organs.

Analysis for statistically significant differences between means was done by Student's *t*-test.

Results and Discussion

For immunohistochemical demonstration of GST-II expression in the liver, two different murine strains

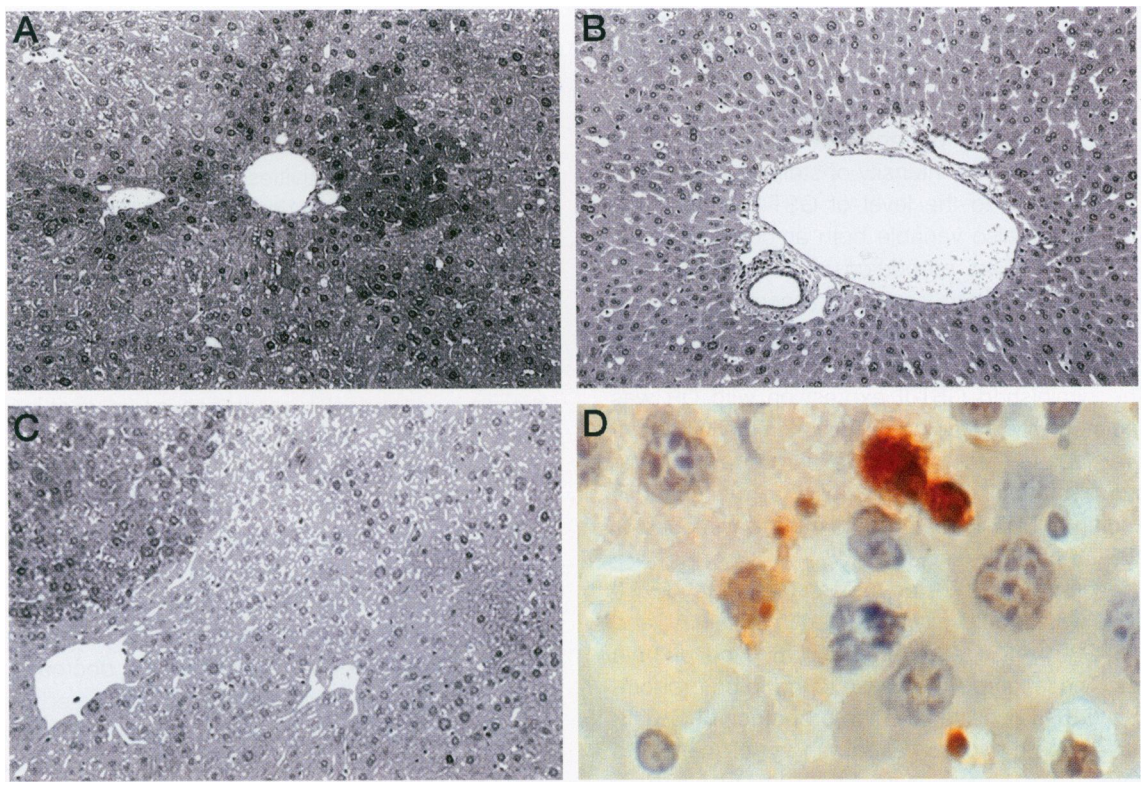

Figure 1. *Immunohistochemical demonstration of GST-II in livers of male AL and DR B6C3F1 mice.* **A:** *Normal liver of a 30-month-old AL mouse. Magnification, ×90.* **B:** *Normal liver of a 30-month-old DR mouse. Magnification, ×90.* **C:** *Spontaneous hepatic adenoma of a 30-month-old AL mouse. Magnification, ×90.* **D:** *Representative example of GST-II-positive ABs in the liver of a 24-month-old mouse at 1 week after DR initiation. Alkaline phosphatase staining is red; magnification, ×700.* **A, B,** *and* **C** *liver sections were processed simultaneously.*

were evaluated. The B6C3F1 murine strain is known to develop a high incidence of spontaneous liver tumors by 18 months of age, whereas C57BL/6 mice have a low incidence of these tumors and a diminished response to chemical carcinogens.[18] In hepatocytes of AL male mice of both strains, GST-II staining was diffuse and occurred in periportal regions of hepatic acinus, whereas perivenous areas were usually weakly stained or were stain-free (Figure 1A). The diffuse pattern of GST-II immunostaining is consistent with previous observations in mice[19] and is different from that observed in aging rats, in which GST-P-positive hepatocytes usually occur in distinct foci.[7,8] GST-II staining was predominantly cytoplasmic; however, nuclear staining was also apparent in some hepatocytes (Figure 1A). GST-II expression in the livers of DR B6C3F1 and C57BL/6 male mice was significantly decreased relative to AL mice; eg, the extensive GST-II immunostaining of periportal regions observed in AL mice was virtually nonexistent in the livers of DR animals (Figure 1B).

Spontaneous liver tumors in AL B6C3F1 male mice were predominantly (75%) GST-II positive (Table 1; Figure 1C), although the staining was uneven.

Table 1. *Proportion of GST-II-Positive Spontaneous Hepatocellular Tumors of B6C3F1 and C57BL/6 Mice as a Function of Age and Diet*

Murine strain	Sex	Diet	Proportion of GST-II$^+$ liver tumors (GST-II$^+$/total in each age group)					Total proportion of GST-II$^+$ tumors
			12 months	18 months	24 months	30 months	36 months	
B6C3F1	M	AL	0/0	1/4	2/3	4/5	8/8	15/22 (75%)
B6C3F1	M	DR	0/0	0/0	0/0	3/3	0/0	3/3 (100%)
B6C3F1	F	AL	0/0	0/1	0/1	1/2	4/6	5/10 (50%)
B6C3F1	F	DR	0/0	0/0	0/0	0/0	5/5	5/5 (100%)
C57BL/6	M	AL	0/0	0/0	0/1	1/1	1/1	2/3 (67 %)
C57BL/6	F	AL	0/0	0/0	0/0	1/1	–*	1/1 (100%)

n = 14 to 15 per each age group. M, male; F, female. No hepatocellular tumors were found in DR C57BL/6 mice.
*No AL C57BL/6 female mice were alive at 36 months.

In many tumors, the positively stained cells occurred in multiple clusters or foci that were interspersed among unstained neoplastic liver cells, without discernible pattern. There was no special tendency for the cells toward the periphery of the tumor to stain more positively. The intensity of staining, inferred to be proportional to the level of GST-II antigen expressed, was also variable both among and within positive foci. The proportion of tumors that expressed GST-II progressively increased with age in AL B6C3F1 male mice, reaching 100% by 36 months of age (Table 1).

The diminished GST-II expression with DR was associated with a marked reduction in the incidence of liver tumors (Table 1). However, the few spontaneous hepatocellular neoplasms that did occur in 30-month-old DR B6C3F1 male mice were found to be GST-II positive.

The proportion of liver tumors that were GST-II positive was additionally evaluated in female AL and DR B6C3F1 mice and AL C57BL/6 mice of both sexes. These tumors were also found to be predominantly GST-II positive, and the proportion of GST-II-positive tumors also increased with age (Table 1). No hepatocellular tumors were found in DR C57BL/6 male and female mice in any age group. The fact that the few liver tumors that developed in AL C57BL/6 mice were GST-II positive indicates that rare initiated cells must express GST-II during tumor promotion.

As most liver tumors in the present study were GST-II positive, it is highly likely that they developed from GST-II-positive initiated hepatocytes. The increase in the proportion of GST-II-positive tumors with age is of particular interest and may be related to oncogene activation in the initiated cells. It is known that the GST gene sequence contains the TPA-(12-*O*-tetradecanoylphorbol-13-acetate)-responsive element, TRE.[20,21] The heterodimer of c-*jun* and c-*fos* products (AP-1) has high affinity for TRE and induces enhanced expression of the corresponding genes.[22,23] Consistent with this notion, simultaneous marked elevations of c-*jun*, c-*fos*, and GST-P gene transcript levels have been observed in the livers of aging rats.[24]

It should be noted that contrary results have been published by Hatayama et al,[19] who found a decrease in GST-II expression in DEN-initiated preneoplastic foci of B6C3F1 male mice. Similarly, Nakano et al[25] have shown decreased GST-II expression in the majority of c-Jun-positive DEN-induced foci in male B6C3F1 mice, as compared with the surrounding c-Jun-negative parenchyma. However, these studies were conducted in relatively young (3- to 9-month-old) F1 hybrids with chemically induced foci that may not be comparable to spontaneous tumors in the highly inbred senescent mice used in the present study. GST-II expression in male mouse liver is known to be specifically regulated by testosterone,[11] and the androgen receptor belongs to the glucocorticoid receptor superfamily.[26] Interestingly, the transcriptional activities of the Jun/Fos complex and the glucocorticoid receptor have been reported to be inhibited by each other due to direct interaction between the receptor and either c-Jun or c-Fos.[27,28] Because the androgen receptor transcriptional activity in the liver declines with age,[29–31] it is likely that the transformed GST-II phenotype has become androgen independent in spontaneous tumors of aging male mice in the present study.

Among the three forms of mouse hepatic glutathione S-transferase, GST-II is the major form found in adult male mice, and the expression of this enzyme has been shown to be specifically regulated by testosterone.[11] Previous studies have shown that DR animals have reduced circulating levels of testosterone.[32,33] Therefore, it is likely that the decrease in GST-II expression in the DR mice is mediated by a decrease in testosterone level. Testosterone withdrawal is also a potent inducer of apoptotic cell death[34] and may additionally be related to the increased apoptotic activity observed in DR mice.[5,35] Recently, we demonstrated that chronic DR of 12-month-old B6C3F1 mice significantly increased the apoptotic activity in preneoplastic liver.[5] We then provided evidence that the reduced rate of aging and tumor incidence with DR may relate to enhanced apoptotic activity.[35] Briefly, the apoptotic activity was increased with aging in the livers of both AL and DR B6C3F1 male mice. We hypothesized that the progressive increase in apoptotic rate observed in mice of both diet groups may reflect a physiological response to the accumulation of genetic aberrations in hepatocytes as a function of age in this model. This explanation would be consistent with the proposal that apoptotic cell death is a protective mechanism for the maintenance of genotypic and phenotypic fidelity in multicellular organisms.[36] A comparison between diet groups revealed that the apoptotic activity was consistently higher in the DR animals relative to AL animals from 12 months to 30 months of age. The increase in the apoptotic activity in the livers of DR mice was associated with a significant decrease in the incidence of spontaneous liver tumors and increase in the life span relative to AL mice. Similarly, Grasl-Kraupp et al[6] have demonstrated that food restriction enhances apoptosis in mitogen-induced and spontaneously occurring putative preneoplastic foci of rat liver.

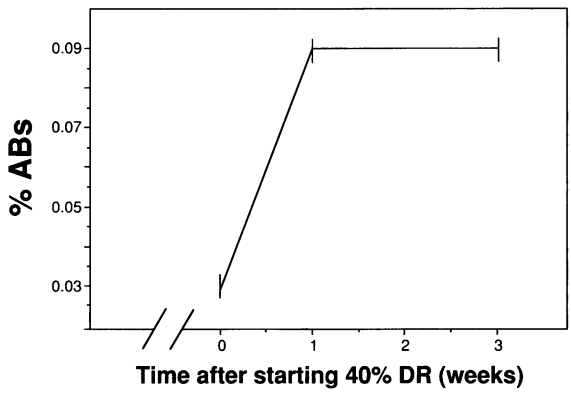

Figure 2. *Effect of 40% DR on apoptotic activity in the livers of 24-month-old male B6C3F1 mice. Data are the means ± SEM from four animals* ($P < 0.01$, *AL mice* versus *DR mice*).

Recent evidence indicates that initiated cells may be more sensitive to apoptosis than normal cells.[4,37,38] For example, hepatocytes in putative preneoplastic foci exhibit an approximately 10-fold higher rate of apoptosis than normal cells.[38] Thus, it is possible that GST-II-positive hepatocytes (potentially preneoplastic in aging mice) could be eliminated through apoptosis induced by DR. To study this possibility, 24-month-old AL B6C3F1 male mice were introduced to 40% DR. When food intake was reduced to 40% for 3 weeks, the average body weight and liver mass declined by 21.4% and 15.2%, respectively. A progressive decline in GST-II immunoreactivity was observed with only a few single GST-II-positive hepatocytes remaining after 3 weeks of DR. The decrease in GST-II labeling was accompanied by a 3-fold increase in apoptotic activity after 1 week of DR; this increase in apoptotic activity with DR was maintained for at least two weeks (Figure 2). It is noteworthy that the apoptotic activity after short-term DR was in the same range as that reported previously with chronic DR.[5,35] The proportion of ABs that were GST-II positive was evaluated at 1 week after DR initiation. At this time, apoptotic activity had already increased, but GST-II immunoreactivity of the livers was still detectable. Quantitative analysis revealed that most ABs (71.9 ± 1.3%) were GST-II positive (Figure 1D). The specificity of GST-II staining of ABs was confirmed by staining of DEN-treated GST-P-positive rat liver foci as positive control. Within preneoplastic foci in these liver sections, a large number of alkaline-phosphatase-stained (GST-P-positive) ABs were present, whereas ABs in neighboring nonhyperplastic liver tissue were not stained. Although it is difficult to determine whether the observed increase in apoptotic activity in the present study represents a selective death of initiated cells, these results indicate that a large proportion of GST-II-positive hepatocytes can be eliminated through apoptosis induced by DR. There are, however, a certain number of apoptosis-resistant subclones of GST-II-positive initiated hepatocytes as evidenced by the GST-II-positive tumors that developed in DR mice (Table 1).

Poole and Drinkwater[39] recently reported that a decrease in testosterone levels appears to promote tumor resistance. The multiplicity of liver tumors in their study was reduced in castrated male mice of different strains, indicating that androgen withdrawal is protective against development of hepatic tumors. Previous studies have established that, in addition to reduced levels of testosterone,[32,33] DR rodents have reduced basal levels of several trophic hormones including insulin, prolactin, and growth hormone.[40,41] Reduced availability of trophic factors increases the incidence of apoptosis in both *in vitro*[42] and *in vivo*[43] systems. Thus, liver tumor resistance and prolongation of life span with chronic DR may be due, in part, to up-regulation of apoptosis induced by the decrease in levels of testosterone and other trophic factors with this dietary intervention.

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