Influence of Ursodeoxycholate-Enriched Diet on Liver Tumor Growth in HBV Transgenic Mice

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Hepatitis B virus (HBV) transgenic mice (official designation, Tg [Alb-1 HBV] Bri 44) invariably develop macroscopically evident tumors within the 20th month of life. Sustained proliferative activity seems to play an important role in the development of these lesions. We previously showed that ursodeoxycholate (UDC) stimulates hepatocyte proliferation in various experimental settings. Herein, we tested the assumption that biological factors able to further increase liver cell proliferation, such as UDC, could accelerate tumor development in this animal model. For this study, 22 eight-week-old male transgenic mice were divided into 2 groups; 11 animals received a standard diet, and 11 received a UDC-enriched diet. The 2 groups were further divided into 2 subgroups of 5 and 6 animals each and were sacrificed at 3 and 15 months of age, respectively. These different times were chosen to exclude diet-related toxicity (in 3-month-old mice) and evaluate tumor growth (in 15-month-old mice). In addition, hepatocyte proliferation was assessed in all animals. In 3-month-old mice receiving UDC, cholestatic and cytolytic indices as well as liver histology were comparable to those in controls. At 15 months, all UDC-treated mice showed large multinodular tumors whereas only 33% of controls developed smaller uninodular neoplasms. Hepatocyte proliferation was increased in all animals receiving UDC compared with controls. In conclusion, the increase in serum UDC (undetectable in mice fed a standard diet), in the absence of any toxic effect on the liver, suggests the involvement of this bile salt in the stimulation of hepatocyte proliferation and tumor growth. (HEPATOLOGY 2003;37:880-886.)

Transgenic mice with hepatitis B virus (HBV) sequences encoding for viral proteins pre-S, S, and X (official designation, Tg [Alb-1 HBV] Bri 44) develop progressive hepatocyte damage due to intracellular accumulation of these proteins.[1-5] During the first months of life, the hepatic damage consists of degenerative alterations.[2] Later, an active inflammatory response commences that induces a marked damage-related compensatory proliferative reaction.[3,5] Such a condition should be considered precancerous because it is constantly followed after the 8th to 9th month of life by the development of dysplastic hepatic lesions[3,4] that progress to overt liver cancer after the 12th month of life.[3] The progressive growth of these neoplastic lesions leads to macroscopic nodules that are invariably observed in all transgenic mice within the 20th month of life.[4]

Interestingly, these transgenic mice, harboring hepatocellular carcinomas, do not manifest any specific alteration of known oncogenes or tumor suppressor genes at any stage of neoplastic transformation.[6] It has therefore been suggested that the damage-related sustained proliferative activity observed in the liver of these animals could play an important role in tumor development.[5]

We have already shown that bile salts can stimulate proliferation in cultured hepatocytes and augment the regenerative response following 40% partial hepatectomy in rats.[7,8] Experimental data have also shown that bile salts increase proliferation in cholangiocytes and colonocytes,[9,10] which, like hepatocytes, are naturally exposed to

Abbreviations: HBV, hepatitis B virus; UDC, ursodeoxycholate; PCNA, proliferating cell nuclear antigen; LI, labeling index.

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the enterohepatic circulation of bile salts. Based on these premises, the aim of this study was to establish whether ursodeoxycholate (UDC) administered with the diet could modify the timing and magnitude of tumor development in HBV transgenic mice.

The rationale for using a UDC-enriched diet was based on the following considerations. (1) In a previous study, this diet significantly increased UDC levels in the circulating bile salt pool.[8] (2) In rats receiving different bile salt–enriched diets, the diet containing UDC determined the highest increase of proliferation in hepatocytes submaximally stimulated by 40% partial hepatectomy.[8] (3) UDC is widely used in the treatment of chronic liver diseases (*i.e.*, in pathologic conditions associated with an increased risk of hepatocellular carcinoma).[11]

To achieve our goal, transgenic mice were fed either a standard or a 0.2% UDC-enriched diet and sacrificed at either (1) 3 months to assess the effect of dietary treatment at the beginning of the hepatitic process in the absence of neoplastic lesions or (2) 15 months to establish whether this dietary treatment could influence the development of neoplastic foci spontaneously occurring in mice of this age.

Materials and Methods

Animals. A total of 22 eight-week-old male transgenic mice designed as Tg (Alb-1HBV) Bri 44 were obtained from Jackson Laboratories (Bar Harbor, ME). On arrival, each mouse was placed in a single cage to avoid animal fights and the possibility of spreading any infectious disease. The mice were kept in temperature-, air-, and light-controlled (light on from 7 AM to 7 PM) conditions and received food and water *ad libitum*. In addition, 10 eight-week-old male C75BL/6J mice were purchased from Charles River (Calco, Italy). All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals.

Chemicals. UDC, cholylglycine hydrolase from *Clostridium perfringens*, and 4-bromo-methyl-7-methoxycoumarin were purchased from Sigma Chemical Co. (St. Louis, MO). Glycoursodeoxycholic acid and 7α,12α-dihydroxy-5β-cholanic acid were purchased from Calbiochem Corp. (La Jolla, CA). Sep-Pak C_{18} cartridge was purchased from Waters (Milford, MA). Proteinase K and all reagents used for programmed cell death detection were purchased from Dako Corp. (Carpinteria, CA).

Dietary Treatment and Sample Collection. Transgenic mice were divided into 2 groups of 11 animals each and received *ad libitum* a standard diet (control group) and a standard diet enriched with 0.2% UDC (treated group), respectively. Similarly, the 10 C75BL/6J mice were divided into 2 groups and received a standard and a 0.2% UDC-enriched diet, respectively. The standard diet was composed of 53.5% carbohydrates, 18.5% proteins, 3% fats, 6% cellulose, 12% moisture, and 7% inorganic compounds and vitamins. This type of diet provided 315 kal/100 g.

The 2 groups of transgenic mice were further divided into 2 different subgroups of 5 and 6 animals each and were sacrificed at 3 and 15 months of age, respectively. All C75BL/6J mice were sacrificed after 1 month of dietary treatment.

Dietary intakes were monitored throughout treatment. At the time the mice were sacrificed, body weight was evaluated. All animals were then anesthetized with metaphane, blood samples were collected, and the entire liver was removed and weighed.

Liver Histopathology and Immunohistochemistry. The whole liver (with the exception of the small triangular lobe, which was snap frozen and stored at −80°C) was fixed in 10% neutral buffered formalin for 12 to 24 hours and sampled according to the step-serial, whole-specimen sectioning technique described by Lam et al.[12] Briefly, the liver was cut into thin (3-mm) serial slices that were progressively numbered and observed under a magnifying lens (×10). Gross findings were recorded in each case. Liver slices were then embedded in paraffin, cut at 4-μm thickness, and stained with hematoxylin-eosin, periodic acid–Schiff, and Gomori's reticulin.

All slides were evaluated for the presence of degenerative (vacuolar, hydropic, fat) changes, necrosis, portal and intralobular inflammation, and nodular regeneration. Each parameter was recorded using an arbitrary scale (absent, 0; focal, 1; diffuse, 2). The occurrence of adenomas (single vs. multiple) with or without hepatocellular dysplasia and/or hepatocellular carcinoma (uninodular vs. multinodular) was assessed in each case.

The liver obtained from C75BL/6J mice was fixed in 10% neutral buffered formalin, embedded in paraffin, cut at 4-μm thickness, and then stained with hematoxylin-eosin.

Proliferating Cell Nuclear Antigen Immunostaining and Labeling Index. The proliferative activity of liver cells was estimated by immunostaining detection of proliferating cell nuclear antigen (PCNA). This nuclear antigen is selectively expressed during the replicative phase of the cell cycle and is preserved in rat and mouse tissues,[13] differently from other well-known cell cycle–associated antigens (*e.g.*, Ki-67) that are not detectable in mouse tissues. PCNA was evaluated by the alkaline phosphatase/anti–alkaline phosphatase immunohistochemical method[14] using a mouse monoclonal antibody anti-PCNA (Dako, Glostrup, Denmark). The PCNA labeling

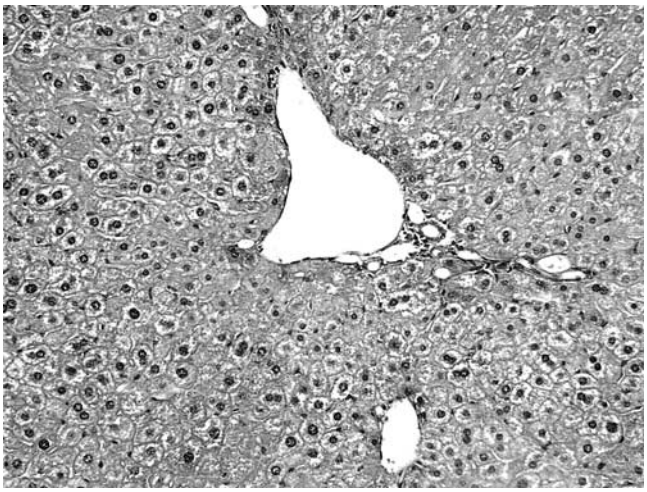

Fig. 1. Histologic appearance of a liver section from a 3-month-old mouse fed a standard diet. Hematoxylin-eosin–stained sections (original magnification ×200).

index (LI) was semiquantitatively evaluated by counting the percentage of immunoreactive hepatocellular nuclei in at least 10 randomly selected high-power (×400) fields. The PCNA-LI was separately assessed in the regenerative nodules, adenomas, and carcinomas.

Biochemical Determinations. Serum transaminase, γ-glutamyl transpeptidase, and alkaline phosphatase levels were determined by standard laboratory methods. Serum bile salts were evaluated using a modification of the methods described by Kamada et al.[15] and Street et al.[16] as described elsewhere.[8]

Statistical Analysis. The statistical evaluation of our results was performed by ANOVA and χ^2 test. When the single-factor ANOVA rejected the hypothesis of the mean equality among the groups, the Tukey test was applied for a comparison of the means in the different groups. In the latter case, only a P value less than .05 was considered significant.

Results

In normal C75BL/6J mice, the administration of a UDC-enriched diet did not produce any modification of histologic features, PCNA-LI, and cytolytic and cholestatic indices compared with a standard diet (data not shown). In 3-month-old transgenic mice fed UDC and standard diets, body and liver weights as well as gross liver features were similar (data not shown). Conventional liver histology showed comparable histopathologic features in the 2 groups. All animals had morphologically nonneoplastic livers with mild and focal degenerative changes, mainly of the vacuolar type. Mild portal inflammatory infiltration, occasionally associated with foci of hepatocellular necrosis, was also observed (Fig. 1). No hepatocytes with enlarged and hyperchromatic/pleomorphic nucleolated nuclei, qualifying for hepatocellular dysplasia, were identified at this stage.

In the 15-month-old UDC-treated transgenic mice, liver weight was significantly increased compared with controls (3.1 ± 0.7 g vs. 2.4 ± 0.1 g, respectively; $P <$ .05) whereas body weight remained unchanged. The increased liver weight observed in UDC-treated animals was due to diffuse enlargement of all hepatic lobes as well as the presence of enlarged nodular areas, altering the lobar profile. The latter feature was confirmed by the observation at low magnification of histologic sections (Fig. 2A). In these areas, higher magnification showed the presence of neoplastic lesions (Fig. 2B and C). Table 1 shows a summary of all pathologic findings observed in the liver from mice fed standard and UDC-enriched diets. Hepatocellular adenomas were detected in 2 standard-fed mice and 1 UDC-fed mouse. Interestingly, all mice fed the UDC-enriched diet developed hepatocellular carcinoma of the diffuse type, infiltrating almost the whole liver, whereas only 2 of 6 (33%) corresponding controls showed malignant tumors, only of the nodular type ($P <$ .01 by χ^2 test).

It is worthwhile noting that a significant increase in PCNA-LI was detected in 3-month-old UDC-treated transgenic mice compared with controls (0.9 ± 0.2 vs. 0.6 ± 0.2, respectively; $P <$ .05) (Fig. 3). A similar increase was also observed in normal tissues from 15-month-old UDC-treated transgenic mice versus controls (9.2 ± 4.9 vs. 6.4 ± 3.5; $P <$ .05) (Fig. 3).

The efficacy of the UDC-enriched diet in modifying serum bile salt levels was studied in 3-month-old transgenic mice (Table 2). Indeed, total bile salt concentration was almost doubled in UDC-treated animals compared with controls (13.3 ± 3.2 vs. 7.5 ± 1.7 μmol/L; $P <$ .05). This increase was primarily due to the appearance in the serum of UDC (3.7 ± 1.3 μmol/L), which was completely absent in animals fed a standard diet. In UDC-fed animals, the modification in the bile salt pool did not induce any toxic effect on the liver, as already shown at the histologic level and confirmed by the slight decrease in the cytolytic and cholestatic indices compared with controls (Fig. 4).

Discussion

Some mechanisms have been proposed to explain the involvement of HBV-related proteins in hepatocarcinogenesis in this animal model: (1) the induction of proliferation by the necroinflammatory process related to the accumulation of pre-S, S, and X proteins in the hepatocytes,[2] (2) the progressive reduction of apoptosis,[17] and (3) the deregulation of G_0 to G_1 cell cycle checkpoints

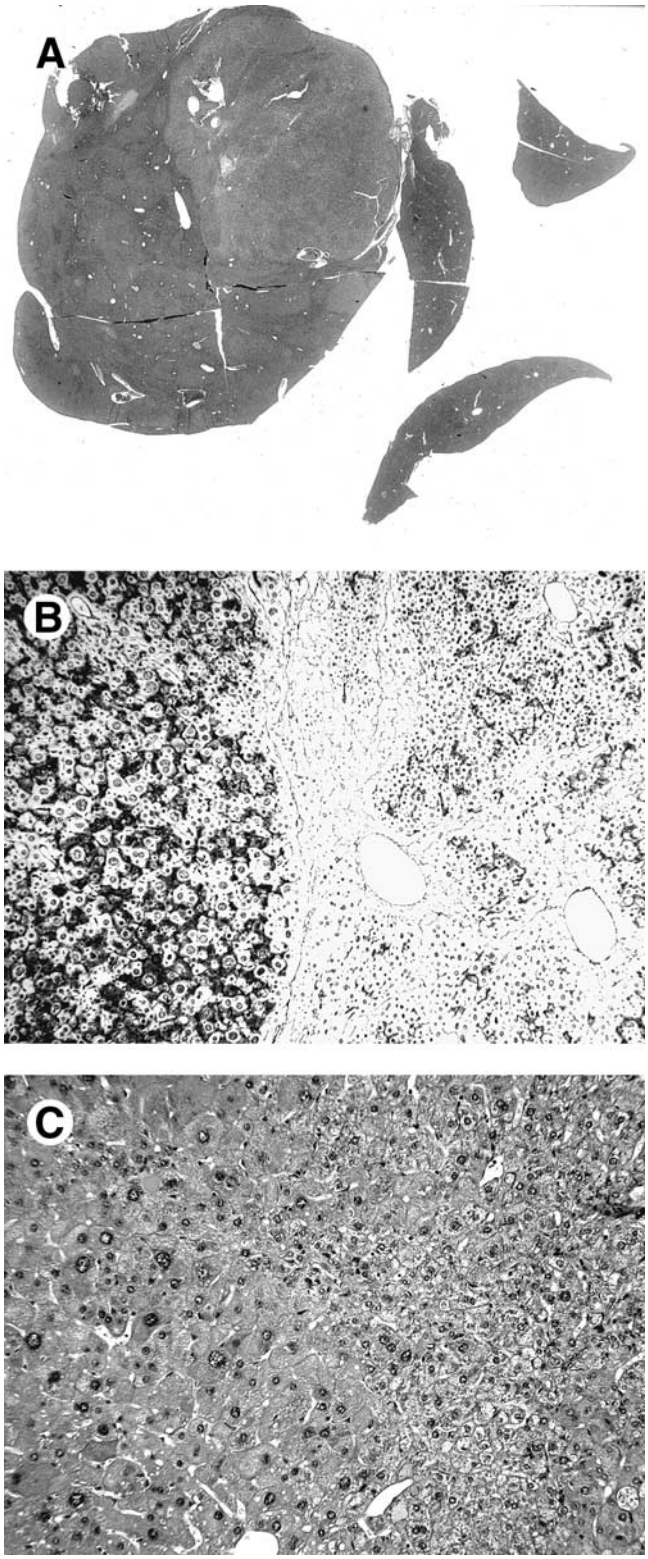

Fig. 2. Histologic appearance at different magnifications of tumoral lesions. (A) Periodic acid–Schiff–stained section (original magnification ×10) with a well-visible macronodule. (B) GOMORI-stained section (original magnification ×200) with a tumoral and adjacent "normal" area on the left and right side, respectively. (C) Hematoxylin-eosin–stained section (original magnification ×200) with a tumoral and adjacent "normal" area on the left and right side, respectively.

Table 1. Pathologic Features in Liver Specimens From 15-Month-Old Mice Fed Either a Standard or a UDC-Enriched Diet

	Standard	UDC
Degenerative changes	1.4 ± 0.5	1.4 ± 0.6
Chronic inflammation	1.1 ± 0.5	1.3 ± 0.4
Liver cell necrosis	1.2 ± 0.4	1.0 ± 0.7
Nodular regeneration	1.8 ± 0.4	2.0 ± 0.0
Adenomas		
Mice (affected/not affected)	2/6	1/5
Number	9	3
Size range (∅ mm)	0.8-3.3	1.2-2.9
Carcinomas		
Mice (affected/not affected)	2/6	5/5*
Number	5	Not evaluable†
Size range (∅ mm)	1.1-7.6	1.0-25‡

NOTE. The values reported were calculated as described in Materials and Methods.

*$P < .01$ by χ^2 test.

†All tumors showed extensive infiltration and involved almost the whole liver.

‡Some of the neoplastic foci were only histologically detectable.

induced by HBV-X protein[18] involving the reduction of $p21^{WAF1/CIP1}$ and the cyclin A promoter activation.[19,20]

We have previously hypothesized that in an organ like the liver, in which hepatocytes usually are in a quiescent state, a persistently elevated proliferative activity would enhance tumor development not only favoring genomic mutations but also inducing immunosuppression of the locoregional immune system. Indeed, the latter mechanism would act by enabling mutated cells to escape immunosurveillance.[21] Based on these considerations, in the present study we attempted to establish whether biological factors able to further increase liver cell proliferation could also accelerate tumor development in this model.

Before testing our hypothesis, we confirmed the high reliability of our animal model showing non-neoplastic histopathologic alterations (in particular, the accumulation of proteins pre-S, S, and X within hepatocytes) at 3 and 15 months as well as neoplastic lesions at 15 months in transgenic mice fed a standard diet, similar to those reported by other investigators in the liver of HBV transgenic mice of equivalent age.[2] In addition, we excluded any direct oncogenic effect of UDC based on a previous phase 2 study showing that mice receiving $1\ g \cdot kg^{-1} \cdot d^{-1}$ UDC (about twice our daily dosage) for 104 weeks did not develop any hepatic neoproliferative lesions.[22]

The first step of the present experimental design was to possibly document the mitogenic effect of UDC, as previously shown in 40% hepatectomized rats[8] and in humans (in patients affected by HCV-related chronic liver disease).[23] For this purpose, we used the pharmacologic dose of UDC previously used to obtain a maximal hepatocyte proliferative response in rats.[8] According to our hypothesis, the results reported in Fig. 3 show that, com-

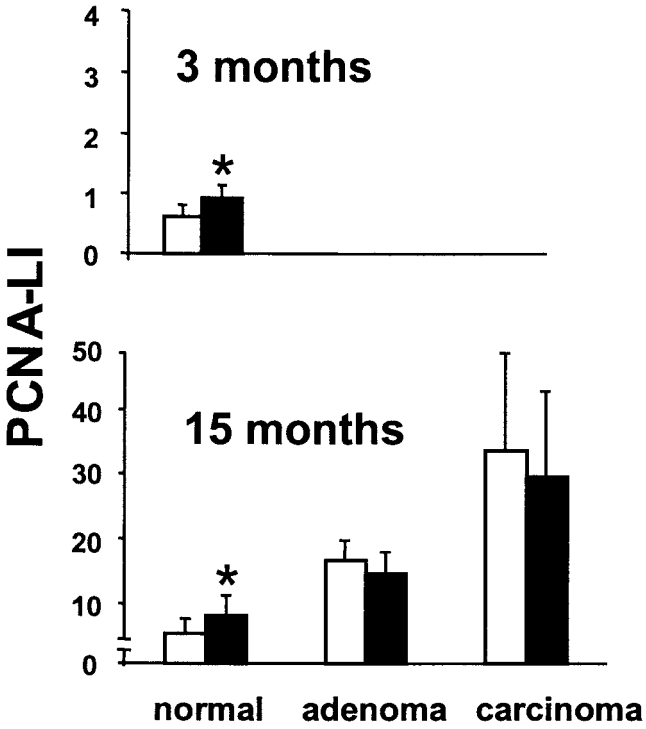

Fig. 3. PCNA-LI in normal and tumoral tissue from 3- and 15-month-old mice fed either standard or UDC-enriched diets. The values reported represent the mean ± SD obtained from all mice fed a standard (□) and UDC-enriched diet (■). *Significantly different compared with controls ($P < .05$)

pared with controls, 3- and 15-month-old UDC-treated mice showed increased hepatocyte proliferative activity. In 15-month-old mice, this increase was evident only in normal hepatocytes but not in malignant cells that manifest *per se* very high proliferative activity and PCNA-LI. In nontransgenic mice, the administration of a UDC-enriched diet did not produce any effect on proliferation (data not shown), confirming our previous findings in normal, untreated rats.[8]

To rule out the possibility that the increased proliferative activity could be related to dietary-induced liver damage and to establish whether there was any relationship between the observed mitotic activity and UDC levels, we evaluated the influence of a UDC-enriched diet on cholestasis and cytolysis indices, liver histology, and serum bile salt levels. These parameters were studied in 3-month-old mice because, at this age, only mild degenerative changes are observed in hepatocytes and no tumoral growth is detected at the microscopic level. In fact, the presence of large or infiltrating tumors, as observed at 15 months, could have caused *per se* liver damage and alterations in serum parameters.

A slight decrease in cytolytic and cholestatic parameters was observed in the UDC group at 3 months (Fig. 4), whereas the histologic findings remained unchanged compared with controls. Regarding the effect of a UDC-enriched diet on the endogenous bile salt pool, a marked increase in UDC level was observed in the serum (undetectable in mice fed the standard diet) that was responsible for 64% of the overall increase in total serum bile salt levels (Table 2). This modification, in the absence of any toxic effect on the liver, suggests an involvement of UDC in the stimulation of hepatocyte proliferation.

Table 2. Total and Single Serum Bile Salt Concentration in 3-Month-Old Transgenic Mice Fed Either Standard or UDC-Enriched Diets

Diet	Total BSs (μmol/L)	Single BSs (μmol/L)			
		CHO	DC	UDC	Others*
Standard	7.5 ± 1.7	7.2 ± 2.2	ND	ND	0.3 ± 0.2
UDC-enriched	13.3 ± 3.2	9.1 ± 4.7	ND	3.7 ± 1.3	0.5 ± 0.4
t Test	$P < .02$	NS	–	–	NS

NOTE. The values reported represent the mean ± SD of free and conjugated bile salt concentration.

Abbreviations: ND, not detectable; NS, not significant.

*This group includes unconjugated and conjugated hyodeoxycholic acid, muricholic acid, and other minor components.

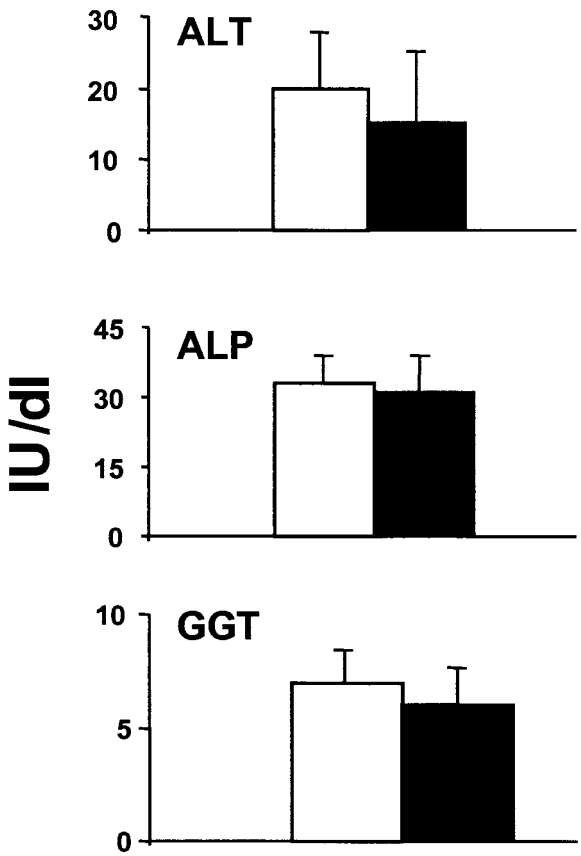

Fig. 4. Serum alanine aminotransferase, alkaline phosphatase, and γ-glutamyl transpeptidase levels in 3-month-old mice fed either standard- or UDC-enriched diets. The values reported represent the mean ± SD obtained from all mice fed standard- (□) and UDC-enriched (■) diets.

Our previous *in vitro* data, showing a dose-related stimulatory effect of UDC in primary cultured hepatocytes, support the hypothesis of a direct mitogenic effect of this bile salt.[7,8] On the other hand, the present findings confirm that, *in vivo*, UDC does not act as an initiator of hepatocyte proliferation but operates as an augmenter.[24] In this case, hepatocyte HBV-related protein accumulation represents the event that triggers proliferation that, in turn, is susceptible to amplification by UDC.

The hypothesis that a further increase in proliferation could accelerate tumor development was clearly shown by our results in 15-month-old mice receiving UDC (Table 1). In fact, these animals were all affected by macroscopically detectable, large multinodular tumors, whereas only 2 of 6 controls (33%) developed smaller neoplasms of the uninodular type.

With regard to the molecular mechanism(s) responsible for the stimulatory effect of UDC, some suggestions come from data in the literature. Our previous findings on rat hepatocyte cytosolic extracts show a direct stimulatory effect of UDC on protein kinase A and C,[25] 2 enzymes involved in signal transduction pathways, including proliferation.[26] More recently, *in vitro* experiments on a human cholangiocarcinoma cell line[27] and rat hepatocytes[28] have shown that bile salts are able to activate phosphorylation cascades stimulated by the epidermal growth factor receptor. Among key targets activated by this cascade, it is worthwhile mentioning cyclooxygenase 2, which seems to be involved in cell growth and colon carcinogenesis,[29] and Raf-1/MEK/ERK, a cascade involved in cell cycle regulation and cell proliferation.[30] Finally, preliminary data obtained in this study indicate a reduction of apoptosis in transgenic mice after the sixth month of life (data not shown). Such findings are in agreement with those reported in the literature on the ability of UDC to reduce programmed cell death[31-33] and also suggest a role for this mechanism in the tumor-promoting effect of UDC.

In conclusion, our data represent a direct demonstration that the stimulation of cell proliferation induced by long-term administration of UDC promotes tumor development in the liver of HBV transgenic mice. It should be noted that the dosage used by us[8] and by others[9] to prove the stimulatory effect of bile salts is far from that used for therapeutic purposes in humans. However, our previous findings showing that long-term administration of tauroursodeoxycholate was able to increase PCNA-LI in patients with chronic liver disease should be taken into account as well.[23] Based on these considerations, further studies with a dose-response curve are necessary to confirm the present findings and establish the lowest UDC tumor-promoting dose.

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