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Decreased Expression of Bcl-x Protein during Hepatocarcinogenesis Induced Exogenously and Endogenously in Rats

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Dysregulations of apoptosis have been widely recognized as important events in multi-stage carcinogenesis. Bcl-x, a member of the Bcl-2 family, is known to act as a regulator of apoptosis. The present study was conducted to assess the role of altered Bcl-x protein expression in exogenous and endogenous hepatocarcinogenesis in rats. In the short-term exogenous models, male Fischer 344 rats, 6 weeks old, were given a single intraperitoneal injection of diethylnitrosamine (DEN) at a dose of 200 mg/kg body weight, partially hepatectomized at the end of week 3, administered phenobarbital at a concentration of 0.05% from the end of week 2 for 6 weeks, and sacrificed. In the livers, glutathione *S*-transferase (GST-P)-positive, putative preneoplastic lesions were induced, and Bcl-x protein expression was decreased in 24.7% of such lesions. The incidence of GST-P-positive lesions with decreased Bcl-x increased depending on the size of the lesions; 18.9%, 32.4% and 86.5% in the lesions smaller than 0.03, between 0.03 and 0.3, and larger than 0.3 mm^2, respectively. In GST-P-positive lesions larger than 0.3 mm^2, both apoptosis induction and cell proliferation activity were enhanced when Bcl-x protein expression was decreased. In the long-term exogenous models, rats were given 10 mg/kg of DEN, partially hepatectomized 4 h after treatment, administered 0.5 mg/kg of colchicine at the end of days 1 and 3, subjected to a selection procedure, and sacrificed at the end of week 45. Hepatocellular carcinomas were induced with the decreased Bcl-x protein expression. In the endogenous model, rats were fed a choline-deficient, L-amino acid-defined diet for 16 or 80 weeks and sacrificed. Bcl-x protein expression was decreased both in GST-P-positive lesions and hepatocellular carcinoma. These results suggest that this decrease of Bcl-x protein might serve as an indicator of the advanced form of preneoplastic lesions, and that this decrease could also be associated with a potential to progress into carcinoma in both exogenous and endogenous hepatocarcinogenesis of rats.

Key words: Bcl-x — Rat — Hepatocarcinogenesis — Apoptosis — Cell proliferation

Dysregulations of apoptosis and cell cycle are important events in multi-stage hepatocarcinogenesis.[1)] The *bcl-x* gene was initially cloned as a homologue of the *bcl-2* gene, and it was later shown that alternative splicing of this gene produces three distinct mRNAs, *bcl-xL, bcl-xβ,* and *bcl-xS* which encode the Bcl-xL, Bcl-xβ, and Bcl-xS proteins, respectively.[2,3)] Bcl-xL protein prevents cells from undergoing apoptosis, Bcl-xS protein functions as a promoter of cell death,[2)] and Bcl-xβ protein promotes apoptosis.[3)] While Bcl-x protein is predominantly expressed in rodent livers, *bcl-x* mRNA is expressed dependent on the cell cycle to serve as a delayed early response gene during rodent liver regeneration.[4–6)] In B6C3F1 mice treated with genotoxic and/or non-genotoxic hepatocarcinogens, Bcl-x and Bcl-2 protein expressions were altered in hepatocellular adenoma and HCC.[5,7,8)] It is thus suggested that the altered expression of Bcl-x protein may be involved in hepatocarcinogenic processes. Little is known, however, about how the altered Bcl-x protein expression dysregu-

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Abbreviations: HCC, hepatocellular carcinoma; DEN, diethylnitrosamine; CDAA diet, choline-deficient, L-amino acid-defined diet; ELISA, enzyme-linked immunosorbent assay; GST-P, glutathione-*S*-transferase placental form; ssDNA, single-stranded DNA; PCNA, proliferating cell nuclear antigen; Topo IIa, topoisomerase II alpha; PH, partial hepatectomy; H & E, hematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling; ROS, reactive oxygen species.

lates apoptotic and cell proliferative processes and, in turn, participates in hepatocarcinogenesis.

In the above context, the present study was conducted to assess the possible involvement of Bcl-x protein expression during rat hepatocarcinogenesis using an exogenous model initiated with DEN, a potent hepatocarcinogen,[9, 10] and an endogenous model, consisting of chronic feeding of a CDAA diet.[11] While HCCs are induced through the development of putatively preneoplastic, focal lesions in both of these two models, DEN causes hepatocarcinogenesis dependent on the formation of genetic damage of specific DNA adducts, but does not induce cirrhosis. In contrast, the CDAA diet, containing no genotoxic or non-genotoxic carcinogens, develops HCC through endogenous mechanisms based on oxidative stress and signaling alterations in the background of induction of continuous liver injury, including hepatocyte death and fibrosis resulting in cirrhosis.[11]

MATERIALS AND METHODS

Animals A total of 80 male Fischer 344 rats (Charles River Japan, Inc., Shizuoka), 5 weeks old weighing 140–160 g, were housed in plastic cages in an air-conditioned room (24±2°C and 55±5% humidity) with a 12-h dark/light cycle, and allowed free access to basal diet (standard rodent chow MF, Oriental Yeast Co., Tokyo) and tap water. After a 1-week acclimation, the animals were allocated to experimental groups.

Chemicals and special diet for animal experiments DEN was purchased from Tokyo Kasei Co. (Tokyo) and diluted with a 0.9% NaCl solution at a concentration of 100 mg/ml. Phenobarbital and 2-acetylaminofluorene, both from Sigma Chemical Co. (St. Louis, MO), were admixed into the basal diet at concentrations of 0.05% and 0.02%, respectively. Colchicine (Sigma Chemical Co.) was dissolved in a 0.9% NaCl solution at a concentration of 0.5 mg/ml. The CDAA diet and a choline-supplemented, L-amino acid-defined diet were obtained from Dyets, Inc. (Bethlehem, PA).

Antibodies A monoclonal anti-rat Bcl-x antibody, clone 35-10, was prepared from an affinity-purified GST-Bcl-x fusion protein as described previously.[12] The ELISA assessment with recombinant proteins revealed that this anti-rat Bcl-x antibody reacted specifically with Bcl-xS, Bcl-xL and Bcl-xβ, but not with Bcl-2.[12] In addition, the western blotting analysis using tissue lysates, spleen, peripheral blood mononuclear cells, cerebrum and cerebellum known to express Bcl-xL but not Bcl-xS,[12–14] demonstrated that this antibody recognized a doublet of proteins with molecular weights of 29–31 kDa (corresponding to Bcl-xL). Anti-rat GST-P and anti-Bcl-xS (also called Bcl-xγ) were purchased from MBL (Nagoya) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Another anti-Bcl-x antibody as reported by Krajewski *et al.*,[13] as well as anti-single stranded DNA (ssDNA), anti-PCNA (clone PC10) and anti-Topo IIa (clone SWT3D1) antibodies, were obtained from DAKO Japan (Kyoto).

Animal treatments Two different protocols, short- and long-term, were used for the exogenous model. To obtain preneoplastic lesions, an 8-week short-term protocol was designed according to the medium term liver bioassay procedure.[15, 16] The DEN treatment group consisting of 10 rats received a single intraperitoneal injection of DEN at a dose of 200 mg/kg body weight, PH at the end of week 3, and a diet containing 0.05% phenobarbital from the end of week 2 for 6 weeks. All rats were sacrificed at the end of week 8 by exsanguination under light ether anesthesia, and their livers were excised. The vehicle group consisting of 10 rats was similarly treated but received vehicle instead of DEN. To obtain HCCs, a 45-week long-term protocol was designed according to Ohashi *et al.*[17] The DEN group consisting of 10 rats received a single intraperitoneal injection of DEN at a dose of 10 mg/kg body weight and were subjected to PH 4 h thereafter. They then received intraperitoneal injections of colchicine at a dose of 0.5 mg/kg body weight 1 and 3 days after DEN, an intragastric injection of carbon tetrachloride at a dose of 1 ml/kg body weight at the end of week 3 and a diet containing 0.02% of 2-acetylaminofluorene between the ends of weeks 2 and 4. All rats were sacrificed at the end of week 45, and their livers excised. The vehicle group consisting of 10 rats was similarly treated, but received vehicle instead of DEN. In the endogenous model, 20 rats were fed the CDAA diet, and were sacrificed, 10 each at the ends of weeks 16 and 80, to obtain preneoplastic lesions and HCCs, respectively. Twenty control rats were fed the choline-supplemented, L-amino acid-defined diet instead of the CDAA diet and similarly sacrificed.

Tissue processing Upon sacrifice, three 2 to 3 mm thick liver slices were prepared from the right anterior, right posterior and caudal lobes, fixed in 10% neutrally buffered formalin, and embedded in paraffin. Serial 4 μm thick specimens were then prepared for histological examination, and were stained with a routine H & E procedure, as well as immunohistochemical and *in situ* enzymatic labeling assessments.

Immunohistochemical and *in situ* enzymatic labeling analyses Paraffin-embedded specimens were deparaffinized in xylene and rehydrated through 100%, 95%, and 90% ethanol. In the cases of Bcl-x and Topo IIa immunohistochemistry, an antigen retrieval procedure, as described by Bankfalvi *et. al.*,[18] by heat treatment with a 10 m*M* citrate buffer, pH 6.0, was performed with an autoclave (Sanyo, Tokyo) to overcome cross-linking due to formalin fixation. All slides were then placed in an automated staining system (DAKO Autostainer, DAKO Japan) for immunohistochemical staining at room temperature

using the following procedure: block endogenous peroxidase activity with 3% hydrogen peroxide solution for 10 min; wash with distilled water; incubate with the primary antibody for 1 h; wash 3 times with 50 m*M* Tris-HCl buffer, pH 7.6, containing 0.1% Tween 20; detect with a labeled streptavidin-biotin system (LSAB2 for rat tissue, DAKO Japan) according to the manufacturer's instruction; wash 3 times with 50 m*M* Tris-HCl buffer, pH 7.6; develop color with 3,3′-diaminobenzidine or, in the case of double immunostaining, new fuchsin (DAKO Japan).

Quantitative analysis of GST-P-positive, putatively preneoplastic lesions was done using a color video camera (MKC-385, Ikegami Tsushin Co., Tokyo) with image processing software (MacSCOPE, Mitani Corp., Tokyo). The lesions were then categorized into 3 classes on the basis of their individual area; <0.03, 0.03–0.3 and >0.3 mm^2.

The proliferative activity of hepatocytes in the preneoplastic lesions and their surrounding areas was assessed by calculating the PCNA and Topo IIa labeling indices, for which positively stained hepatocytes were counted within a total of 400 hepatocytes in randomly selected fields under a light microscope. Hepatocyte apoptosis in the preneoplastic lesions and their surrounding areas was assessed by calculating the ssDNA positive index, for which positively stained hepatocytes were counted within a total of 400 hepatocytes in randomly selected fields under a light microscope. In addition, apoptotic cells in the tissue sections were stained and detected with ApopDETEK system (Enzo Diagnostics, NY) according to the TUNEL method based on the preferential binding of terminal deoxynucleotidyl transferase to the 3′-hydroxyl ends of DNA.[19] Briefly, after deparaffinization and rehydration, the specimens were immersed in 50 m*M* Tris-HCl buffer, pH 7.6, containing 1% saponin overnight at 4°C. The specimens were washed with 50 m*M* Tris-HCl buffer, pH 7.6, and the endogenous peroxidase activity was inactivated by treatment with 3% hydrogen peroxide. Then, the ends of the DNA fragments were labeled by incubation with biotinylated deoxyuridine triphosphate and terminal deoxynucleotidyl transferase. After end-labeling, the specimens were incubated with streptavidin-conjugated horseradish peroxidase and developed with 3,3′-diaminobenzidine. The estimation for hepatocyte apoptosis was conducted similarly to the ssDNA immunohistochemical analysis.

Statistical analysis Statistical analysis for the numbers of GST-P-positive foci, PCNA and Topo IIa Labeling indices, and apoptotic indices was done using Student's *t* test. A correlation between the area size and the frequency of Bcl-x-decreasing foci in each focus area-based category was determined by the χ^2 test using an $m \times n$ matrix contingency table analysis. The intergroup difference was considered significant when the *P* value was less than 0.05.

RESULTS

Bcl-x protein expression in preneoplastic lesions and HCCs in the exogenous model Normal parenchymal hepatocytes exhibited strong immunoreactivity for the presently used anti-Bcl-x antibody, clone 35-10, and the expressed Bcl-x protein was confirmed to correspond mainly to Bcl-xL, but not Bcl-xS or Bcl-xβ, by western blot analysis (Fig. 1).

GST-P-positive, preneoplastic lesions were significantly induced in the DEN group of the short-term experiment, numbering 33.8±8.5 per cm^2, while that in the vehicle group was 0.2±0.3. In 201 out of 815 (24.7%) GST-P-positive lesions, Bcl-x protein expression was markedly diminished (Table I, Fig. 2). When the lesions were categorized on the basis of their sizes, the incidences of the lesions with decreased Bcl-x protein expression in the classes of <0.03, 0.03–0.3 and >0.3 mm^2 were 19% (103

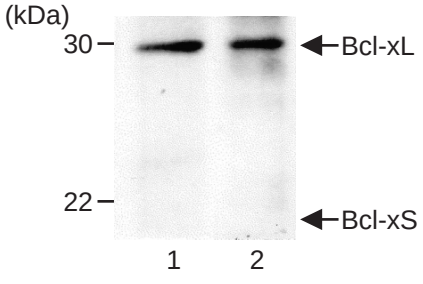

Fig. 1. Western blot analysis of Bcl-x protein in tissue lysates from normal liver. Twenty micrograms of total cellular protein, prepared from thymus as a positive control (lane 1) and liver (lane 2) of the rat, was separated using 15% SDS-PAGE, transferred to nitrocellulose membrane, and then analyzed with specific antibody to Bcl-x, clone 35-10.

Table I. The Incidence of GST-P-positive Lesions with Decreased Bcl-x Protein Expression

Size-based category (mm^2)[a]	Number of examined GST-P-positive lesions	Incidence of the lesions with decreased Bcl-x protein expression		
		No.	Incidence (%)	
Total	815	201	24.7	
<0.03	546	103	18.9	
0.03–0.3	250	81	32.4	$P<0.001$[b]
>0.3	22	19	86.5	

a) The foci were categorized into 3 classes (<0.03, 0.03–0.3, >0.3 mm^2) on the basis of their area size.
b) Significant correlation between incidence of foci with decreased Bcl-x protein expression and their focal area in each group using the χ^2 test.

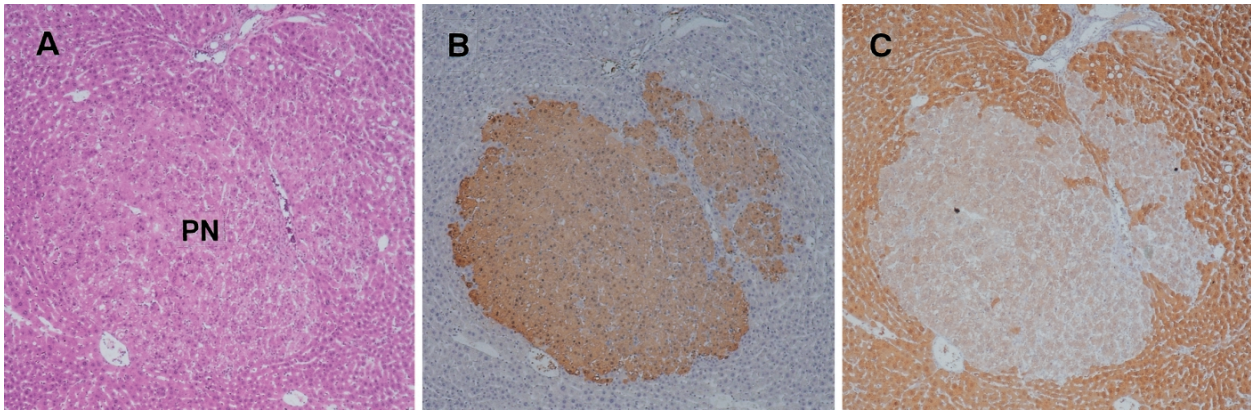

Fig. 2. Representative appearance of a preneoplastic focal lesion (PN) induced exogenously by DEN (×40). (A) H & E staining, (B) GST-P, and (C) Bcl-x immunohistochemical staining.

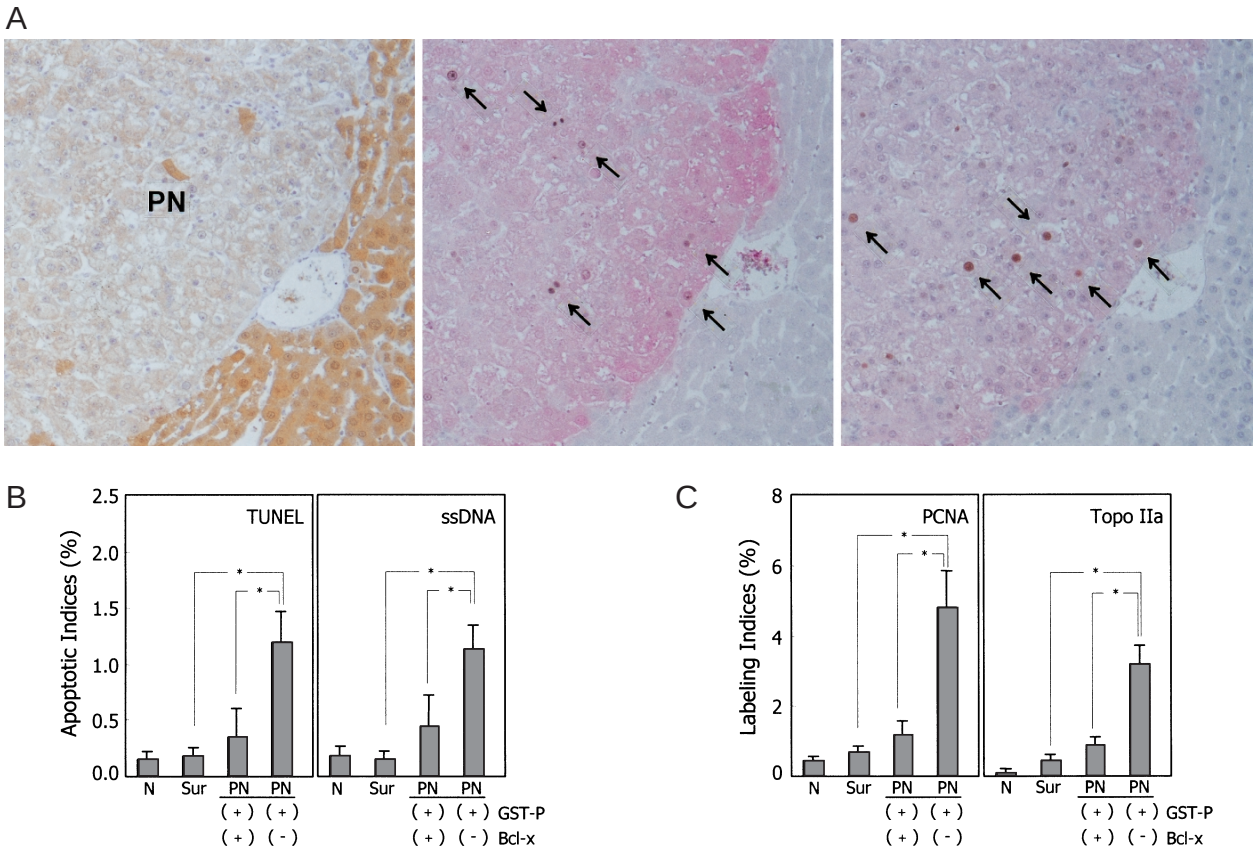

Fig. 3. Apoptosis and proliferative activities of hepatocytes in preneoplastic focal lesions, induced exogenously by DEN, and their surrounding areas (×100). (A) Representative Bcl-x immunohistochemistry (left), TUNEL/GST-P double staining (center) and PCNA/GST-P double immunohistochemistry (right) of a liver preneoplastic lesion (PN). Arrows indicate TUNEL- or PCNA-positive hepatocytes. (B) Hepatocyte apoptotic indices in control non-treated liver (N), preneoplastic focal lesions (PN), and their surrounding areas (Sur). The results obtained using the TUNEL method and ssDNA immunohistochemistry are shown in the left and right panels, respectively. Asterisk represents the presence of a significant difference. (C) Hepatocyte proliferative activities. Labeling indices obtained using PCNA and Topo IIa immunohistochemistry are shown in the left and right panels, respectively.

of 546 lesions), 32% (81 of 250 lesions) and 86% (19 of 22 lesions), respectively. Statistical analysis revealed that the incidences of GST-P-positive lesions with decreased Bcl-x protein expression significantly increased with the increase of the size of the lesions ($P<0.001$). Moreover, the degree of decreased expression in lesions clearly depended on their size. All of the 103 lesions smaller than 0.03 mm^2 exhibited a weak decrease, while in contrast, strongly decreased expression was observed in lesions larger than 0.03 mm^2. These results were confirmed by obtaining a similar staining pattern using Krajewski's anti-Bcl-x antibody, whereas no immunoreactivities for anti-Bcl-xS antibody were observed in either normal liver tissue or preneoplastic lesions (data not shown).

Using the TUNEL method, hepatocyte apoptosis was detected more frequently in GST-P-positive lesions with decreased Bcl-x protein expression than in their surrounding area (Fig. 3A, left and center panels). The hepatocyte apoptotic index in such lesions was 1.19%, which was significantly higher than the indices in GST-P-positive lesions without decreased Bcl-x protein expression (0.36%), and in the surrounding areas (0.18%) (Fig. 3B, left panel). These results were confirmed by ssDNA immunohistochemistry (Fig. 3B, right panel). PCNA immunohistochemistry demonstrated higher hepatocyte proliferative activity in GST-P-positive lesions with decreased Bcl-x protein expression than in their surrounding areas (Fig. 3A, left and right panels). The hepatocyte labeling index in such lesions was 4.52%, and was significantly higher than the indices in GST-P-positive lesions without decreased Bcl-x protein expression (1.20%) and in the surrounding area (0.80%) (Fig. 3C, left panel). These results were confirmed by Topo IIa immunohistochemistry.

In the livers of rats treated with DEN, well- or moderately-differentiated HCCs were induced. All of these HCCs were heterogeneously positive for GST-P immunohistochemistry (Fig. 4A) and showed decreased expression of Bcl-x protein (Fig. 4B).

Bcl-x protein expression in preneoplastic lesions and HCCs in the endogenous model Preneoplastic, focal lesions and well- or moderately differentiated HCCs were induced with intrahepatocellular fat accumulation and cirrhosis in the livers of all rats fed the CDAA diet for 16 and 80 weeks, respectively, while no such lesions were observed in any control rats fed the choline-supplemented, L-amino acid-defined diet (data not shown). Most of the preneoplastic lesions over 0.3 mm^2, and all of the HCCs were positive for GST-P immunohistochemistry (Fig. 5, A and C, respectively) and had decreased Bcl-x protein expression (Fig. 5, B and D, respectively).

DISCUSSION

Alteration of signal transduction systems regulating the apoptotic and cell proliferative process is frequently involved in carcinogenic mechanisms, and ROS are known to be major second messengers in such systems.[20] Oxidative stress caused by ROS generation also plays an important role in both exogenous and endogenous hepatocarcinogenesis in rats.[11, 21–23] Among the Bcl-2 family, Bcl-xL protein negatively regulates the apoptotic processes,[2] and inhibits apoptosis induced by oxidative stress and proinflammatory cytokines that trigger ROS generation.[24, 25] From these findings, we have inferred that alteration of the expression level of Bcl-x protein may be involved in carcinogenic processes in rat liver. In this study, we focused on this point, and found that the expression of Bcl-xL protein was decreased in GST-P-positive preneoplastic lesions induced both exogenously by DEN initiation, and endogenously by the chronic feeding of the

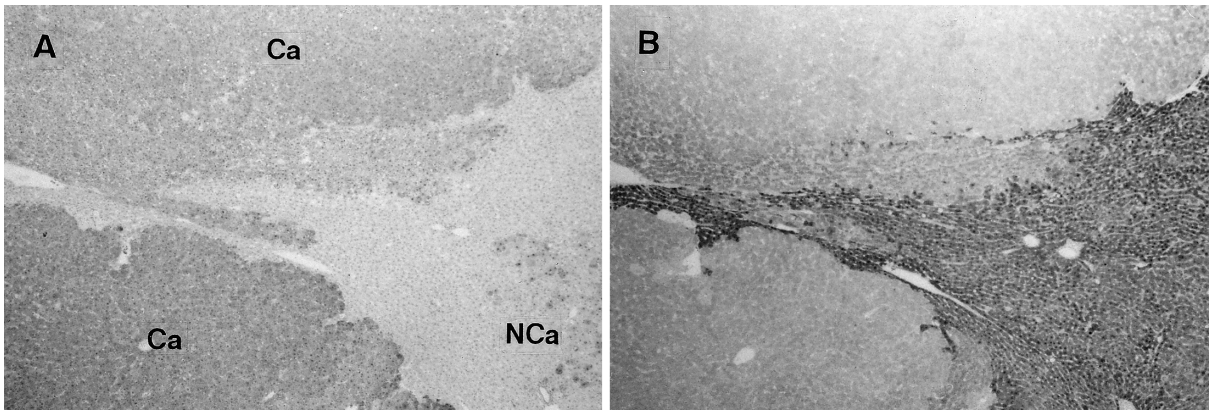

Fig. 4. Representative immunohistochemical appearance of HCCs induced exogenously by DEN (×40). (A) GST-P and (B) Bcl-x immunohistochemical staining. Ca and NCa represent HCC and its surrounding area, respectively.

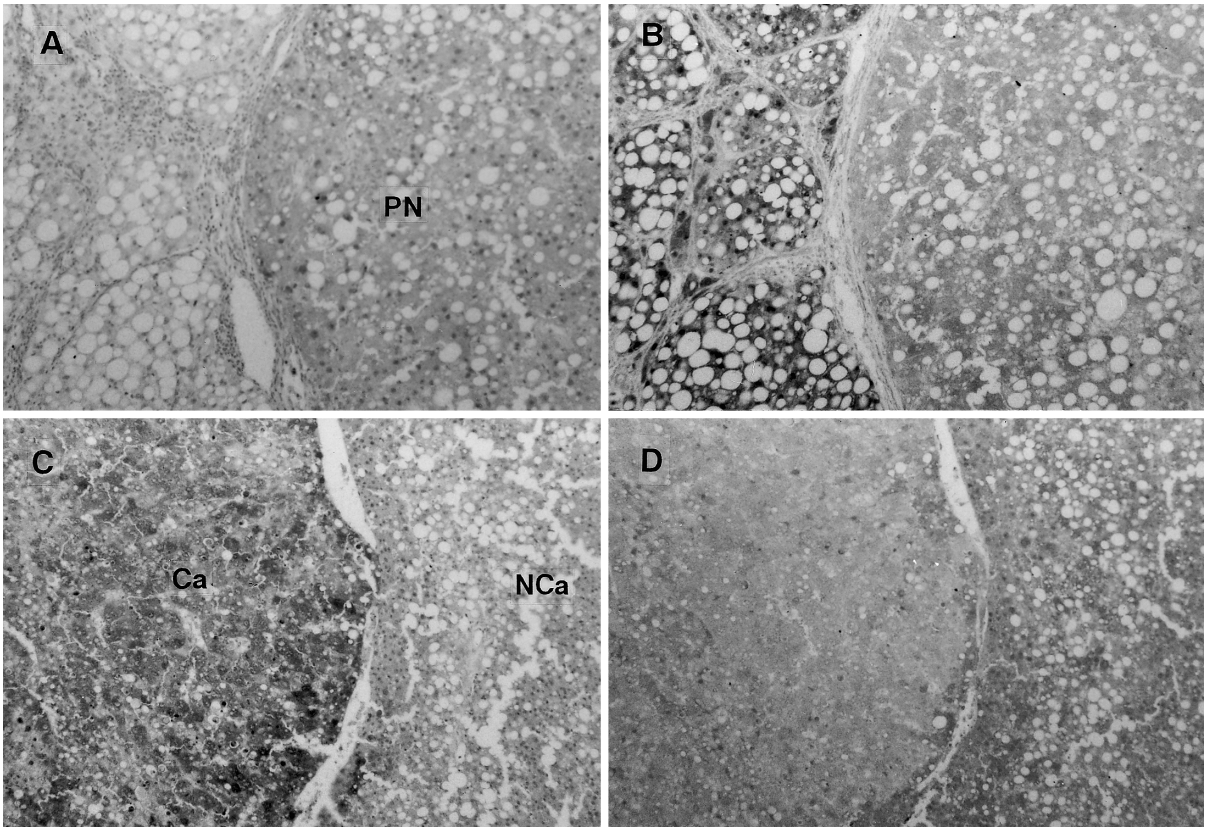

Fig. 5. Representative immunohistochemical appearances of a preneoplastic focal lesion and HCC induced endogenously by chronic feeding of the CDAA diet (×100). (A) Preneoplastic lesion (PN) by GST-P and (B) by Bcl-x immunohistochemistry. (C) HCC (Ca) by GST-P immunohistochemistry. NCa represents the surrounding area. (D) HCC by Bcl-x immunohistochemistry.

CDAA diet. Moreover, the decreased Bcl-x expression was observed in HCCs induced both exogenously and endogenously. These results suggest that the reduction of Bcl-x protein expression is one of many pathologic events during experimentally-induced rat hepatocarcinogenesis.

As for the expression patterns of the *bcl-2* gene family, extensive biochemical and immunohistochemical studies have been reported in various normal and tumor tissues. In some carcinomas, the expressions of these proteins exhibited diverse patterns among histological types or malignancies.[26–28] Therefore, it is now widely thought that the difference in their expression patterns is tissue-specific and depends on the stage of carcinogenesis. Bcl-x expression in a rodent hepatocarcinogenesis model has recently been reported in B6C3F1 mice. In contrast to our observation, increased Bcl-xL, as well as Bcl-2, expression was seen in both preneoplastic focal lesions and hepatocellular adenomas induced spontaneously or by non-genotoxic hepatocarcinogens, such as phenobarbital.[5] B6C3F1 mice have a high susceptibility to hepatocarcinogens,[5, 7, 8] and exhibit strongly increased Bcl-2 expression in basophilic tumors induced by DEN alone, but not eosinophilic tumors induced by the combination of DEN with PB, in spite of having no expression in normal liver.[7] Conversely, we observed that there was no expression of Bcl-2 protein in preneoplastic lesions after similar treatments (data not shown). Furthermore, in human livers, Bcl-2 is considered clinically as a diagnostic marker for cholangiocarcinoma, but not HCC.[29] Thus, the discrepancy between the above mouse cases[5, 7, 8] and our data may be attributed to the differences among experimental models or species. Moreover, we have recently found that there are differences between exogenous and endogenous mechanisms underlying rat hepatocarcinogenesis.[30, 31] However, the present results showed that the decreased Bcl-x expression was observed in the experimental models with both DEN and CDAA diet, which have been widely used in studies on hepatocarcinogenesis, suggesting that, similar to the increase in GST-P protein, the decrease in Bcl-x protein may be a common event for at least rat preneoplastic

lesions induced exogenously and endogenously. The appearance of preneoplastic cells with decreased Bcl-x expression also seems to be more delayed than that of the increased GST-P expression.

When the relationship between decreased Bcl-x expression and the induction of apoptosis in large GST-P-positive lesions was investigated, the hepatocyte apoptotic index in these lesions was significantly higher than that of lesions with normally expressed Bcl-xL protein. These observations suggest that enhanced apoptosis of hepatocytes in such lesions could be involved in the reduced expression level of Bcl-xL protein that can block apoptosis.

On the other hand, Bcl-x protein has recently been shown to play a role in cell proliferation and cell cycle regulation. The modulation of Bcl-xL expression level by transgene into breast and ovarian carcinoma cells affects tumor cell growth *in vivo*,[32, 33] and this event exhibits an organospecificity when the cells injected to form the primary lesion metastasize to a variety of organs.[34] Moreover, it has been demonstrated that the *bcl-x* gene may be involved in the progression stage of the cell cycle and acts as a delayed early response gene in rat liver.[4, 6] Interestingly, in preneoplastic lesions arising in the exogenous model, there was a significant correlation between the incidence of preneoplastic lesions with decreased Bcl-x protein expression, and the expansion of such lesions. Our results raise the possibility that there could be a relationship between decreased Bcl-x protein expression and the function of Bcl-x associated with cell proliferation, as mentioned above, although the mechanisms involved are entirely unclear.

In conclusion, decreased expression of Bcl-x protein might serve as an indicator of the advanced form of preneoplastic lesions, possibly with a potential to progress to HCCs in rat hepatocarcinogenesis induced exogenously and endogenously. Further studies are necessary to understand the biological meanings of the present results and the molecular mechanisms involved.

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