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Altered bcl-2 family expression during non-genotoxic hepatocarcinogenesis in mice

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Dysregulation of apoptosis is an important component of multistage hepatocarcinogenesis. Members of the bcl-2 protein family are important in the regulation of apoptosis and their expression is altered in several cancers. The objectives of the present study were to determine whether the expression of members of the bcl-2 protein family are altered in mouse liver during acute treatment with non-genotoxic carcinogens and throughout non-genotoxic hepatocarcinogenesis. Acute treatment of B6C3F1 mice with phenobarbital resulted in increased levels of bcl-2 and decreased levels of bax protein, while acute treatment with WY-14,643 resulted in increased bcl-2 and BAG-1 protein in the liver. Following chronic treatment, altered hepatic foci and adenomas were classified as: small-cell, heterogeneous basophilic lesions (spontaneous or tetrachlorodibenzo-*p*-dioxin-induced); large-cell, homogeneous basophilic lesions (WY-14,643-induced); acidophilic lesions (phenobarbital- or chlordane-induced). Of the small-cell heterogeneous basophilic lesions, 86% of foci (31/36) and 85% of adenomas (35/41) exhibited increased bcl-2 protein levels compared with surrounding normal hepatocytes, whereas only 12.5% of foci (4/36) and 12% of adenomas (5/41) exhibited increased bcl-X_L levels. Of the large-cell, homogenous, basophilic lesions, 100% of foci (3/3) and 90% of adenomas (9/10) expressed bcl-2 protein, whereas 100% of foci (3/3) and 80% of adenomas (8/10) exhibited increased bcl-X_L protein levels compared with surrounding normal hepatocytes. Of the acidophilic lesions, the majority of foci (28/32, 88%) and adenomas (47/50, 94%) expressed increased bcl-X_L, whereas increased bcl-2 was observed in only 12.5% of acidophilic preneoplastic foci (4/32) and 14% of acidophilic adenomas (7/50). Of the carcinomas analyzed, 81% expressed increased bcl-2 (54/67), 78% expressed increased bcl-X_L (52/67) and 69% expressed increased levels of both bcl-2 and bcl-X_L (46/67). Collectively, only 8% of preneoplastic foci, 3% of adenomas and 1.5% of carcinomas did not express either bcl-2 or bcl-X_L. These results suggest that regulation of apoptotic proteins is altered during non-genotoxic carcinogenesis in mouse liver. Furthermore, there were both chemical- and lesion-specific aspects of expression of apoptotic proteins during hepatocarcinogenesis in mice.

Abbreviations: EGF, epidermal growth factor; H&E, hematoxylin and eosin; PB, phenobarbital; PMSF, phenylmethylsulfonyl fluoride; TBS-T, Tris-buffered saline containing 0.1% Tween 20; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TGF, transforming growth factor.

Introduction

Assessment of human cancer risk often relies heavily on results from long-term rodent cancer bioassays of selected chemicals. The most frequently used strain of mouse in rodent bioassays is B6C3F1. This strain is highly susceptible to liver tumor formation, the most commonly observed end-point in rodent bioassays (1–4). Approximately 30% of male B6C3F1 mice develop liver tumors in the absence of chemical treatment (1,4). Many chemicals cause an increased number of liver tumors in rodent bioassays but do not directly damage DNA and are classified as non-genotoxic (5,6). Whether chemicals that cause liver tumors via non-genotoxic mechanisms in highly susceptible mouse strains should be considered potential human carcinogens is unclear. An improved mechanistic understanding of non-genotoxic hepatocarcinogenesis in rodents will aid in assessing human health risks associated with this class of chemicals.

One proposed mechanism for non-genotoxic carcinogenesis is an alteration of apoptosis regulation. Dysregulation of apoptosis may result in the decreased ability of cells to undergo apoptosis and provide a selective survival advantage for those cells, thus leading to altered growth, cellular transformation and tumor progression. During hepatocarcinogenesis, altered hepatic foci, hepatocellular adenomas and hepatocellular carcinomas exhibit increased rates of cell proliferation and cell death relative to surrounding hepatocytes (7–9). The balance of these factors determines cell growth and clonal expansion. Some non-genotoxic carcinogens were found to reduce relative rates of cell death in both normal liver and hepatic neoplasia and thus accelerate growth and progression of cancer in rodents (10–13). Withdrawal of certain non-genotoxic carcinogens [i.e. phenobarbital (PB), WY-14,643 and chlordane] in mice or rats has been shown to result in regression of hyperplastic liver, preneoplastic foci, adenomas and some carcinomas with a concomitant increase in apoptosis (10–15). In contrast, the tumor promoter 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been shown to inhibit apoptosis in putative preneoplastic hepatocytes while having only a minimal affect on normal hepatocytes (16,17). These studies indicate that apoptosis is pivotal in the modulation of rodent hepatocarcinogenesis.

Much attention has recently been given to the identification of molecular targets that are involved in the regulation of

apoptosis and that are altered in tumors. *bcl*-2 is a gene that was originally identified at a breakpoint of translocations commonly occurring in follicular B cell lymphomas that resulted in its overexpression (18–20). The bcl-2 protein was later found to play a role in cell survival and inhibition of apoptosis rather than regulation of cell replication (21–23). Subsequent studies have resulted in the identification of a family of bcl-2-related homologs that contribute to the regulation of programmed cell death in many species and cell types. Family members include apoptosis-inducing proteins (e.g. bax and bak) and inhibiting proteins (e.g. bcl-X_L and BAG-1) that form homodimers and heterodimers with themselves and other family members (24–28). The precise mechanism by which these proteins regulate cell death is currently under investigation. However, the cellular equilibrium between inducing and inhibiting family members is known to be important in determining cell fate (25,29). Because of their important role in apoptosis and their regulation by a number of cell growth regulatory pathways, it is not surprising that the expression of several bcl-2 family members has been shown to be altered in a number of cancers (30).

We recently reported that bcl-2 family members were involved in the inhibition of apoptosis by non-genotoxic carcinogens in primary hepatocytes (31). In addition, bcl-2 was recently shown to be overexpressed in diethylnitrosamine-induced mouse neoplasia but not in acidophilic neoplasia promoted by PB (32,33). The objectives of the present investigation were to determine whether expression of bcl-2 family members was altered *in vivo* following acute treatment with non-genotoxic carcinogens and in mouse neoplasms induced by non-genotoxic carcinogens in long-term carcinogenesis experiments. Results from the present study indicate that expression of bcl-2 family members were altered during acute treatment with PB or WY-14,643. In addition, bcl-2 was expressed at high levels in most basophilic lesions that arose spontaneously or were induced by TCDD, whereas both bcl-2 and bcl-X_L were expressed in the majority of basophilic lesions induced by WY-14,643. Most acidophilic lesions induced by PB or chlordane expressed increased bcl-X_L. An increased fraction of carcinomas relative to foci and adenomas expressed both bcl-2 and bcl-X_L. These results suggest that proteins that are involved in the regulation of apoptosis are altered during carcinogenesis and tumor progression in mouse liver and genes that regulate apoptosis may represent molecular targets for non-genotoxic carcinogens.

Materials and methods

Mice

Male B6C3F1 hybrid mice, ~4 weeks of age, were obtained from Charles River (Portage, MI and Raleigh, NC) or the National Toxicology Program (National Cancer Institute, Fredrick, MD). SV/129 mice of 7–8 weeks of age were used in the chronic WY-14,643 study (34). Mice were housed in polystyrene cages, four to five per cage. All animal husbandry and care procedures conformed to the NIH *Guide for the Care and Use of Laboratory Animals*. All mice were housed in humidity and temperature controlled, HEPA-filtered facilities accredited by the American Association of Laboratory Animal Care.

Acute treatment with PB or WY-14,643

Male B6C3F1 mice were fed PB (0.05% in their drinking water), WY-14,643 (1000 p.p.m., 0.1% of diet) or standard diet and water starting at age 42 days. At days 1, 3, 5, 10 and 30 after treatment mice (three per time point per treatment) were killed with CO_2, necropsies were performed and mouse livers were removed for molecular analysis of gene or protein expression. PB concentration in drinking water, WY-14,643 concentration in diet and control drinking water and diet were analyzed at the beginning and end of the studies.

Chronic mouse carcinogenesis studies

Protocols for chronic mouse carcinogenesis studies of chlordane (55 p.p.m. continuously in diet *ad libitum*, starting at 9 weeks of age for 13–19 months), WY-14,643 (0.1% continuously in diet *ad libitum* starting at 7–8 weeks of age for 11 months) and spontaneous tumor formation used in this study have been described previously (14,34,35). Other studies of TCDD- or PB-induced neoplasia involved the following: 2.5 μg/kg body wt TCDD (Chemsyn Science Laboratories, Lenexa, KS) by gavage in corn oil starting at 6 weeks of age and every 2 weeks for 12–17 months; 0.05% PB (Sigma Chemical, St Louis, MO) continuously in drinking water starting at 6 weeks of age for 12–18 months. The concentration of PB was determined weekly throughout the study by HPLC analysis. For each of the studies, mice were weighed and killed by isofluorane anesthesia and exanguination at necropsy. Whole liver was excised, weighed and examined for presence of gross lesions. Neoplasms were dissected from surrounding non-neoplastic tissue, snap frozen in liquid nitrogen and stored at –70°C; corresponding sections of liver lesions and surrounding tissue were fixed in neutral buffered formalin for subsequent analysis.

Protein isolation and immunoblotting

To isolate total liver or tumor protein, a portion of frozen tissue (~0.1 g) was placed in a microfuge tube containing cell lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM Na_2VO_3, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml pepstatin A and 1 mM dithiolthreitol). Tissues were homogenized, aspirated through a 21 gauge needle on ice and incubated for 45 min to lyse cells and release proteins. Lysates were collected and microcentrifuged at 12 000 *g* for 15 min to remove cellular debris. Equal amounts of protein (50 μg/treatment) were added to each well and proteins were separated by (12.5–18%) SDS–PAGE. Transfer of proteins to a PVDF membrane (Immobilon-P; Millipore, Bedford, MA) was achieved using a Bio-Rad (Richmond, CA) transfer apparatus. Membranes were then blocked for 30–60 min with 2–4% commercial non-fat dry milk and 1% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBS-T). After blocking, membranes were incubated with primary antibody (0.1–1 μg/ml primary antibody diluted in TBS-T, 1% BSA, 1% milk) for 1 h to overnight, washed and exposed to the appropriate secondary antibody (1:7500–10 000 dilution). Specific proteins were then detected using an enhanced chemiluminescence system (Amersham, Arlington Heights, IL). Equal protein loading and transfer was verified by staining of PVDF membranes with Ponceau Red. Densitometric analysis was performed on blots using NIH Image Analysis Software. Changes in protein expression that were ⩾2-fold, as indicated by densitometric analysis of a series of blots (minimum of two), were considered to be significantly different from the control protein expression at any given time point.

Immunohistochemistry and pathology

Liver samples were fixed in formalin and routinely processed for embedding in paraffin. For each sample, liver serial sections (4–5 μm thick) were cut and one section was stained with hematoxylin and eosin (H&E). Others were paraffin coated and stored for immunohistochemical analysis. Liver lesions were diagnosed as altered hepatocellular foci, adenomas or carcinomas and classified as acidophilic, basophilic, clear, vacuolated or mixed lesions according to previously established criteria (36–38). To immunohistochemically detect bcl-2 family members, sections were deparaffinized in xylene and endogenous peroxidases were blocked with 3% hydrogen peroxide. To enhance antigen retrieval, sections were microwaved for 20 min in 0.01 M citrate buffer (pH 6.0). To block non-specific protein binding, sections were incubated with 6% goat serum, 1% BSA for 15 min. Antigens were detected using primary antibodies listed below and a supersensitive detection kit (BioGenex, San Ramon, CA). Sections were counterstained in hematoxylin. Specific staining for both bcl-2 or bcl-X_L was validated using two distinct antibodies that recognized different epitopes of each protein. Furthermore, addition of competing peptides for each of the epitopes resulted in loss of immunohistochemical staining.

Antibodies

Antibodies used were anti-bcl-2 [Armenian hamster clone 3F11 (1:400) for immunoblots and polyclonal rabbit anti-mouse/rat (13456E) or polyclonal rabbit anti-mouse (15616E) (1:750) for immunohistochemistry; Pharmingen, San Diego, CA], anti-bcl-X_L [mouse YTH-2H12 (1:500) for immunoblots and rabbit polyclonal sc-634 or goat polyclonal sc-1599 (1:400) for immunohistochemistry; Trevigen, Gaithersburg, MD and Santa Cruz Biotechnology, Santa Cruz, CA], anti-BAG-1 [rabbit polyclonal sc-939 (1:400); Santa Cruz Biotechnology]. Secondary antibodies used were sheep anti-mouse, donkey anti-rabbit (Amersham) or goat anti-Armenian hamster (Jackson Immunoresearch, West Grove, PA) immunoglobulin coupled to horseradish peroxidase.

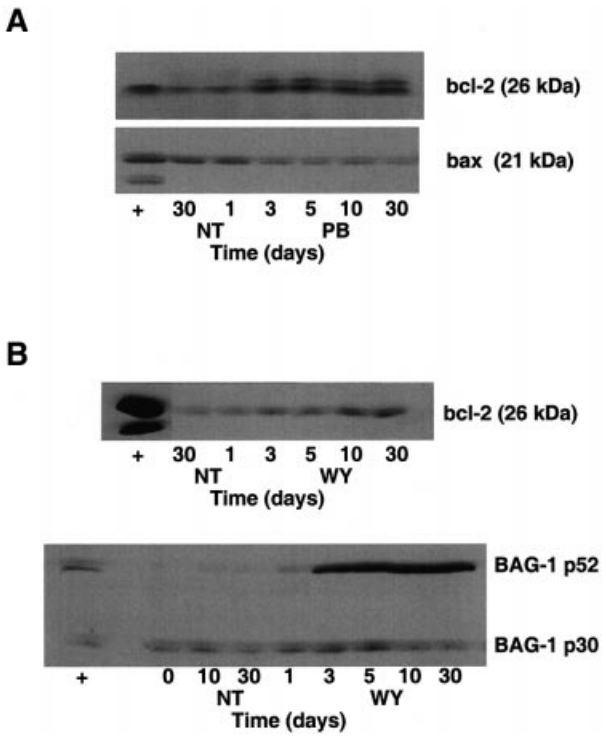

Fig. 1. (**A**) Expression of bcl-2 (top) and bax (bottom) protein in mouse liver at days 1, 3, 5, 10 and 30 following acute PB treatment. (**B**) Expression of bcl-2 (top) or BAG-1 (bottom) protein in mouse liver at days 0, 10 and 30 following standard diet (not treated, NT) and at days 1, 3, 5, 10 and 30 following acute WY-14,643 (WY) treatment. Male B6C3F1 mice were fed PB (0.05% in their drinking water), WY-14,643 (1000 p.p.m., 0.1% of diet) or standard diet and water starting at age 29 days. At days 1, 3, 5, 10 and 30 after treatment mouse livers were removed for analysis of protein expression. Protein lysates from mouse liver were normalized for protein content (50 μg/lane) and analyzed by immunoblotting using antibodies against the aforementioned proteins. Positive controls were M1 mouse myeloma cell lysate (Pharmingen, San Diego, CA) (bcl-2), NIH 3T3 cell lysate (bax) or Ag8 lysate (BAG-1). Negative controls were mouse brain lysate (bcl-2 or BAG-1) or HL60 lysate (bax). Protein expression in treated livers was compared with protein expression in non-treated livers of the same time course on the same gel. Representative blots are shown.

Results

Altered expression of bcl-2 family during acute treatment with non-genotoxic carcinogens

Alterations in the expression of *bcl*-2 family genes during acute treatment with non-genotoxic carcinogens or in non-genotoxic carcinogen-induced neoplasia were screened using the RNase protection assay (data not shown) or cDNA microarray screening (S.P.Anderson, R.C.Cattley and J.C.Corton, personal communication). Subsequently, standard immunoblotting and immunohistochemical techniques were used to analyze expression of bcl-2 family proteins that were found to be altered during screening in frozen tissues or fixed sections, respectively. Acute treatment of B6C3F1 mice with PB resulted in an ~3-fold induction of bcl-2 protein levels (along with an additional band that migrated at ~27 kDa) compared with non-treated mouse liver at days 3–30 of treatment, as shown by immunoblotting (Figure 1A). Immunohistochemical staining for bcl-2 in liver sections from PB-treated mice was observed to be increased in the cytoplasm of hypertrophic centrilobular hepatocytes compared with periportal hepatocytes (Figure 2A). The apoptosis inhibitor bcl-X_L was expressed at similar levels in both normal and PB-treated liver, as shown by immunoblotting (data not shown), and its expression also appeared to be greater in the cytoplasm of centrilobular hepatocytes compared with periportal hepatocytes, as shown by immunohistochemistry (Figure 2B). Acute treatment of mice with PB also resulted in a gradual decrease in the levels of the apoptosis-inducing protein bax, with a 2-fold decrease at day 5 and a 3-fold reduction at day 30 compared with bax expression in non-treated mouse liver at these time points (Figure 1A).

Acute treatment of B6C3F1 mice with WY-14,643 resulted in a gradual increase in bcl-2 levels in liver, with a 2-fold increase at day 5 and a 4-fold increase at day 30 compared with bcl-2 expression in non-treated mouse liver at these time points (Figure 1B). In contrast to PB, the increase in bcl-2 protein expression was not localized to centrilobular hepatocytes following acute treatment with WY-14,643. Treatment with WY-14,643, but not PB, also resulted in an 8-fold increase in a mouse isoform of the apoptosis inhibitor BAG-1 (39) that migrated at ~52–53 kDa compared with BAG-1 expression in non-treated mouse liver at days 3–30 (Figure 1B).

Expression of bcl-2 and bcl-X_L in hepatic neoplasia

Lesion phenotype. Lesions arising spontaneously or following treatment with PB, TCDD, WY-14,643 or chlordane were diagnosed as altered hepatocellular foci, adenomas or carcinomas. In addition, foci and adenomas were classified as: small-cell, heterogeneous basophilic lesions; large-cell, homogeneous basophilic lesions; or acidophilic lesions. Lesions that did not display a basophilic or acidophilic phenotype (e.g. clear cell) generally were not positive for bcl-2 or bcl-X_L and were not included in the analysis. Most of the spontaneous lesions (60% of foci, 89% of adenomas) or TCDD-induced lesions (82% of foci, 82% of adenomas) were a small-cell, heterogeneous basophilic phenotype. The majority of WY-14,643-induced lesions (60% of foci, 83% of adenomas) were a large-cell, homogeneous basophilic phenotype, whereas the remainder of lesions were a small-cell, heterogeneous basophilic phenotype. The majority of PB-induced lesions (82% of foci, 93% of adenomas) and chlordane-induced lesions (90% of adenomas) were an acidophilic phenotype.

Immunohistochemistry. Most of the small-cell, heterogeneous basophilic foci (31/36, 86%) and adenomas (35/41, 85%) exhibited increased staining for bcl-2 protein compared with surrounding normal hepatocytes, whereas increased bcl-X_L was observed in only 12% of small-cell, heterogeneous basophilic foci (4/36) and adenomas (5/41) (Tables I and II and Figure 2C–E). Of the large-cell, homogenous, basophilic lesions, 100% of foci (3/3) and 90% of adenomas (9/10) expressed bcl-2 protein and 100% of foci (3/3) and 80% of adenomas (8/10) exhibited increased bcl-X_L protein levels compared with surrounding normal hepatocytes (Tables I and II and Figure 2F–H). The majority of acidophilic foci (28/32, 88%) and adenomas (47/50, 94%) exhibited increased staining for bcl-X_L protein, whereas increased bcl-2 was observed in only 12.5% of acidophilic foci (4/32) and 14% of adenomas (7/50) (Tables I and II and Figure 2I–K). Although bcl-2 was not increased in most acidophilic foci and adenomas induced by PB, it was expressed at levels comparable with those observed in centrilobular hepatocytes following acute PB treatment. Some adenomas (eight in total) displayed both basophilic and acidophilic characteristics and appeared to stain heterogeneously for both bcl-2 and bcl-X_L. Large carcinomas were often multilobular and were composed of aggregates of

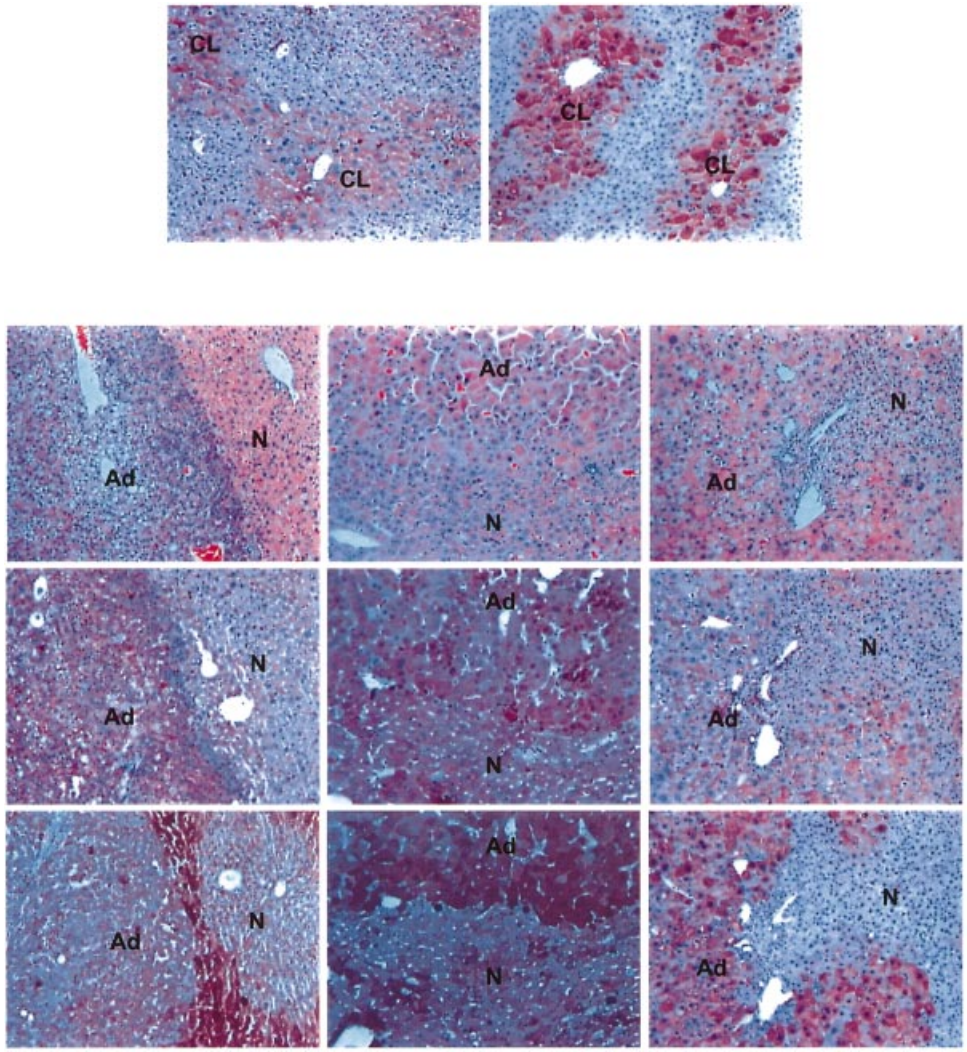

Fig. 2. Immunohistochemical analysis of bcl-2 and bcl-X_L in distinct hepatocellular lesions. Tissue sections from mouse liver were stained with H&E or immunohistochemically stained for bcl-2 or bcl-X_L and counterstained with hematoxylin. (**A**) Bcl-2 staining in cytoplasm of centrilobular hepatocytes (CL) following acute phenobarbital treatment (30 days). (**B**) Bcl-X_L staining in cytoplasm of centrilobular hepatocytes (CL) following acute PB treatment (30 days). (**C–E**) Step sections through a spontaneous adenoma. (C) H&E staining. Note cytoplasmic basophilia of adenoma (Ad) cells compared with surrounding normal hepatocytes (N). (D) bcl-2 staining. Adenoma cells stained positively compared with the surrounding normal hepatocytes. (E) bcl-X_L staining. Adenoma cells stained negatively compared with immediately surrounding and centrilobular hepatocytes. (**F–H**) Step sections through a WY-14,643-induced adenoma. (F) H&E staining. Note cytoplasmic basophilia, increased cell size in adenoma compared with surrounding hepatocytes and homogeneous appearance. (G) bcl-2 staining. Positive staining of adenoma cells compared with surrounding normal hepatocytes. (H) bcl-X_L staining. Positive staining of adenoma cells compared with surrounding normal hepatocytes. (**I–K**) Step sections through a PB-induced adenoma. (I) H&E staining. Note cytoplasmic acidophilia of adenoma cells compared with surrounding normal hepatocytes. (J) bcl-2 staining. Adenoma cells stained positively compared with periportal hepatocytes and equal to centrilobular hepatocytes. (K) bcl-X_L staining. Adenoma cells stained positively compared with the surrounding hepatocytes.

Table I. Expression of bcl-2 in mouse hepatocellular lesions

Group	Altered hepatic foci			Adenomas			Carcinomas		
	Small-cell basophilic	Large-cell basophilic	Acidophilic	Small-cell basophilic	Large-cell basophilic	Acidophilic	Positive[a]	Mixed[b]	Negative[c]
Spontaneous	5/6 (83%)	n/a	1/4 (25%)	15/17 (88%)	n/a	0/2 (0%)	10/12 (83%)	2/12 (17%)	0/12 (0%)
WY-14,643	2/2 (100%)	3/3 (100%)	n/a	2/2 (100%)	9/10 (90%)	n/a	4/4 (100%)	0/4 (0%)	0/4 (0%)
TCDD	21/23 (91%)	n/a	0/5 (0%)	15/18 (83%)	n/a	0/4 (0%)	10/19 (53%)	7/19 (37%)	2/19 (11%)
PB	3/5 (60%)	n/a	3/23 (13%)	2/3 (67%)	n/a	7/40 (18%)	12/28 (43%)	8/28 (29%)	8/28 (29%)
Chlordane	n/a	n/a	n/a	1/1 (100%)	n/a	0/4 (0%)	0/4 (0%)	1/4 (87%)	3/4 (14%)
Totals	31/36 (86%)	3/3 (100%)	4/32 (12%)	35/41 (85%)	9/10 (90%)	7/50 (13%)	54/67 (81%)		13/66 (20%)

n/a denotes that no lesions of the indicated phenotype were present or that they were unavailable for analysis.
[a]Positive denotes a lesion that stained positive relative to surrounding tissue.
[b]Mixed denotes a lesion that stained heterogeneously and contained both positive and negative cells.
[c]Negative denotes a lesion that stained negative relative to surrounding tissue.

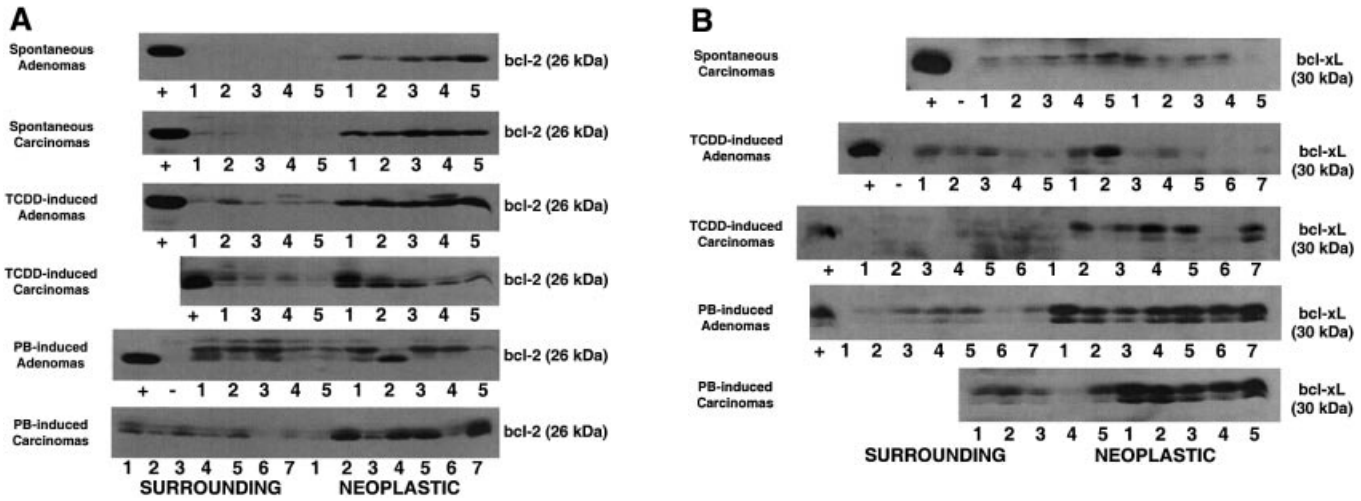

Fig. 3. (**A**) Expression of bcl-2 protein in spontaneous, TCDD-induced and PB-induced adenomas and carcinomas and surrounding tissue. (**B**) Expression of bcl-X_L protein in spontaneous, TCDD-induced and PB-induced adenomas and carcinomas and surrounding tissue. Protein expression in neoplastic tissue (right) was compared with protein expression in surrounding tissue (left). Normal and neoplastic tissue from the same liver are denoted with corresponding numbers. Study protocols were described in Materials and methods. Protein lysates from neoplasias and surrounding non-neoplastic mouse liver were normalized for protein content (50 mg/lane) and analyzed by immunoblotting using antibodies against the aforementioned proteins. Positive controls were M1 mouse myeloma cell lysate (bcl-2) and HeLa cell lysate (bcl-X_L) and the negative control was mouse brain lysate (bcl-2 and bcl-X_L). Representative blots are shown.

Table II. Expression of bcl-X_L in mouse hepatocellular lesions

Group	Altered hepatic foci			Adenomas			Carcinomas		
	Small-cell basophilic	Large-cell basophilic	Acidophilic	Small-cell basophilic	Large-cell basophilic	Acidophilic	Positive[a]	Mixed[b]	Negative[c]
Spontaneous	0/6 (0%)	n/a	4/4 (100%)	2/17 (12%)	n/a	2/2 (100%)	3/12 (25%)	2/12 (17%)	7/12 (58%)
WY-14,643	0/2 (0%)	3/3 (100%)	n/a	0/2 (0%)	8/10 (80%)	n/a	4/4 (100%)	0/4 (0%)	0/4 (33%)
TCDD	3/23 (13%)	n/a	3/5 (60%)	3/18 (17%)	n/a	3/4 (75%)	6/19 (32%)	8/19 (42%)	5/19 (26%)
PB	1/5 (20%)	n/a	21/23 (91%)	0/3 (0%)	n/a	38/40 (95%)	19/28 (68%)	7/28 (25%)	2/28 (7%)
Chlordane	n/a	n/a	n/a	0/1 (0%)	n/a	4/4 (100%)	4/4 (100%)	0/4 (0%)	0/4 (0%)
Totals	4/36 (12%)	3/3 (100%)	28/32 (88%)	5/41 (12%)	8/10 (80%)	47/50 (94%)	52/67 (78%)		14/67 (21%)

n/a denotes that no lesions of the indicated phenotype were present or that they were unavailable for analysis.
[a]Positive denotes a lesion that stained positive relative to surrounding tissue.
[b]Mixed denotes a lesion that stained heterogeneously and contained both positive and negative cells.
[c]Negative denotes a lesion that stained negative relative to surrounding tissue.

cells with heterogeneous morphology. Expression of bcl-2 was increased in 80% of these neoplasms (53/66) and 77% (51/66) showed an increase in bcl-X_L expression (Tables I and II). Expression of bcl-2 and bcl-X_L correlated with regions of increased relative basophilia or acidophilia, respectively. Many carcinomas (67%, 44/66) expressed both bcl-2 and bcl-X_L or heterogeneously stained for bcl-2 and bcl-X_L in different regions of the carcinoma. Collectively, only 8% of foci, 3% of adenomas and 1.5% of carcinomas did not express either bcl-2 or bcl-X_L. Although most carcinomas were not exclusively distinguishable as either basophilic or acidophilic, carcinomas from mice that developed a majority of basophilic neoplasias (i.e. spontaneous, TCDD or WY-14-643) were more likely to express increased amounts of bcl-2 (94%, 32/34) than carcinomas from mice that developed a majority of acidophilic neoplasia (66%, 21/32) (i.e. PB and chlordane) (Tables I and II). Similarly, carcinomas from animals that developed a majority of acidophilic neoplasias were more likely to express increased amounts of bcl-X_L (94% 30/32) than carcinomas from animals that developed a majority of basophilic neoplasia (62%, 21/34) (Tables I and II).

Immunoblotting. Bcl-2 and bcl-X_L expression was also analyzed in neoplasms by immunoblotting. Analysis of protein expression by immunoblotting indicated that 100% of adenomas (10/10) and carcinomas (10/10) arising spontaneously and 90% of adenomas (9/10) and carcinomas (9/10) from TCDD-treated mice expressed increased levels of bcl-2 protein compared with surrounding tissue (Figure 3A). In contrast, only 21% of adenomas (3/14) and 43% of carcinomas (6/14) from PB-treated mice expressed increased levels of bcl-2 protein compared with surrounding tissue (Figure 3A). Immunoblot analysis also indicated that 10% of carcinomas (1/10) arising spontaneously and 21% of adenomas (3/14) and 58% of carcinomas (7/12) from TCDD-treated mice expressed increased levels of bcl-X_L protein compared with surrounding tissue (Figure 3B). In contrast, 86% of adenomas (12/14) and 93% of carcinomas (14/15) from PB-treated mice expressed increased levels of bcl-X_L protein compared with surrounding tissue (Figure 3B). Both immunoblotting and immunohistochemical analysis demonstrated similar alterations of bcl-2 and bcl-X_L, which correlated with lesion phenotype and stage of progression.

Discussion

Previous studies have suggested that suppression of apoptosis is an important mechanism of induction of cancer by non-genotoxic hepatocarcinogens (10–13). Data generated in the present study indicate that important molecular mediators of apoptosis (i.e. the bcl-2 family) are altered in mouse liver by acute treatment with certain non-genotoxic carcinogens. Furthermore, molecular mediators of apoptosis are altered throughout lesion development during spontaneous or non-genotoxic carcinogen-induced hepatocarcinogenesis. The hypothesis that dysregulation of apoptosis is a critical component of mouse non-genotoxic hepatocarcinogenesis is supported by the following observations: (i) acute treatment with non-genotoxic carcinogens in the present studies resulted in altered expression of bcl-2 family proteins; (ii) bcl-2 or bcl-X_L expression was increased in most lesions in the present studies; (iii) previous studies in rodents that demonstrated that non-genotoxic carcinogens inhibit apoptosis in normal and neoplastic liver (10–13,40). However, there are few quantitative data on apoptosis in mouse liver and little is known about its regulation in hepatocytes. Continued investigation of the dysregulation of apoptosis by non-genotoxic carcinogens and linkage to apoptotic indices in mouse liver may be useful in understanding mechanisms by which cancer arises in mouse liver.

The induction of bcl-2 and suppression of bax expression by PB and the induction of bcl-2 and BAG-1 by the peroxisome proliferator WY-14,643 in mouse liver following acute treatment extends our previous *in vitro* studies demonstrating that PB and peroxisome proliferators inhibit apoptosis through altered regulation of the bcl-2 family proteins in primary hepatocytes (31). These previous studies in primary hepatocytes indicated that these distinct non-genotoxic carcinogens differentially inhibit apoptosis induced by different stimuli and through distinct mechanisms. It is possible that the increase in bcl-2 and decrease in bax protein levels following PB treatment and the increase in bcl-2 and BAG-1 following WY-14,643 treatment are related to effects on hepatocyte growth and survival pathways that are specific to each non-genotoxic carcinogen. The differences in the mechanisms by which these diverse non-genotoxic carcinogens inhibit apoptosis may provide insight into why certain non-genotoxic chemicals promote distinct cell populations. Hepatocytes differ in function and gene expression depending on their location within specific lobular regions of the liver. The potential ability of non-genotoxic carcinogens to selectively cooperate with expressed gene products and signaling pathways in the inhibition of apoptosis in distinct cell populations could result in a selective survival advantage for that population of cells. In the present study, increases in bcl-2 expression induced by PB in mice were shown to be localized to hypertrophic centrilobular hepatocytes known to result from PB treatment (41). The induction of bcl-2 expression and inhibition of bax expression by PB, along with the expression of bcl-X_L in centrilobular hepatocytes, could potentially result in a selective survival advantage for this cell population. Treatment of mice with PB has previously been shown to increase cell proliferation in centrilobular hepatocytes (42). In addition, PB induced the expression of transforming growth factor (TGF)-α and epidermal growth factor (EGF)-r and inhibited expression of TGF-β and M6P-R in centrilobular hepatocytes (43). The TGF-α and TGF-β pathways have been shown to regulate both proliferation and apoptosis as well as expression of bcl-2 family members in hepatocytes (31,44–47). The induction of proliferation and the inhibition of apoptosis in centrilobular hepatocytes along with expression of genetic markers that are common to both centrilobular hepatocytes and neoplasms induced by PB suggest that the neoplasms arise from this cell population (42,43,48). There is also evidence that suggests that neoplasia induced by peroxisome proliferators (e.g. deyhydroepiandosterone) in rodent liver may arise from specific cell lineages in the periportal region of the liver (49,50). The present observations may delineate mechanisms by which non-genotoxic carcinogens promote certain cell populations and may also differentiate effects of non-genotoxic agents on normal cell populations versus genetically altered cell populations (13,51,52).

In addition to promoting the clonal expansion of discrete subpopulations of hepatocytes, diverse non-genotoxic carcinogens are known to elicit phenotypically different types of liver preneoplastic and neoplastic lesions. In the present study, TCDD induced primarily heterogeneous, small-cell basophilic lesions, WY-14,643 induced primarily homogeneous, large-cell basophilic lesions and PB and chlordane induced primarily acidophilic lesions. In addition to distinct staining characteristics, phenotypically distinct lesions also differ with respect to expression of growth factors, alteration of cellular oncogenes and reversibility upon withdrawal of the non-genotoxic carcinogen (13,53–56). The differences in bcl-2 and bcl-X_L expression between distinct lesion phenotypes in the present study support previous findings that these types of lesions might arise via distinct molecular events. Genetic events that are commonly observed in each neoplastic phenotype, such as the mutation of *ras* genes in certain basophilic lesions or overexpression of TGF-α and EGF-r in acidophilic lesions, may contribute to the changes in expression of apoptosis genes in neoplastic lesions (53,57). Activation of both Ras- and TGF-α-mediated signal transduction pathways has been shown to enhance cell survival and affect regulation of both bcl-2 and bcl-X_L expression *in vitro* (58,59). The cooperative interaction between certain oncogenes that are activated in initiated cells and the changes in gene expression induced by non-genotoxic carcinogens may increase the susceptibility of initiated cells to tumor promotion by altering regulation of cell replication and apoptosis. Furthermore, the inhibition of apoptosis and induction of cell replication in selected initiated cells could result in clonal expansion of the initiated cell population and, thus, contribute to determining the lesion phenotype. The inhibition of apoptosis by TCDD in certain preneoplastic cell populations, while having a minimal affect on apoptosis in normal hepatocytes, may illustrate the cooperation between activated oncogene pathways and induced changes in gene expression in the inhibition of apoptosis during tumor promotion (16,17).

The alteration in bcl-2 family member expression following acute treatment with PB and WY-14,643 and the phenotype-dependent expression of bcl-2 and bcl-X_L in hepatic preneoplasms and neoplasms suggest that there are both agent- and lesion-specific components to altered apoptosis regulation during non-genotoxic hepatocarcinogenesis. In addition to the expression of bcl-X_L observed in most acidophilic lesions, lesions induced by PB were observed to express bcl-2 at levels similar to centrilobular hepatocytes but greater than periportal hepatocytes. Bcl-2 expression was not observed in most acidophilic lesions or in centrilobular hepatocytes in mice not

treated with PB, indicating that the observed changes in bcl-2 expression were specific to PB. Both bcl-2 and BAG-1 were shown to be induced by WY-14,643 and were observed to be expressed in large-cell, homogeneous basophilic lesions induced by WY-14,643. Bcl-2 and BAG-1 have been shown to be co-regulated and cooperate in the inhibition of apoptosis in a number of cell types. The expression of bcl-2 and BAG-1 in WY-14,643-induced lesions may also suggest that alterations in expression in lesions are related to both the presence of the agent as well as the neoplastic phenotype.

It should also be noted that alterations in protein expression that are not related to the presence of the non-genotoxic carcinogen do not clearly increase throughout the course of progression during hepatocarcinogenesis. Similar percentages of foci, adenomas and carcinomas occurring spontaneously or induced by TCDD were bcl-2-positive, whereas similar percentages of foci, adenomas and carcinomas induced by PB or chlordane were bcl-X_L-positive. These observations suggest that signaling pathways or molecular mediators that regulate bcl-2 or bcl-X_L are altered at an early stage in hepatocarcinogenesis. Furthermore, the expression of bcl-2 early and in a high percentage of spontaneous and TCDD-induced lesions and expression of bcl-X_L early and in a high percentage of PB- and chlordane-induced lesions may suggest that alteration of certain obligatory or redundant pathways that regulate bcl-2 or bcl-X_L expression in each type of lesion is necessary for the initiation of hepatocarcinogenesis.

The understanding of agent- and lesion-specific components to altered apoptosis regulation may aid in the understanding of mechanisms by which neoplastic cells undergo apoptosis and lesions regress upon withdrawal of the promoting stimulus. Upon withdrawal of the stimulus, the expression and equilibrium of bcl-2 family members may be altered, favoring initiation of apoptosis pathways in target cell populations. Once certain lesions reach a particular state of progression or malignancy, however, they may no longer be dependent on the presence of the promoting stimulus. The increased number of carcinomas, as compared with adenomas and foci, that express both bcl-2 and bcl-X_L may indicate the accumulation of further genetic alterations in these lesions and be used as a marker of progression in mouse liver. The expression of both bcl-2 and bcl-X_L in certain carcinomas may indicate the dysregulation of signaling pathways that are altered in both basophilic and acidophilic neoplasia and signify a point in malignancy when these lesions become independent of the promoting stimulus.

The present study illustrates an alteration in apoptosis-related protein expression by non-genotoxic carcinogens and during mouse hepatocarcinogenesis and tumor progression. The alteration in apoptosis regulation by certain non-genotoxic carcinogens in distinct cell populations may contribute to a selective growth advantage and promotion of these cell populations. The alteration of apoptosis regulation by certain non-genotoxic carcinogens in distinct cell populations may contribute to a selective growth advantage and promotion of these cell populations. The selective effects of certain non-genotoxic carcinogens on distinct cell populations may also contribute to determination of the neoplastic phenotype. Bcl-2 expression, along with other genetic events (e.g. *ras* mutations) observed in certain mouse liver tumors, are rare in human hepatocellular carcinomas (60,61). The understanding of dysregulation of apoptosis in mouse liver tumors in the context of events that occur in human cancer may aid in the interpretation of rodent bioassay results for selected chemicals that cause liver tumors.

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