Virchows Arch (1999) 434:333–339

ORIGINAL ARTICLE

Balthasar J. Stähelin · Ulrich Marti
Heinz Zimmermann · Jürg Reichen

The interaction of Bcl-2 and Bax regulates apoptosis in biliary epithelial cells of rats with obstructive jaundice

Received: 9 July 1998 / Accepted: 16 December 1998

Abstract A complex molecular network controls cell homeostasis by inducing apoptosis or proliferation. The balance of Bcl-2 and Bax, members of a protein family, determines whether a cell will become immortal (Bcl-2) or will undergo apoptosis (Bax). To determine the role of Bcl-2 and Bax during proliferation of biliary epithelial cells (BEC) after bile duct ligation (BDL) and their regression after biliary decompression we induced hyperplasia of BEC by BDL in male rats. Regression of hyperplastic BEC by way of apoptosis was induced by biliary decompression through a Roux-en-Y biliodigestive anastomosis. To quantify apoptosis a modified TUNEL assay was used. Expression of Bcl-2 and Bax was visualized by immunohistochemistry and quantified stereologically. BEC increased from <1% to >20% after BDL; this increase was associated with overexpression of Bcl-2 in up to 30% of hyperplastic BEC. After biliodigestive anastomosis, apoptotic BEC increased from <0.1% to a peak of 5.4% after 1 day to reach baseline again 1 week after decompression. This was associated with de novo appearance of Bax. The interaction between Bcl-2 and Bax triggers apoptosis in BEC and acts as a cell rheostat in BEC hyperplasia and its involution after biliary decompression.

Key words Apoptosis · Programmed cell death · Ductular proliferation · Biliary decompression · Immunohistochemistry

B.J. Stähelin · U. Marti
Department of Internal Medicine, Inselspital, CH-3010 Berne, Switzerland

H. Zimmermann · J. Reichen (✉)
Department of Clinical Pharmacology, University of Berne, Murtenstrasse 35, CH-3010 Berne, Switzerland
e-mail reichen@ikp.unibe.ch
Tel.: +41-31-632-8711, Fax: +41-31-632-4997

Introduction

Cell homeostasis is regulated by a balance between apoptosis (programmed cell death), proliferation and growth arrest [30, 31, 42]. Like proliferation, apoptosis is an active process requiring an intracellular signalling cascade of protein interactions activated through a broad variety of stimuli [19, 38, 42].

The protein Bcl-2, first characterized in a B cell leukaemia cell line [10], shows marked homology with *ced-3*, an anti-apoptotic gene in *Caenorhabditis elegans* [12]. By co-precipitation with Bcl-2 an associated protein, Bax, was characterized [27]; various other homologues of Bcl-2 have since been identified. This protein family is an important modulator of cell homeostasis, which it influences by promoting (Bax) or inhibiting (Bcl-2) apoptosis. The Bcl-2 protein family is characterized by two highly conserved regions and their ability to form homo- or heterodimers [32]. The ratio of homo- and heterodimers apparently decides the fate of the cell. Thus, in the presence of an excess of Bcl-2 its homodimers dominate and protect cells from apoptosis [33]. Conversely, Bax inhibits Bcl-2 by forming a heterodimer and thereby opposing the antiapoptotic properties of Bcl-2 [27].

Different mechanisms to explain this rheostat – including an influence on the intracellular calcium level, the production of reactive oxygen species (ROS) and changes in the mitochondrial membrane permeability – have been proposed [32]. Recently, Antonsson et al. demonstrated that Bax forms channels and that this formation is inhibited by Bcl-2 [1]. Furthermore, in Bcl-2/Bax heterodimers, Bcl-2 serves as a target for Raf-1 to inactivate Bax by phosphorylation [40]. The situation is certainly more complex than this simple model, since other family members can also interact with Bcl-2 and Bax [5, 14, 16, 19], but the balance between Bcl-2 and Bax has been clearly shown to act as a cell rheostat in different cell lines in vitro [32].

Ligation of the common bile duct in the rat induces marked proliferation of bile duct epithelial cells [11, 17] which is reversible upon relief of the obstruction by

biliodigestive anastomosis [4, 43]. The removal of these cells has been shown to occur by apoptosis [4] but the signals mediating the maintenance of hyperplasia and its removal have yet to be defined. To investigate whether the cellular rheostat described above is also functional in this model, we investigated the expression of Bcl-2 and Bax in livers after bile duct obstruction and its relief by Roux-en-Y biliodigestive anastomosis.

Materials and methods

Male Sprague-Dawley rats were obtained from Süddeutsche Versuchstierfarm Hartmut and Voss (Tuttlingen Germany) and allowed free access to standard chow and tap water; they were kept in temperature-controlled animal quarters under a 12 h:12 h light–dark cycle. Secondary biliary cirrhosis was induced by bile duct ligation (BDL) and dissection according to Kontouras et al. [17] as previously described in a publication from our laboratories [11]; sham-operated animals served as controls. Biliary decompression was performed by Roux-en-Y biliodigestive anastomosis as previously described [43]. At 3 weeks after BDL and on days 1, 2, 3 and 7 after R-Y anastomosis animals were killed by exsanguination under pentobarbital anaesthesia; liver tissue was in part homogenized for immunoblotting and in part fixed in 4% buffered formalin; 4 animals per group were studied. All animal experiments were approved by the State Supervisory Board on Animal Experimentation and were performed according to federal and international guidelines regulating animal experimentation.

The TUNEL assay was performed according to the original description of Gavrieli et al. [9] modified by preincubation with diethylpyrocarbonate, a slight but essential modification to inhibit false-positive staining of nuclei [35]. Briefly, formalin-fixed paraffin-embedded liver tissue was cut into 4- to 6-µm sections and glued with Cementit (Merz & Benteli, Niederwangen, Switzerland) to regular glass slides. After the section had been dewaxed in xylene it was incubated for 30 min in 4% diethylpyrocarbonate in ethanol [35]. Thereafter, the slides were rehydrated in a series of decreasing ethanol concentrations and then washed in phosphate-buffered saline (PBS). Nuclei were stripped from proteins by incubation with proteinase K 10 µg/ml (Boehringer Mannheim, Germany) in Tris-HCl 20 mM/EDTA (pH 8.1) 5 mM at 37°C for up to 60 min. Digestion was stopped with H_20 and the slides were washed four times in H_20. Then the sections were incubated for 90 min at 37°C with terminal deoxyribonucleotidyl transferase (75 U/ml) and digoxigenin-11-dUTP (5 nmol/1 ml) in 200 mM potassium cacodylate, 50 µg/ml of bovine serum albumin and 2.5 mM of $CoCl_2$ (pH 8). The slides were washed with SSC (NaCl 150 mM, Na_3Citrate 15 mM, pH 7.0), followed by Tris-HCl 10 mM /NaCl 150 mM, pH 8.2. Unspecific binding was blocked with a blocking reagent for nucleic acid, hybridization and detection (Boehringer Mannheim, Germany) for 30 min at room temperature. Alkaline phosphatase-labelled Fab fragments against digoxigenin (Boehringer Mannheim, Germany) were used to detect the labelled nick end of the DNA strands diluted 1:1000 in PBS. Fast red chromogen (Boehringer Mannheim) was used as substrate. The reaction was stopped after 15–30 min by washing with H_20.

For Western blotting one lobe of the liver was immediately homogenized in 4 volumes of ice-cold 5 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM $MgCl_2$, 1 mM phenyl-methyl-sulfonyl fluoride, 25 mM benzamidine, 5 µg/ml leupeptine using a Polytron (twice, each time for 15 s), followed by 15 strokes in a Dounce glass homogenizer with loose-fitting pestle. Protein concentration was measured by the Lowry method [23], using bovine serum albumin as a standard. Fifty micrograms of protein per lane was run in 10% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which where then blocked with 5% nonfat milk in TBS-T (TBS; 20 mM Tris HCl, 500 mM NaCl, pH 7.5 and Tween 1%). The washed membranes were incubated for 1 h at room temperature with antibodies against Bcl-2 protein (murine monoclonal against human Bcl-2, DAKO-Bcl-2/124, purchased from DAKO, High Wycombe, Bucks., UK) and Bax (polyclonal antibody from rabbit, purchased from Santa Cruz Biotechnology, Santa Cruz Calif.), respectively, at a dilution of 1 : 250. After being washed three times in TBS-T, membranes were incubated with a peroxidase-conjugated F(ab)2 secondary antibody goat anti-mouse IgG for Bcl-2 and goat anti-rabbit IgG for Bax for 1 h. Membranes were washed again in TBS-T and then incubated in enhanced chemiluminescent (ECL) detection reagents (Amersham International, Amersham, Bucks., UK) for 30 s at room temperature and immediately thereafter exposed to X-Omat film (Eastman Kodak, Rochester, N.Y.).

To purify the polyclonal antibody against Bax we performed a regular Western blot as described above. After transfer of the proteins to the nitrocellulose membrane, the band with the appropriate weight for Bax (21 kDa) was cut out and the resulting strip was blocked in nonfat milk in PBS, washed with TBS-T and then incubated with the primary antibody against murine Bax overnight at RT. After three washes with TBS-T the bound antibodies on the membrane strip were washed off with 1 ml of KSCN 3M. The membrane strip was then removed and washed with 19 ml of nonfat milk diluted in PBS, and 1 ml of 3 M KSCN was added to the nonfat milk–PBS. This solution was used to incubate the nitrocellulose membranes from which the strip was taken for 24 h at RT. This membrane was then processed through the standard Western blotting protocol given above.

For the immunohistochemical detection of Bcl-2 and Bax in liver tissue sections, formalin-fixed liver sections were glued with Cementit to regular glass slides to detect Bax and mounted on poly-L-lysine-coated glass slides (Sigma, St. Louis, Mo.) for detection of Bcl-2. The use of poly-L-lysine adhesive was found earlier to be crucial for the antigen retrieval process.

For the detection of Bcl-2 antigen retrieval enhancement was necessary: the sections were exposed in a microwave oven at 95°C for 15 min in citrate buffer (pH 4.0). To inhibit endogenous peroxidase the tissue sections were incubated for 10 min with an aqueous solution of H_20_2 (1:20 v/v). All samples were routinely blocked for 30 min in 1:10 (v/v) normal goat serum (Zymed, San Francisco, Calif.) diluted in PBS, pH 8.1, prior to the addition of antibody. The antibody to human Bcl-2 was diluted 1:250 in PBS plus 0.5% bovine serum albumin, then incubated with the section for 90 min. After washing twice in PBS, the preparations were covered with a biotinylated goat anti-mouse IgG (Zymed, San Francisco, Calif.) used at a 1:200 dilution in PBS for 60 min. Samples were then sequentially washed in PBS and exposed for 60 min to streptavidin–peroxidase diluted 1:200 in PBS. Peroxidase-stained sections

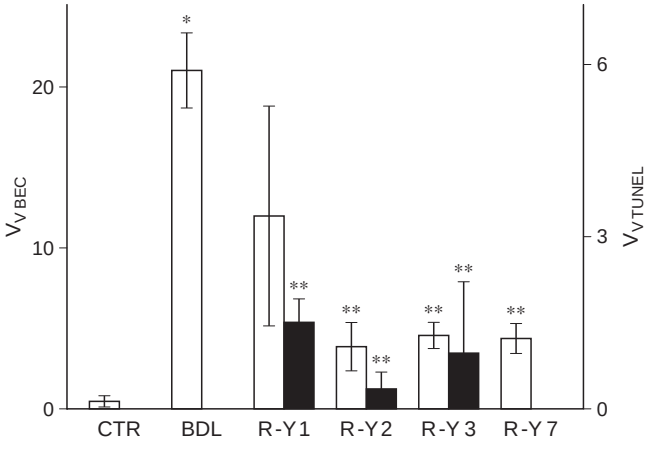

Fig. 1 Volume fraction of biliary epithelial cells (❑) and of apoptotic cells by TUNEL (■) in control animals 21 days after ligation of the common bile duct (BDL) and following Roux-en-Y anastomosis (*R-Y*; the figures refer to days after biliary decompression). Mean±1 standard deviation are shown; *n*=4/group. *Asterisks* (*) denote a statistically significant difference compared with controls (ANOVA). A wave of apoptosis accompanies the rapid involution in the 3 days after RY anastomosis

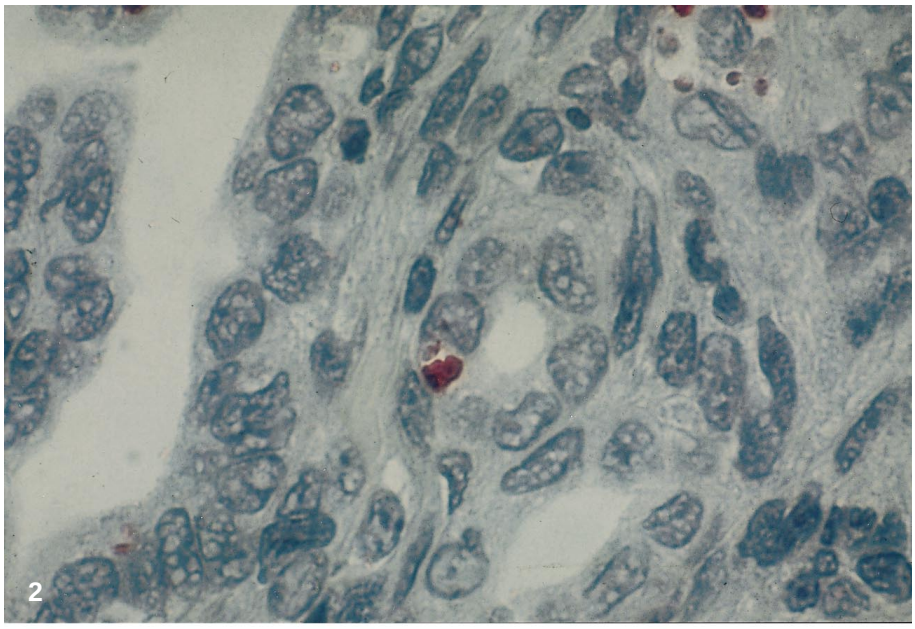

Fig. 2 TUNEL-positive biliary epithelial cell in a rat liver on day 1 after Roux-en-y anastomosis. Fast red, counterstained with haemalaum, ×600

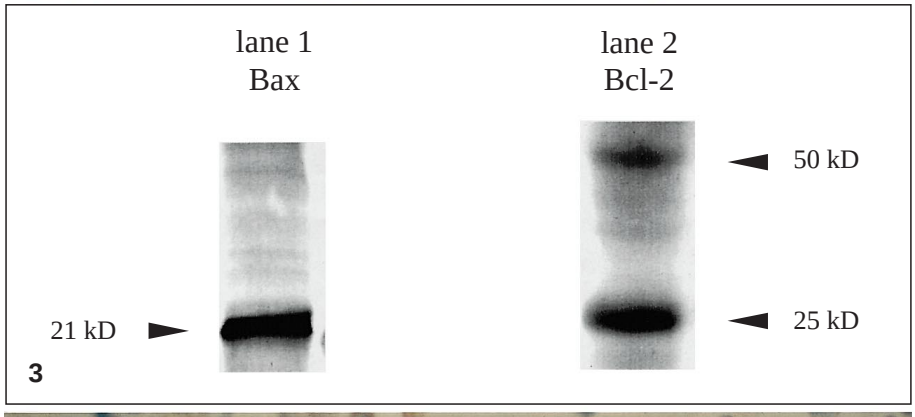

Fig. 3 Immunoblot with monoclonal antibodies against Bcl-2 and polyclonal antibodies against Bax. Both antibodies show binding to the respective protein of the appropriate size. In *lane 2* the specific staining of a lane at exactly twice the expected weight indicates that homodimers of Bcl-2 are present

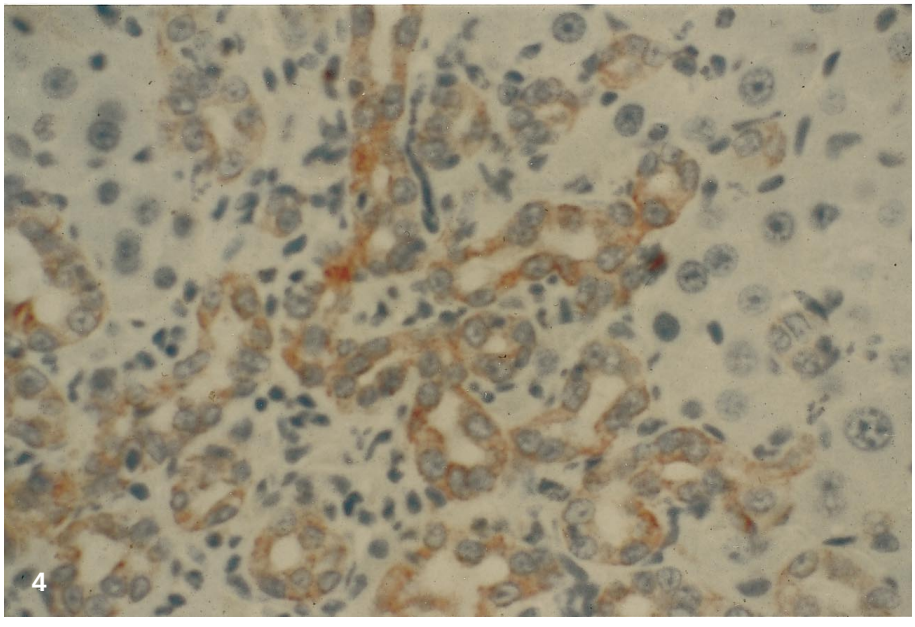

Fig. 4 Periportal zone of a rat liver on day 1 after Roux-en-Y anastomosis. There is still marked ductular proliferation with positive staining of the cytoplasm of cholangiocytes for Bcl-2. Other cells (including hepatocytes) do not stain for Bcl-2. ×400

were developed with diaminobenzidine. Control preparations were incubated in the absence of primary antibody.

To detect Bax, the purified antibody described above was used. Essentially the same procedure as described for the detection of Bcl-2 was used. The antigen-retrieval step was omitted; biotinylated goat anti-rabbit antibodies were used as secondary antibody.

After systematic random sampling [6] livers were characterized stereologically by the point counting procedure of Weibel [41] as described from our laboratories for conventional [11] and immunohistochemical sections [26]. Point counting was performed at a magnification of 40x; points were assigned to hepatocytes, bile duct epithelial cells or other structures. Results were expressed as volume fractions (V_v) taking the whole liver as the reference space [11]. Additionally, in appropriate sections, points characterized as positive for Bcl-2, Bax or TUNEL were counted. Only bile duct epithelial cells with clear-cut staining of the cytoplasm or nuclei for the specific antibodies against Bcl-2 or Bax were scored. In the TUNEL assay, only positive nuclei were scored.

The results were expressed as mean±1 standard deviation. Means of the different groups were compared with analysis of variance [34], with $P<0.05$ being considered statistically significant.

Results

Bile duct ligation led to marked ductular proliferation as previously described [11], exceeding 20% V_v after 3 weeks. Following biliary decompression by means of a Roux-en-Y biliodigestive anastomosis, bile duct epithelial cells rapidly decreased, to about 5% by day 7 (Fig. 1).

In livers from BDL rats, hardly any biliary epithelial cells exhibited features of apoptosis or were TUNEL positive (<1/1,000). As previously described by Bhathal et al. [4], biliary decompression was immediately followed by a wave of apoptosis, peaking as soon as day 1 after surgery, with 6.7% of bile duct epithelial cells being TUNEL positive (Fig. 1). This wave was over 1 week after biliary decompression, when the baseline value for TUNEL-positive cells was recovered (Fig. 1). Most TUNEL-positive cells met further criteria of apoptosis [15], such as chromatin condensation or fragmentation into apoptotic bodies (Fig. 2).

Both antigens of interest, Bcl-2 and Bax, were seen on Western blots of liver homogenates as proteins of the appropriate weight (Fig. 3). For both proteins, two bands – one with the expected molecular weight of the monomer, the other exactly twice its weight – could be visualized, a finding described previously [27].

Bcl-2 could not be detected in control livers (data not shown); it was not expressed in hepatocytes in control, biliary cirrhotic or biliary decompressed livers. In contrast, expression of Bcl-2 increased markedly in biliary epithelial cells after ligation of the common bile duct (Fig. 4), one third of biliary epithelial cells expressing this protein in the liver from a BDL rat (Fig. 5). Somewhat surprisingly, the percentage of Bcl-2-positive bile duct epithelial cells increased up to 80% after biliary decompression, (during the wave of apoptosis). On day 7 after biliary decompression Bcl-2 remained elevated, with close to 50% of biliary epithelial cells staining positive for Bcl-2 (Fig. 5).

In contrast to Bcl-2, hepatocytes expressed Bax under all experimental conditions: in control (Fig. 6A), biliary cirrhotic (Fig. 6B) and decompressed livers (Fig. 6C). Another pattern could be seen in biliary epithelial cells: Bax was not expressed in bile duct epithelial cells after ligation of the common bile duct, but appeared immediately after biliary decompression (Fig. 6C). Its appearance coincided with the wave of apoptosis that removed the hyperplastic cholangiocytes (Figs. 1, 5). There, it can be seen that the expression of Bax was restricted to the 3 days following the Roux-en-Y biliodigestive anastomosis, roughly 80% of cholangiocytes staining positive for Bax. At day 7 – when no apoptosis was detectable by TUNEL – the expression of Bax had vanished (Fig. 5).

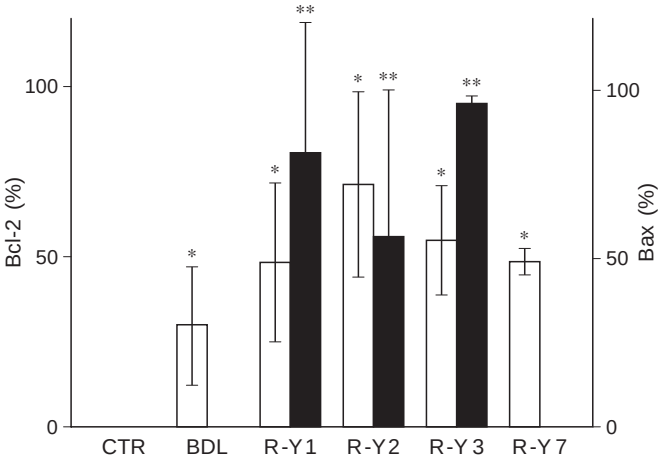

Fig. 5 Percentage of bile duct epithelial cells staining positive for Bcl-2 (❑) or Bax (■) in BDL rats after 21 days and during involution of secondary biliary cirrhosis after Roux-en-Y anastomosis (*R-Y*). In hyperplastic bile ductules BEC express Bcl-2 lacking Bax. The involution of hyperplastic BEC by apoptosis (see Fig. 1) is accompanied by de novo expression of Bax and persistent/increasing expression of BCL-2

Discussion

Our studies confirm that proliferating bile duct epithelial cells are removed by apoptosis. They provide the first evidence that this may be achieved by the balance between Bcl-2 and Bax, the main inhibitor and promoter, respectively, of cell removal by apoptosis. This cellular rheostat [27] has been well described in other epithelia, but to our knowledge not so far in biliary epithelial cells.

Three weeks after BDL there is marked proliferation of epithelial cells [11, 17]. The signal for this proliferation remains unknown, but we have previously reported overexpression of nuclear EGF receptor in proliferating bile ductules after biliary obstruction [4]. The de novo expression of Bcl-2, a protein protecting cells from apoptosis [13, 25, 32, 39] suggests that inhibition of apoptosis could also contribute to ductular proliferation in extrahepatic obstruction.

The signal for the expression of Bcl-2 in bile duct epithelial cells in obstructed liver remains unclear. It is tempting to speculate that bile acids could be the signal,

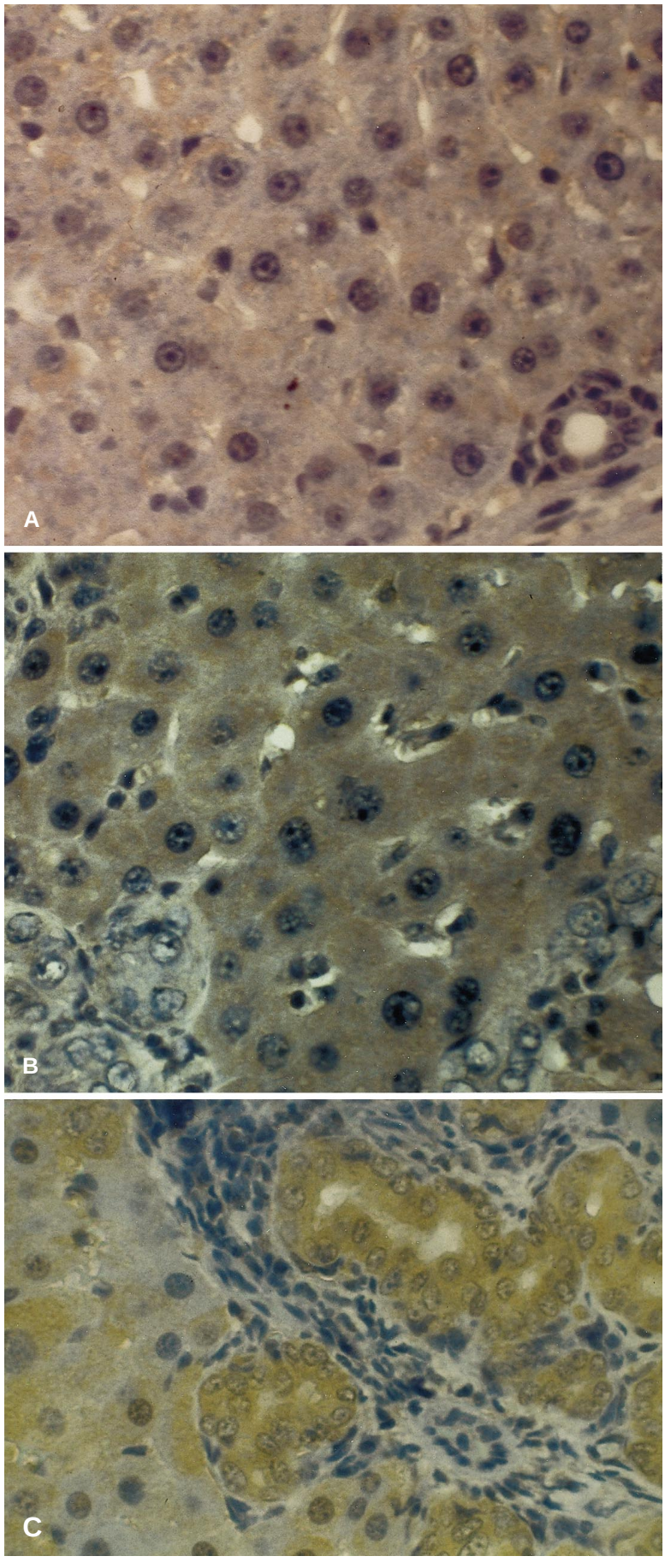

Fig. 6A–C Expression of BAX in liver. **A** In normal liver there is staining of hepatocytes but not of bile duct epithelial cells. **B** Three weeks after BDL there is ductular proliferation. As in control livers, hepatocytes but not cholangiocytes stain positively for BAX. **C** After biliary decompression there is a marked increase in staining for BAX in bile duct epithelial cells

since they have been shown to induce apoptosis at least in hepatocytes [29]. Another explanation for the overexpression of Bcl-2 could be a role in cell growth: Bcl-2 inhibits cells from promoting through the cell cycle [10, 24]; thus, its expression might reflect an inhibition of ductular proliferation. Overexpression of Bcl-2 has also been described in human liver after massive hepatic necrosis [7] and the opposite, down-regulation, has been reported in human liver allografts, in which bile ductules are prone to apoptotic cell death in the setting of acute rejection [8].

Our data on Bax clearly demonstrate its key role in initiating apoptosis in cholangiocytes: regardless of the expression of Bcl-2 – which actually increases after removal of the stimulus for hyperplasia – the immunohistochemical detection of Bax coincides with the wave of apoptosis. Again, the stimulus for the expression of Bax remains to be identified. In contrast, the mechanism whereby the balance of Bcl-2 and Bax could induce or inhibit apoptosis has recently been elucidated: Antonsson et al. have demonstrated that Bcl-2 inhibits channel formation initiated by Bax [1]. This is in line with the mechanism of apoptosis in hepatocytes, where a ceramide-mediated mitochondrial membrane transition has been identified as a key event in apoptosis [2].

The removal of excess biliary epithelial cells by apoptosis was first described by Bhathal et al. [4] and has been quantitated with a modified TUNEL assay in this study. Compared with the small number of apoptotic bile duct epithelial cells at any given time, the velocity of involution (a 5-fold decrease in the volume fraction of bile duct epithelial cells within only 3 days) is astonishing at first sight. However, this is explained by the fact that apoptosis needs only a few hours to be completed and early loss of cell–cell contact will inevitably promote shedding of apoptotic cells into the biliary lumen [3, 28]. Apoptotic bile duct epithelial cells can be collected in bile after decompression of the common bile duct [4].

In contrast to bile duct epithelial cells, apoptosis among hepatocytes in BDL rats and after bile duct decompression does not differ in amount from that in hepatocytes in sham-operated control animals, in agreement with the observations of other authors [3]. The mechanism of apoptosis in cholangiocytes has yet to be unravelled; early investigations by the Mayo Clinic group demonstrated that most usual pro-apoptotic signals were unable to elicit apoptosis in this cell type but demonstrated the presence of calcium-dependent ICE-like protease, which could be activated by K^+ ionophores and thereby elicit apoptosis [2]. During development, Bcl-2 also plays an essential part in organogenesis in the liver and is expressed in bile ductules [36]; its counterpart in this situation could be Fas, but Bax was not sought in the study cited. An involvement of Fas in human disease is suggested by a study by Kuroki et al., which found Fas expression in livers from patients with primary biliary cirrhosis [20]. Interestingly, Bcl-2 protects the liver from anti-Fas-induced massive hepatocellular necrosis [22, 33].

In agreement with previous studies [18], hepatocytes under normal conditions express Bax. In cholangiocytes, however, Bax is detectable immunohistochemically neither under basal conditions nor after BDL. The presence of Bax in hepatocytes without evidence of an increased rate of apoptosis is explained by the fact that other members of the Bcl-2 family can provide protection against Bax-induced apoptosis. Thus, resting hepatocytes express Bcl-x and Bak, among other proteins [16, 18, 37]. Recently, Bcl-2 – which is not expressed in normal rat hepatocytes – has been detected in hepatocytes of BDL animals by reverse transcriptase–polymerase chain reaction [21]. Immunohistochemically Bcl-2 was not detectable in hepatocytes in the present investigation.

Even though Bcl-2 and Bax are under extensive investigations and the modality of their interaction has been elucidated, this is only the second in vivo model in which the interaction of these two proteins fits in with the model predicted by studies in cell cultures. The other situation in which a similar mechanism of action could be at work in the liver is regeneration, where Bcl-2 and Bcl-x appear earlier than Bax [18]; in this study, however, apoptosis has not been quantified. The up-regulation of members of the Bcl family in liver regeneration has recently been confirmed [37]. Interestingly, in the former study the changes in mRNA suggested regulation at the translational level [18].

In conclusion, we have demonstrated that Bcl-2/Bax may act as a rheostat in maintaining or removing excess biliary epithelial cells in the situation of obstructive jaundice and its reversion by biliodigestive anastomosis.

Acknowledgements This work was supported by a grant from the Swiss National Foundation for Scientific Research (92.45349.95) to J.R.

References

1. Antonsson B, Conti F, Ciavatta AM, Montessuit S, Lewis S, Martinou I, Bernasconi L, Bernard A, Mermod JJ, Mazzei G, Maundrell K, Gambale F, Sadoul R, Martinou JC (1997) Inhibition of Bax channel forming activity by Bcl-2. Science 277:370–373
2. Arora AS, Jones BJ, Patel TC, Bronk SF, Gores GJ (1997) Ceramide induces hepatocyte cell death through disruption of mitochondrial function in the rat. Hepatology 25:958–963
3. Benedetti A, Jezequel AM, Orlandi F (1988) A quantitative evaluation of apoptotic bodies in rat liver. Liver 8:172–177
4. Bhathal PS, Gall JAM (1985) Deletion of hyperplastic biliary epithelial cells by apoptosis following removal of the proliferative stimulus. Liver 5:311–325
5. Chao DT, Linette GP, Boise LH, White LS, Thompson CB, Korsmeyer SJ (1995) Bcl-XL and Bcl-2 repress a common pathway of cell death. J Exp Med 182:821–828
6. Cruz-Orive LM, Weibel ER (1981) Sampling design for stereology. J Microsc 122:235–257
7. Demetris AJ, Seaberg EC, Wennerberg A, Ionellie J, Michalopoulos G (1996) Ductular reaction after submassive necrosis in humans – special emphasis on analysis of ductular hepatocytes. Am J Pathol 149:439–448
8. Gapany C, Zhao M, Zimmermann A (1997) The apoptosis protector, bcl-2 protein, is downregulated in bile duct epithelial cells of human liver allografts. J Hepatol 26:535–542

9. Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. J Cell Biol 119:493–501
10. Gottardi D, Alfarano A, De Leo AM, Stacchini A, Bergui L, Caligaris-Cappio F (1995) Defective apoptosis due to Bcl-2 overexpression may explain why B-CLL cells accumulate in G0. Curr Top Microbiol Immunol 194:307–312
11. Gross JB, Reichen J, Zeltner T, Zimmermann A (1987) The evolution of changes in quantitative liver function tests in a rat model of cirrhosis: correlation with morphometric measurement of hepatocyte mass. Hepatology 7:457–463
12. Hengartner MO, Horvitz HR (1994) Programmed cell death in *Caenorhabditis elegans*. Curr Opin Genet Dev 4:581–586
13. Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 75:241–251
14. Kernohan NM, Cox LS (1996) Regulation of apoptosis by Bcl-2 and its related proteins: immunochemical challenges and therapeutic implications. J Pathol 179:1–3
15. Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257
16. Kiefer MC, Brauer MJ, Powers VC, Wu JJ, Umansky SR, Tomei LD, Barr PJ (1995) Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. Nature 374:736–739
17. Kontouras J, Billing BH, Scheuer PJ (1984) Prolonged bile duct obstruction: a new experimental model for cirrhosis in the rat. Br J Exp Pathol 65:305–311
18. Kren BT, Trembley JH, Krajewski S, Behrens TW, Reed JC, Steer CJ (1996) Modulation of apoptosis-associated genes bcl-2, bcl-x, and bax during rat liver regeneration. Cell Growth Differ 7:1633–1642
19. Kroemer G, Petit P, Zamzami N, Vayssière JL, Mignotte B (1995) The biochemistry of programmed cell death. FASEB J 9:1277–1287
20. Kuroki T, Seki S, Kawakita N, Nakatani K, Hisa T, Kitada T, Sakaguchi H (1996) Expression of antigens related to apoptosis and cell proliferation in chronic nonsuppurative destructive cholangitis in primary biliary cirrhosis. Virchows Arch [B] 42: 119–129
21. Kurosawa H, Que FG, Roberts LR, Fesmier PJ, Gores GJ (1997) Hepatocytes in the bile duct ligated rat express Bcl-2. Am J Physiol 272:G1587-G1593
22. Lacronique V, Mignon A, Fabre M, Viollet B, Rouquet N, Molina T, Porteu A, Henrion A, Bouscary D, Varlet P, Joulin V, Kahn A (1996) Bcl-2 protects from lethal hepatic apoptosis induced by an anti-Fas antibody in mice. Nat Med 2:80–86
23. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
24. Mazel S, Burtrum D, Petrie HT (1996) Regulation of cell division cycle progression by bcl-2 expression: a potential mechanism for inhibition of programmed cell death. J Exp Med 183:2219–2226
25. Newmeyer DD, Farschon DM, Reed JC (1994) Cell-free apoptosis in *Xenopus* egg extracts. Inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. Cell 79:353–364
26. Oguey D, Marti U, Reichen J (1992) Epidermal growth factor receptor: its possible implication in the maintenance of hepatocellular mass in biliary cirrhosis in the rat. Eur J Cell Biol 59:187–195
27. Oltvai ZN, Milliman CL, Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74:609–619
28. Patel T, Gores GJ (1995) Apoptosis and hepatobiliary disease. Hepatology 21:1725–1741
29. Patel T, Bronk SF, Gores GJ (1994) Increases of intracellular magnesium promote glycodeoxycholate-induced apoptosis in rat hepatocytes. J Clin Invest 94:2183–2192
30. Raff MC (1992) Social control on cell survival and cell death. Nature 356:397–399
31. Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y, Jacobson MD (1993) Programmed cell death and the control of cell survival: lessons from the nervous system. Science 262: 695–700
32. Reed JC (1994) Bcl-2 and the regulation of programmed cell death. J Cell Biol 124:1–6
33. Rodriguez I, Matsuura K, Khatib K, Reed JC, Nagata S, Vassalli P (1996) A bcl-2 transgene expressed in hepatocytes protects mice from fulminant liver destruction but not from rapid death induced by anti-Fas antibody injection A bcl-2 transgene expressed in hepatocytes protects mice from fulminant liver destraction hat not from rapid death induced by anti-Fas injection. J Exp Med 183:1031–1036
34. Snedecor GW, Cochran WG (1967) Statistical methods. Iowa State University Press, Ames
35. Stähelin BJ, Marti U, Solioz M, Zimmermann H, Reichen J (1998) False positive staining in the tunel assay to detect apoptosis in liver and intestine is due to endogenous nucleases and inhibited by diethyl pyrocarbonate. J Clin Pathol Mol Pathol 51:204–208
36. Terada T, Nakanuma Y (1995) Detection of apoptosis and expression of apoptosis-related proteins during human intrahepatic bile duct development. Am J Pathol 146:67–74
37. Tzung SP, Fausto N, Hockenbery DM (1997) Expression of Bcl-2 family during liver regeneration and identification of Bcl-x as a delayed early response gene. Am J Pathol 150: 1985–1995
38. Vaux DL, Strasser A (1996) The molecular biology of apoptosis. Proc Natl Acad Sci USA 93:2239–2244
39. Vaux DL, Weissman IL, Kim SK (1992) Prevention of programmed cell death in *Caenorhabditis elegans* by human bcl-2. Science 258:1955
40. Wang HG, Rapp UR, Reed JC (1996) Bcl-2 targets the protein kinase Raf-1 to mitochondria. Cell 87:629–638
41. Weibel ER (1979) Stereological methods. Academic Press, London
42. Williams GT, Smith C (1993) Molecular regulation of apoptosis: genetic control of cell death. Cell 74:777–779
43. Zimmermann H, Reichen J, Zimmermann A, Sägesser H, Tenisch B, Höflin F (1992) Reversibility of secondary biliary fibrosis by biliodigestive anastomosis in the rat. Gastroenterology 103:579–589
44. Zimmermann H, Ganz P, Zimmermann A, Oguey D, Marti U, Reichen J (1995) The overexpression of proliferating cell nuclear antigen in biliary cirrhosis in the rat and its relationship with epidermal growth factor receptor. J Hepatol 23:459–464