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CLINICAL STUDIES

Activation of FoxO3a/Bim axis in patients with Primary Biliary Cirrhosis

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Abstract

Background/Aims: Impaired regulation of apoptosis has been suggested to play a role in the pathogenesis of Primary Biliary Cirrhosis (PBC). In this study, we analysed a signalling pathway that comprises the transcription factor FoxO3a and its downstream target Bim, a Bcl-2 interacting mediator of apoptosis. *Materials & Methods:* The tissues examined included livers explanted from patients with cirrhotic PBC, primary sclerosing cholangitis (PSC), alcoholic liver disease (ALD) and liver biopsies from patients with non-cirrhotic PBC. Large margin resections of hepatocellular carcinoma were used as controls. *Results:* Expression of FoxO3a and Bim mRNA was significantly enhanced in both non-cirrhotic and end-stage PBC (2.2-fold and 4.3-fold increases, respectively), but not in the other disorders. Similarly, FOXO3a protein level was increased in end-stage PBC ($P < 0.05$ vs. control). A significant increase in Bim mRNA in non-cirrhotic and cirrhotic PBC was observed (2.2-fold and 8.2-fold respectively). In addition, the most pro-apoptotic isoform of Bim dominated in livers of PBC patients (2.5- fold increase vs. control; $P < 0.05$). Enhanced FoxO3a and Bim expression was associated with a substantial activation of caspase-3 in PBC (2-fold increase vs. controls; $P < 0.0001$), whereas it was decreased in both ALD and PSC (46% and 67% reductions respectively). The relationship between FoxO3a and Bim was further investigated in the livers of FoxO-deficient mice. The somatic deletion of FoxO3a caused a significant decrease in Bim, but not caspase-3 protein expression confirming the crucial role of FoxO3a in induction of Bim gene transcription. *Conclusions:* Our results imply that the FoxO3/Bim signalling pathway can be of importance in the livers of patients with PBC.

Primary biliary cirrhosis (PBC) is a chronic cholestatic condition of autoimmune origin characterized by progressive destruction of small intrahepatic bile ducts and the presence of antimitochondrial antibodies (AMA). The etiopathogenesis of this disease remains poorly understood. The immunological breakdown can be triggered by genetic susceptibility, immune disregulation or exposure to enivronmental agents such as xenobiotics, hormones or viruses (1–3). Apoptosis has been implicated in PBC development (4). In most circumstances, nearly all autoantigens created during this process are cleaved by caspases or granzyme B. Subsequent phagocytosis of the cleaved autoantigens by dendritic cells facilitates the formation of peripherial self-tolerance (5). However, in patients with PBC, it was suggested that aberrant apoptosis, with subsequent expression of the E2 subunit of pyruvate dehydrogenase complex (PDC-E2) on the surface of the apoptosome, may play an important role in the pathogenesis of the disease (6, 7).

Recent studies uncovered a critical role for the forkhead box (FOX) family of transcription factors in coupling extracellular signals to downstream changes in gene expression (8, 9). The members of this family are distinguished by a conserved 100–amino acid domain called the 'forkhead box' and have been divided into 19 subgroups. In response to external stimuli such as insulin, growth factors, oxidative stress and cytokines, the forkhead-box O (FoxO) subfamily promotes cell cycle arrest, repair of damaged DNA, gluconeogenesis and apoptosis (10, 11). Three major members of the mam-

malian FoxO subfamily have been identified, i.e. FoxO1 (FKHR), FoxO3a (FKHRL1) and FoxO4 (AFX) (12). Recently, FoxO3a was found to activate pro-apoptotic pathways via induction of Bim transcription (13, 14).

Members of the Bcl-2 family of interacting proteins are critical players in apoptosis. Pro-survival members (Bcl-2, Bcl-XL) are in opposition to pro-apoptotic proteins such as Bax and Bim (15). The Bim protein exists as extra long (BimEL), long (BimL) and short (BimS) isoforms. They differ in their apoptotic potential, with BimS being the most potent (16). In mitochondria, Bim either forms a heterodimer with pro-apoptotic Bax, which leads to mitochondrial membrane dysfunction and release of cytochrome c, or it antagonizes the protective function of Bcl-2 by direct binding. Bcl-2, as an anti-apoptotic protein, hinders apoptosis and restrains oxidative damage via inhibition of glutathiolation of protein (16–18). Healthy intrahepatic cholangiocytes, unlike other cells, express high levels of Bcl-2, which makes these cells unaccustomed to exposure to a high concentration of unmodified PDC-E2. It was demonstrated that recognition of apoptotic cell-derived PDC-E2 by AMAs is owing to the failure of linking this molecule to a cytoprotective glutathione (6).

In our study, we hypothesized that activated Bim molecules counter Bcl-2 proteins making them ineffective in reducing the protein glutathiolation induced by apoptosis. Therefore, we have analysed the expression of factors engaged in activation of apoptosis such as FoxO3a, Bim and caspase-3 in patients with cirrhotic and non-cirrhotic PBC, primary sclerosing cholangitis, alcoholic cirrhosis and control liver tissue. We postulated that determining whether a FoxO3a/Bim axis directly affect the activity of Bcl-2 in patients with PBC would help in our understanding of the pathogenesis of the disease. These human biospecimen studies were reinforced with examination of this pathway in mice harbouring a conditional deletion of FoxO1,3a,4.

Material and methods

Human study

This study was performed on 37 patients with histologically proven cirrhosis who underwent liver transplantation as a result of Primary Biliary Cirrhosis (PBC; $n = 21$), Primary Sclerosing Cholangitis (PSC; $n = 8$) and Alcoholic Cirrhosis (ALD; $n = 8$). In addition, non-cirrhotic patients with PBC ($n = 19$) undergoing percutaneous liver biopsy for histological assessment were included in this study. Seven patients from this group received ursodeoxycholic acid (URSO) in the dose recommended for PBC, i.e. 13 – 15mg/kg b.w. before obtaining liver tissue. The samples of liver tissue ($n = 19$) from large margin of hepatocellular carcinoma resections which had no macroscopic changes served as controls. Patients and controls were matched for age and sex. Clinical and laboratory data on analysed patients are summarized in Table 1. An informed written consent was obtained from each patient. The study protocol was approved by the ethics committee of Pomeranian Medical University and was conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Human liver tissue preparation

Tissue specimens were in the form of either blocks of tissue (~1 cm^3; controls, ALD, PSC, cirrhotic-PBC) or obtained by percutaneous needle liver biopsy (2–3 mm^2; non-cirrhotic PBC). Blocks of liver tissue were immediately frozen in liquid nitrogen and kept at −75°C until used. Subsequently, samples were powdered and homogenized in an appropriate lysis buffer to extract either total RNA or protein. Human specimens from non-cirrhotic PBC (percutaneous needle biopsy) were cut into two pieces. One part was stored in RNA *later* (Applied Biosystems, Carlsbad, CA, USA) for gene expression analysis, and the second one was immersed in formalin and later embedded in paraffin. Histological assessments of liver tissue from non-cirrhotic PBC patients were performed by a pathologist who was blinded to the clinical and laboratory data on the included patients (Table 2).

Animal study: Generation of Mx-Cre$^+$: FoxO1/3/4 $^{L/L}$ Mice

Mice harbouring the interferon-inducible transgene Mx-Cre in a FoxO1/3/4$^{L/L}$ background were generated as previously described (19). Cre expression and subsequent FoxO1/3/4 excision were induced in 4- to 5-week-old mice by three intraperitoneal injections of

Table 1. Clinical and laboratory data on analyzed patients

	PBC non-cirrhotic* $n = 19$	PBC cirrhotic $n = 21$	End-stage PSC $n = 8$	End-stage ALD $n = 8$
Gender (M/F)	0/19	4/17	5/3	7/1
Age (years)	54 ± 2	56 ± 2	48 ± 5	54 ± 2
Total Bilirubin (μmol/L)(N 9-17)	23 ± 7	114 ± 25	133 ± 34	67 ± 32
ALP (IU/L) (N 30-120)	269 ± 42	644 ± 83	541 ± 88	275 ± 38
AST (IU/L) (N 4-40)	65 ± 14	493 ± 169	449 ± 153	216 ± 138
UDCA (Y/N)	7/12	21/0	4/4	0/8

Data shown are mean ±SEM.

*Tissue was obtained during percutaneous liver biopsy.

Table 2. Clinical data on non-cirrhotic patients with PBC who were and were not treated with UDCA

	UDCA (+)	UDCA (−)	*P* value
Gender (M/F)	0/7	0/12	0.99
Age (years)	60 ± 2	51 ± 3	0.06
Total bilirubin (μmol/L) (N 9-17)	25 ± 7	22 ± 10	0.82
ALP (U/L) (N 30 -120)	314 ± 96	243 ± 39	0.43
AST (U/L) (N 4-40)	92 ± 28	50 ± 13	0.13
Degree of fibrosis 0-1/2-3	2/5	8/4	0.027

Data shown are mean ±SEM.

polyinosinepolycytidylic acid (pIpC, 300 μg; Invivogen, San Diego, CA, USA) administered every other day over 5 days. pIpC was also administered to Mx-Cre^{-} littermate controls. All mice were housed in a pathogen-free animal facility in microisolator cages. Animals were monitored daily and were sacrificed at designated times after induction with pIpC. All experiments were conducted with the ethical approval of the York University Animal Care Committee. Extraction of mouse livers was performed under aseptic conditions, at a minimum of 2 weeks following the final injection of pIpC. Total RNA or proteins were extracted directly from the fresh tissue.

RNA extraction and quantification of gene expression using Real-Time PCR

Total RNA from human and murine liver tissue was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. cDNA synthesis was carried out using Superscript II RT kit (Invitrogen, Carlsbad, CA, USA) according to the protocol previously described (20). Quantification of gene expression was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). FAM-labelled probes: human FoxO3a (Hs00818121_m1); human Bim (Hs00708019_s1); murine FoxO3a (Mm01185722_m1); murine Bim (Mm00437796_m1) and control human GAPDH (Hs99999905_m1) and murine GAPDH (Mm99999915_g1) were obtained from Applied Biosystems. PCR reactions were performed in duplicate in a total volume of 20 μl containing 10 μL of TaqMan® Gene Expression PCR Master Mix (Applied Biosystems, USA), 2 μl diluted first strand cDNA and 1 μl of the probe/primer mix. The fluorescence data were analysed with 7500 Software v2.0.2. The ΔΔCt method was used to compare the amount of target gene in experimental vs control conditions as described in the Applied Biosystems manual.

Protein expression analysis

Proteins from human (control $n = 9$; ALD $n = 8$; PSC $n = 8$; and cirrhotic PBC $n = 9$) and murine liver tissue were extracted using homogenization in ice-cold RIPA buffer containing protease inhibitor cocktail and PhosSTOP (Roche, Applied Science, Penzberg, Germany). Proteins were quantified using the bicinchoninic acid assay (Micro BCA Protein Assay Kit; Thermo Scientific). 80 μg of each sample was electrophoresed through SDS polyacrylamide gels and then transferred to a PVDF membrane (Thermo Scientific, Rockford, IL, USA) under semi-dry transfer conditions. Membranes were blocked overnight (4°C) with TBST containing 5% (w/v) milk (Merck, Darmstadt, Germany). The following primary antibodies were used: anti-FOXO3A (07-702, Millipore, 1:500 dilution), anti-Bim (AAP-330, Stressgen, 1:1000 dilution), Caspase-3 (9662; Cell Signalling, 1:500 dilution), Cleaved Caspase-3 (9664; Cell Signalling, 1:500 dilution) and anti-α/β-tubulin (2148, Cell Signalling, 1:1000 dilution). To detect antigen–antibody complexes, peroxidase-conjugated anti-rabbit secondary antibody was used (NA9340, Amersham, GE Healthcare; 1:5000 dilution). The enhanced chemiluminescence detection system (SuperSignal West Pico Chemiluminescent, Thermo Scientific) was used and the membranes were exposed to autoradiographic film to visualize immunocomplexes (Kodak Biomax; Amersham Hyperfilm ECL). Band densities were assessed using GeneSnap 7.01 (SynGene) after normalization to α/β-tubulin.

Statistical Analysis

Statistical significance was determined by ANOVA, Fisher's PSLD and Spearman Rank Correlation using StatView software version 5.0. Results are expressed as mean ± SE for at least nine separate experiments. *P* values less than 0.05 were considered statistically significant.

Results

Expression of FOXO3a

To investigate the role of FOXO transcription factors in the regulation of apoptosis in PBC, we assessed the expression profile of FoxO3a mRNA in human liver specimens. Expression of FOXO3a mRNA showed a statistically significant increase in both non-cirrhotic PBC (2.2-fold increase vs. control; $P < 0.05$) and end-stage PBC (4.3-fold increase vs. control; $P < 0.005$; Fig. 1A). The levels of FOXO3a mRNA in PSC and ALD were not significantly different from control. Furthermore, protein expression of FOXO3a in end-stage PBC was enhanced in comparison with controls (50% increase vs. control; $P < 0.05$; Fig. 1B). Treatment with UDCA had no significant effect on FOXO3a mRNA expression in patients with non-cirrhotic PBC (data not shown; $P = 0.77$).

Expression of Bim

As FoxO3a transcription factor is known to regulate expression of Bim, Bim mRNA and protein levels were

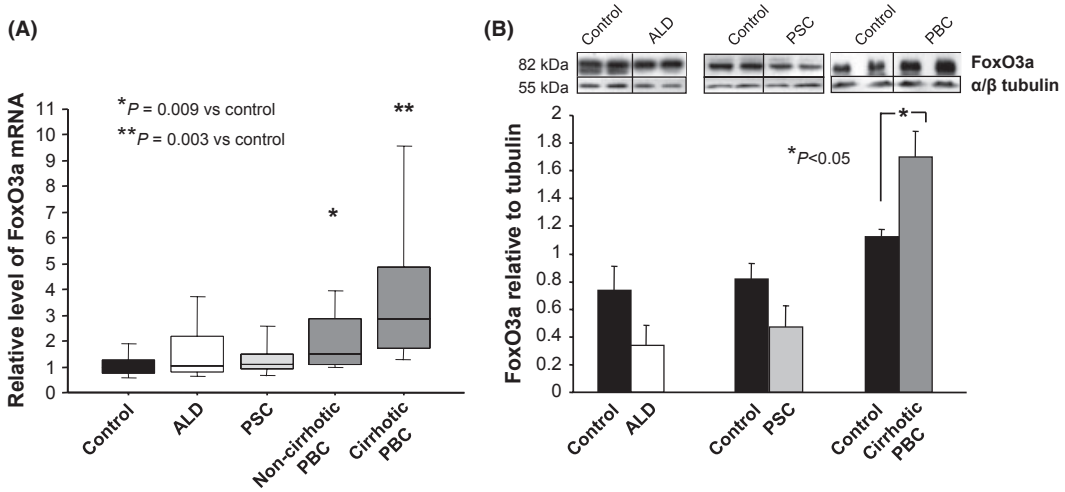

Fig. 1. FoxO3a mRNA (A) and protein (B) expression in early-stage PBC, end-stage PBC, ALD, PSC and controls. Levels of gene expression were normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and presented as a fold-change relative to control. Changes in FoxO3a protein levels were determined by densitometry after normalization to α/β tubulin as a control for loading. Images are representative of at least eight experiments. Bars indicate the mean ± SEM (*$P < 0.05$; **$P < 0.005$ vs. control).

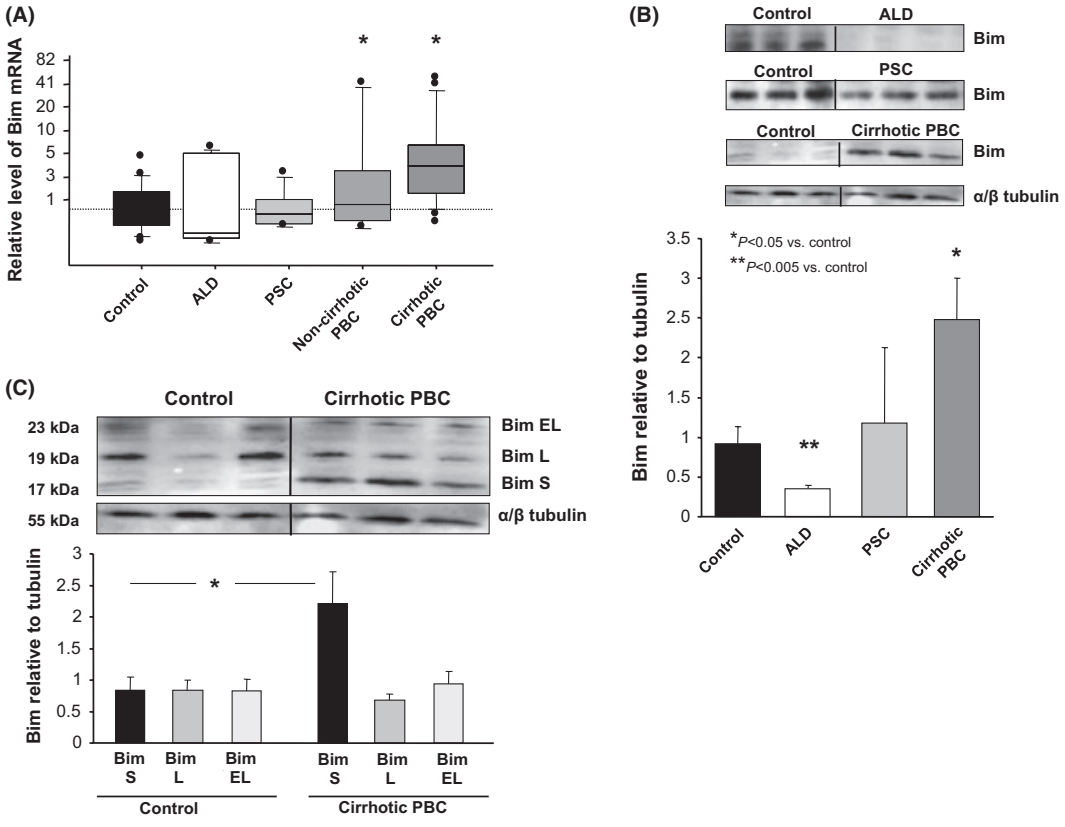

Fig. 2. Bim mRNA (A) and BimS protein (B) expression in ALD, PSC, PBC and controls. Representative images of BimS isoform expression in ALD, PSC, PBC put side by side to controls. The exposure time is different among three blots to bring out the difference (1min; 2min; 30 s respectively) (B). The expression of BimS isoform was the strongest in cirrhotic PBC livers when compared with controls. A representative image of Bim isoforms (EL, L, S) expression in cirrhotic PBC (C). Changes in the expression of Bim isoforms were determined by densitometry after normalization to α/β tubulin. Bars indicate the mean ±SE (*$P < 0.05$; **$P < 0.0005$ vs. control).

evaluated in human liver specimens. Real-time PCR analyses showed a significant increase in Bim mRNA in patients with non-cirrhotic and cirrhotic PBC (2.2-fold and 8.2-fold increase vs. controls, respectively, both $P < 0.05$; Fig. 2A). The level of Bim mRNA in another type of cholestatic disease, PSC, was not significantly different from control (Fig. 2A). In patients with non-cirrhotic PBC, treatment with UDCA had no significant effect on Bim mRNA expression (data not shown; $P = 0.78$).

Western blot analysis showed an increased level of BimS protein in end-stage PBC livers when compared with control liver tissue (2.5- fold increase; $P < 0.05$; Fig. 2B). On contrary, in liver tissue from patients with ALD, BimS protein level was lower than in controls (0.5-fold decrease, $P < 0.0005$; Fig. 2B). In addition, the blots revealed the three different isoforms of Bim known as EL, L and S. Analysis of these splice variants of Bim showed a clear, significant increase in the S isoform in patients with cirrhotic PBC compared with controls (Fig. 2C).

Expression of caspase-3

Caspase-3 is an important apoptotic protein, which once synthesized is maintained in a latent state as a zymogen. Cleavage of the interdomain linker segment of the zymogen results in conformational rearrangement of two remaining subunits that leads to the formation of spontaneously active enzyme**.** The expression of an apoptotic protein caspase-3 was significantly increased in patients with cirrhosis because of PBC (1.96 ± 0.18 vs. 0.98 ± 0.07 in controls; $P = 0.0013$). On the other hand, the levels of caspase-3 protein were substantially decreased in ALD (0.54 ± 0.04 vs.0.9 ± 0.1 in controls; $P = 0.01$) and PSC liver tissue (0.33 ± 0.04 vs.0.9 ± 0.04 in controls; $P < 0.0001$; Fig. 3A). Moreover, evaluation of the levels of cleaved (activated) caspase-3 in cirrhotic liver tissues of patients with PBC showed a significant increase in this form of caspase (2-fold increase vs. controls; $P < 0.0001$) (Fig. 3B).

Expression of FoxO3a and Bim in mice following somatic deletion of FoxO genes

Pharmacologically induced somatic deletion of FoxOs in Mx-Cre$^{+/-}$: FoxO1/3/4$^{L/L}$ mice was validated by the substantial reduction in Foxo3a mRNA and protein levels when compared with control Mx-Cre^{-} mice (Fig. 4A, B). In addition, a subsequent decrease in the expression of a downstream target gene Bim both at mRNA and protein levels was clearly observed. (Fig. 4A, B). The lack of change in the expression of other apoptotic protein, caspase-3 confirms that its expression remains unrelated to FoxO3a.

Discussion

This study provides further insights into the signalling pathways in the liver of patients with PBC. We assessed the activation of FoxO3a/Bim axis and demonstrated that upregulation of pro-apoptotic factors such as caspase-3, FoxO3a and Bim was specific for non-cirrhotic and cirrhotic PBC. Interestingly, these changes did not occur in other cholestatic (PSC) and non-cholestatic (ALD) conditions and thus appear to be disease-specific. Moreover, using a mouse model of conditional genetic deletion of FoxO gene, we confirmed that FoxO3a modulates expression of Bim in liver tissue.

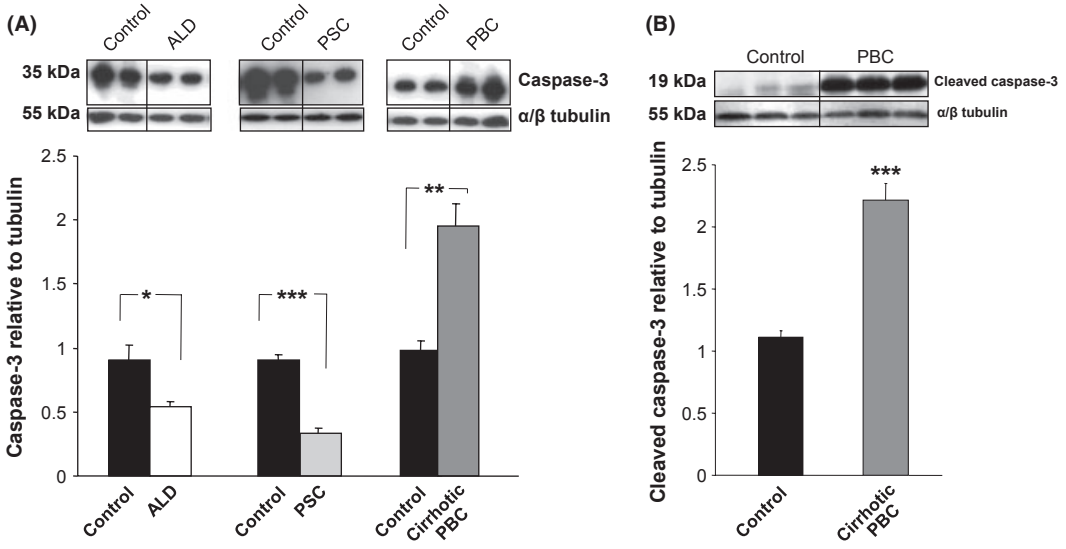

Fig. 3. Caspase-3 protein expression in ALD, PSC, cirrhotic PBC and controls (A) and cleaved caspase-3 protein expression in cirrhotic PBC and controls (B). Protein levels were determined by densitometry after normalization to α/β tubulin as a control for loading. Images are representative of at least eight experiments. Bars indicate the mean ±SEM *$P = 0.010$; **$P = 0.0013$; ***$P < 0.0001$ vs. control.

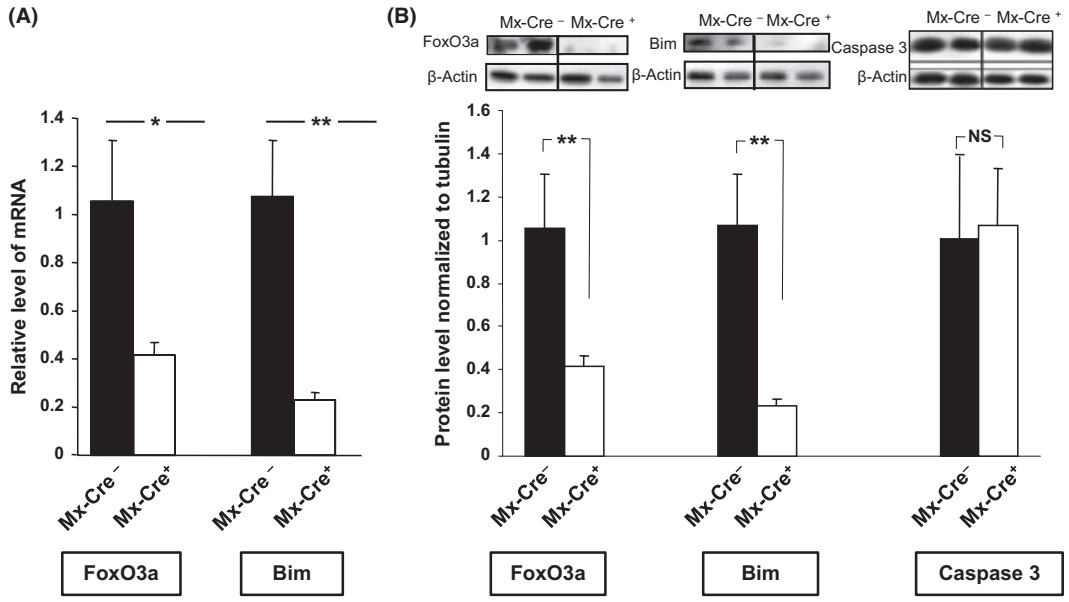

Fig. 4. Regulation of Bim expression by FoxOs. Functional studies confirming the direct relationship between FoxO3a and Bim in liver tissue. Genetic inactivation of FoxO3a results in suppression of FoxO3a and Bim mRNA expression in the experimental Mx-Cre^{+}FoxO1,3,4$^{L/L}$ mice in comparison to Mx-Cre^{-} FoxO1,3,4$^{L/L}$ mice (A). Similarly, FoxO3a and Bim protein levels were strongly reduced in the experimental Mx-Cre^{+} mice when compared with control Mx-Cre^{-} mice (B). Somatic deletion of FoxO gene did not affect the caspase-3 protein level (B). Results represent 5-6 independent experiments. Data are mean ± SEM (*P = 0.0003; **P < 0.001 vs. control).

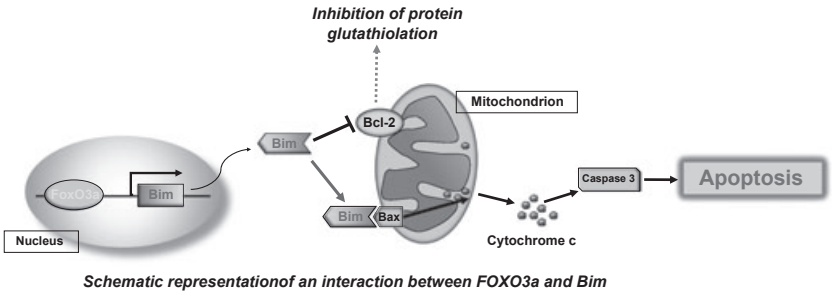

Fig. 5. Schematic representation of the interaction between FoxO3a and Bim.

Apoptosis takes place ubiquitously; yet in contrast to necrosis, the process does not induce a severe inflammatory response and elimination of remnants of apoptotic cells is efficiently achieved. In this study, we confirmed that apoptosis is enhanced in the livers of patients with PBC and this phenomenon was specific to PBC and did not occur in other diseases tested. The observed apoptosis in livers of PBC patients corresponds with findings by Pelli *et al.* who have described a significantly increased concentration of soluble Fas ligand in patients with PBC when compared with subjects with chronic hepatitis C (21). Moreover, in our analysis, the level of activated form of caspase-3, i.e. a cleaved form of the protease, was significantly enhanced in liver tissue from PBC patients. These findings are consistent with the reports showing a strong positive caspase-3 staining in PBC liver sections, which was not present in PSC and ALD sections or healthy controls (7). Likewise, Koga *et al.*, by means of the examination of nuclear DNA fragmentation and Bcl-2 expression, demonstrated the enhanced apoptosis in biliary epithelial cells (BECs) and hepatocytes in the livers of PBC patients (4).

Deregulation of apoptosis has been associated with cancer, degenerative diseases and autoimmune disorders. The triggering of autoimmune reactions by impaired apoptosis was described in the pathogenesis of antibody-mediated myocarditis in infants born to mothers with anti-SSA/Ro-SSB/La. (22). A similar phenomenon was observed in patients with SLA (22, 23). Data which have accumulated over the last decade suggest that impaired apoptosis may play a role in the development of PBC, although it remains controversial whether it is specific to the early or late stage of the disease (24).

It has been shown that PDC-E2, a major antigen for AMA, remains recognizable by autoantibodies following apoptosis (6). In apoptotic cholangiocytes, the anticipated loss of antigenicity is impaired because of inhibition of glutathiolation of its sulfhydryl group and the process is related to the expression of Bcl-2 (6). We hypothesized that the activity of Bcl-2, which hinders glutathiolation of proteins, is blocked or suppressed by the increased expression of Bim protein. In accordance with our initial assumption, we observed enhanced Bim expression specifically in PBC. Three isoforms of Bim differ in their apoptotic potential with BimS being the most potent (16). We demonstrated that the BimS isoform dominated in the livers of patients with PBC, but found no difference in the expression pattern of any Bim isoform in other diseases we examined. The interactions between Bim and Bcl-2 are well documented. Apoptotic signals induce Bim dissociation from the microtubule-associated motor complex and stimulate interaction with Bcl-2 (25). It was clearly shown in a transgenic mouse model that mutation of Thr112 causes decreased binding of Bim to Bcl-2 and increased cell survival. On the contrary, mutation at the Ser55, 65 and 73 sites increased apoptosis as a result of reduced proteasomal degradation of Bim (26). To our knowledge, Bim expression has never been analysed in cirrhotic livers. However, induction of Bim protein synthesis was observed in cultured hepatocytes after exposure to toxic saturated free fatty acids (27).

Our study further investigated the FoxO3a/Bim signalling pathway by looking at the expression of FoxO3a, which directly modulates the transcription of Bim gene. In concordance with our data regarding Bim protein, the strongly induced expression of FoxO3a was observed only in non-cirrhotic and cirrhotic livers from PBC patients, and not in the other diseases examined. As this factor has not previously been examined in human liver tissue, these are novel observations. Transcriptional regulation of Bim by FoxO3a was only demonstrated during lipoapoptosis in cultured hepatocytes (27). Studies in other cell types showed, for instance, that in pro-B and T cells, tropic factor deprivation causes FoxO3a dephosphorylation that leads to enhanced Bim expression and apoptosis (13, 28, 29). Similarly, an involvement of FoxO3a in Bim induction was shown in neurons where Bim activation in cerebellar granule neurons correlated with dephosphorylation of FOXO3a, although a direct requirement for FOXO3a has not been demonstrated (30, 31). To confirm that FoxO3a is a transcriptional regulator of Bim in liver tissue, we utilized a mouse model in which expression of FoxO genes was restrained by inducible somatic deletion (32). Silencing of FoxOs exerted a direct negative effect on Bim expression confirming the interaction of transcription factor FoxO3a with its downstream target, i.e. Bim protein. This effect was not observed when the expression of caspase-3 was analysed in Mx-Cre$^{+/-}$ mice.

In our study, treatment with UDCA, which in the past was found to have anti-apoptotic properties, had no significant effect on FoxO3a or Bim mRNA expression. This unexpected finding will require additional investigation.

Conclusions

Collectively, our data have demonstrated increased apoptosis in livers of patients with PBC, but not in the other diseases we examined. Our results showed that the process is likely to be related to the activation of FoxO3a – Bim – caspase-3 signalling pathway (Fig. 5). Seeing that all presented analyses were carried out in extract from human livers, further detailed studies into apoptotic pathways specific for bile duct lesions are necessary particularly in the context of PBC pathogenesis.

Further understanding of the molecular mechanisms responsible for modulation of the Bcl-2 family activities may generate the potential for pharmaceutical intervention to control apoptosis as inhibition of apoptosis with specific antibodies has proved to be beneficial in other autoimmune diseases (33, 34).

Acknowledgements

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