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Epithelial Cell Specificity and Apoptosis Recognition by Serum Autoantibodies in Primary Biliary Cirrhosis

Guanghua Rong^{1,2}, Renqian Zhong², Ana Lleo^{1,3}, Patrick S.C. Leung¹, Christopher L. Bowlus¹, Guo-Xiang Yang¹, Chen-Yen Yang¹, Ross L. Coppel⁴, Aftab A. Ansari⁵, Dean A. Cuebas⁶, Howard J. Worman⁷, Pietro Invernizzi^{1,3}, Gregory J. Gores⁸, Gary Norman⁹, Xiao-Song He¹, and M. Eric Gershwin¹

¹Division of Rheumatology, Allergy, and Clinical Immunology, University of California Davis, Davis, CA, USA

²Department of Laboratory Medicine, Chang Zheng Hospital, Second Military Medical University, Shanghai, China

³Hepatobiliary Center for Autoimmune Liver Diseases, IRCCS-Istituto Clinico Humanitas, Rozzano, Italy

⁴Department of Medical Microbiology, Monash University, Melbourne, Australia

⁵Department of Pathology, Emory University School of Medicine, Atlanta, GA, USA

⁶Department of Chemistry, Missouri State University, Springfield, MO, USA

⁷Departments of Medicine and of Pathology and Cell Biology, College of Physicians and Surgeons, Columbia University, New York, NY, USA

⁸Division of Gastroenterology and Hepatology, The Mayo Clinic, Rochester, Minnesota

⁹INOVA Diagnostics, Inc., San Diego, CA, USA.

Abstract

A major enigma of primary biliary cirrhosis (PBC) is the selective targeting of biliary cells. Our laboratory has reported that following apoptosis human intrahepatic biliary epithelial cells (HiBEC) translocate the E2 subunit of the pyruvate dehydrogenase complex immunologically intact into apoptotic bodies, forming an apoptope. However, the cell type and specificity of this reaction has not been fully defined. To address this issue we have investigated whether PDC-E2, BCOADC-E2, OGDC-E2, four additional inner mitochondrial enzymes and four nuclear antigens remain immunologically intact with respect to post-apoptotic translocation in HiBEC and 3 additional control epithelial cells. We report that all three 2-oxo acid dehydrogenase enzymes share the ability to remain intact within the apoptope of HiBEC. Interestingly the E2 subunit of the branched chain 2-oxo acid dehydrogenase complex also remained intact in the other cell types tested. We extended the data using 95 AMA⁺ and 19 AMA⁻ PBC and 76 control sera for reactivity against the 7 mitochondrial proteins studied herein and also the ability of AMA⁻ sera to react with HiBEC apoptopes. Sera from 3/95 AMA⁺ sera, but none of the controls, reacted with 2, 4-dienoyl

Corresponding author: M. Eric Gershwin MD, Division of Rheumatology, Allergy and Clinical Immunology, University of California Davis School of Medicine, Genome and Biomedical Sciences Facility, 451 Health Sciences Drive, Suite 6510, Davis, CA 95616; Telephone: 530-752-2884; Fax: 530-752-4669; megershwin@ucdavis.edu or Renqian Zhong, rqzhong@yahoo.com. Guanghua Rong, shengfandayeren@126.com Ana Lleo, ana.lleo@unimi.it Patrick S.C. Leung, psleung@ucdavis.edu Christopher L. Bowlus, clbowlus@ucdavis.edu Guo-Xiang Yang, gxyang@ucdavis.edu Chen-Yen Yang, chnyang@ucdavis.edu Ross L. Coppel, ross.coppel@monash.edu Aftab A. Ansari, pathaaa@emory.edu Dean A. Cuebas, DeanCuebas@missouristate.edu Howard J. Worman, hjw14@columbia.edu Pietro Invernizzi, pietro.invernizzi@unimi.it Gregory J. Gores, gores.gregory@mayo.edu Gary Norman, glnorman@inovadx.com Xiao-Song He, xiaosong@stanford.edu.

CoA reductase 1 (DECR1), an enzyme also present intact only in the HiBEC apoptosome; DECR1 has not been previously associated with any autoimmune disease. Finally the specificity of HiBEC apoptosome reactivity was confined to AMA⁺ sera. In conclusion, we submit that the biliary specificity of PBC is secondary to the unique processes of biliary apoptosis.

Keywords

Apoptosis; human intrahepatic biliary epithelial cells; E2 subunit of 2-oxoacid dehydrogenase complex; nuclear antigens; autoimmunity

A major deficiency in our understanding of primary biliary cirrhosis (PBC) is the identification of mechanisms that lead to the selective destruction of small intrahepatic bile ducts. PBC is characterized by a multi-lineage T and B cell response against the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) (1, 2), which is contained in the mitochondria of all nucleated cells. However, only the epithelial cells of small bile ducts and, to a lesser extent, salivary glands are targeted in this autoimmune disease. PBC can also reoccur following liver transplantation, even in the absence of MHC matching, suggesting that there is no restricted phenotype of the target cells and that bile ducts from any host can be destroyed (3).

We have recently shown that following apoptosis, human intrahepatic biliary epithelial cells (HiBEC), but not other epithelial cells, translocate immunologically intact PDC-E2 into the apoptotic body (AB), forming an apoptosome. This could explain the biliary selectivity of autoimmune damage in PBC (4, 5). While our previous study focused only on PDC-E2, it has been reported that 23% and 57% patients with PBC also produce autoantibodies against two other 2-oxo acid dehydrogenase enzymes, the E2 subunit of the oxo-glutarate dehydrogenase complex (OGDC-E2) and the E2 subunit of the branched chain 2-oxo acid dehydrogenase complex (BCOADC-E2), respectively (1, 6-9). Furthermore, AMA⁻ PBC patients do not have autoantibodies against PDC-E2, OGDC-E2 or BCOADC-E2 but often have autoantibodies to nuclear autoantigens (8, 10-13). This raises the possibility that an immune response against other proteins in ABs of HiBEC could also cause selective biliary damage in PBC. We therefore determined whether OGDC-E2 or BCOADC-E2, as well as other potential mitochondrial autoantigens or candidate nuclear autoantigens are also immunologically intact in the ABs of HiBEC. We report that PDC-E2, OGDC-E2 and BCOADC-E2 are all translocated immunologically intact to the ABs of HiBEC. Furthermore, another mitochondrial enzyme, 2, 4-dienoyl CoA reductase 1 (DECR1), is also found intact within the ABs of HiBEC and a small number of patients with PBC have serum autoantibodies against this protein.

MATERIALS AND METHODS

Patient Samples

Serum samples were obtained from human subjects diagnosed with PBC (n=114), systemic lupus erythematosus (SLE; n=23), primary sclerosing cholangitis (PSC; n=22) or unaffected controls (n=31). The diagnosis in all cases was based on established criteria (1, 14, 15). Patients with PBC and the three control groups were matched by gender and age. The 114 PBC patients include 108 females and 6 males. Ninety-five patients had serum antimitochondrial antibodies (AMA) while 19 were AMA⁻. The AMA⁺ serum samples were randomly selected from a sera bank maintained at the University of California Davis. The presence or absence of serum AMA was confirmed by both immunofluorescence microscopy and immunoblotting against recombinant antigens (see below). The clinical and

pathological features of patients with PBC are summarized in **Table 1**. The protocol was approved by the Institutional Review Board of the University of California Davis.

Antigens

Recombinant human PDC-E2, OGDC-E2 and BCOADC-E2 were prepared in our laboratory as previously described (9, 16). Partial recombinant human DECR1 fused to glutathione-S-transferase (GST) was purchased from Abnova (Taipei, Taiwan). Recombinant ubiquinol cytochrome c reductase complex core protein II (UQCRC2), cytochrome C oxidase IV (COX-IV) and adenosine-5'-triphosphate synthase subunit beta (ATPB) were purchased from Abcam Inc. (Cambridge, MA). The antigens studied herein were selected based on their ubiquitous mitochondrial nature and conserved sequence across species.

Antibodies

Mouse monoclonal antibodies against PDC-E2, OGDC-E2 and BCOADC-E2 (clones 2H-4C8, 2H-5A12 and 2H-2D3, respectively) have been described previously (17). Mouse monoclonal antibodies against ATPB (clone 3D5), DECR1, UQCRC2 (clone 13G12), COX-IV (clone 20E8), and SSA/Ro were purchased from Abcam. Rb72 (rabbit antisera against gp210) was a gift of Dr. Larry Gerace, Scripps Research Institute (La Jolla, CA) (18). Rabbit polyclonal antibody against SSB/La was purchased from Abcam. Rabbit polyclonal antibody against Sp100 was purchased from R&D Systems (Minneapolis, MN). Horseradish peroxidase (HRP) conjugated secondary antibodies anti-human IgG, anti-rabbit IgG, and anti-mouse IgG were purchased from Jackson Immuno-Research (West Grove, PA).

Cell Cultures

HiBEC, human bronchial epithelial cells (BrEPC), and human mammary epithelial cells (MaEPC) were purchased from ScienCell (San Diego, CA). Human keratinocytes were kindly donated by Dr. Rivkah Isseroff (University of California Davis). All cells were primary cultures isolated from normal human tissue and cryopreserved immediately after purification. The HiBEC cells were derived from two separate healthy donors. HiBEC were cultured in epithelial cell medium (ScienCell) supplemented with 2% fetal bovine serum (FBS), epithelial cell growth supplement (ScienCell) and 1% penicillin in flasks coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO). HiBEC were characterized using a previously described immunofluorescence microscopic method with antibodies to cytokeratin 7, cytokeratin 19 and vimentin, which labeled >90% of the cells in culture (19, 20). The other epithelial cells were cultured under the same conditions without FBS. All cells were cultured at 37°C in a humidified 5% CO₂ incubator and experiments were performed using cells between passage 2 and 5 (4, 5).

Induction of Apoptosis and Isolation of ABs

Initially apoptosis was induced using three different methods. Firstly we induced apoptosis with bile salts as described previously (4, 5, 21), with minor modifications. Briefly, cell cultures were incubated in serum-free medium containing 1 mM sodium glycochenodeoxycholate (GCDC; Sigma-Aldrich) for 10 hours at 37°C. Apoptosis was also induced by UVB irradiation (1650 J/m²) followed by incubation in fresh medium for 6 hours and alternatively using anti-Fas antibodies (Clone EOS9.1, eBioscience, San Diego, CA) at 1 µg/ml to the culture medium for 16 hours with the confirmation of surface expression of Fas in all cell lines. We selected bile salts as in our previous work (4). After induction of apoptosis, cell culture supernatants were collected and two additional centrifugation steps (500g for 5 minutes) were performed to remove remaining viable cells. Supernatants were then passed through a 1.2 µm nonpyrogenic hydrophilic syringe filter. After centrifugation at 100,000g for 45 minutes, the pellets containing apoptotic bodies were

resuspended in RIPA lysis buffer (Cell Signaling Technology, Boston, MA) containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN).

Quantification of Apoptosis by Flow Cytometry

The rate of apoptosis was determined by flow cytometry using the PE Annexin V Apoptosis Detection Kit (BD Pharmingen, San Jose, CA). Briefly, 1×10^6 cells were washed twice with cold phosphate buffered saline and resuspended in 1X Annexin V Binding Buffer (BD Pharmingen, San Jose, CA) followed by double-staining with PE-conjugated anti-Annexin V and 7-amino-actinomycin D for 15 minutes at room temperature in the dark. The samples were immediately analyzed by flow cytometry with at least 10,000 events counted. Stained cells were assessed on a FACScanflow cytometer (BD Immunocytometry Systems, San Jose, CA). Acquired data were analyzed with FlowJo Software (TreeStar, Inc., San Carlos, CA).

Detection of Mitochondrial Proteins and Nuclear Proteins in ABs

Non-apoptotic cells and apoptotic bodies were resuspended in the RIPA lysis buffer (Cell Signaling Technology) with protease inhibitor cocktail and incubated on ice for 30 minutes. Total protein contents of the lysates were determined by the bicinchoninic acid assay (Thermo Scientific, Rockford, IL). Samples were then diluted 1:4 in NuPAGE SDS Sample Buffer (Invitrogen, Carlsbad, CA) containing dithiothreitol (5 mM). Lysates equivalent to 5 μ g of total protein per lane were loaded on 10% NuPAGE gels (Invitrogen) and electrophoresed at 150 volts for 2 hours, then electro-transferred onto nitrocellulose membranes. The membranes were stained with Ponceau S solution (Sigma-Aldrich) to visualize protein bands. After blocking with 5% skim milk in phosphate buffered saline for 2 hours, membranes were incubated with primary monoclonal or polyclonal antibodies or antisera against each individual mitochondrial and nuclear proteins overnight at 4°C, washed and then incubated with HRP-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG diluted 1:5,000. Antibody binding was detected by chemiluminescence using the Supersignal chemiluminescent substrate (ThermoScientific, Rockford, IL) as described (4).

Detection of Autoantibodies in Serum Samples

Autoantibodies were detected by immunoblotting using a triple hybrid recombinant protein containing the immunodominant domains of PDC-E2, OGDC-E2 and BCOADC-E2, or using individual recombinant mitochondrial proteins (7, 17, 22). In brief, 15 μ g of purified recombinant protein was loaded onto a 4-12% NuPAGE Zoom gel with immobilized pH gradient wells (Invitrogen, Carlsbad, CA), and electrophoresed at 150 V for 2 hours. Separated proteins were electro-transferred onto nitrocellulose membranes, which were then cut into 30 strips (0.5 μ g/strip). Serum samples were diluted 1:500 and incubated with the nitrocellulose strips containing individual antigens overnight at 4°C. Strips were washed and incubated with HRP-conjugated anti-human IgA+M+G at a 1:5,000 dilution. Antibody binding was detected by chemiluminescence (4). Antibodies to gp210 and Sp100 were measured using the QUANTA LITE™gp210 and QUANTA Lite™ Sp100 ELISA kit (INOVA Diagnostic Inc., San Diego, CA). Positive and negative controls were included throughout.

RESULTS

PDC-E2, OGDC-E2, BCOADC-E2 and DECR1 Are Present Intact in ABs from HiBEC

We first set out to determine whether the 7 mitochondrial and 4 nuclear antigens were present in ABs from HiBEC or other epithelial cells. We confirmed by immunoblotting that all 11 proteins were present in naive unmanipulated cultures of human primary epithelial

cells studied herein, HiBEC, BrEPC, MaEPC and keratinocytes. Next, we incubated these 4 cell lines with GCDC and demonstrated that apoptosis was efficiently induced in each (**Figure 1**).

We then isolated ABs from each of the 4 cell types and their non-apoptotic counterparts to determine using immunoblotting whether the 7 mitochondrial enzymes and the 4 nuclear antigens remained intact following apoptosis (**Figure 2**). As expected, PDC-E2 was only detected in ABs from HiBECs as we previously reported (4). It was not detected in ABs of the three other epithelial cell lines (**Figure 2A**). The protein recognized by anti-PDC-E2 antibodies migrated with an apparent molecular mass of 74 kDa, consistent with the full-length PDC-E2. OGDC-E2, another lipoyl-domain containing enzyme of the mitochondrial inner membrane that is structurally related to PDC-E2, also appeared to be intact exclusively within ABs from HiBEC (**Figure 2B**). DECR1, a 36-kDa subunit-sized homotetrameric protein of the mitochondrial matrix, was also present intact in ABs from HiBEC but not the other epithelial cells (**Figure 2C**). Among the other proteins we examined, BCOADC-E2 and UQCRC2 were detected intact in ABs from HiBEC, BrEPC and MaEPC but not keratinocytes (**Figure 2D and 2E**). SSA/Ro (**Figure 2F**) was detected in ABs from HiBEC and BrEPC whereas SSB/La (**Figure 2G**) was detected only in ABs from BrEPC. Antibodies specific to gp210 recognized only a fragment of the full length protein, a 170kDa band and a 100kDa in the ABs from HiBEC and BrEPC, respectively. No gp210 reactivity was detected in the ABs from keratinocytes and MaEPC (**Table 2**). ATPB (**Figure 2H**) COX-IV and Sp100 were not found in apoptotic bodies from any of the cell types examined (see supplementary data).

Detection of Serum Autoantibodies Against Mitochondrial Enzymes in ABs from HiBEC

To examine the autoantibody reactivity of patients against the autoantigens that were detected intact in the ABs from HiBEC, we tested 190 serum samples from patients with PBC and controls for autoantibodies against the 7 mitochondrial enzymes (**Table 3**). As expected, autoantibodies against PDC-E2, OGDC-E2 and BCOADC-E2 were detected only in AMA⁺ patients with PBC (**Table 3**). However, we also detected antibodies against DECR1 in 3 patients with PBC who also had serum antibodies against PDC-E2 (**Figure 3 and Table 3**). There was no reactivity of sera from AMA⁻ patients against any of the mitochondrial proteins studied herein. Further there was no reactivity of the AMA⁻ PBC sera nor any of the control sera against the ABs of HiBEC cells. Thus the specificity against HiBEC ABs is confined to AMA⁺ patients.

DISCUSSION

Apoptosis, or programmed cell death, occurs ubiquitously in human cells and is a process by which the remnants of dead cells are eliminated efficiently without the induction of overt inflammatory responses. Dysfunction of apoptosis has been linked to the onset of autoimmunity (23-26). In particular, defective clearance of apoptotic cells have been implicated in the initiation and pathogenesis of autoimmunity (23, 27, 28). While serum autoantibodies against the E2 subunits of mitochondrial 2-oxo-dehydrogenase have been well characterized in PBC (1, 6), the discovery that these proteins are intact only in ABs from HiBEC helps to explain the selective destruction of biliary cells in the disease.

We have previously reported a HiBEC-specific failure in the post-apoptotic degradation (PAD) of antigenic PDC-E2, the major autoantigen (4).

In this study, we have now demonstrated that defective PAD in HiBEC is not limited to PDC-E2, but also involves OGDC-E2 and BCOADC-E2 that are also intact in HiBEC ABs. We identified in ABs from HiBEC the other PBC-specific mitochondrial autoantigens,

OGDC-E2 and BCOADC-E2, which are recognized by serum antibodies in approximately 23% and 57% of patients, respectively (9). Thus, all 3 mitochondrial 2-oxo acid dehydrogenase complexes that are autoantigens in PBC can be traced to HiBEC ABs. These findings highlight the involvement of inappropriate PAD as the source of autoantigens and perhaps in the pathogenesis of biliary-selective damage in PBC.

The present study focused on the tissue specificity of the apoptosome. Upon being taken up by local professional phagocytotic cells, these incompletely processed proteins may critically challenge the balance between tolerance and autoimmunity, thus providing a structural basis for the eventual biliary epithelial cell (BEC)-selective immune response of PBC. However, we hypothesize that the unique PAD pattern of HiBEC is not sufficient to initiate the pathologic damage in PBC, not only because the HiBEC studied here are from donors without PBC and the autoantigen-loaded ABs may therefore occur in anyone, but because PBC frequently recurs even after allogenic liver transplantation. In addition, the constant leakage of intact cellular components may cause antigen accumulation in regions near BECs.

Epithelial cells can either uptake and process ABs from their neighboring apoptotic cells as non-professional phagocytes (29, 30) or present pathologic epitopes onto their surface as non-professional antigen presenting cells (31-33). In PBC, the atypical distribution of PDC-E2 on the surface of BECs in patients has been described (34-37). The presence of pathological epitopes on the surface of BEC may serve as targets to attract the autoantibody mediated immunologic attack if tolerance has been broken.

Our data suggest that the defect of cellular protein PAD is not unique to HiBECs. We found several intact autoantigens in ABs of different epithelial cells, implying human epithelial cells variably process their apoptotic leftovers due to factors yet to be determined. We found BCOADC-E2, a PBC autoantigen, to be immunologically intact in epithelial cells other than HiBEC. This finding would suggest that cells other than biliary epithelium could be targeted by the immune response in patients with PBC and anti-BCOADC-E2 autoantibodies. However, more complex factors may regulate and determine the immune response to epithelial ABs, such as quantity of antigen expressed and its access to immune cells (38). Furthermore, only patients with PBC and serum AMA react with HiBEC ABs; yet patients with PBC without detectable AMA still have biliary damage. This suggests that biliary damage in PBC may not only be mediated by autoantibodies but also be cell mediated responses, which would not have been detected in the experimental approach used herein.

Data from this study reinforces the hypothesis of apoptosis-related immune tolerance as a mechanism in the initiation and perpetuation in PBC. Clearly, the etiology of PBC is unknown. However, both genetic susceptibility and environmental factors contribute to the onset of disease. Interestingly, a number of candidate gene studies have reported critical links involving both MHC and non-MHC genes (39-44). More recently, genome-wide case-control association studies in PBC have identified a significant association with *IL-12A*, *IL-12RB2*, and *STAT-4* polymorphisms (45, 46). Interestingly, *IL12A* polymorphism is associated with celiac disease (47) and multiple sclerosis (48) and *STAT-4* polymorphism is also found in patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (49). The association of these pleiotropic immune function related genes in PBC and other autoimmune diseases illustrates that multiple genes are shared between clinical immune related diseases and the immune-mediated pathogenesis may be secondary upon breaking of tolerance by environmental xenobiotics (50). The challenge is to translate these genetic differences with functional human immunopathology.

The pattern of antigens found within ABs is determined not by disease but rather by the evolutionary characters of each cell type. Given this perspective, no cell of an autoimmune attack is really an innocent victim. Rather, development of disease, whether systemic or organ-specific, is largely dependent on the genetics and/or environment-induced susceptibility of each individual to the loss of tolerance of a specific epitope. Thus, in the case of PBC, autoimmunity does not target epithelial cells of the bronchia or mammary glands despite the failure of these epithelial cells to completely clear all self-antigens under the same experimental conditions. HiBECs are targeted and destroyed for the selective presence of special apoptotic antigens, PDC-E2 and sometimes others, which are sensitive to the pre-existing immunologic defect in PBC patients.

In addition to the three known mitochondrial autoantigens in PBC, we identified another mitochondrial enzyme, DECR1, as exclusively intact within HiBEC ABs. DECR1 was also immunologically recognized by antibodies in a small number of serum samples from PBC patients. DECR1, also known as 2,4-dienoyl-CoA reductase 1, participates in the beta-oxidative metabolism of unsaturated fatty enoyl-CoA esters in mitochondria (51). Neither DECR1 nor antibodies against it have so far been reported to be involved in the pathogenesis of any autoimmune disease. Studies to trace and compare the apoptotic pathway of PDC-E2 and DECR1 may provide more details about the defect of antigen preservation in BECs.

Although anti-gp210 and anti-Sp100 are also prominent in patients in PBC, Sp100 was not detected in AB, whereas gp210 was detected in AB of BrEPC and HiBEC. Furthermore, our data showed that neither AMA⁻ PBC patients nor any of the control sera reacted with ABs of HiBECs. Thus, the specificity against HiBECs is confined to AMA⁺ patients. The role of gp210 and other nuclear antigens in PBC thus still remains unclear. The immunological differences between AMA⁺ and AMA⁻ patients also remain enigmatic. Interestingly, AMA⁻ negative patients have been detected to have T cell reactivity to the mitochondrial antigens but clearly do not have the same properties against ABs as found in AMA⁺ patients.

Our study provides additional insights into the apoptosis-related immune tolerance breakdown in PBC. We have obtained data supporting the hypothesis that the incompletely cleaved cellular components specifically generated in biliary epithelium are potential sources of autoantigens and thus contribute to the formation of PBC. Tolerance to all the four identified HiBEC specific epitopes, PDC-E2, OGDC-E2, BCOADC-E2, and DECR1, were proved to be broken by the detection of their autoantibodies and/or antigen-specific T cells PBC (1, 6-8, 52). However, the immunogenicity of each epitope, from 95% for PDC-E2 to 3% for DECR1, shows great diversity indicating the process is determined by multiple factors that require further investigation. The current results also extend our knowledge about the immunological properties of HiBECs, which indicate that they are more than an innocent victim in the pathogenesis of PBC. A further systematic assessment of the immunobiological features of HiBECs may therefore lead to a better understanding of the biliary-selective damage in PBC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

PBC	primary biliary cirrhosis
PDC-E2	E2 subunit of the pyruvate dehydrogenase complex
HiBEC	human intrahepatic biliary cells
AB	apoptotic body
OGDC-E2	E2 subunit of the oxo-glutarate dehydrogenase complex
BCOADC-E2	E2 subunit of the branched chain 2-oxo acid dehydrogenase complex
AMA	antimitochondrial antibodies
DECRI	2,4- dienoyl CoA reductase 1
PSC	primary sclerosing cholangitis
SLE	systemic lupus erythematosus
GST	glutathione-S-transferase
UQCR2	ubiquinol cytochrome C reductase complex core protein II
COX-IV	cytochrome C oxidase IV
ATPB	adenosine-5'-triphosphate synthase subunit beta
SSA/Ro	Sjögren's syndrome antigen A
gp210	glycoprotein 210 kDa
SSB/La	Sjögren's syndrome antigen B
Sp100	speckled 100 kDa autoantigen
HRP	horseradish peroxidase
BrEPCs	bronchial epithelial cells
MaEPCs	mammary epithelial cells
FBS	fetal bovine serum
GCDC	glycochenodeoxycholate
PAD	post apoptotic degradation
BEC	biliary epithelial cells

REFERENCES

1. Kaplan MM, Gershwin ME. Primary biliary cirrhosis. *N Engl J Med*. 2005; 353:1261–1273. [PubMed: 16177252]
2. Gershwin ME, Mackay IR, Sturgess A, Coppel RL. Identification and specificity of a cDNA encoding the 70 kd mitochondrial antigen recognized in primary biliary cirrhosis. *J Immunol*. 1987; 138:3525–3531. [PubMed: 3571977]
3. Van de Water J, Gerson LB, Ferrell LD, Lake JR, Coppel RL, Batts KP, Wiesner RH, et al. Immunohistochemical evidence of disease recurrence after liver transplantation for primary biliary cirrhosis. *Hepatology*. 1996; 24:1079–1084. [PubMed: 8903379]
4. Lleo A, Selmi C, Invernizzi P, Podda M, Coppel RL, Mackay IR, Gores GJ, et al. Apoptoses and the biliary specificity of primary biliary cirrhosis. *Hepatology*. 2009; 49:871–879. [PubMed: 19185000]
5. Lleo A, Bowlus CL, Yang GX, Invernizzi P, Podda M, Van de Water J, Ansari AA, et al. Biliary apoptoses and anti-mitochondrial antibodies activate innate immune responses in primary biliary cirrhosis. *Hepatology*. 2010; 52:987–998. [PubMed: 20568301]

6. Kikuchi K. Immunoreactivity of recombinant human oxo-glutarate dehydrogenase complex (OGDC)-E2 protein in primary biliary cirrhosis. *Hepatology Research*. 2000; 16:211–223.
7. Moteki S, Leung P, Dickson E, Van Thiel D, Galperin C, Buch T, Alarcon Segovia D, et al. Epitope mapping and reactivity of autoantibodies to the E2 component of 2 oxoglutarate dehydrogenase complex in primary biliary cirrhosis using recombinant 2 oxoglutarate dehydrogenase complex. *Hepatology*. 1996; 23:436–444. [PubMed: 8617422]
8. Miyakawa H, Tanaka A, Kikuchi K, Matsushita M, Kitazawa E, Kawaguchi N, Fujikawa H, et al. Detection of antimitochondrial autoantibodies in immunofluorescent AMA-negative patients with primary biliary cirrhosis using recombinant autoantigens. *Hepatology*. 2001; 34:243–248. [PubMed: 11481607]
9. Oertelt S, Rieger R, Selmi C, Invernizzi P, Ansari AA, Coppel RL, Podda M, et al. A sensitive bead assay for antimitochondrial antibodies: Chipping away at AMA-negative primary biliary cirrhosis. *Hepatology*. 2007; 45:659–665. [PubMed: 17326160]
10. Muratori P, Muratori L, Ferrari R, Cassani F, Bianchi G, Lenzi M, Rodrigo L, et al. Characterization and clinical impact of antinuclear antibodies in primary biliary cirrhosis. *Am J Gastroenterol*. 2003; 98:431–437. [PubMed: 12591064]
11. Liu H, Norman GL, Shums Z, Worman HJ, Krawitt EL, Bizzaro N, Vergani D, et al. PBC screen: an IgG/IgA dual isotype ELISA detecting multiple mitochondrial and nuclear autoantibodies specific for primary biliary cirrhosis. *J Autoimmun*. 2010; 35:436–442. [PubMed: 20932720]
12. Agmon-Levin N, Shapira Y, Selmi C, Barzilai O, Ram M, Szyper-Kravitz M, Sella S, et al. A comprehensive evaluation of serum autoantibodies in primary biliary cirrhosis. *J Autoimmun*. 2010; 34:55–58. [PubMed: 19897339]
13. Hu CJ, Zhang FC, Li YZ, Zhang X. Primary biliary cirrhosis: what do autoantibodies tell us? *World J Gastroenterol*. 2010; 16:3616–3629. [PubMed: 20677333]
14. Hochberg M. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis & Rheumatism*. 1997; 40:1725. [PubMed: 9324032]
15. Wiesner R, Grambsch P, Dickson E, Ludwig J, Maccarty R, Hunter E, Fleming T, et al. Primary sclerosing cholangitis: natural history, prognostic factors and survival analysis. *Hepatology*. 1989; 10:430–436. [PubMed: 2777204]
16. Leung PS, Rossaro L, Davis PA, Park O, Tanaka A, Kikuchi K, Miyakawa H, et al. Antimitochondrial antibodies in acute liver failure: implications for primary biliary cirrhosis. *Hepatology*. 2007; 46:1436–1442. [PubMed: 17657817]
17. Migliaccio C, Nishio A, Van de Water J, Ansari A, Leung P, Nakanuma Y, Coppel R, et al. Monoclonal antibodies to mitochondrial E2 components define autoepitopes in primary biliary cirrhosis. *The Journal of Immunology*. 1998; 161:5157. [PubMed: 9820485]
18. Greber UF, Senior A, Gerace L. A major glycoprotein of the nuclear pore complex is a membrane-spanning polypeptide with a large luminal domain and a small cytoplasmic tail. *EMBO J*. 1990; 9:1495–1502. [PubMed: 2184032]
19. Strain A, Wallace L, Joplin R, Daikuhara Y, Ishii T, Kelly D, Neuberger J. Characterization of biliary epithelial cells isolated from needle biopsies of human liver in the presence of hepatocyte growth factor. *The American journal of pathology*. 1995; 146:537. [PubMed: 7856763]
20. Yang Y, Raper S, Cohn J, Engelhardt J, Wilson J. An approach for treating the hepatobiliary disease of cystic fibrosis by somatic gene transfer. *Proceedings of the National Academy of Sciences of the United States of America*. 1993; 90:4601. [PubMed: 7685107]
21. Drudi Metalli V, Mancino M, Mancino A, Torrice A, Gatto M, Attili A, Alpini G, et al. Bile salts regulate proliferation and apoptosis of liver cells by modulating the IGF1 system. *Digestive and Liver Disease*. 2007; 39:654–662. [PubMed: 17531559]
22. Leung PS, Chuang DT, Wynn RM, Cha S, Danner DJ, Ansari A, Coppel RL, et al. Autoantibodies to BCOADC-E2 in patients with primary biliary cirrhosis recognize a conformational epitope. *Hepatology*. 1995; 22:505–513. [PubMed: 7543435]
23. Nagata S, Hanayama R, Kawane K. Autoimmunity and the clearance of dead cells. *Cell*. 2010; 140:619–630. [PubMed: 20211132]

24. Savill J, Dransfield I, Gregory C, Haslett C. A blast from the past: clearance of apoptotic cells regulates immune responses. *Nature Reviews Immunology*. 2002; 2:965–975.
25. Ravichandran K, Lorenz U. Engulfment of apoptotic cells: signals for a good meal. *Nature Reviews Immunology*. 2007; 7:964–974.
26. Mahoney J, Rosen A. Apoptosis and autoimmunity. *Current opinion in immunology*. 2005; 17:583–588. [PubMed: 16214321]
27. Chekeni FB, Ravichandran KS. The role of nucleotides in apoptotic cell clearance: implications for disease pathogenesis. *J Mol Med*. 2011; 89:13–22. [PubMed: 20809090]
28. Munoz LE, Lauber K, Schiller M, Manfredi AA, Herrmann M. The role of defective clearance of apoptotic cells in systemic autoimmunity. *Nat Rev Rheumatol*. 2010; 6:280–289. [PubMed: 20431553]
29. Monks J, Rosner D, Geske F, Lehman L, Hanson L, Neville M, Fadok V. Epithelial cells as phagocytes: apoptotic epithelial cells are engulfed by mammary alveolar epithelial cells and repress inflammatory mediator release. *Cell Death & Differentiation*. 2005; 12:107–114. [PubMed: 15647754]
30. Parnaik R, Raff M, Scholes J. Differences between the clearance of apoptotic cells by professional and non-professional phagocytes. *Current Biology*. 2000; 10:857–860. [PubMed: 10899007]
31. Gereke M, Jung S, Buer J, Bruder D. Alveolar type II epithelial cells present antigen to CD4+ T cells and induce Foxp3+ regulatory T cells. *American journal of respiratory and critical care medicine*. 2009; 179:344. [PubMed: 19096007]
32. Fahey J, Wallace P, Johnson K, Guyre P, Wira C. Antigen presentation by human uterine epithelial cells to autologous T cells. *American Journal of Reproductive Immunology*. 2006; 55:1–11. [PubMed: 16364006]
33. Mulder DJ, Pooni A, Mak N, Hurlbut DJ, Basta S, Justinich CJ. Antigen Presentation and MHC Class II Expression by Human Esophageal Epithelial Cells Role in Eosinophilic Esophagitis. *Am J Pathol*. 2011; 178:744–753. [PubMed: 21281807]
34. Joplin, R.; Gershwin, M. Ductular expression of autoantigens in primary biliary cirrhosis.; 1997: GEORG THIEME VERLAG. 1997. p. 97-104.
35. Tsuneyama K, Van de Water J, Leung PS, Cha S, Nakanuma Y, Kaplan M, De Lellis R, et al. Abnormal expression of the E2 component of the pyruvate dehydrogenase complex on the luminal surface of biliary epithelium occurs before major histocompatibility complex class II and BB1/B7 expression. *Hepatology*. 1995; 21:1031–1037. [PubMed: 7535733]
36. Cha S, Leung PS, Van de Water J, Tsuneyama K, Joplin RE, Ansari AA, Nakanuma Y, et al. Random phage mimotopes recognized by monoclonal antibodies against the pyruvate dehydrogenase complex-E2 (PDC-E2). *Proc Natl Acad Sci U S A*. 1996; 93:10949–10954. [PubMed: 8855289]
37. Leung PS, Cha S, Joplin RE, Galperin C, Van de Water J, Ansari AA, Coppel RL, et al. Inhibition of PDC-E2 human combinatorial autoantibodies by peptide mimotopes. *J Autoimmun*. 1996; 9:785–793. [PubMed: 9115581]
38. Gregory CD, Pound JD. Microenvironmental influences of apoptosis in vivo and in vitro. *Apoptosis*. 2010; 15:1029–1049. [PubMed: 20237956]
39. Bassendine MF, Dewar PJ, James OF. HLA-DR antigens in primary biliary cirrhosis: lack of association. *Gut*. 1985; 26:625–628. [PubMed: 3859460]
40. Invernizzi P, Selmi C, Poli F, Frison S, Floreani A, Alvaro D, Almasio P, et al. Human leukocyte antigen polymorphisms in Italian primary biliary cirrhosis: a multicenter study of 664 patients and 1992 healthy controls. *Hepatology*. 2008; 48:1906–1912. [PubMed: 19003916]
41. Juran BD, Atkinson EJ, Larson JJ, Schlicht EM, Lazaridis KN. Common genetic variation and haplotypes of the anion exchanger SLC4A2 in primary biliary cirrhosis. *Am J Gastroenterol*. 2009; 104:1406–1411. [PubMed: 19491853]
42. Kimura Y, Selmi C, Leung PS, Mao TK, Schauer J, Watnik M, Kuriyama S, et al. Genetic polymorphisms influencing xenobiotic metabolism and transport in patients with primary biliary cirrhosis. *Hepatology*. 2005; 41:55–63. [PubMed: 15690482]

43. Wassmuth R, Depner F, Danielsson A, Hultcrantz R, Loof L, Olson R, Prytz H, et al. HLA class II markers and clinical heterogeneity in Swedish patients with primary biliary cirrhosis. *Tissue Antigens*. 2002; 59:381–387. [PubMed: 12144621]
44. Agarwal K, Jones DE, Daly AK, James OF, Vaidya B, Pearce S, Bassendine MF. CTLA-4 gene polymorphism confers susceptibility to primary biliary cirrhosis. *J Hepatol*. 2000; 32:538–541. [PubMed: 10782900]
45. Hirschfield GM, Liu X, Xu C, Lu Y, Xie G, Gu X, Walker EJ, et al. Primary biliary cirrhosis associated with HLA, IL12A, and IL12RB2 variants. *N Engl J Med*. 2009; 360:2544–2555. [PubMed: 19458352]
46. Liu X, Invernizzi P, Lu Y, Kosoy R, Bianchi I, Podda M, Xu C, et al. Genome-wide meta-analyses identify three loci associated with primary biliary cirrhosis. *Nat Genet*. 2010; 42:658–660. [PubMed: 20639880]
47. Hunt KA, Zhernakova A, Turner G, Heap GA, Franke L, Bruinenberg M, Romanos J, et al. Newly identified genetic risk variants for celiac disease related to the immune response. *Nat Genet*. 2008; 40:395–402. [PubMed: 18311140]
48. IL12A, MPHOSPH9/CDK2AP1 and RGS1 are novel multiple sclerosis susceptibility loci. *Genes Immun*. 2010; 11:397–405. [PubMed: 20555355]
49. Remmers EF, Plenge RM, Lee AT, Graham RR, Hom G, Behrens TW, de Bakker PI, et al. STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. *N Engl J Med*. 2007; 357:977–986. [PubMed: 17804842]
50. Amano K, Leung PS, Rieger R, Quan C, Wang X, Marik J, Suen YF, et al. Chemical xenobiotics and mitochondrial autoantigens in primary biliary cirrhosis: identification of antibodies against a common environmental, cosmetic, and food additive, 2-octynoic acid. *J Immunol*. 2005; 174:5874–5883. [PubMed: 15845458]
51. Alphey MS, Yu W, Byres E, Li D, Hunter WN. Structure and Reactivity of Human Mitochondrial 2,4-Dienoyl-CoA Reductase. *Journal of Biological Chemistry*. 2005; 280:3068–3077. [PubMed: 15531764]
52. Kita H, Matsumura S, He X, Ansari A, Lian Z, Van de Water J, Coppel R, et al. Quantitative and functional analysis of PDC-E2-specific autoreactive cytotoxic T lymphocytes in primary biliary cirrhosis. *Journal of Clinical Investigation*. 2002; 109:1231–1240. [PubMed: 11994412]

Figure 1. GCDC-induced apoptosis in HiBEC and three other epithelial complete cell types. A. Representative dot plots of HiBEC incubated with complete medium or medium with 1 mM GCDC. Apoptosis was examined by flow cytometry after double staining for Annexin V and 7-amino-actinomycin D and the apoptotic cells were detected as Annexin V positive and 7-amino-actinomycin D negative (lower right quadrant). The rate of apoptosis was defined as the percentage of apoptotic cells of total cells counted. B. Rate of apoptosis induced by GCDC in different cell types. Each experiment was performed in triplicate; data expressed as $\pm$ SEM. experiments.

Figure 2.

Detection of antigenically intact mitochondrial proteins in ABs. Lysates of non-apoptotic cells (lanes 1 – 4) and their ABs (lanes 5 – 8) were immunoblotted on same membranes with monoclonal or polyclonal antibodies against individual mitochondrial proteins (detailed in Materials and Methods). The cell types tested were: BrEPCs (lanes 1 and 5), HiBECs (lanes 2 and 6), keratinocytes (lanes 3 and 7) and MaEPCs (lanes 4 and 8).

Figure 3.

Detection of anti-DEC1 antibodies in the serum of a PBC patient. Representative blots of DEC1-GST fusion protein (rh-DEC1; 36 kDa) were probed with the patient serum (lane 1), anti-DEC1 monoclonal antibody (lane 2) and normal human IgG (lane 3). Control blots of GST (r-GST; 26 kDa) were probed with the same patient serum (lane 5) or goat-anti GST polyclonal antibodies (lane 4) to eliminate the possibility of false positive caused by anti-GST reactivity in the patient serum.

Table 1

Features of patients with PBC at the time of enrollment

Histological stage	All Stages	I	II	III	IV
Number	114	30	36	15	33
Female	108	27	34	15	32
Male	6	3	2	0	1
Mean Age (years)	59.5	60.1	54.7	60.2	63.0
Age Range (years)	39-80	49-75	39-80	54-70	49-78
AMA positive	95	25	29	12	29
AMA negative	19	5	7	3	4

Presence of mitochondrial inner membrane enzymes and nuclear antigens in apoptotic bodies of various cell types undergoing apoptosis

Table 2

	Non-apoptotic cells				Apoptotic bodies			
	BrEPC	HIBEC	Keratinocytes	MaEPC	BrEPC	HIBEC	Keratinocytes	MaEPC
PDC-E2 *	+	+	+	+	-	+	-	-
OGDC-E2 *	+	+	+	+	-	+	-	-
DECRI *	+	+	+	+	-	+	-	-
BCOADC-E2 *	+	+	+	+	+	+	-	+
UQCRC2 *	+	+	+	+	+	+	-	+
SSA/Ro **	+	+	+	+	+	+	-	-
SSB/La **	+	+	+	+	+	-	-	-
COX-IV *	+	+	+	+	-	-	-	-
ATPB *	+	+	+	+	-	-	-	-
SP100 **	+	+	+	+	-	-	-	-
Gp210 **	+	+	+	+	+	+	-	-

* Mitochondrial inner membrane enzymes.

** Nuclear antigens.

Table 3

Detection of autoantibodies in PBC patients and control

Antigens	PBC patients				Controls		
	AMA Positive (n=95)	AMA Negative (n=19)	Healthy (n=31)	PSC (n=22)	SLE (n=23)		
PDC-E2 *	95/95	0/19	0/31	0/22	0/23		
OGDC-E2 *	19/95	0/19	0/31	0/22	0/23		
BCOADC-E2 *	25/95	0/19	0/31	0/22	0/23		
DECRI *	3/95	0/19	0/31	0/22	0/23		
UQCRC2 *	0/95	0/19	0/31	0/22	0/23		
ATPB *	0/95	0/19	0/31	0/22	0/23		
COX-IV *	0/95	0/19	0/31	0/22	0/23		
Sp100 **	17/95	2/19	0/31	0/22	0/23		
Gp210 ***	11/95	3/19	0/31	0/22	0/23		

Autoantibodies to PDC-E2, OGDC-E2, BCOADC-E2, DECRI, UQCRC2, ATPB, COX-IV were detected by immunoblotting. Antibodies to Sp100 and gp210 were detected by ELISA (see Materials and Methods). Data was presented as positive subject number/total subject number.

* Mitochondrial inner membrane enzymes.

** Nuclear antigens.