American Journal of Pathology, Vol. 161, No. 6, December 2002

Bile Acid-Induced Mallory Body Formation in Drug-Primed Mouse Liver

Peter Fickert,* Michael Trauner,*
Andrea Fuchsbichler,† Conny Stumptner,†
Kurt Zatloukal,† and Helmut Denk†

From the Departments of Medicine and Pathology,† Karl-Franzens University, Graz, Austria*

Chronic cholestasis is associated with retention of bile acids and profound cytoskeletal alterations in hepatocytes including Mallory body (MB) formation. The mechanisms responsible for MB formation in cholestatic liver diseases are unclear. The aim of our study was to determine the relevance of cholestasis and bile acids for MB formation. For this purpose mice received a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-supplemented diet for 2.5 months to induce MB formation. After recovery from DDC intoxication for 4 weeks followed by disappearance of MBs, these drug-primed mice were subjected to DDC refeeding, common bile duct ligation (CBDL), and feeding of a cholic acid (CA)-supplemented diet for 7 days, respectively. Cytokeratin (CK) 8 and CK 18 expression was studied by competitive reverse transcriptase-polymerase chain reaction and Western blot analysis. Cytoskeletal alterations of hepatocytes and MB formation were monitored by immunofluorescence microscopy and immunohistochemistry using CK-, ubiquitin-, and MB-specific antibodies. Like DDC refeeding, both CBDL and CA feeding of drug-primed mice significantly increased CK 8 and CK 18 mRNA and protein levels (with excess of CK 8) and resulted in ubiquitination and abnormal phosphorylation of CKs. Furthermore, CBDL and CA feeding resulted in rapid neoformation of MBs in drug-primed mice. It is concluded that MB formation in cholestatic liver diseases may be triggered by the action of potentially toxic bile acids. ***(Am J Pathol 2002, 161:2019–2026)***

Cytokeratin (CK) intermediate filaments (IFs) are major cytoskeletal components and are concentrated in the perinuclear and submembraneous regions of epithelial cells.[1] The CK subfamily has more than 20 members forming heteropolymers of type I and type II CKs.[1] CK 8 and CK 18 are subunits of the IFs of hepatocytes and were also identified as major components of Mallory bodies (MBs) associated with certain human liver diseases and related mouse models.[2] MBs are characteristic cytoplasmic hyaline inclusions in hepatocytes reflecting a peculiar morphological manifestation of chronic liver cell injury.[2,3] Their appearance is related to alterations of the CK-IF cytoskeleton including overexpression and post-translational modifications of CKs (eg, cross-linking, abnormal phosphorylation, ubiquitination).[2,4–10] In humans, MBs are typically associated with alcoholic and nonalcoholic steatohepatitis, but are also found in chronic cholestatic conditions such as primary biliary cirrhosis and primary sclerosing cholangitis.[2,8,11] A common denominator of these etiologically different liver diseases is their association with cholestasis and elevated serum bile acid levels. In mice, MBs can be induced by chronic griseofulvin (GF) or 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) intoxication.[12–15] Administration of these porphyrinogenic agents also induces cholestasis in mice indicated by elevated serum bile acid levels that may at least partly result from the formation of protoporphyrin plugs and stones obstructing the bile drainage system.[16,17] MB formation requires prolonged intoxication (~2.5 months) with GF or DDC.[2,4] Interestingly, after recovery from intoxication with disappearance of MBs, which takes ~4 weeks (primed mouse liver), MBs are reinduced within days by reintoxication with DDC or GF as well as application of colchicine.[4,18–21]

We recently demonstrated that obstructive cholestasis or cholic acid (CA) feeding leads to CK overexpression accompanied by abnormal phosphorylation in the mouse liver;[22] nonetheless, the causal relationship between cholestasis with retention of potentially toxic bile acids and MB formation remained unclear. This study was designed to clarify whether cholestasis and bile acids by themselves represent causative factors in MB formation. We therefore assessed the influence of obstructive cholestasis by common bile duct ligation (CBDL) and CA feeding (to mimic retention of a major primary bile acid) on the IF cytoskeleton and MB formation in a well-defined experimental mouse model (ie, the drug-primed mouse liver).[2,4,18–21] Evidence that cholestasis and bile acids play a central role in MB formation is reported.

Supported by the Austrian Science Foundation (grant S 7401-MOB to K. Z. and P-15502 to M. T.), the Jubilee Fund of the Austrian National Bank (grants 7171 and 8522 to M. T.), and by the Joseph Skoda Prize from the Austrian Society of Internal Medicine (to M. T.).

Accepted for publication August 16, 2002.

Address reprint requests to Helmut Denk, M.D., FRCPath, Department of Pathology, Karl-Franzens University, Auenbruggerplatz 25, A-8036 Graz, Austria. E-mail: helmut.denk@kfunigraz.ac.at.

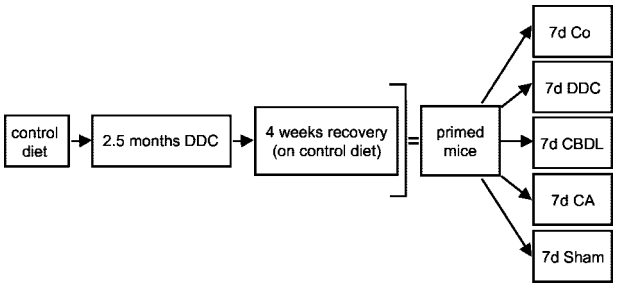

Figure 1. Experimental design to study the role of cholestasis and bile acids in MB formation in drug-primed mice. Mice were fed a control diet or 0.1% DDC-supplemented diet for 2.5 months to induce MBs. One group of animals was sacrificed to study DDC-induced cytoskeletal alterations (including MB formation), whereas another group was sacrificed 4 weeks after discontinuation of DDC feeding (recovery) to assess the reversibility of these changes. In addition, recovered (primed) mice received control diet (Co) or were subjected to DDC refeeding (DDC), CBDL, CA feeding, and sham operation for 7 days, respectively. Five animals were studied in each group.

Materials and Methods

Animals

Male Swiss albino mice (strain Him OF1 SPF) were obtained from the Institute for Laboratory Animal Research, University of Vienna School of Medicine, Himberg, Austria, housed with a 12:12 hour light-dark cycle and permitted *ad libitum* consumption of water and a standard mouse diet (Marek, Vienna, Austria). Experiments were performed with 2-month-old mice weighing 25 to 30 g. The experiments were approved by the local ethics committee and followed the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the United States National Academy of Sciences, as published by the National Institutes of Health (NIH publication 86-23, revised 1985). CA and DDC were obtained from Aldrich (Steinheim, Germany).

DDC Intoxication

Mice were fed a diet containing 0.1% DDC for 2.5 months to induce MBs.[2,4] After this time period one group of animals was sacrificed to assess DDC-induced cytoskeletal alterations including MB formation, whereas another group was sacrificed 4 weeks after discontinuation of DDC feeding to study the reversibility of these changes as described previously.[4] In addition, recovered primed mice were refed a diet containing 0.1% DDC for 7 days or subjected to CBDL or CA feeding (see Figure 1 for experimental design).

CBDL

All surgical procedures were performed under sterile conditions. To study the effects of obstructive cholestasis on CK expression and MB formation in drug-primed mice, the common bile duct was ligated close to the liver hilum immediately below the bifurcation and dissected between the ligatures as described previously.[23] In addition, cholecystectomy was performed after ligation of the cystic duct. Controls underwent a sham operation with exposure but without ligation of the common bile duct and removal of the gallbladder. The livers were excised under general anesthesia (10 mg Avertin i.p.) 7 days after surgery. Liver tissue samples were frozen in liquid nitrogen for molecular analysis and immunofluorescence microscopy or fixed in 4% neutral buffered formaldehyde solution for light microscopy and immunohistochemistry. Serum samples from each mouse were stored at −70°C for analysis of aspartate aminotransferase/alanine aminotransferase, alkaline phosphatase, and total bile acid levels.

Bile Acid Feeding

To study the effects of bile acids on CK expression and MB formation, drug-primed mice were fed a diet supplemented with CA (1%) for 7 days.[22,24] Livers and sera were processed as described above.

Determination of mRNA Copy Numbers

mRNA copy numbers for CK 8, CK 18, and glyceraldehyde-3-phosphate dehydrogenase were determined by competitive reverse transcriptase-polymerase chain reaction.[10]

Western Blotting of CK 8 and CK 18

CK 8 and CK 18 protein levels were determined by Western blot analysis.[4,22]

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed on frozen liver sections (3-μm thick, fixed in acetone at −20°C for 10 minutes) using the monoclonal antibody M_M120-1 specifically recognizing MBs and the polyclonal rabbit CK antibody 50K160 against CK 8 and CK 18 as described previously.[4,22,25] In addition, phosphorylation of CK 8 was assessed with the antibody 5B3 against a hyperphosphorylated epitope of CK 8.[4,26] Double immunolabeling was performed combining the monoclonal antibodies M_M120-1 or 5B3 with the polyclonal antibody 50K160. As secondary antibodies, Cy2-conjugated goat anti-mouse IgG (Amersham, Buckinghamshire, UK) and tetramethylrhodamine isothiocyanate-conjugated swine anti-rabbit Ig (DAKO, Glostrup, Denmark) were used. For control the primary antibodies were omitted or replaced by isotype-matched immunoglobulins (DAKO). Immunofluorescent specimens were analyzed with a MRC 600 (Bio-Rad, Richmond, CA) laser-scanning confocal device attached to a Zeiss Axiophot microscope. The fluorescent images were collected using the confocal photomultiplier tube at full frame 768 × 512 pixels).

Histology

Mouse livers were fixed in 4% neutral buffered formaldehyde solution and embedded in paraffin. Sections 4 μm thick were stained with hematoxylin and eosin.