A Biliary HCO_3^- Umbrella Constitutes a Protective Mechanism Against Bile Acid-Induced Injury in Human Cholangiocytes

Simon Hohenester,[1,2]* Lucas Maillette de Buy Wenniger,[1]* Coen C. Paulusma,[1] Sandra J. van Vliet,[3] Douglas M. Jefferson,[4] Ronald P. Oude Elferink,[1] and Ulrich Beuers[1]

Human cholangiocytes are continuously exposed to millimolar levels of hydrophobic bile salt monomers. We recently hypothesized that an apical biliary HCO_3^- umbrella might prevent the protonation of biliary glycine-conjugated bile salts and uncontrolled cell entry of the corresponding bile acids, and that defects in this biliary HCO_3^- umbrella might predispose to chronic cholangiopathies. Here, we tested *in vitro* whether human cholangiocyte integrity in the presence of millimolar bile salt monomers is dependent on (1) pH, (2) adequate expression of the key HCO_3^- exporter, anion exchanger 2 (AE2), and (3) an intact cholangiocyte glycocalyx. To address these questions, human immortalized cholangiocytes and cholangiocarcinoma cells were exposed to chenodeoxycholate and its glycine/taurine conjugates at different pH levels. Bile acid uptake was determined radiochemically. Cell viability and apoptosis were measured enzymatically. AE2 was knocked down by lentiviral short hairpin RNA. A cholangiocyte glycocalyx was identified by electron microscopy, was enzymatically desialylated, and sialylation was quantified by flow cytometry. We found that bile acid uptake and toxicity in human immortalized cholangiocytes and cholangiocarcinoma cell lines *in vitro* were pH and AE2 dependent, with the highest rates at low pH and when AE2 expression was defective. An apical glycocalyx was identified on cholangiocytes *in vitro* by electron microscopic techniques. Desialylation of this protective layer increased cholangiocellular vulnerability in a pH-dependent manner. *Conclusion:* A biliary HCO_3^- umbrella protects human cholangiocytes against damage by bile acid monomers. An intact glycocalyx and adequate AE2 expression are crucial in this process. Defects of the biliary HCO_3^- umbrella may lead to the development of chronic cholangiopathies.
(HEPATOLOGY 2012;55:173-183)

Hydrophobic bile salts induce cytotoxicity in many cell types, including hepatocytes, already at low micromolar concentrations.[1-4] In striking contrast, human biliary epithelial cells are exposed to high millimolar concentrations of hydrophobic bile salts under physiologic conditions[5] without signs of cytotoxicity. This remarkable resistance against bile-salt–induced toxicity is incompletely understood. Formation of mixed micelles of bile salts with phospholipids[5] or "flushing" of bile by secretion of

Abbreviations: AE2, anion exchanger 2; BSA, bovine serum albumin; CDC, chenodeoxycholate; CFTR, cystic fibrosis transmembrane conductance regulator; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; GCDC, glycochenodeoxycholate; HBSS, Hank's balanced salt solution; MEM, minimal essential medium; PBC, primary biliary cirrhosis; PDC-E2, pyruvate dehydrogenase complex; PI3K, phosphatidylinositol 3-kinase; E2; PSC, primary sclerosing cholangitis; RPMI, Roswell Park Memorial Institute medium; shRNA, short hairpin RNA; SNA, Sambucus nigra *bark agglutinin; TCDC, taurochenodeoxycholate; TEM, transmission electron microscopy; UDCA, ursodeoxycholic acid; WST, water-soluble tetrazolium salt.*

From the [1]Tytgat Institute for Liver and Intestinal Research, Department of Gastroenterology and Hepatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; [2]Department of Internal Medicine III, University of Aachen, Aachen, Germany; [3]Department of Molecular Cell Biology and Immunology, VU Medical Center, Amsterdam, The Netherlands; and [4]Tufts University School of Medicine, Boston, MA.

Received May 21, 2011; Revised August 25, 2011; accepted September 8, 2011.

This work was supported by a research scholarschip from the Deutsche Forschungsgesellschaft (to S.H.; HO 4460/1-1), a Ph.D. scholarship from the University of Amsterdam Academic Medical Center (to L.M.W.), and research grants "Primary sclerosing cholangitis" from the Deutsche Crohn-Colitis-Vereinigung and the Norwegian Primary Sclerosing Cholangitis Foundation (to U.B.).

Data were, in part, reported at the Annual Meeting of the American Association for the Study of Liver Diseases in Boston, MA, in November 2010, at the Annual Meeting of the European Association for the Study of the Liver in Berlin, Germany, in April 2011, and at the Falk® International Bile Acid Meeting in Freiburg, Germany, in October 2010 and were published, in part, in abstract form in HEPATOLOGY 2010;52(S1):923A, J Hepatol 2011;54(S1):S279, J Hepatol 2011;54(S1):S280, and Dig Dis 2011;29:62.

HCO_3^--rich fluid[5,6] were proposed as protective mechanisms, but are unable to sufficiently reduce the concentration of free hydrophobic bile salt monomers to less than low millimolar levels in bile, as recently discussed in detail.[7]

Human biliary HCO_3^- secretion accounts for 25%-40% of total bile flow and, by far, exceeds that of rodents.[8] The physiologic role of the enormous difference in biliary HCO_3^- secretion between humans and rodents has remained obscure. We recently hypothesized[7] that biliary HCO_3^- secretion might constitute a biliary HCO_3^- umbrella on the apical cholangiocyte surface, a, so far, unrecognized protective mechanism of cholangiocytes against bile-salt–induced toxicity.

Intracellular accumulation of hydrophobic bile salts is a prerequisite for their cytotoxic effects.[9] Uncontrolled, carrier-independent membrane traffic and cell invasion of bile salts is determined by their polarity and degree of protonation.[10] Glycine conjugates account for the majority of bile salts in human bile, have a pK_a of $\sim$4,[11] and, at a physiologic pH of $\sim$7.4, are partially protonated, apolar, and thus cell permeable at micromolar amounts. Even small changes in local biliary pH close to the apical membrane of cholangiocytes will thus have a dramatic effect on glycine-conjugated bile salt:bile acid ratio and, thereby, a sensitivity of cholangiocytes toward glycine-conjugated bile acids. In rodents, which have a more hydrophilic, less toxic bile salt pool with mainly taurine conjugates[5] (pK_a of $\sim$1-2),[11] changes in biliary pH would have a minor, negligible effect on bile salt protonation and toxicity. pH dependency of carrier-independent cell entry and toxicity of bile acids has been established in gastric and esophageal mucosa cells.[12-14]

Cholangiocyte apoptosis has been shown to drive autoimmunity and inflammation in primary biliary cirrhosis (PBC).[15,16] By altering sensitivity toward bile salt toxicity and increasing frequency of apoptotic events in cholangiocytes, genetic and acquired defects disrupting the biliary HCO_3^- umbrella may be a common pathogenetic factor in various cholangiopathies. Genetic variants of key modulators of the biliary HCO_3^- umbrella[7] have been associated with the manifestation or progression of chronic cholestasis, as demonstrated for anion exchanger 2 (AE2) in PBC,[17-20] TGR5 in primary sclerosing cholangitis (PSC),[21] or cystic fibrosis transmembrane conductance regulator (CFTR)[22,23] in cystic-fibrosis–associated liver disease.

The exterior surface of the cell membrane of eukaryotic cells carries a glycocalyx, consisting of various glycosylated proteins, proteoglycans, glycosphingolipids, and membrane-bound and soluble glycosaminoglycans.[24] This "sugary" layer mediates cell-cell recognition, receptor-ligand interactions, and adds to the barrier function of the plasma membrane, as best characterized for the endothelial[25] and intestinal glycocalyx.[26-28] This juxtamembranous layer may locally alter the pH near, but exterior, to the lipid bilayer. In melanoma cells, the pH measured in the glycocalyx was reported to be slightly higher than the pH of the medium, and the absolute number of H^+ molecules differed up to 40% between different domains of the glycocalyx of a single cell.[29] HCO_3^- molecules are effectively trapped in the bile-acid–resistant[30] mucus gel layer of the gastric mucosa, thus establishing a local environment with a relatively high pH of $\sim$6-7 at the cell surface, whereas the bulk pH of gastric juice hovers around 1-2.[31-33] A similar function may be attributed to a glycocalyx of the biliary tree. However, an apical glycocalyx on cholangiocytes has never been described. This may, in part, be because of the common use of preparation and staining methods, which do not allow the visualization of a glycocalyx when applying transmission electron microscopy (TEM).[34] We hypothesize that cholangiocytes form an apical glycocalyx layer, which stabilizes the alkaline pH microclimate close to the apical membrane, thus contributing to the biliary HCO_3^- umbrella to prohibit apolar hydrophobic bile acids from entering the cell.

It was the aim of the present study to test the hypothesis on the human biliary HCO_3^- umbrella in cholangiocytes *in vitro* by various experimental approaches.

Materials and Methods

Cell Culture. The human cholangiocarcinoma cell lines, TFK-1 and EGI-1, were cultured in Roswell

*These authors contributed equally.

Address reprint requests to: Ulrich Beuers, M.D., Ph.D., Department of Gastroenterology and Hepatology, Tytgat Institute for Liver and Intestinal Research, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. E-mail: u.h.beuers@amc.nl; fax: +31-20-6917033.

Copyright © 2011 by the American Association for the Study of Liver Diseases.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.24691

Potential conflict of interest: Dr. Beuers is a consultant for Intercept. He received grants from Zambon. He is on the speakers' bureau of Gilead, Falk, Roche, and Schering-Plough.

Additional Supporting Information may be found in the online version of this article.

Park Memorial Institute (RPMI) 1640 medium (Lonza, Basel, Switzerland) with 4 mM of glutamine and 10% fetal calf serum (FCS) or Dulbecco's minimal essential medium (MEM) (Lonza) with 4 mM of glutamine, 10% FCS, and MEM essential and nonessential amino acids (Gibco, Carlsbad, CA), respectively. The immortalized nonmalignant human intrahepatic cholangiocyte cell line, H69,[35] was cultured in a DMEM/F12 (3:1)-based medium, enriched with 4 mM of glutamine, 180 μM of adenin, 865 nM of insulin, 10 μg/L of epidermal growth factor, 5 mg/L of triiodothyronin, 1.1 μM of hydrocortisone, and 5 mg/L of transferrin. All media contained 40 U/mL of penicillin, 40 ug/mL of streptomycin, and 0.1 ug/mL of amphotericin, and cells were kept at 37°C in a 5% CO_2 atmosphere and passaged twice-weekly. Under these culture conditions, H69 cells consistently formed a monolayer with clear signs of polarization forming microvilli, desmosomes, and tight junctions (Supporting Fig. 1).

Short Hairpin RNA-Mediated Knockdown of AE2. Plasmids encoding for short hairpin RNA (shRNA) against human SLC4A2 (AE2) or nontargeting shRNA, and containing a puromycin resistance gene, were obtained from the Sigma Mission shRNA library (Sigma-Aldrich, St. Louis, MO), and recombinant lentivirus was produced as previously described.[36] For lentiviral transduction, H69 cells were grown to 30%-40% confluence and incubated with virus-containing supernatants/DMEM (1:1) supplemented with 10 μg/mL of diethylaminoethyl-dextran for 4 hours. Subsequently, medium was refreshed, cells were cultured for 48 hours, and then selected for cells that integrated the viral DNA by adding 10 ug/mL of puromycin. After two passages, cells were further cultured without puromycin and knockdown was confirmed by real-time polymerase chain reaction.

Measurement of Bile Acid Uptake. Confluent native or AE2 knockdown H69 cells were washed three times with Hank's balanced salt solution (HBSS) and incubated for 1 hour at 37°C in HBSS containing 100 mM of glycochenodeoxycholate (GCDC) and ^{14}C-GCDC (1 uCi/mL) at indicated pH. Subsequently, cells were washed in HBSS containing 1% bovine serum albumin (BSA) to wash away membrane-bound bile acids and lysed in 0.2 M of NaOH/1% sodium dodecyl sulfate. Accumulated bile acids were determined by liquid scintillation counting and expressed as disintegrations per minute.

Induction of Cholangiotoxicity. Confluent native TFK-1, EGI-1, and H69 cells and $AE2^{KD}$- or control-transfected H69 cells were cultured in the presence of dimethyl sulfoxide (DMSO) (control), chenodeoxycholate (CDC), GCDC, taurochenodeoxycholate (TCDC), or etoposide at indicated concentrations for 4 (bile salts) or 18 hours (etoposide) at a pH of 8.0, 7.4, 7.1, 6.7, or 6.4 (20 mM of HEPES). To study the role of intact glycocalyx, native confluent H69 cells were washed twice with HBSS and incubated with neuraminidase (1U/mL) or hyaluronidase (2,140 U/mL) in serum-free H69 medium (pH 6.7) for 2.5 hours. Medium was replaced by full H69 medium at a pH of 7.1 or 8.0 (20 mM of HEPES) containing bile salts (CDC, GCDC, TCDC) or DMSO and incubated for 18 hours. Subsequent to respective treatments, apoptosis and viability were assessed as previously described.

Assessment of Cell Viability and Apoptosis. After the induction of cholangiotoxicity, metabolic activity was determined as a marker of cell viability by water-soluble tetrazolium salt (WST)-1 assays (Roche, Basel, Switzerland) and apoptosis was quantified by activity assays for the effector caspase-3/-7 in ApoOne assays (Promega, Madison, WI), following the manufacturer's instructions.

Determination of Intracellular pH. Confluent native H69 cells were washed 3 times with HBSS (Lonza) and were subsequently incubated with 5 μM of [2′,7′-bis(2-carboxyethyl1)-5,6-carboxy-fluorescein-acetoxy-methyl ester] (Sigma-Aldrich) in HBSS for 30 minutes. Cells were washed and incubated with DMSO (control), CDC, GCDC, or TCDC at indicated concentrations in indicator-free HBSS buffered with 20 mM of HEPES to different pH levels to cover the range from pH 6.4-7.4, as was used for the bile salt toxicity experiments for 1 hour at 37°C and atmospheric CO_2. For calibration, cells were incubated in calibration medium (containing 105 mM of potassium gluconate, 5 mM of magnesium chloride, 0.441 mM of potassium phosphate monobasic, 0.345 mM of potassium phosphate dibasic, 50 mM of N-methyl-d-glucamine, 10 mM of ethylene glycol tetraacetic acid, 4.46 mM of D-glucose, and 10 μM of nigericin; Sigma-Aldrich; buffered with 20 mM of HEPES to different pH levels to obtain a standard curve. pH levels of all buffers were determined simultaneously during the final measurement of fluorescence. Fluorescence intensity was measured after 1 hour of incubation using a Novostar analyzer (BMG Labtech GmbH, Offenburg, Germany) at excitation wavelengths of 490 and 440 nm and emission wavelength of 530 nm. Intracellular pH was determined from the ratio of fluorescence intensity obtained at excitation at 490 nm over excitation at 440 nm.[37]

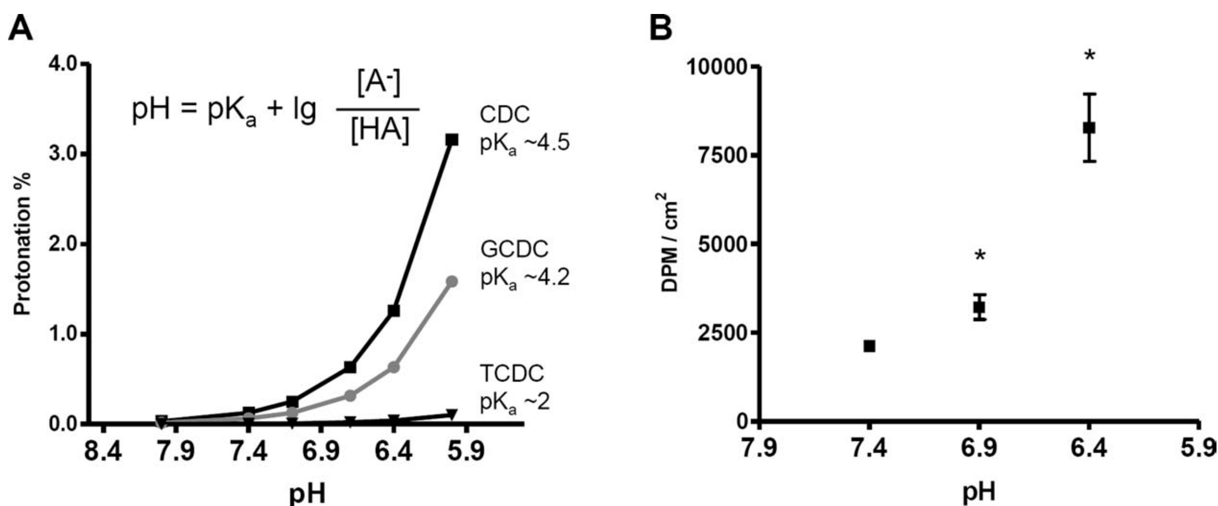

Fig. 1. Cholangiocellular bile salt accumulation is pK_a and pH dependent. (A) Applying pK_a estimates from the literature,[11] pH-dependent bile salt protonation was calculated by the Henderson-Hasselbalch equation (pK_a values applied for calculation were 4.5, 4.2, and 2.0 for CDC, GCDC, and TCDC, respectively). The figure illustrates the variability of unconjugated and glycine-conjugated bile salt protonation in a physiologic pH range. Because of low pK_a, taurine-conjugated bile salts stay virtually unprotonated, even at low pH. (B) Human H69 cholangiocytes were incubated with ^{14}C-GCDC for 1 hour at different pH levels, and intracellular GCDC accumulation was determined via liquid scintillation counting (n = 3; *$P < 0.05$ versus pH 7.4; Kruskal-Wallis).

Electron Microscopy of Cultured Cholangiocytes and Liver Sections. Cultured cells were fixed in McDowell fixative (4% paraformaldehyde, 1% glutaraldehyde, 0.1 M of cacodylate, and 4.4 mM of $CaCl_2$; pH 7.4), containing 0.2% ruthenium red (Sigma-Aldrich), and counterstained with 1% OsO_4 and 0.05% ruthenium red. Stained cells were embedded in the epoxy resin, Epon, and analyzed on a Philips EM420 transmission electron microscope (Philips, Eindhoven, The Netherlands), equipped with an SIS Megaview II camera (SIS, Münster, Germany).

Assessment of Sialylation of Glycocalyx by Flow Cytometry. To assess the efficiency of desialylation by neuraminidase on the apical cell surface, cells were quickly trypsinated after treatment with neuraminidase, as described above, and brought into suspension in serum-containing RPMI medium. Cells were incubated with 5 μg/mL of biotinylated SNA lectin (*Sambucus nigra* bark agglutinin; Vector Laboratories, Burlingame, CA) or control in TSM buffer (0.5% BSA, 20 mM of Tris-HCl, 150 mM of NaCl, 2 mM of $CaCl_2$, and 2 mM of $MgCl_2$; pH 7.4) for 30 minutes at 37°C, followed by staining with an Alexa 488–conjugated streptavidin (Molecular Probes, Carlsbad, CA) and analysis on a FACSCalibur (BD Biosciences, Franklin Lakes, NJ).

Statistical Analysis. All results are expressed as mean ± standard deviation of three to eight independent experiments. Data were analyzed in GraphPad Prism (GraphPad Software, San Diego, CA). Data were not normally distributed, and the Mann-Whitney or Kruskal-Wallis test were applied, as indicated and appropriate. A level of $P < 0.05$ was considered statistically significant.

Results

Bile Salt Entry Into Cholangiocytes Is Determined by Their pK_a and Extracellular pH. Bile salts mediate their toxic, apoptotic effects via specific signaling pathways, prominently from an intracellular platform. Carrier-independent membrane permeability of bile salts largely depends on their polarity, thus their protonation. Protonation of bile salts, as calculated from the Henderson-Hasselbach equation, is an exponential function of pH (Fig. 1A). Because of their high pK_a, CDC and GCDC are protonated at considerable amounts already in the physiologic pH range, whereas TCDC remains deprotonated. By use of ^{14}C-labeled GCDC, we tested the concept of pH dependency of bile salt uptake in biliary epithelial cells: ^{14}C-GCDC uptake increased exponentially with decreasing pH (Fig. 1B). Measured ^{14}C-GCDC uptake strongly correlated with values calculated from the Henderson-Hasselbach equation ($R^2 = 0.996$). Thus, bile salt diffusion into human cholangiocytes is pH and pK_a dependent.

Bile-Salt–Induced Cholangiocyte Toxicity Is pH Dependent. Viability of immortalized human cholangiocytes (H69 cells) was hardly affected by exposure to even millimolar concentrations of conjugated bile salts at physiologic pH (pH 7.4) (Fig. 2A). Only

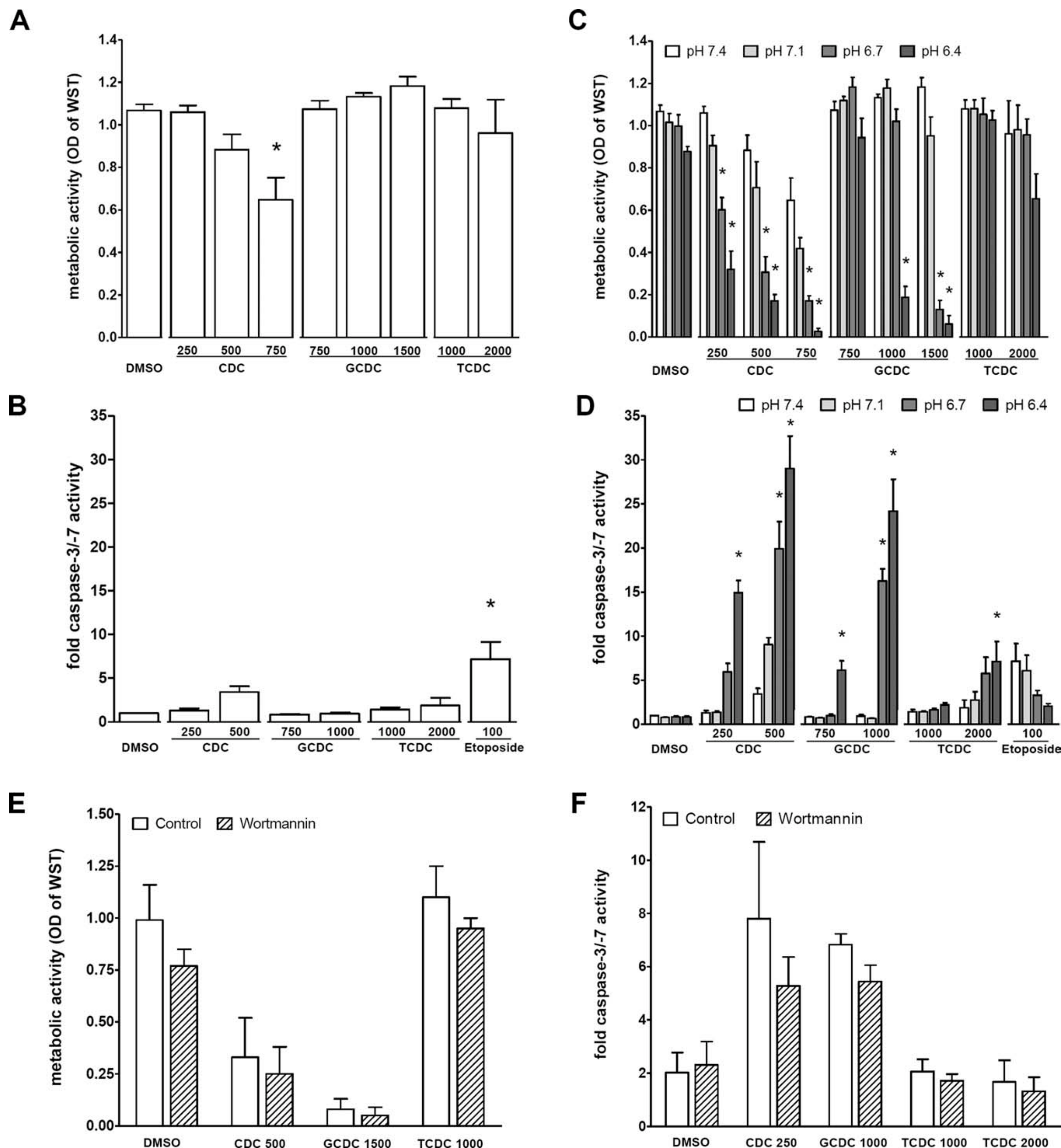

Fig. 2. Bile-salt–induced toxicity in the human cholangiocyte cell line, H69, is pK_a and pH dependent. (A) Cell viability and (B) induction of apoptosis were determined by WST-1 and caspase-3/-7 activity assays, respectively, in H69 cells stimulated with the bile salts, CDC, GCDC, and TCDC, or etoposide at indicated concentrations at a physiological pH of 7.4 for 4 hours (*$P < 0.05$ versus DMSO; Kruskal-Wallis; n = 5-6). (C) Cell viability and (D) induction of apoptosis were determined at otherwise identical experimental conditions (A and B) when pH was lowered from 7.4 to 7.1, 6.7, and 6.4 (*$P < 0.05$ versus pH 7.4; Kruskal-Wallis; n = 5-6). To test PI3K-dependent survival signaling after TCDC stimulation, (E) cell viability and (F) induction of apoptosis were determined in the presence or absence of 100 nM of wortmannin during bile acid stimulation at indicated concentrations for 4 hours at pH 6.7.

unconjugated CDC at a supraphysiologic concentration of 750 μM reduced cell viability from 1.1 ± 0.1 to 0.6 ± 0.2 (optical density) in WST-1 assays ($P < 0.05$ versus DMSO; n = 5). Correspondingly, at pH 7.4, 100 μM of etoposide caused a 7.2 ± 2.9-fold increase of caspase-3/-7 activity ($P < 0.05$ versus DMSO; n = 6), but bile salts were without effect (Fig. 2B). These results demonstrate the resistance of

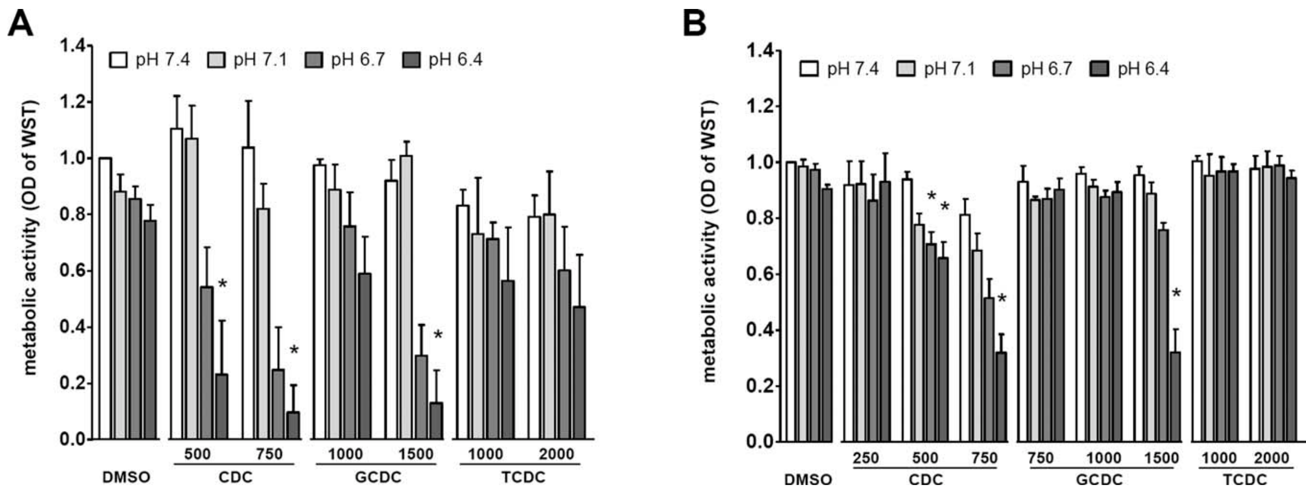

Fig. 3. pH dependency of bile-salt–induced cholangiotoxicity was confirmed in cholangiocarcinoma cell lines. Cholangiocarcinoma cell lines EGI-1 (A) and TFK-1 (B) cells were stimulated with CDC, GCDC, and TCDC at indicated concentrations for 4 hours at pH 7.4, 7.1, 6.7, and 6.4, respectively. Cell viability was determined by WST-1 assays (* $P < 0.05$ versus pH 7.4; Kruskal-Wallis; n = 3-6).

cholangiocytes against bile salt toxicity at physiologic pH. Viability of immortalized human cholangiocytes was progressively reduced when extracellular pH was lowered to 7.1, 6.7, or 6.4 during incubation with CDC or GCDC, but not TCDC or vehicle (DMSO), underlining pH and pK_a dependency of bile salt toxicity (Fig. 2C). Apoptosis was a key mechanism of pH-dependent bile salt cytotoxicity in human cholangiocytes: Caspase-3/-7 activity as a readout of apoptosis was enhanced by CDC and GCGC in a pH-dependent way (Fig. 2D). This effect was virtually absent for TCDC. Incubation of cells at indicated acidic pH moderately affected intracellular pH, whereas incubation with bile salts had no effect (Supporting Fig. 2). These data support the concept that bile salt cytotoxicity in cholangiocytes is pH and pK_a dependent and depends on the protonation state and diffusion of hydrophobic bile salts. TCDC has been described to coactivate phosphatidylinositol 3-kinase (PI3K)-dependent survival pathways in hepatocytes.[38] This molecular mechanism could be an alternative explanation for reduced toxicity of TCDC, in comparison to CDC and GCDC, in cholangiocytes. Therefore, the experiments described above were repeated in the presence or absence of wortmannin, a potent PI3K inhibitor. They showed that even millimolar concentrations of TCDC did not induce pH-dependent toxicity, despite PI3K inhibition (Fig. 2E,F). For further confirmation, experiments were repeated in two independent cholangiocarcinoma cell lines, EGI-1 and TFK-1. Using WST-1 viability assays, these experiments confirmed the pH dependency of bile-salt–induced cholangiotoxicity (Fig. 3A,B). Together, these data show that hydrophobic bile salts may damage human cholangiocytes in a pH- and pK_a-dependent fashion.

Knockdown of AE2 Sensitizes H69 Cells Towards Bile-Salt–Induced Apoptosis. We proposed that HCO_3^--mediated alkalization of bile close to the apical membrane is a protective mechanism of human cholangiocytes against bile salt toxicity.[7] A candidate transporter for biliary apical HCO_3^- secretion in humans is AE2.[18,39] We knocked down AE2 expression in H69 cells and tested their sensitivity to bile salt toxicity in comparison to control-transduced cells. We achieved an 80% knockdown of AE2. This resulted in markedly increased intracellular bile salt accumulation under physiologic conditions, indicating that AE2-mediated HCO_3^- secretion protects against uncontrolled bile salt uptake in cholangiocytes (Fig. 4A). Accordingly, the apoptotic effect of 500 μM of CDC, 750 μM of CDC, and 2,000 μM of GCDC was enhanced 2.5 $\pm$ 1.0, 3.2 $\pm$ 1.4, and 2.0 $\pm$ 0.9-fold, respectively, under physiologic conditions (Fig. 4B: $P < 0.05$ versus control-transfected cells; n = 5-7). To test the hypothesis that this increase in bile salt sensitivity correlates with altered extracellular alkalization, we exposed $AE2^{KD}$-H69 cells to bile salts at pH 8.0. Such treatment rescued $AE2^{KD}$-H69 cells from bile-salt–induced toxicity: Apoptosis induced by 750 μM of CDC and 2,000 μM of GCDC was reduced 6.8 $\pm$ 2.6 and 3.4 $\pm$ 1.1-fold, respectively ($P < 0.05$ versus pH 7.4; n = 5-7) (Fig. 4C). In $AE2^{KD}$-H69 cells, sensitivity toward etoposide was increased 1.9 $\pm$ 0.5-fold ($P < 0.05$ versus control cells; n = 4-5). Up-regulation of the apoptotic pathway was expected because of the increase of intracellular pH after the knockdown of AE2. However,

etoposide-induced cell death was not sensitive to increase in extracellular pH, indicating that the effects observed on bile-salt–induced damage were bile salt specific and independent of global up-regulation of apoptosis. Together, these data suggest that AE2 has a crucial function in the protection of cholangiocytes against bile-salt–induced cytotoxicity.

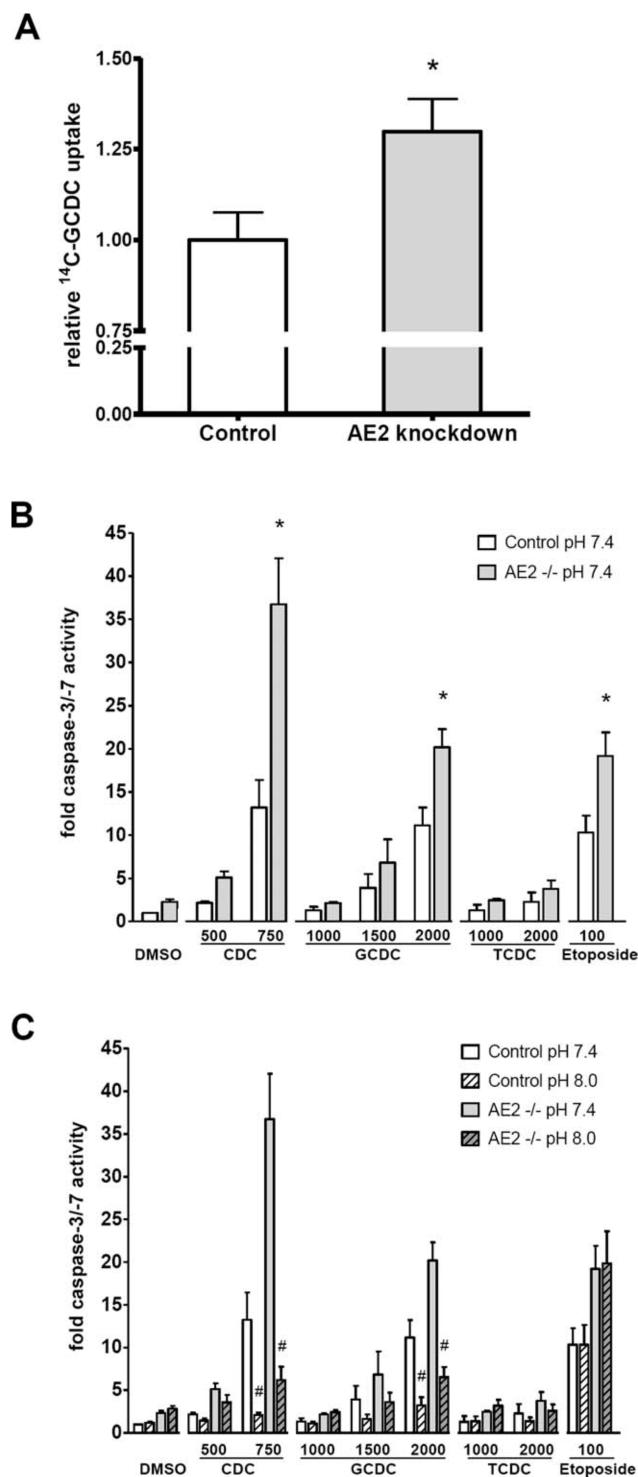

Fig. 4. The human cholangiocyte cell line, H69, is sensitized to increased bile salt load and bile-salt–induced cell death after AE2 knockdown and is rescued by alkalization of medium. The Cl^-/HCO_3^- exchanger, AE2, was knocked down via stable shRNA transduction. (A) AE2 knockdown and control cells were incubated with ^{14}C-GCDC for 1 hour, and intracellular accumulation of GCDC was determined via liquid scintillation counting (n = 4; *$P < 0.05$ versus control; Mann-Whitney). (B) AE2 knockdown and sham-transduced cells were stimulated with CDC, GCDC, TCDC, and etoposide, as indicated, for 4 hours, and induction of apoptosis was determined by caspase-3/-7 activity assays (*$P < 0.05$ versus control-transduced cells; Mann-Whitney; n = 3-7). (C) Identical experiments were conducted after increase of extracellular pH to 8.0 and were compared to the results of (B) (#$P < 0.05$ versus pH 7.4; Kruskal-Wallis; n = 3-7).

Biliary Epithelial Cells Express an Apical Glycocalyx. An apical glycocalyx has barrier function against the extracellular milieu in various eukaryotic cells, including intestinal cells. An apical glycocalyx has, so far, not been described on bile duct epithelia. We applied electron microscopic techniques known to visualize this layer, which is translucent in normal electron microscopic protocols. Using TEM, a ~20-30-nm-thick glycocalyx layer was consistently identified on the outer leaflet of the apical membrane of monolayers of cultured cholangiocytes. Bridging of glycocalyx material between microvilli was observed, suggesting that the intravillous space may be effectively dominated by glycocalyx (Fig. 5A). In analogy with the glycocalyx of the stomach mucosa, the biliary glycocalyx is assumed to form a local environment with chemical properties different from the bulk bile fluid.

Enzymatic Modification of the Glycocalyx Sensitizes Cholangiocytes Toward Bile-Salt–Induced Apoptosis. We tested whether the newly identified glycocalyx on human cholangiocytes has a barrier function against toxic bile constituents. We treated monolayers of H69 cells with neuraminidase, an established intervention for investigating the physiologic function of the glycocalyx.[40,41] Neuraminidase cleaves off negatively charged sialic acids, the predominant terminal residues on complex N-linked glycans and on many O-linked glycans. Desialylation was confirmed by flow cytometry for membrane binding of SNA lectin, a plant sugar-binding protein specifically recognizing α2-6 linked sialic acid. Flow cytometry showed a decreased median fluorescence intensity for SNA lectin binding in neuraminidase-treated cells, as compared to sham-treated cells, confirming the effectiveness of enzymatic desialylation (Fig. 5B). After neuraminidase treatment, cells were incubated with 500 μM of CDC and 1,000 μM of GCDC or TCDC at pH 7.1, and viability was assessed by WST-1 assays after 18 hours. Neuraminidase treatment increased the susceptibility

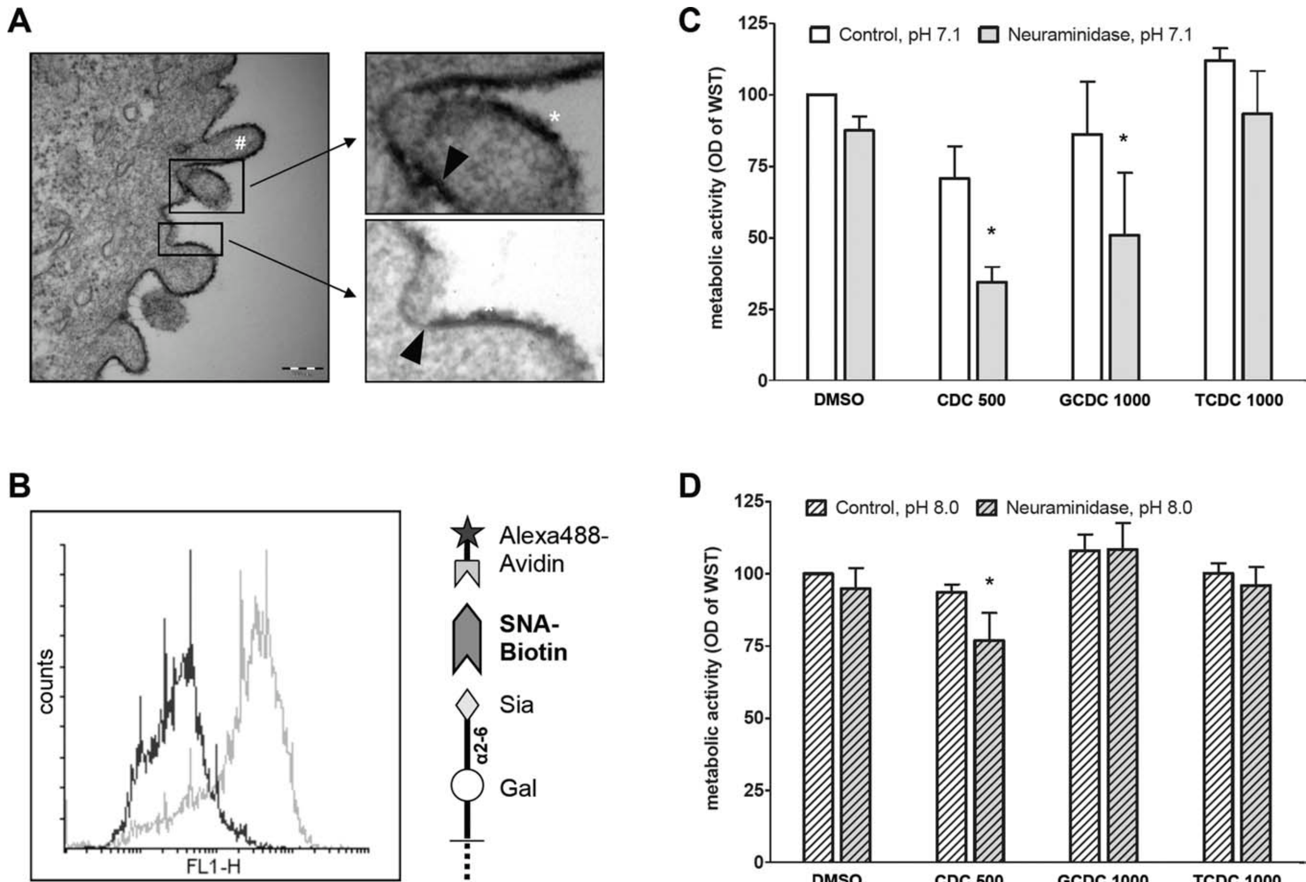

Fig. 5. Enzymatic desialylation of the glycocalyx turns H69 cells vulnerable to bile acid toxicity. (A) An apical glycocalyx is visualized by TEM on the human cholangiocyte cell line, H69, after staining with ruthenium red (hash: microvillus; white asterisk: glycocalyx; black arrow head: cell membrane lipid bilayer; bar: 200 nm). (B) Neuraminidase-mediated desialylation of the glycocalyx of H69 cells was assessed using an SNA lectin-binding assay. SNA specifically binds α2-6 bound sialic acid (Sia) and was detected here by applying Alexa 488–conjugated avidin. Efficient desialylation by neuraminidase was reflected by the strongly reduced median cell fluorescence (FL1-H), as determined by flow cytometry of neuraminidase-treated (dark gray) or sham-treated (light gray) cells. (C and D) H69 cells were pretreated with neuraminidase or carrier medium (control) for 2 hours for desialylation of the glycocalyx. Subsequently, cells were stimulated with CDC, GCDC, and TCDC, at indicated concentrations for 18 hours at pH 7.1 (C) or pH 8.0 (D), and cell viability was determined by WST-1 assays (*$P < 0.05$ vs. control; Kruskal-Wallis; n = 4-8).

to CDC- and GCDC-induced toxicity, but not TCDC-induced toxicity, and decreased H69 viability by 66% ± 5%, as compared to 29% ± 11% for control upon exposure to CDC, and by 49% ± 22% as compared to 14% ± 18% upon exposure to GCDC (Fig. 5C). At pH 8.0, neuraminidase treatment hardly altered CDC- and GCDC-induced toxicity, indicating that desialylation sensitized cells to hydrophobic bile-salt–induced toxicity (Fig. 5D). Neuraminidase treatment alone did not significantly affect cell viability. Moreover, treatment with hyaluronidase, an enzyme specifically degrading hyaluronic acid in the glycocalyx and used as a control, did not induce susceptibility to bile-salt–induced toxicity (data not shown). These data suggest that sialic acid residues in the apical glycocalyx layer on cholangiocytes may contribute to stabilization of the biliary HCO_3^- umbrella and to protection against bile-salt–induced cell injury, possibly by stabilization of the biliary HCO_3^- umbrella, but it may also represent an independent mechanism.

Discussion

The present data provide the first experimental evidence *in vitro* for the presence of a biliary HCO_3^- umbrella,[7] which may protect human cholangiocytes (and hepatocytes) against bile-acid–induced cytotoxicity. Our data indicate that carrier-independent penetration of bile salts/acids into cholangiocytes is dependent on their protonation, thus pH dependent, as predicted from their different pK_a (Fig. 1). Accordingly, we demonstrate that bile-salt–induced toxicity and apoptosis in human cholangiocytes is determined by extracellular pH, and that alkalization of bile close to the apical membrane of cholangiocytes via secretion of HCO_3^- may be a key protective mechanism against bile salt

toxicity (Figs. 2 and 3). Knockdown of the Cl^-/HCO_3^- exchanger, AE2, by shRNA sensitized cholangiocytes toward bile acid toxicity, underlining a key role of AE2 in biliary HCO_3^- secretion of humans (Fig. 4). By TEM, an apical glycocalyx was identified on the human cholangiocyte cell line, H69 (Fig. 5). Destabilization of this glycocalyx by enzymatic desialylation sensitized cholangiocytes to bile acid toxicity (Fig. 5). Thus, the biliary HCO_3^- umbrella may be a key protective mechanism against bile-acid–induced injury of cholangiocytes, but, putatively, also other cell types, such as hepatocytes, delimiting the body against millimolar bile salt monomers in human bile.

Our experimental findings delineate, for the first time, why biliary HCO_3^- secretion in man by far exceeds that in rodents[8]: Protonation of glycine-conjugated bile salts with a $pK_a \geq 4$,predominantly found in the human bile salt pool,[11] but not of taurine-conjugated bile salts with a pK_a of 1-2, predominantly found in the rodent bile salt pool,[11] is sensitive to changes in biliary pH near the physiologic range. Protonation led to diffusional penetration and subsequent apoptotic cell death in the experimental setting used by us. Thus, glycine-conugated, but not taurine-conjugated, bile salts may represent a potential threat for human cholangiocytes when minor acidification of biliary pH occurs because of genetic, endogenous, or exogenous factors.

Our hypothesis of a human biliary HCO_3^- umbrella,[7] supported by the data provided here, may provide a unifying pathogenetic link between various cholangiopathies. Genetic variants of the Cl^-/HCO_3^- exchanger, AE2, which is regarded as a key HCO_3^- exporter in humans, have been associated with improved prognosis of PBC[20]; genetic variants of the bile salt sensor, TGR5, which modulates biliary HCO_3^- secretion, have been associated with PSC.[21] Alteration of the transmembrane Cl^- gradient, driving HCO_3^- secretion, or altered flux of HCO_3^- via a dysfunctional cystic fibrosis transmembrane conductance regulator, CFTR, might be the basis for the development of cystic-fibrosis–associated liver disease, which may resemble sclerosing cholangitis.[23] Ischemia-type biliary lesions and nonanastomotic bile duct strictures after liver transplantation with denervation in man[42] may be a consequence of disrupted vagal acetylcholine signaling, a physiologic driving force of biliary HCO_3^- secretion. Thus, destabilization of the biliary HCO_3^- umbrella by genetic, endogenous, or exogenous factors might predispose humans to various types of cholangiopathies.

Our data may have particular impact on the understanding of the pathogenesis and treatment of PBC.[17-20,39,43] Increasing evidence supports the view that cholangiocyte apoptosis is a driving force in the pathogenesis of PBC: It has long been demonstrated that cholangiocyte apoptosis is associated with ductular inflammation in PBC, but not PSC.[44] Furthermore, cholangiocytes of small bile ducts expose immunologically reactive PDC-E2 or PDC-E2-like protein during apoptosis,[16] which might explain, in part, the organ specificity of the immune reaction in PBC. Most recently, it has been demonstrated, *in vitro*, that apoptotic cholangiocytes, in the presence of anti-PDC-E2 antibodies, drive innate inflammatory response in PBC.[15] Based on these observations, genetic or acquired dysfunction of protective mechanisms against bile-salt–induced apoptosis, such as the biliary HCO_3^- umbrella, may play a key role in the pathogenesis of PBC. Impaired expression of the Cl^-/HCO_3^- exchanger, AE2, and impaired HCO_3^- secretion have earlier been recognized as potential pathogenetic factors in PBC.[17-20,39,43] Our data may provide a missing pathophysiologic link in the pathogenesis of PBC, which, in our view, is based on the interplay between genetic, exogenous, and endogenous predisposing factors.[17-20,39,43] Future therapeutic approaches might, therefore, aim to further strengthen the weakened biliary HCO_3^- umbrella in PBC.[7]

Advances have been made in understanding the mechanisms and sites of action of ursodeoxycholic acid (UDCA) in the therapy of chronic cholangiopathies.[45,46] Our data might emphasize its putative beneficial action as a hepatocyte[47,48] and cholangiocyte[49,50] HCO_3^- secretagogue, particularly for small intrahepatic bile ductules affected in PBC. Our data may also explain, in part, the superiority of norUDCA above UDCA in the treatment of experimental cholangiopathy in $Mdr2^{-/-}$ mice, a disease affecting also medium- and large-sized ducts, such as PSC[51]: Exogenous norUDCA has been shown to represent the strongest biliary HCO_3^- secretagogue, so far, tested in humans.[52]

We report, for the first time, on the presence and potential function of a biliary glycocalyx. We consistently detected a 20-30-nm-thick glycocalyx layer on the outer leaf of the apical membrane of cholangiocytes. In the experimental setup chosen, the intact glycocalyx clearly contributed to the protection of cholangiocytes against pH-dependent bile salt toxicity (Fig. 5). The results support our previous speculation that a cholangiocyte glycocalyx layer may stabilize the alkaline pH microclimate close to the apical membrane, as

earlier described for melanoma cells,[29] thus contributing to the biliary HCO_3^- umbrella to prohibit apolar hydrophobic bile acids from entering the cell.[7]

A glycocalyx influences the permeation of charged proteins over the endothelial wall.[53,54] The striking effect of desialylation of the glycocalyx by neuraminidase treatment on pH-dependent cholangiocyte toxicity induced by CDC and GCDC, but not TCDC, supports the idea that polar, negatively charged bile salts are prohibited from entering cholangiocytes and from inducing cell death. We assume that this protective effect of the intact glycocalyx is the result of local stabilization of the biliary HCO_3^- umbrella, enabling the deprotonation of bile acids to bile salts in the close vicinity to the membrane. In this context, the potential role of membrane-bound and secretory mucins, particularly in medium- and large-sized ducts, for stabilization of the biliary HCO_3^- umbrella deserves attention, considering that HCO_3^- molecules are effectively trapped in the bile-acid–resistant mucus gel layer of the gastric mucosa.[30] In addition, the recent identification of fucosyltransferase 2, an enzyme involved in the maintenance of the glycocalyx, as a susceptibility gene for PSC,[55] deserves further investigation and is a subject of our future experimental efforts.

In conclusion, our actual data support our hypothesis on the presence of a biliary HCO_3^- umbrella,[7] which protects human cholangiocytes (and hepatocytes) against bile-acid–induced cytotoxicity. These data may have major implications for future pathogenetic and therapeutic considerations related to a variety of chronic cholangiopathies.

Acknowledgment: The authors gratefully acknowledge the support of Prof. A.F. Hofmann (San Diego, CA) in providing ^{14}C-GCDC and Dr. H.A. van Veen (Amsterdam, The Netherlands) in assisting with electron microscopy.

References

1. Rust C, Wild N, Bernt C, Vennegeerts T, Wimmer R, Beuers U. Bile acid-induced apoptosis in hepatocytes is caspase-6-dependent. J Biol Chem 2009;284:2908-2916.
2. Perez MJ, Briz O. Bile-acid-induced cell injury and protection. World J Gastroenterol 2009;15:1677-1689.
3. Spivey JR, Bronk SF, Gores GJ. Glycochenodeoxycholate-induced lethal hepatocellular injury in rat hepatocytes. Role of ATP depletion and cytosolic free calcium. J Clin Invest 1993;92:17-24.
4. Hohenester S, Gates A, Wimmer R, Beuers U, Anwer MS, Rust C, Webster CR. Phosphatidylinositol-3-kinase p110gamma contributes to bile salt-induced apoptosis in primary rat hepatocytes and human hepatoma cells. J Hepatol 2010;53:918-926.
5. Hofmann AF. Bile acids: trying to understand their chemistry and biology with the hope of helping patients. HEPATOLOGY 2009;49:1403-1418.
6. Boyer JL. It's all about bile. HEPATOLOGY 2009;49:711-723.
7. Beuers U, Hohenester S, de Buy Wenniger LJ, Kremer AE, Jansen PL, Elferink RP. The biliary HCO(3)(-) umbrella: a unifying hypothesis on pathogenetic and therapeutic aspects of fibrosing cholangiopathies. HEPATOLOGY 2010;52:1489-1496.
8. Boyer JL. Mechanisms of bile secretion and hepatic transport. In: Andreoli TE, Hoffman JF, Fanestil DD, Schultz SG, eds. Physiology of Membrane Disorders. New York: Plenum; 1986:609-636.
9. Komichi D, Tazuma S, Nishioka T, Hyogo H, Une M, Chayama K. Unique inhibition of bile salt-induced apoptosis by lecithins and cytoprotective bile salts in immortalized mouse cholangiocytes. Dig Dis Sci 2003;48:2315-2322.
10. Amelsberg A, Schteingart CD, Ton-Nu HT, Hofmann AF. Carrier-mediated jejunal absorption of conjugated bile acids in the guinea pig. Gastroenterology 1996;110:1098-1106.
11. Carey MC. Physical-chemical properties of bile acids and their salts. In: Danielsson H, Sjövall J, eds. Sterols and Bile Acids. Amsterdam: Elsevier; 1985:345-403.
12. Eastwood GL. Effect of pH on bile salt injury to mouse gastric mucosa. A light- and electron-microscopic study. Gastroenterology 1975;68:1456-1465.
13. Duane WC, Wiegand DM, Sievert CE. Bile acid and bile salt disrupt gastric mucosal barrier in the dog by different mechanisms. Am J Physiol 1982;242:G95-G99.
14. Batzri S, Harmon JW, Schweitzer EJ, Toles R. Bile acid accumulation in gastric mucosal cells. Proc Soc Exp Biol Med 1991;197:393-399.
15. Lleo A, Bowlus CL, Yang GX, Invernizzi P, Podda M, Van de Water J, et al. Biliary apotopes and anti-mitochondrial antibodies activate innate immune responses in primary biliary cirrhosis. HEPATOLOGY 2010;52:987-998.
16. Lleo A, Selmi C, Invernizzi P, Podda M, Coppel RL, Mackay IR, et al. Apotopes and the biliary specificity of primary biliary cirrhosis. HEPATOLOGY 2009;49:871-879.
17. Prieto J, Garcia N, Marti-Climent JM, Penuelas I, Richter JA, Medina JF. Assessment of biliary bicarbonate secretion in humans by positron emission tomography. Gastroenterology 1999;117:167-172.
18. Medina JF, Martinez A, Vazquez JJ, Prieto J. Decreased anion exchanger 2 immunoreactivity in the liver of patients with primary biliary cirrhosis. HEPATOLOGY 1997;25:12-17.
19. Prieto J, Qian C, Garcia N, Diez J, Medina JF. Abnormal expression of anion exchanger genes in primary biliary cirrhosis. Gastroenterology 1993;105:572-578.
20. Poupon R, Ping C, Chrétien Y, Corpechot C, Chazouillères O, Simon T, et al. Genetic factors of susceptibility and of severity in primary biliary cirrhosis. J Hepatol 2008;49:1038-1045.
21. Hov JR, Keitel V, Laerdahl JK, Spomer L, Ellinghaus E, ElSharaway A, et al. Mutational characterization of the bile acid receptor TGR5 in primary sclerosing cholangitis. PLoS ONE 2010;5:e12403.
22. Colombo C, Battezzati PM, Strazzabosco M, Podda M. Liver and biliary problems in cystic fibrosis. Semin Liver Dis 1998;18:227-235.
23. Herrmann U, Dockter G, Lammert F. Cystic fibrosis-associated liver disease. Best Pract Res Clin Gastroenterol 2010;24:585-592.
24. Lee GM, Johnstone B, Jacobson K, Caterson B. The dynamic structure of the pericellular matrix on living cells. J Cell Biol 1993;123:1899-1907.
25. Reitsma S, Slaaf DW, Vink H, van Zandvoort MA, oude Egbrink MG. The endothelial glycocalyx: composition, functions, and visualization. Pflugers Arch 2007;454:345-359.
26. Horiuchi K, Naito I, Nakano K, Nakatani S, Nishida K, Taguchi T, Ohtsuda A. Three-dimensional ultrastructure of the brush border glycocalyx in the mouse small intestine: a high resolution scanning electron microscopic study. Arch Histol Cytol 2005;68:51-56.
27. Patsos G, Corfield A. Management of the human mucosal defensive barrier: evidence for glycan legislation. Biol Chem 2009;390:581-590.
28. McGuckin MA, Linden SK, Sutton P, Florin TH. Mucin dynamics and enteric pathogens. Nat Rev Microbiol 2011;9:265-278.
29. Stock C, Mueller M, Kraehling H, Mally S, Noël J, Eder C, Schwab A. pH nanoenvironment at the surface of single melanoma cells. Cell Physiol Biochem 2007;20:679-686.

30. Bell AE, Sellers LA, Allen A, Cunliffe WJ, Morris ER, Ross-Murphy SB. Properties of gastric and duodenal mucus: effect of proteolysis, disulfide reduction, bile, acid, ethanol, and hypertonicity on mucus gel structure. Gastroenterology 1985;88:269-280.
31. Bhaskar KR, Garik P, Turner BS, Bradley JD, Bansil R, Stanley HE, LaMont JT. Viscous fingering of HCl through gastric mucin. Nature 1992;360:458-461.
32. Varki A. Biological roles of oligosaccharides: all of the theories are correct. Glycobiology 1993;3:97-130.
33. Allen A, Flemstrom G. Gastroduodenal mucus bicarbonate barrier: protection against acid and pepsin. Am J Physiol Cell Physiol 2005;288: C1-C19.
34. Huang BQ, Masyuk TV, Muff MA, Tietz PS, Masyuk AI, Larusso NF. Isolation and characterization of cholangiocyte primary cilia. Am J Physiol Gastrointest Liver Physiol 2006;291:G500-G509.
35. Grubman SA, Perrone RD, Lee DW, Murray SL, Rogers LC, Wolkoff LI, et al. Regulation of intracellular pH by immortalized human intrahepatic biliary epithelial cell lines. Am J Physiol 1994;266:G1060-G1070.
36. Seppen J, Rijnberg M, Cooreman MP, Oude Elferink RP. Lentiviral vectors for efficient transduction of isolated primary quiescent hepatocytes. J Hepatol 2002;36:459-465.
37. Mardones P, Medina JF, Oude Elferink RP. Activation of cyclic AMP signaling in Ae2-deficient mouse fibroblasts,. J Biol Chem 2008;283: 12146-12153.
38. Rust C, Bauchmuller K, Fickert P, Fuchsbichler A, Beuers U. Phosphatidylinositol 3-kinase-dependent signaling modulates taurochenodeoxycholic acid-induced liver injury and cholestasis in perfused rat livers. Am J Physiol Gastrointest Liver Physiol 2005;289:G88-G94.
39. Melero S, Spirlì C, Zsembery A, Medina JF, Joplin RE, Duner E, et al. Defective regulation of cholangiocyte Cl-/HCO3(-) and Na+/H+ exchanger activities in primary biliary cirrhosis. HEPATOLOGY 2002;35: 1513-1521.
40. Meesmann HM, Fehr EM, Kierschke S, Herrmann M, Bilyy R, Heyder P, et al. Decrease of sialic acid residues as an *eat-me* signal on the surface of apoptotic lymphocytes. J Cell Sci 2010;123:3347-3356.
41. Ensinck A, Biondi CS, Marini A, García Borrás S, Racca LL, Cotorruelo CM, Racca AL. Effect of membrane-bound IgG and desialysation in the interaction of monocytes with senescent erythrocytes. Clin Exp Med 2006;6:138-142.
42. Hoekstra H, Porte RJ, Tian Y, Jochum W, Stieger B, Moritz W, et al. Bile salt toxicity aggravates cold ischemic injury of bile ducts after liver transplantation in Mdr2+/- mice. HEPATOLOGY 2006;43:1022-1031.
43. Hohenester S, Oude-Elferink RP, Beuers U. Primary biliary cirrhosis. Semin Immunopathol 2009;31:283-307.
44. Tinmouth J, Lee M, Wanless IR, Tsui FW, Inman R, Heathcote EJ. Apoptosis of biliary epithelial cells in primary biliary cirrhosis and primary sclerosing cholangitis. Liver 2002;22:228-234.
45. Beuers U. Drug insight: mechanisms and sites of action of ursodeoxycholic acid in cholestasis. Nat Clin Pract Gastroenterol Hepatol 2006; 3:318-328.
46. Wimmer R, Hohenester S, Pusl T, Denk GU, Rust C, Beuers U. Tauroursodeoxycholic acid exerts anticholestatic effects by a cooperative cPKC alpha-/PKA-dependent mechanism in rat liver. Gut 2008;57:1448-1454.
47. Beuers U, Nathanson MH, Isales CM, Boyer JL. Tauroursodeoxycholic acid stimulates hepatocellular exocytosis and mobilizes extracellular Ca++ mechanisms defective in cholestasis. J Clin Invest 1993;92: 2984-2993.
48. Benedetti A, Strazzabosco M, Ng OC, Boyer JL. Regulation of activity and apical targeting of the Cl-/HCO3- exchanger in rat hepatocytes. Proc Natl Acad Sci U S A 1994;91:792-796.
49. Minagawa N, Nagata J, Shibao K, Masyuk AI, Gomes DA, Rodrigues MA, et al. Cyclic AMP regulates bicarbonate secretion in cholangiocytes through release of ATP into bile. Gastroenterology 2007;133: 1592-1602.
50. Fiorotto R, Spirli C, Fabris L, Cadamuro M, Okolicsanyi L, Strazzabosco M. Ursodeoxycholic acid stimulates cholangiocyte fluid secretion in mice via CFTR-dependent ATP secretion. Gastroenterology 2007;133: 1603-1613.
51. Fickert P, Wagner M, Marschall HU, Fuchsbichler A, Zollner G, Tsybrovskyy O, et al. 24-norUrsodeoxycholic acid is superior to ursodeoxycholic acid in the treatment of sclerosing cholangitis in Mdr2 (Abcb4) knockout mice. Gastroenterology 2006;130:465-481.
52. Hofmann AF, Zakko SF, Lira M, Clerici C, Hagey LR, Lambert KK, et al. Novel biotransformation and physiological properties of norursodeoxycholic acid in humans. HEPATOLOGY 2005;42:1391-1398.
53. Huxley VH, Williams DA. Role of a glycocalyx on coronary arteriole permeability to proteins: evidence from enzyme treatments. Am J Physiol Heart Circ Physiol 2000;278:H1177-H1185.
54. Singh A, Satchell SC, Neal CR, McKenzie EA, Tooke JE, Mathieson PW. Glomerular endothelial glycocalyx constitutes a barrier to protein permeability. J Am Soc Nephrol 2007;18:2885-2893.
55. Folseraas T, Melum E, Juran B, Weersma RK, Schramm C, Weismüller TJ, et al. Novel susceptibility loci for primary sclerosing cholangitis identified by genome-wide association and replication analysis. J Hepatol 2011;54:S46-S46.