

The Cholangiocyte Glycocalyx Stabilizes the 'Biliary HCO₃⁻ Umbrella': An Integrated Line of Defense against Toxic Bile Acids

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Key Words

Glycocalyx · Cholangiocyte · Bile salts

Abstract

Background: Destruction of cholangiocytes is the hallmark of chronic cholangiopathies such as primary biliary cirrhosis. Under physiologic conditions, cholangiocytes display a striking resistance to the high, millimolar concentrations of toxic bile salts present in bile. We recently showed that a 'biliary HCO₃⁻ umbrella', i.e. apical cholangiocyte HCO₃⁻ secretion, prevents cholangiotoxicity of bile acids, and speculated on a role for extracellular membrane-bound glycans in the stabilization of this protective layer. This paper summarizes published and thus far unpublished evidence supporting the role of the glycocalyx in stabilizing the 'biliary HCO₃⁻ umbrella' and thus preventing cholangiotoxicity of bile acids.

Key Messages: The apical glycocalyx of a human cholangiocyte cell line and mouse liver sections were visualized by electron microscopy. FACS analysis was used to characterize the surface glycan profile of cultured human cholangiocytes. Using enzymatic digestion with neuraminidase the cholan-

giocyte glycocalyx was desialylated to test its protective function. Using lectin assays, we demonstrated that the main N-glycans in human and mouse cholangiocytes were sialylated biantennary structures, accompanied by high expression of the H-antigen (α_{1-2} fucose). Apical neuraminidase treatment induced desialylation without affecting cell viability, but lowered cholangiocyte resistance to bile acid-induced toxicity: both glycochenodeoxycholate and chenodeoxycholate ($pK_a \geq 4$), but not taurochenodeoxycholate ($pK_a < 2$), displayed cholangiotoxic effects after desialylation. A 24-hour reconstitution period allowed cholangiocytes to recover to a pretreatment bile salt susceptibility pattern. **Conclusion:** Experimental evidence indicates that an apical cholangiocyte glycocalyx with glycosylated mucins and other glycan-bearing membrane glycoproteins stabilizes the 'biliary HCO₃⁻ umbrella', thus aiding in the protection of human cholangiocytes against bile acid toxicity.

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Lucas J. Maillette de Buy Wenniger and Simon Hohenester contributed equally to this work.

Toxic Bile Salts

Destruction of biliary epithelial cells, cholangiocytes, is the hallmark of chronic cholangiopathies such as primary biliary cirrhosis [1], with subsequent loss of bile ducts and chronic cholestasis [2, 3]. Under chronic cholestatic conditions, hydrophobic, i.e. potentially toxic, bile salts accumulate in the body [4] and are thought to drive disease progression [2]. Reflecting upon cholangiocellular physiology, it is striking that cholangiocytes are continuously exposed, without major damage, to the most toxic of all body fluids: bile. Hydrophobic bile salts, which are present in bile at high, millimolar concentrations, induce apoptosis in other cell types such as hepatocytes already at low, micromolar concentrations via specific signaling pathways [5, 6] and may even elicit necrosis in several cell types [7]. In striking contrast, human cholangiocytes are exposed to millimolar concentrations of hydrophobic bile salts under physiologic conditions [1] without significant cytotoxicity.

Several protective mechanisms help cholangiocytes endure their constant exposure to bile. Secretion of phospholipids by hepatocytes allows for the formation of mixed micelles, decreasing the concentration of free bile salt monomers. Failure to form such mixed micelles, for example due to impaired phospholipid secretion in the setting of ABCB4 deficiency, leads to ductular damage and cholestasis. However, formation of mixed micelles insufficiently reduces the concentration of free hydrophobic bile salt monomers to low millimolar levels in bile, as discussed earlier [8].

The 'Biliary HCO₃⁻ Umbrella'

We have recently hypothesized that excessive biliary HCO₃⁻ secretion in humans is a key protective mechanism to prevent bile salt-induced cholangiocellular damage [8]. Intracellular accumulation of hydrophobic bile salts is a prerequisite for their cytotoxic effects. Passive, carrier-independent cell invasion of bile salts is determined by their polarity and degree of protonation [9]. Conjugates of chenodeoxycholate (CDC), the predominant toxic bile salts in human bile during cholestasis, are among the most hydrophobic bile salts in humans. The glycine conjugate (GCDC) with a pK_a of ~4 is partially protonated, apolar, and thus cell permeable at micromolar amounts at a physiologic pH of 7.4. Even minor changes in local biliary pH affect the glycine-conjugated bile salt:bile acid ratio and, thereby, the sensitivity of cholangiocytes to-

ward glycine-conjugated bile acid-induced damage. Experimental evidence recently published by our group supports our hypothesis of a protective 'biliary HCO₃⁻ umbrella' [10]: we found that GCDC uptake and GCDC-induced cholangiocellular apoptosis is pH-dependent in vitro and contained by expression of the main biliary HCO₃⁻ exporter, anion exchanger 2. These insights opened up new avenues for the development of therapeutic strategies for patients in whom loss of biliary barrier function causes cholestatic disease [11].

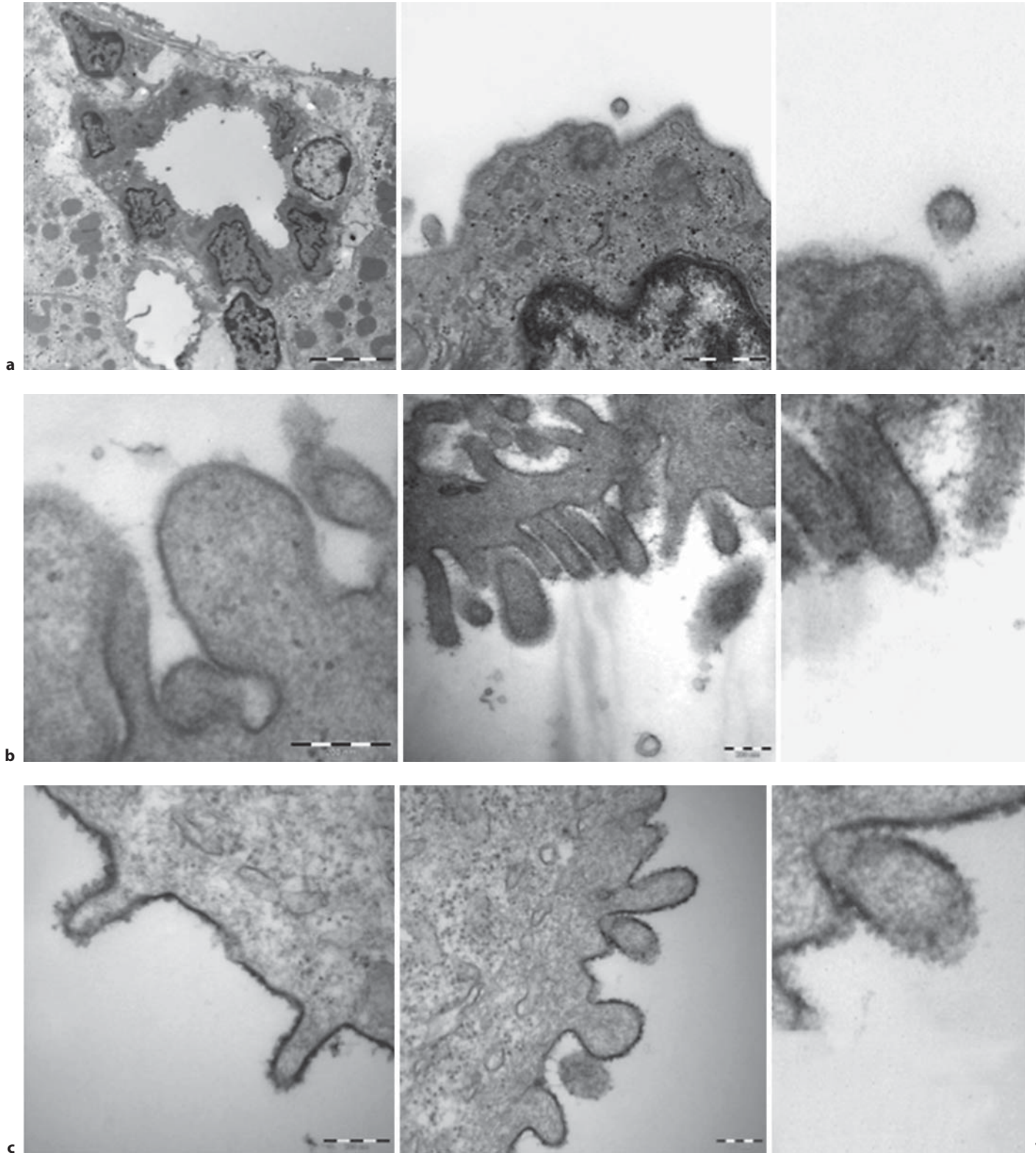
Having gathered evidence supporting the existence of a 'biliary HCO₃⁻ umbrella' we hypothesized that physical/cellular structures are needed to help stabilize the pericellular alkaline milieu. 'Trapping' of the HCO₃⁻-rich pericellular fluid would help the cholangiocyte to maintain an alkaline pH close to its apical membrane without the need to alkalize the bulk of mainstream bile. In the stomach, a mucous layer scaffolds a pH-gradient ranging from pH 1–2 within the lumen to ~6–7 just near the cell membrane [12]. We thus reasoned that a similar principle could apply for cholangiocytes. We hypothesized that a distinct glycocalyx layer on the apical surface of cholangiocytes could be the physical structure stabilizing the pericellular pH in the biliary tree.

The Biliary Glycocalyx

A glycocalyx is defined as an extracellular, juxtamembranous layer of glycoproteins, glycans, glycosphingolipids and polysaccharides. Some components of the glyco-

Fig. 1. Visualization of the biliary glycocalyx in a mouse (a), human (b) and biliary epithelial cell line (c) using transmission electron microscopy. In the cultured cells in c, ruthenium red was deployed to selectively stain the glycocalyx (c adapted from Hohenester et al. [10]). Fixation of mouse liver tissue was performed in McDowell fixative (4% paraformaldehyde, 1% glutaraldehyde, 0.1 M cacodylate and 4.4 mM CaCl₂; pH 7.4), and counterstaining was performed with 1% OsO₄. The immortalized nonmalignant human intrahepatic cholangiocyte cell line H69 [26] was cultured in a DMEM/F12- (3:1) based medium as described earlier [10]. Fixation of cultured cells was performed in McDowell fixative, either containing 0.2% ruthenium red or not (Sigma-Aldrich, St. Louis, Mo., USA), and counterstaining was performed with 1% OsO₄ either supplemented with 0.05% ruthenium red or not. Fixed tissue and cells were embedded in epoxy resin, Epon, cut and analyzed on a Philips EM420 transmission electron microscope (Philips, Eindhoven, The Netherlands), equipped with an SIS Megaview II camera (SIS, Münster, Germany).

(For figure see next page.)



calyx are structurally coupled to the lipid bilayer as they are bound to transmembrane protein domains or sphingolipids. Other constituents, such as secreted glycans and hyaluronan, are excreted and loosely associated to the layer. The glycocalyx is mostly known for its presence on the luminal wall of the endothelium, where it forms a thick polysaccharide cushion that serves to reduce shear stress between passing blood cells and the vessel wall [13]. A role of the glycocalyx in the physiology and pathophysiology of other organs has also been described: in the kidney it contributes to the filtering properties of the glomeruli [14] and in the gut it is a mediator of homeostasis of the gut flora [15, 16]. Variations in the molecular composition of the glycocalyx present on enterocytes may affect the gut microbiome as has been implicated by the recent reports on fucosyltransferase 2 in Crohn's disease [17]. A thick glycocalyx also plays a central role as a foundation for the mucous layer that stabilizes the tear film on the cornea, and may be deficient in patients with dry eye symptoms. Artificial tears containing hyaluronic acid, one of the structural components of the normal glycocalyx, may provide relief from these symptoms [18].

Fucosyltransferase 2 modifies the composition of the glycocalyx by mediating the inclusion of fucose in sugar moieties of glycoproteins and glycolipids. Intriguingly, recent genome-wide association studies identified inactivating variants at the fucosyltransferase 2 locus to be associated with primary sclerosing cholangitis [19]. These results are indirect evidence for a role of the glycocalyx in cholangiopathies as discussed earlier [20].

Speculating on a role for a glycocalyx on the luminal membrane of cholangiocytes, we recently reported that using specialized electron microscopy protocols a glycocalyx-like layer could consistently be visualized on these cells in normal mouse biliary tree tissue [8]. This prompted us to further investigate the properties of this biliary glycocalyx.

Tools to Investigate the Glycocalyx

Supplemental to electron microscopy and specific staining methods, quantitative and qualitative staining with lectins allows for the biochemical characterization of the glycocalyx layer. Lectins form a class of proteins that bind to specific carbohydrate groups on the cell membranes, and can be used to assess the composition of the glycocalyx [21]. Lectins are highly specific, e.g. the lectin *Sambucus nigra* agglutinin (SNA) binds to α_{2-6} -bound sialic acid, while *Maackia amurensis* agglutinin II (MAA) binds α_{2-3} -bound sialic acid. Cells stained with lectins can be analyzed, e.g. by

flow cytometry for quantitative analysis of percentage of positive cells and average intensity of lectin binding. As a result, a carbohydrate landscape can be identified, providing insight into the composition of the glycocalyx.

In addition to specific imaging and biochemical characterization of the biliary glycocalyx, we also experimented with functional analyses of its protective properties by established enzymatic modification of its composition [22]. In our current efforts, we focused on the use of sialidases, which cleave off the terminal, negatively charged sialic acid residues.

Key Messages

Human and Mouse Cholangiocytes Carry a Glycocalyx in vivo and in vitro

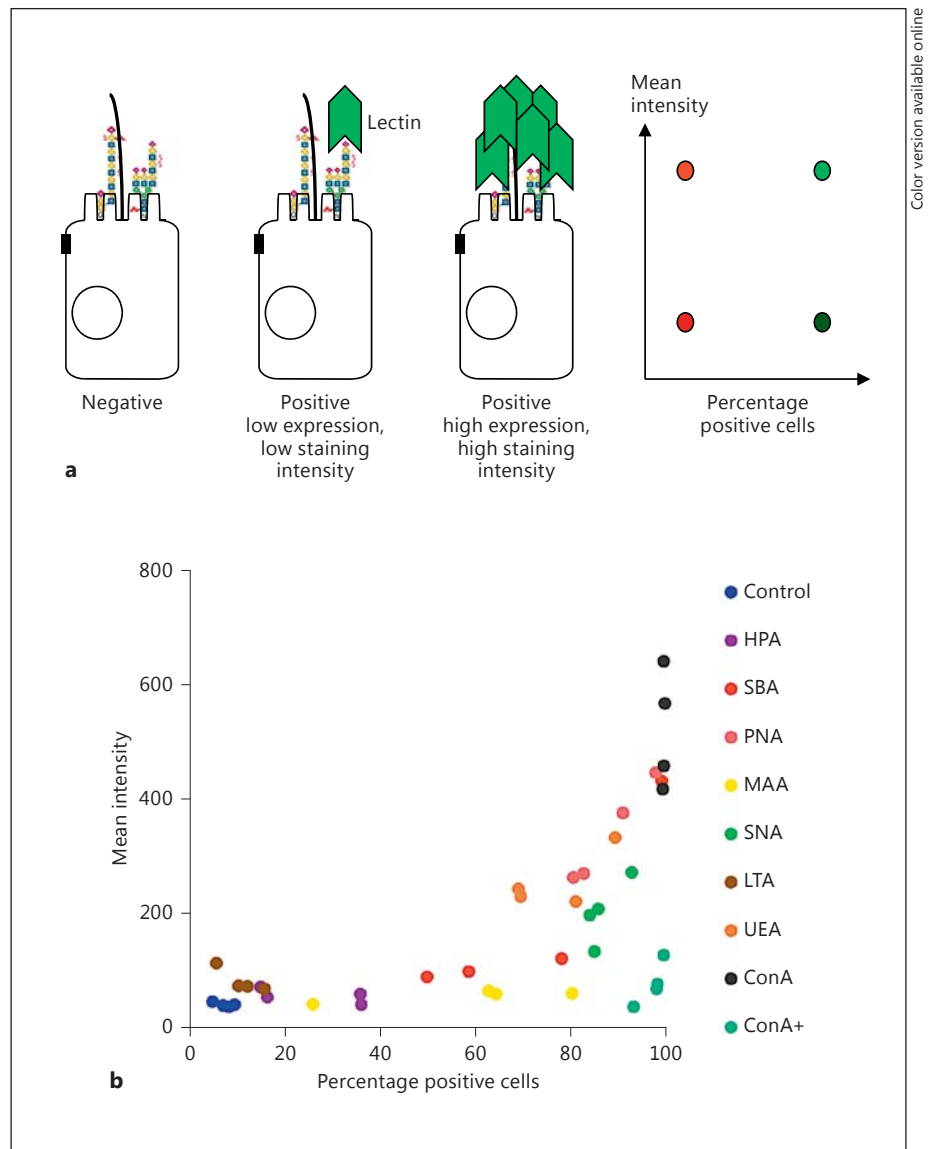
Tailored fixation and specific staining transmission electron microscopy on the biliary epithelium of both wild-type BL/6 mice and human samples obtained from liver biopsy showed the consistent presence of a 20- to 40-nm-thick juxtamembranous glycocalyx layer on the apical membrane of the biliary epithelium. Representative stainings of tissues from a BL/6 mouse (fig. 1a) and from a patient with a history of a primary biliary cirrhosis in a stable condition under ursodeoxycholic acid treatment (fig. 1b) are shown. We next used an established method to enhance glycocalyx visibility by staining with ruthenium red and identified the apical glycocalyx on cultured human immortalized cholangiocytes (fig. 1c).

Thus, both human and mouse cholangiocytes carry an apical glycocalyx, both in vivo and in vitro.

Sialic Acids Are Dominantly Present in the Biliary Glycocalyx

After verification of the presence of an apical glycocalyx on cholangiocytes, we biochemically characterized its composition by applying a preestablished lectin panel (fig. 2). Among O-linked glycans, human cholangiocytes predominantly expressed Core 1 (also known as T antigen), which is probably largely not sialylated as it is bound by peanut agglutinin. Its precursor Tn antigen was not found in significant amounts. The most abundant N-linked glycan was the mannose diantenna, which is recognized by ConA only in the absence of α -methylmannoside. We found the sialic acids on these glycans to be abundant, and mostly α_{2-6} linked instead of α_{2-3} linked (as revealed by more prominent binding of SNA than MAA).

Furthermore, we identified several more epitopes on these N-glycans. Normally, these could also be found on

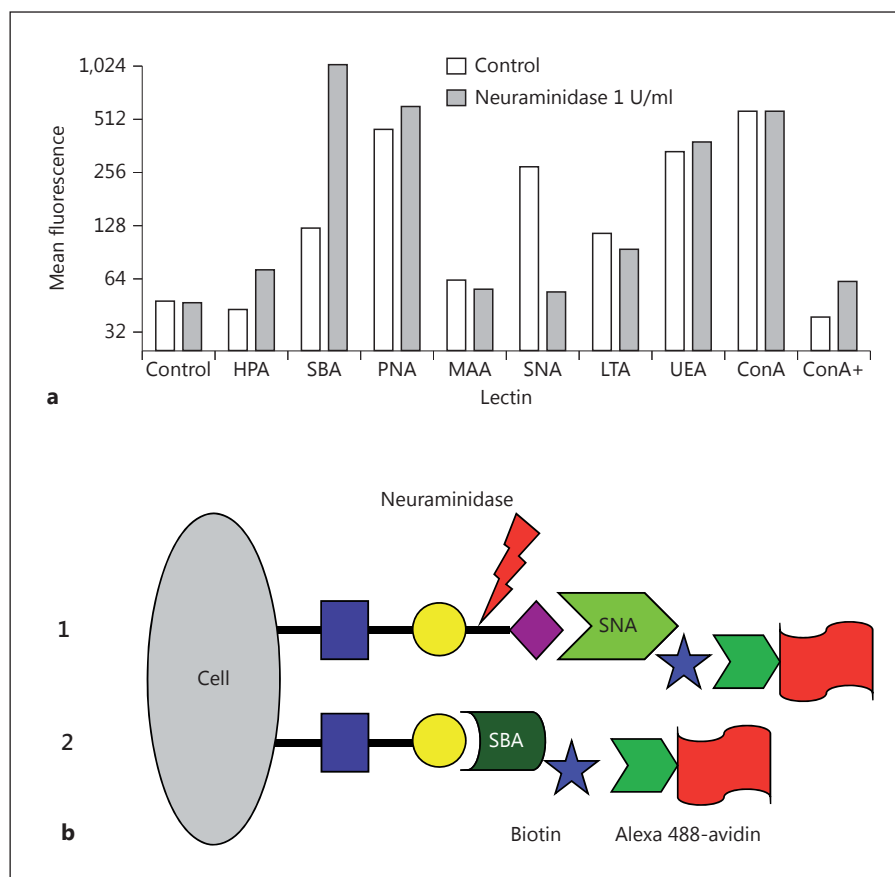


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Fig. 2. A lectin panel can be used to investigate the biliary glycocalyx. To assess the respective contributions of different glycans to the chemical composition of the biliary glycocalyx, we used a lectin panel as described in detail earlier [21]. In short, cells were brought into suspension in serum-containing RPMI medium after short trypsinization and were incubated with 5 µg/ml of biotinylated lectins MAA, SNA, and UEA-1 (Vector Laboratories, Burlingame, Calif., USA), ConA (*Canavalia ensiformis*), *Helix pomatia* agglutinin (HPA), peanut agglutinin (PNA) (*Arachis hypogaea*), soybean agglutinin (SBA) (*Glycin max*) and *L. tetragonolobus* agglutinin (LTA) (Sigma-Aldrich). Subsequent incubation with Alexa 488-conjugated streptavidin (Molecular Probes, Carlsbad, Calif., USA) allowed for quantitative detection applying the FACSCalibur system (BD Biosciences, Franklin Lakes, N.J., USA). For each

measurement the system was carefully calibrated using Alexa 488-conjugated streptavidin only to control for potential aspecific staining. **a** Diagram of flow cytometry-based lectin-binding assay, yielding information about the percentage of positive cells and about the mean intensity of staining. Data can be plotted on an xy axis. Each dot is the average of 5,000 analyzed cells. **b** Lectin landscape of cultured human immortalized cholangiocytes. The cells show a high expression of terminal sialic acid residues, detected by SNA (α₂₋₆-bound) and MAA (α₂₋₃-bound). Each dot shows the average staining intensity of 5,000 analyzed cells on the y-axis and the percentage of positively staining cells on the x-axis. For all lectins, measurements were repeated in four independent experiments as shown by dots in identical colors/shades.

Fig. 3. Sialic acids are an important constituent of the biliary glycocalyx and can be enzymatically removed using neuraminidase. To assess the efficacy of desialylation by neuraminidase, cells were quickly trypsinated after treatment with neuraminidase, as described above, and brought into suspension in serum-containing RPMI medium. Effects on glycocalyx composition were assessed by FACS, applying the lectin panel as described in figure 2. **a** Treatment with neuraminidase results in a strong decrease in exposition of terminal sialic acids as represented by a strong reduction in SNA (α_{2-6} -bound sialic acids) and a smaller reduction in MAA (α_{2-3} -bound sialic acids). Desialylation leads to an increase of binding of SBA lectin (specifically binding N-acetylgalactosamine), most likely as the residues bound by SBA are ‘opened up’ after removal of the sialic acid groups. Each bar indicates the mean fluorescence intensity of 5,000 analyzed cells. **b** Diagram of the detection of N-acetylgalactosamine by SBA lectin after neuraminidase-mediated desialylation.



O-glycans, but given that these O-linked glycans apparently are rather short (as represented by a high intensity of peanut agglutinin staining) it is likely that these epitopes are mostly linked to N-glycans. We found a high expression of α_{1-2} fucose, also known as the H-antigen. This epitope, recognized by UEA-1, is expressed at significant quantities on the endothelium. The Lewis epitope, recognized by *Lotus tetragonolobus* agglutinin, was barely identified. Sialylated LacdiNAc also appears to be present.

In conclusion, we established that lectins can be used to characterize the composition of the glycocalyx of biliary epithelial cells and showed, amongst other things, the presence of substantial amounts of terminal sialic acids on its glycocalyx.

Alteration of Sialylation Status Can Be Reproducibly Achieved Enzymatically in vitro

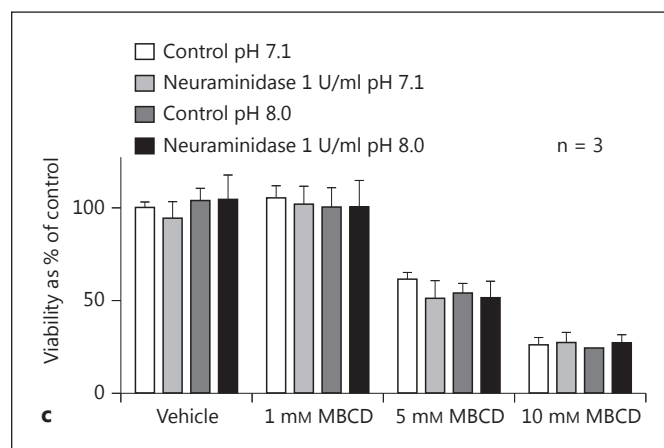
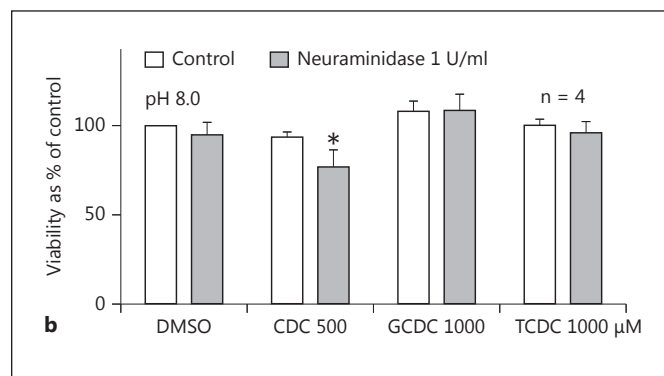
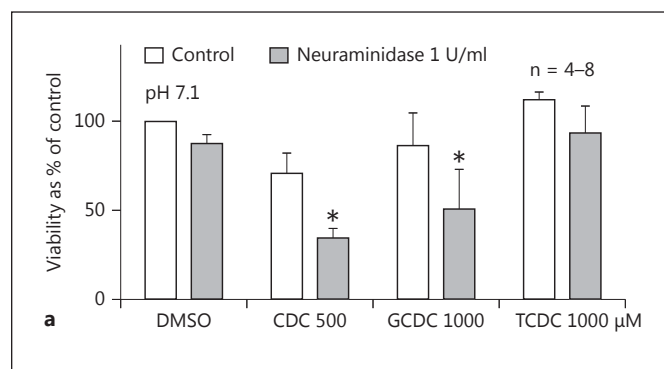
Next, we investigated the feasibility to enzymatically alter the composition of the biliary glycocalyx. Given the observed dominance of sialic acids as terminal sugars on the glycans, neuraminidase was applied, an en-

zyme known to cleave sialic acid groups off apical glycans. Treatment of cultured H69 cells, an immortalized human cholangiocyte cell line, with the well-characterized enzyme neuraminidase caused a strong desialylation, as documented by flow cytometry (fig. 3a), without significantly affecting H69 viability. Most likely due to ‘opening up’ of the sugar residues normally covered by terminal sialic acids (fig. 3b), we observed a strong increase in binding of soybean agglutinin (SBA) lectin upon desialylation. Thus, application of neuraminidase was efficient in cleaving off sialic acid residues in the periphery of the glycocalyx without inflicting serious toxicity.

Negatively Charged Sialic Acids in the Glycocalyx May Stabilize the ‘Biliary HCO₃⁻ Umbrella’ That Protects against Apical Protonated Bile Salt Monomers

After showing that enzymatic desialylation using neuraminidase was feasible in vitro, we compared the toxicity of bile salts on cultured H69 cells pretreated with neuraminidase or vehicle control. We hypothesized that

Fig. 4. Desialylation reduces H69 resistance to toxicity induced by bile acids as measured by WST-1 viability assays. Confluent native H69 cells were cultured in the presence of dimethyl sulfoxide (DMSO, control), CDC, GCDC, taurochenodeoxycholate (TCDC) or etoposide at indicated concentrations for 4 h at a pH of 8.0, 7.4, 7.1, 6.7 or 6.4 (20 mM HEPES). To study the role of intact glycocalyx, native confluent H69 cells were washed twice with HBSS and incubated with neuraminidase (1 U/ml) in serum-free medium (pH 6.7) for 2.5 h. Subsequently, medium was replaced by full culture medium and cells were either allowed reconstitution for 24 h or immediately treated with bile salts (CDC, GCDC, TCDC) or DMSO at a pH of 7.1 or 8.0 (20 mM HEPES) and incubated for 18 h. 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) following the manufacturer's instructions. All results are expressed as means \pm SD of at least three independent experiments. Data were analyzed in GraphPad Prism (GraphPad Software, San Diego, Calif., USA). Data were not normally distributed, and a Mann-Whitney or Kruskal-Wallis test was applied as indicated and appropriate. A level of $p < 0.05$ was considered statistically significant. **a** Desialylation significantly increases susceptibility to toxicity of protonated bile salts (* $p < 0.05$ vs. control, Kruskal-Wallis; adapted from Hohenester et al. [10]). **b** The enhanced toxicity can be nearly fully reversed by increasing the pH to 8.0 (* $p < 0.05$ vs. control, Kruskal-Wallis; adapted from Hohenester et al. [10]). **c** Sialylation status does not affect toxicity by methyl- β -cyclodextrin (MBCD). MBCD toxicity is also not modulated by pH. Values are expressed as a percentage of DMSO control (bar not shown).



the terminal sialic acids would add to the barrier function of the glycocalyx. Sialic acid residues in glycans are known to stabilize molecules and membranes, and to modulate their interactions with the environment. Their comparatively strong electronegativity stabilizes the viscosity of mucins on the glycocalyx and may regulate the access of molecules to the glycans or underlying membrane molecules such as receptors and transporters. We thus investigated if desialylation would lead to an enhanced toxic effect of luminal bile salts.

As shown in figure 4a, desialylation increased the susceptibility to CDC and GCDC, which are mainly unprotonated at the experimental pH. In contrast, desialylation did not significantly affect the toxicity caused by TCDC, which at a pH of 7.1 is nearly fully present in its unprotonated form due to its low pK_a . At an experimental pH of 8.0, at which also the majority of CDC molecules is expected to be present in its unprotonated form, desialylation no longer caused a significant increase in H69 toxicity. In a control experiment we showed that si-

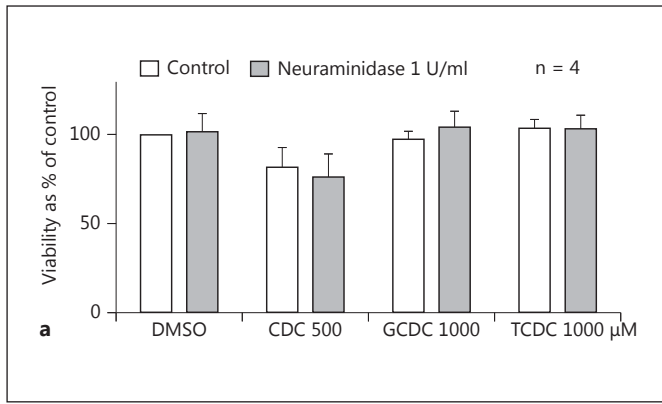
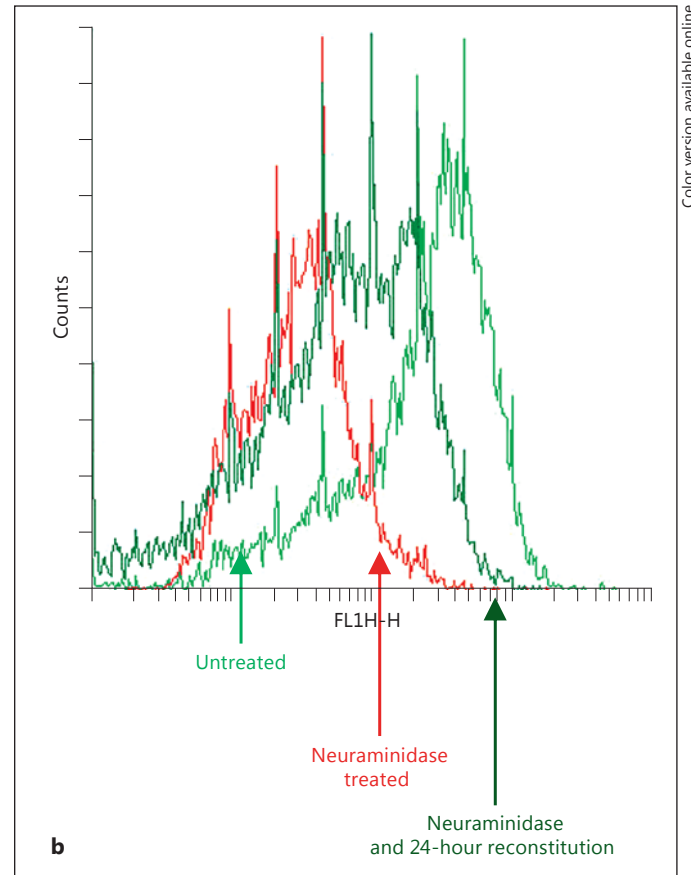


Fig. 5. Enhanced bile acid susceptibility by enzymatic desialylation can be reversed by reconstitution of the biliary glycocalyx. **a** An 18-hour reconstitution of the glycocalyx after desialylation reverses the enhanced toxicity of bile acids upon desialylation. **b** Reconstitution of the glycocalyx after desialylation by neuraminidase, shown on a flow cytometry plot depicting SNA lectin binding. Data of 5,000 analyzed cells.



alation status does not affect the toxicity of methyl- β -cyclodextrin, a known disruptor of biological membranes due to its ability to extract cholesterol molecules from the lipid bilayer (fig. 4c). Desialylation thus increased H69 susceptibility to bile salt toxicity in a pH-dependent, specific fashion. This supports the hypothesis that the glycocalyx stabilizes a local HCO_3^- -rich milieu at the apical membrane of the cholangiocyte.

The Cholangiellular Apical Glycocalyx Is Rapidly Reconstituted and Regains Its Protective Properties following Artificial Desialylation

To test the reversibility of the desialylation caused by neuraminidase treatment, H69 cells were allowed to reconstitute their glycocalyx before they were used in bile salt toxicity assays. An 18-hour incubation in normal growth medium after neuraminidase treatment led to a nearly full recovery of the amount of terminal sialic acids on H69 cells as measured by FACS (fig. 5b). In addition, after this reconstitution period, the neuraminidase-treated cells were no longer more susceptible to bile acid-in-

duced toxicity as compared to cells treated with vehicle control (fig. 5a). Insults to the glycocalyx composition can thus be overcome by the epithelium by reconstitution of its original glycan composition.

Conclusions

In chronic cholangiopathies such as primary biliary cirrhosis, the contribution of the pathogenetic factors that have been identified so far remains controversial. While rarefaction of bile ducts is a histopathologic hallmark of the disease, it is under debate whether cholangiellular damage is the cause or consequence of the autoimmune attack towards bile ducts [2]. However, there is increasing evidence that biliary damage triggers autoimmunity in this disease [23, 24]. It therefore seems crucial to better understand and characterize cholangiellular pathophysiology in order to further unravel the pathogenesis of chronic cholangiopathies.

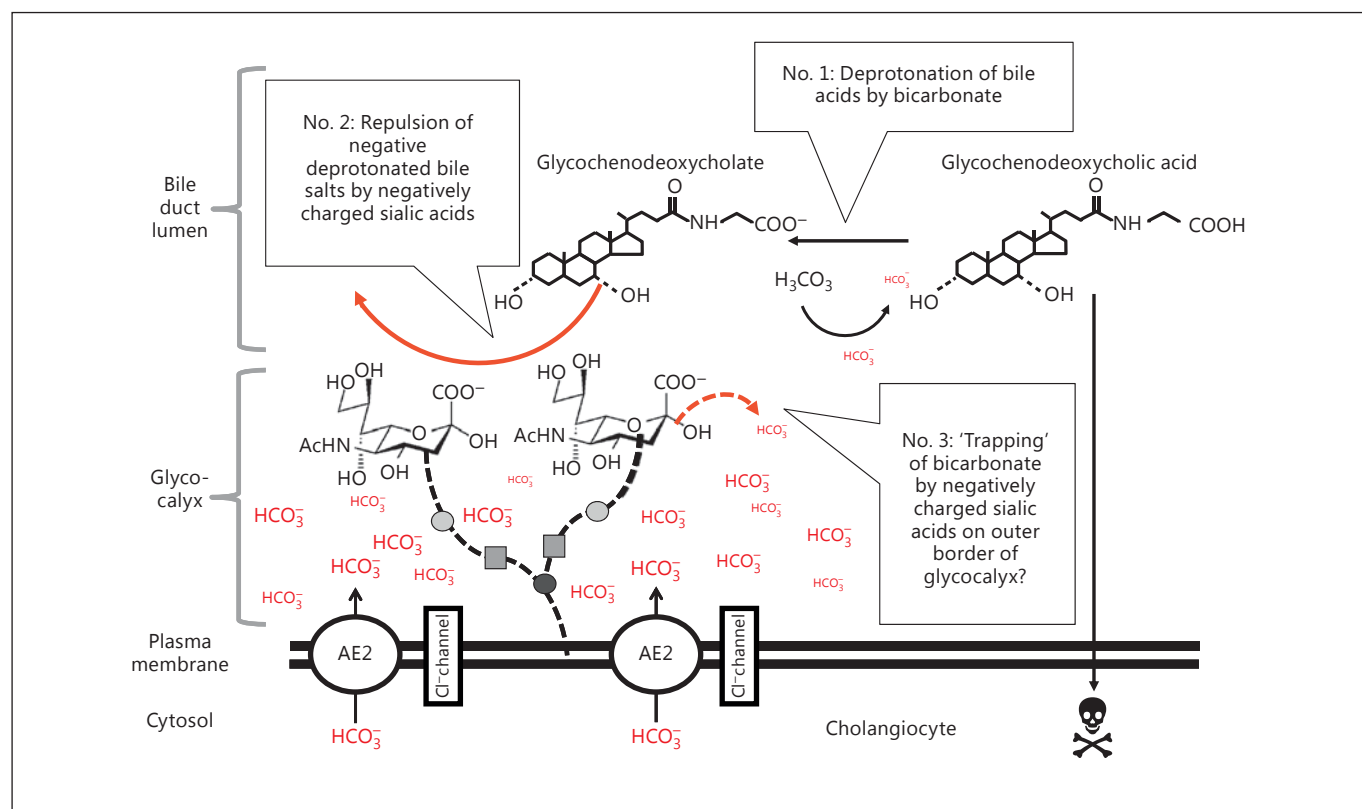


Fig. 6. Proposed model of the biliary glycocalyx supporting the biliary bicarbonate umbrella. Electrochemical repulsion by electronegative charges, shown in red in the figure (color in online version only), may both prohibit access of bile salts to the membrane and could 'trap' secreted HCO_3^- near the plasma membrane. No. 1: cholangiocytes prevent the uptake of potentially harmful bile salts from the bile duct lumen by secreting large amounts of HCO_3^- , which shifts the balance in the acid-base reaction of bile acids towards their salt form. As shown in No. 2, the biliary glycocalyx forms a physical barrier that limits the access of the deprotonated bile salts to the plasma membrane, in which the electro-

negative charge of the sialic acids plays a crucial role. Lastly in No. 3, we speculate that the relative negativity of the outer part of the glycocalyx, due to the high amounts of terminal sialic acids, may form a repulsive barrier to HCO_3^- secreted by the cholangiocyte, effectively 'trapping' HCO_3^- within the glycocalyx which causes a higher local pH near the cell membrane. This more alkaline pH will lead to rapid deprotonation of bile acids that despite other protective mechanisms did manage to get close to the plasma membrane, thus prohibiting them from easily transferring to the cytosol. AE2 = Anion exchanger 2.

We have recently proposed that the striking resistance of the biliary epithelium against the toxic bile salts that physiologically are present in bile at high, millimolar concentrations at least partly can be explained by the presence of a 'biliary HCO_3^- umbrella' [8]. This 'biliary HCO_3^- umbrella' alkalizes the bile close to the apical membrane, fosters the deprotonation of apolar bile acids to charged bile salts and thus prevents their uncontrolled cell entry and toxicity [10]. Genetic defects causing malfunctioning of this HCO_3^- -rich protective layer may predispose individuals to develop bile salt-induced cholangiocyte toxicity. In case of cell necrosis, apoptosis or senescence, this may cause autoantigens to become available to the im-

mune system, thus providing a possible starting point for biliary autoimmune disease.

Here, we reviewed the current evidence supporting the hypothesis that a cholangiocellular glycocalyx, expressed on the apical membrane of biliary epithelial cells, is another protective mechanisms and that it might act, in part, via physical stabilization of the 'biliary HCO_3^- umbrella'.

To the best of our knowledge, we are the first to visually and biochemically describe the presence and function of a glycocalyx layer on the apical membrane of cholangiocytes [8, 10]. By specific staining and electron microscopy, we identified a glycocalyx layer amounting to ~20–

40 nm on the apical membrane of cholangiocytes. This was confirmed on both liver tissues of mouse and man and on cultured immortalized human cholangiocytes. This layer of ~20–40 nm should suffice to allow for the generation of a local, pericellular microenvironment that may differ greatly from the milieu in bulk luminal bile, e.g. by retaining secreted compounds.

Thus, the pericellular pH may be alkalized by stabilization of the 'biliary HCO_3^- umbrella', i.e. physically preventing secreted HCO_3^- to be flushed away by bulk luminal bile. Although the production of HCO_3^- by biliary epithelial cells may effectively alkalize the pH in the bile duct lumen, altering the pericholangiocyte milieu would be much more efficient when stabilized by a glycocalyx. In addition to such physical protection, the positive charge of sugar moieties in the inner part of the glycocalyx might help in retaining negatively charged HCO_3^- (fig. 6). In concert, the cholangiocyte glycocalyx may stabilize a juxtacellular alkaline layer in a similar fashion as has been described for the stomach epithelium [12].

The glycan composition of the biliary glycocalyx as defined using lectin-binding assays identified abundant sialic acid residues among the most terminal components of the glycocalyx. The functional interpretation of this finding could be that the negative charge of this outer part of the glycocalyx may form a repulsive barrier to unprotonated bile salts, which equally carry a negative load, and minimize their pericellular concentration.

This notion is supported by the experiments that showed a physiologic function of the cholangiocyte glycocalyx in preventing bile salt-induced toxicity. As expected, at basal conditions human cholangiocytes were resistant to cellular damage inflicted by GCDC, which is the most abundant hydrophobic, toxic bile salt in human bile. After desialylation of the glycocalyx by neuraminidase treatment, GCDC ($\text{pK}_a > 4$) toxicity was exacerbated about 4-fold as shown by viability assays. CDC ($\text{pK}_a > 4$)

but not TCDC ($\text{pK}_a < 2$) toxicity was also significantly increased by neuraminidase pretreatment, which supports our hypothesis that the glycocalyx specifically adds to the barrier function of the 'biliary HCO_3^- umbrella' against protonated bile acids. TCDC has a similar hydrophobicity as GCDC but, with a lower pK_a value, it is fully deprotonated and thus electrically charged at physiologic pH. This prevents its passive cell entry almost independently of local pH. These findings are in line with earlier reports showing that the transgression of charged particles is modulated by the glycocalyx on the endothelial wall [14, 25].

In summary, we brought together all published and thus far unpublished evidence that a glycocalyx is actively maintained on the apical side of cholangiocytes. This biliary glycocalyx with glycosylated mucins and other glycan-bearing membrane proteins may physically and chemically stabilize the 'biliary HCO_3^- umbrella'. Biochemical analysis of the composition of the glycocalyx identified negatively charged sialic acid residues that dominate the outer branches of the glycocalyx tree. This may add another protective effect against bile salt-induced cell injury by repelling charged bile salts from the pericellular space, minimizing their effective concentration at the biliary epithelium.

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Disclosure Statement

The authors report no conflicts of interest.

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