Research Article

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Actomyosin contractility drives bile regurgitation as an early response during obstructive cholestasis

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Background & Aims: A wide range of liver diseases manifest as biliary obstruction, or cholestasis. However, the sequence of molecular events triggered as part of the early hepatocellular homeostatic response in obstructive cholestasis is poorly elucidated. Pericanalicular actin is known to accumulate during obstructive cholestasis. Therefore, we hypothesized that the pericanalicular actin cortex undergoes significant remodeling as a regulatory response to obstructive cholestasis.

Methods: *In vivo* investigations were performed in a bile duct-ligated mouse model. Actomyosin contractility was assessed using sandwich-cultured rat hepatocytes transfected with various fluorescently labeled proteins and pharmacological inhibitors of actomyosin contractility.

Results: Actomyosin contractility induces transient deformations along the canalicular membrane, a process we have termed inward blebbing. We show that these membrane intrusions are initiated by local ruptures in the pericanalicular actin cortex; and they typically retract following repair by actin polymerization and actomyosin contraction. However, above a certain osmotic pressure threshold, these inward blebs pinch away from the canalicular membrane into the hepatocyte cytoplasm as large vesicles (2–8 μm). Importantly, we show that these vesicles aid in the regurgitation of bile from the bile canaliculi.

Conclusion: Actomyosin contractility induces the formation of bile-regurgitative vesicles, thus serving as an early homeostatic mechanism against increased biliary pressure during cholestasis.

Lay summary: Bile canaliculi expand and contract in response to the amount of secreted bile, and resistance from the surrounding actin bundles. Further expansion due to bile duct blockade leads to the formation of inward blebs, which carry away excess bile to prevent bile build up in the canaliculi.

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Introduction

The biliary function of the liver is critical for survival, serving to eliminate toxic endo- and xenobiotics, cholesterol, and inflammatory mediators [1]. The apical membranes of adjacent hepatocytes form the bile canalicular lumen, an intercellular structure surrounded by a dynamic pericanalicular actin cortex (PAC), which actively contracts to propel secreted biliary fluid towards the bile ducts [2,3]. A variety of liver diseases result in impaired bile flow, or obstructive cholestasis [4–7]. These include extrahepatic etiologies such as biliary strictures, stones and biliary atresia in infants; as well as intrahepatic causes that include primary biliary cirrhosis, vanishing duct syndrome, and alcoholic and viral hepatitis. Bile stasis and backpressure increases liver and serum bile acid levels, resulting in liver toxicity and fibrosis, which may eventually progress to decompensated cirrhosis, mandating the need for a liver transplant [7].

Several pathological changes that are consequent to increased biliary pressure have been identified, including changes in transporter expression, which reduce the uptake and increase the basolateral export of bile acids [6], and the accumulation of actin along

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the canalicular membrane [8,9]. Indeed, the thickening of the PAC observed in common bile duct ligation (BDL) rodent models has also been observed in patients with biliary atresia [10]. However, these reports have largely been correlative; little is known about the exact sequence of events that is triggered to effect homeostatic regulation of abnormal elevations in biliary pressure.

More than 40 years ago, Matter and colleagues reported the presence of vacuoles containing horseradish peroxidase (HRP) in the hepatocyte cytoplasm upon retrograde injection of HRP through the common bile duct [11]. Though not proven, these results led to a proposed model whereby these vacuoles were part of a diacytotic-based process of bile regurgitation that enable the transport of bile from the canalicular to the sinusoidal surface during increased biliary pressure. In another study, Watanabe and colleagues monitored the process of bile regurgitation from the canalicular space through the hepatocyte cytoplasm into the sinusoids following common BDL, thus establishing the transcellular pathway as the main homeostatic mechanism that protects hepatocytes from bile toxicity during increased biliary pressure [12]. Together, these two studies implicate the transcellular transport of bile as an immediate homeostatic mechanism triggered by increased biliary pressure. However, the precise molecular machinery and sequential events underlying this phenomenon is still poorly understood.

Approaching obstructive cholestasis as a disease of aberrant cellular mechanics, the known motility of the canalicular network and reported involvement of the PAC in bile flow and bile stasis suggest that cytoskeletal changes in the PAC may be involved as an early homeostatic mechanism to counteract elevations in biliary pressure; the failure of which then results in further adaptive changes [3]. In this study, we detailed in real-time the sequence of events that occur immediately following induced elevations in biliary pressure, and demonstrated the role of actomyosin contractility in facilitating bile regurgitation. A mechanistic understanding of the early homeostatic response triggered to relieve intracanalicular pressure (ICP) may lead to the identification of therapeutic targets to prevent or treat obstructive cholestasis.

Materials and methods

Maintenance and in vivo imaging of LifeAct mice

All animal experiments were approved and in accordance to the guidelines by the Institutional Animal Care and Use Committee of the Agency for Science, Technology and Research (A*STAR) in Singapore. Transgenic LifeAct-green fluorescent protein (GFP) mice [13] (20 weeks old, average body weight of 28 g) were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). An intravital imaging window chamber was mounted on the abdomen as previously described [14]. Intravital imaging was performed with a titanium-sapphire laser (Tsunami, SpectraPhysics, Mountain View, California) with a 488 nm output. The laser was scanned using a x-y mirror scanning system (Model 6220, Cambridge Technology, Cambridge, Massachusetts) and guided towards the modified inverted microscope. A high-power objective (Plan Fluor ELWD water 40x, NA 0.45, Nikon) was used. After passing through the primary dichroic mirror, the GFP fluorescence signal was detected with a 488 nm bandwidth using additional band pass filters (HQ590/80, ChromaTechnology). Each optical scan is composed of 512 by 512 pixels and took approximately 1 s to complete.

Bile duct ligation

Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The common bile duct was ligated using double surgical knots below the bifurcation and one single knot above the pancreas. Following ligation, the window chamber was attached to the animal and imaging was performed 1 h after the procedure as described above. Three mice from each of the control and bile duct-ligated groups were imaged in this study.

Isolation and sandwich culture of hepatocytes

Hepatocytes were isolated from male Wistar rats using a previously described two-step *in situ* collagenase perfusion method.[15] We used rat hepatocytes instead of mouse hepatocytes in all *in vitro* studies as the transfection efficiency in rat hepatocytes is higher than that in mouse hepatocytes. Transfection of primary hepatocytes *in vitro* is challenging. In our hands, the transfection efficiency for mouse hepatocytes was only <1%, whereas we were able to get transfection efficiencies of >5% in rat hepatocytes. Transfection efficiency for mouse hepatocytes is poorer than rat hepatocytes because the yield of healthy mouse hepatocytes is generally much lower than that of rat hepatocytes. Transfection efficiency was particularly important in this study to enable the spatiotemporal study of multiple proteins. Isolated hepatocytes were cultured in collagen sandwich configuration. Hepatocytes were either transfected with various fluorescently-tagged proteins or were exposed to different concentration of blebbistatin, cytochalasin D or ursodeoxycholic acid (UDCA) to alter canalicular dynamics. For investigations of the role of bile canaliculi-derived vesicles (BCV), hepatocytes were exposed to cholyl-lysyl-fluorescein (CLF). Details of hepatocyte isolation, culture and treatment of hepatocytes are discussed in the Supplementary material.

Statistical analysis

Data values in this report are presented as average ± standard error of the mean (SEM). One-way ANOVA, Student's *t* test and sign test were used to analyze significance. Refer to the Supplementary material and CTAT table for additional details.

Results

Observation of inward bleb formation in normal bile canaliculi (BC) in vivo

Using intravital microscopy (Fig. 1A–C) to image the LifeAct probe of filamentous (f)-actin in the mouse liver (Figs. 1–4), we imaged the liver surface (2–3 cell layers in depth). Besides the epithelium and sinusoidal network, we were also able to identify the BC network which had a greater intensity as actin is highly expressed at the apical surface of hepatocytes [16]. As the actin in the BC network gave a much stronger signal than the surrounding tissue, we segmented the BC using simple threshold (Fig. S1). By visual inspection, we observed that the average diameter of BC in the control group was 1.48 ± 0.14 μm ($n = 20$), excluding the possibility that these structures were bile ducts, which are known to have diameters greater than 10 μm (Fig. 1D and E) [17–19]. Based on our knowledge, this study demonstrates for the first time the ability to live image the BC network with such resolution. With the ability to study the *in vivo* BC network in real-time, we first sought to characterize the periodic cycles of bile canalicular expansion and contraction. In agreement with previous literature [3,20–22], BC were observed to be highly dynamic structures. However, beyond the previously described global motility of BC, we discovered that the bile canalicular surface is also remarkably dynamic on the local scale. During the BC expansion phase, we observed the formation of membrane herniations (1–2 μm in diameter with lifetime of approximately 2 min) which occur randomly and dynamically on the bile canalicular surface. These bleb-like structures intrude inwardly into the hepatocyte cytoplasm and then

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