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Depletion of Apoptosis Signal-Regulating Kinase 1 Prevents Bile Duct Ligation—Induced Necroinflammation and Subsequent Peribiliary Fibrosis

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Apoptosis signal-regulating kinase 1 (ASK1), also known as mitogen-activated protein kinase kinase kinase (MAP3K), is ubiquitously expressed and situated in an important upstream position of many signal transduction pathways. ASK1 plays a pivotal role in stressor-induced cell survival and inflammatory reactions. To ascertain the regulatory functions of ASK1 in bile duct ligation (BDL)—induced liver injury, we examined the net effects of ASK1 depletion on hepatic necroinflammation and/or fibrosis. We subjected C57BL/6 wild-type (WT) or *ASK1*-deficient (*ASK1*$^{-/-}$) mice to sham or BDL surgery for 14 days. In day 3 BDL animals, *ASK1*$^{-/-}$ mice had significantly fewer bile infarcts along with more reduced interlobular or portal inflammatory infiltrate of various immune cells, including neutrophils, compared with WT mice in which ASK1 expression was markedly activated. Morphologically apoptotic hepatocytes or cholangiocytes were negligible in both the sham and BDL animals. In contrast, *ASK1*$^{-/-}$ mice had significantly less proliferating activity of not only hepatocytes but also large cholangiocytes than WT mice. Day 14 BDL *ASK1*$^{-/-}$ mice manifested potential antifibrogenic aspects of *ASK1* deficiency, characterized by significantly fewer activated peribiliary fibrogenic cells and peribiliary fibrosis. These observations indicate that ASK1-mediated hepatic necroinflammation and proliferation, but not apoptosis, are closely linked to liver fibrosis and fibrogenesis. A specific ASK1 pathway blocker or inhibitor might offer a therapeutic strategy against human cholestatic diseases. *(Am J Pathol 2014, 184: 644—661; http://dx.doi.org/10.1016/j.ajpath.2013.11.030)*

It was previously proposed that hepatocytes undergoing apoptosis might provide a critical hit to drive progression from chronic inflammation to cirrhosis, especially in cholestatic injury, such as primary biliary cirrhosis, primary sclerosing cholangitis, biliary atresia, or chronic cholelithiasis, with reference to a single laboratory.[1—4] In striking contrast, several other groups[5—9] have recently suggested that there is no evidence of hepatocellular apoptosis using morphologic criteria, but not terminal deoxynucleotidyl transferase end-labeling (TUNEL) staining, in or around necrotic foci (ie, bile infarcts) after bile duct ligation (BDL), which is used to induce cholestasis in rodents.[1—3,5—9] They also concluded that BDL-induced oncotic necrosis but not apoptosis of hepatocytes is closely correlated with the severity of the inflammatory response, including high chemokine and/or cytokine expression.[6,8,9] Meanwhile, although cholangiocytes are a minor component of liver cells, comprising merely 3% to 4% of the rodent liver, cells lining the large bile ducts (large cholangiocytes) are the

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main target in animal models of cholestasis and intractable human cholestatic diseases.[10,11] Previous studies revealed that rodent cholangiocytes are mitotically dormant under basal conditions, whereas large but not small cholangiocytes undergo significant proliferation, leading to subsequent liver fibrosis after BDL injury, even though morphologic or functional heterogeneity is noted in large versus small cholangiocytes.[12–14] Nevertheless, the regulation of biliary injury and proliferation during cholestasis is multifactorial and complex, and the various roles of large cholangiocytes in the initiation and progression of cholestatic liver diseases are still debatable.[12,14] Hence, key factors and signaling pathways that control the responses to injury in not only hepatocytes but also cholangiocytes need to be identified.

Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase kinase kinase (MAP3K) family member that is activated through distinct mechanisms in response to various cytotoxic stressors, including oxidative stress mediated by hydrogen peroxide, endoplasmic reticulum (ER) stress, and immune system mediators, such as tumor necrosis factor (TNF)-α, IL-1β, or Fas ligands.[15,16] ASK1 is situated in an important upstream position for many signal transduction pathways, such as the c-Jun N-terminal kinase (JNK) and p38 MAP kinase (MAPK), which subsequently induce inflammation and intrinsic apoptotic signaling through mitochondria-dependent caspase activation.[15–17] Previous reports using *ASK1*$^{-/-}$ mice or *ASK1* overexpressing transgenic mice confirmed that ASK1 plays a pivotal role in the regulation of cardiomyocyte apoptosis in ischemia-reperfusion injury models.[18,19] Moreover, we recently found that activation of ASK1 signaling enhances hyperlipidemia-induced necrotic lipid core formation by inducing macrophage apoptosis and accelerates mechanical injury–induced vascular remodeling via increased neovascularization and proinflammatory reaction and by stimulating apoptosis of smooth muscle cells and/or endothelial cells.[17,20] ASK1 plays a significant role in the regulation of vascular cell apoptosis and inflammatory signaling *in vivo* and is correlated with plaque vulnerability in atherosclerosis.[20] Other studies report that ASK1 expression plays a critical role *in vivo* in the regulation of apoptosis in type 2 pneumocytes in lung injury, with both antiapoptotic and anti-inflammatory properties.[21] However, few studies have investigated the relationship between ASK1 signaling pathway and cholestasis-induced injury, even though ASK1 is ubiquitously expressed.

In the current study, we examined the roles of ASK1 in BDL-induced cholestatic liver injury using mice genetically deficient for *ASK1* (*ASK1*$^{-/-}$). Furthermore, we aimed to determine the net effects and key factors of ASK1 in liver necroinflammation or subsequent fibrosis.

Materials and Methods

Animals and BDL Model

Experiments were performed using 6- to 8-week-old, male, C57BL/6 wild-type (WT) and *ASK1*$^{-/-}$ mice,[17,20] weighing 18 to 22 g, maintained in a temperature and light-controlled facility with free access to standard rodent chow diet and water. *ASK1*$^{-/-}$ mice were developed on a C57BL/6 background.[17,20] To produce a ligation-induced cholestatic liver injury (BDL) model, the peritoneal cavity was opened after a midline upper-abdominal incision, and the common bile duct was double-ligated with sterile surgical 7-0 silk sutures (Alfresa Pharma Corp., Tokyo, Japan) and cut between the ligatures in two groups of mice at 6 to 8 weeks of age under anesthesia [intraperitoneal injection of ketamine (100 mg/kg) (Daiichi Sankyo Co., Tokyo, Japan) and medetamidine (2 mg/kg) (Meiji Yakuhin Co., Tokyo, Japan)], as previously described.[20] Sham-operated mice, as controls, underwent laparotomy with exposure but no ligation of the common bile duct. The fascia and skin of the midline abdominal incision were closed with sterile surgical 6-0 silk sutures (Alfresa Pharma Corp.). After a defined period of BDL or sham operation at 3 or 14 days, animals were euthanized by exsanguination under reanesthetization with an i.p. injection of ketamine-medetamidine, as follows: the peritoneal cavity was reopened, and blood samples were taken from the inferior vena cava, followed by immediate cannulation of the suprahepatic vena cava. In all animals, after blood was flushed out of the liver via the suprahepatic vena cava catheter, livers were excised and cut into small pieces and used for various experiments as described below. The 24-hour urine of day 3 BDL mice was collected using mouse metabolic cages (Sugiyama-Gen Co., Ltd., Tokyo, Japan).

The Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health (Japan), approved the protocols. They were performed according to the Institutional Guidelines for Animal Experiments and the Law (no. 105) and Notification (no. 6) of the Japanese government. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.

Histopathology

Liver specimens were stained with H&E, Masson's trichrome, or picrosirius red stain, or immunohistochemistry (IHC) preparations in sequential sections, after fixation in 10% neutral buffered formalin for 24 hours.[17,20,22–25] Analyses were performed in BDL-induced cholestatic livers in all experiments, whereas the sham-operated livers served as controls.

Livers embedded in paraffin for histologic examination were cut systematically in sequential sections of 4-μm thickness using a sliding microtome (Leica SM2010R; Leica Microsystems, Wetzler, Germany). For histologic analyses of the liver, images of H&E and special stained sections or IHC sections were captured and quantified using NanoZoomer Digital Pathology Virtual Slide Viewer software version 2.0 (Hamamatsu Photonics Corp., Hamamatsu, Japan). H&E-stained liver sections were used to measure the areas of bile infarcts (hepatic necrosis) and calculate the