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Review

Critical and diverse *in vivo* roles of apoptosis signal-regulating kinase 1 in animal models of atherosclerosis and cholestatic liver injury

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Summary. Apoptosis plays pivotal *in vivo* roles in not only vital processes, such as cell turnover and embryonic development, but also various inflammatory disorders. However, the role of apoptosis by vascular and hepatic cells in the respective progression of atherosclerosis and liver injury remains controversial. Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase kinase kinase family member that is activated through distinct mechanisms in response to various cytotoxic stressors. ASK1, ubiquitously expressed, is situated in an important upstream position for many signal transduction pathways, which subsequently induce inflammation and/or apoptosis. Our serial *in vivo* studies have uniquely reported that the expression of phosphorylated ASK1 is variably seen in atherosclerotic lesions or bile-duct-ligation (BDL)-induced injury livers. In mice genetically deficient of *ASK1* (*ASK1*$^{-/-}$), activated ASK1 signaling accelerates high-cholesterol-diet-induced necrotic lipid core formation by inducing macrophage apoptosis and enhances ligation injury-induced vascular remodeling via pro-inflammatory reactions and by stimulating apoptosis of smooth muscle cells. In contrast, in models of BDL-induced cholestatic liver injury, the pathogenic roles of ASK1-mediated early necro-inflammation, but not apoptosis, and the proliferation of hepatocytes and cholangiocytes are crucial in subsequent peribiliary fibrosis/fibrogenesis. These animal models of acute to chronic inflammatory diseases show that stimulated ASK1 signaling critically and diversely regulates not only hypercholesterolemia-induced atherosclerosis and injury-induced arteriosclerosis, but also the acute and subacute-to-chronic phase of BDL-induced cholestasis. We herein review the diverse, key *in vivo* roles of ASK1 signaling in the pathogenesis of inflammatory disorders closely related to metabolic syndrome.

Key words: ASK1, Animal model, Atherosclerosis, Bile duct ligation (BDL), Cholestasis

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Introduction

Apoptotic cell death is considered not only a vital component of various processes, including normal cell turnover, immune system functioning and embryonic development, but also a critical factor in many human detrimental conditions, such as inflammatory and/or autoimmune disorders, neurodegenerative diseases and many types of cancer (Elmore, 2007). However, the i*n vivo* roles of the apoptosis of vascular and hepatic cells in the respective progression of atherosclerosis and cholestatic liver injury, representing the majority of human inflammatory disorders, remain unclear.

Our previous findings have suggested that, given their biochemical/molecular mechanisms, lipid and bile acid (BA) metabolism as well as inflammatory processes are likely involved in the pathogenesis of both atherosclerosis and cholestasis to some degree; as such, these two diseases can be said to fall within the same

spectrum of human metabolic syndrome (Guo et al., 2012; Noguchi et al., 2014; Nawata et al., 2016; Yamada et al., 2016). Previous studies in atherosclerotic lesions have reported the presence of both appropriately and inappropriately apoptotic vascular cells, including macrophages (Mϕs), smooth muscle cells (SMCs) and endothelial cells (ECs) (Ross, 1999; Lusis, 2000). However, in contrast to atherosclerosis, studies in rodent models of bile duct ligation (BDL)-induced cholestasis have found no evidence of apoptotic cells in the cholestatic liver, characteristic of human primary biliary cirrhosis, primary sclerosing cholangitis, biliary atresia and chronic cholelithiasis (Georgiev et al., 2008; Noguchi et al., 2014).

Despite these conflicting findings between the two disorders, their mechanisms, including the regulation of vascular or biliary injury and migration/proliferation of SMCs or hepatobiliary cells, is multifactorial and complex, and the roles of EC, Mϕ, SMC, hepatocytes and/or cholangiocytes in the initiation of progression of atherosclerosis or cholestatic liver diseases remain debatable (Glaser et al., 2009; Yamada et al., 2011; Tasaki et al., 2013; Noguchi et al., 2014). As such, the key factors and signaling pathways controlling the responses to injury in not only vascular but also liver cells need to be determined.

Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase kinase kinase (MAPKKK) family member activated through distinct mechanisms in response to various cytotoxic stressors (Fig. 1), including immune system mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β or Fas ligands, and oxidative stressors mediated by hydrogen peroxide (H_2O_2) or endoplasmic reticulum (ER) stress (Ichijo et al., 1997). ASK1 is ubiquitously expressed by multiple cell types and situated upstream of many signal transduction pathways, such as the c-Jun N-terminal kinase (JNK) and p38 MAP kinase (MAPK) pathways, which subsequently induce not only intrinsic apoptotic signaling via mitochondria-dependent caspase activation but also inflammation or cell proliferation and differentiation (Ichijo et al., 1997; Tobiume et al., 2001). As shown in the schematic illustration in Fig. 1, ASK1 activates both the mitogen-activated protein kinase kinase 4 (MKK4)/MKK7-JNK and MKK3/MKK6-p38 pathways. Using mice genetically deficient of *ASK1* (*ASK1*$^{-/-}$), previous studies by other groups have shown that the response to left ventricular remodeling in *ASK1*$^{-/-}$ mice, caused by angiotensin II infusion, myocardial infarction and pressure-overload stimulation, is markedly attenuated compared with wild-type (WT) mice (Izumiya et al., 2003; Yamaguchi et al., 2003; Watanabe et al., 2005), suggesting that *ASK1*$^{-/-}$ cardiomyocytes have higher resistance to H_2O_2 or Ca^{2+}-induced apoptotic cell death. Conversely, ASK1-overexpressing transgenic mice have shown increased rates of cardiomyocyte apoptosis following ischemia-reperfusion injury compared with WT mice (Liu et al., 2009). These data indicate that ASK1 plays a pivotal role in the regulation of cardiomyocyte apoptosis. Furthermore, a thorough examination of the literature turned up reports that ASK1 expression plays a critical role regarding the *in vivo* function of apoptosis in neural cells or type II pneumocytes during different types of tissue injury, with anti-apoptotic and/or anti-inflammatory properties (Harada et al., 2006; Makena et al., 2012). However, very few studies have so far investigated the relationship between ASK1/apoptosis-signaling pathway and the initiation of the progression of atherosclerosis or cholestasis-induced liver injury.

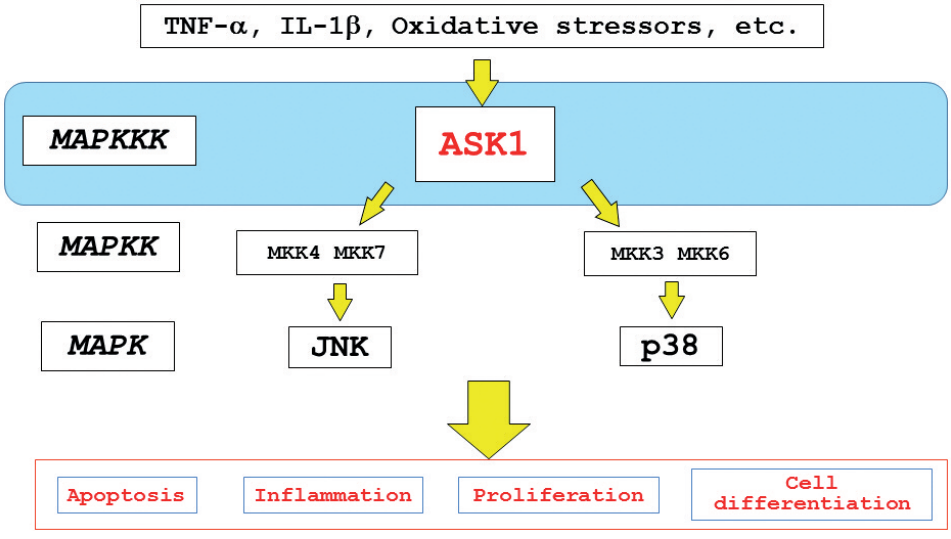

Fig. 1. Schematic illustration of the relationship between ASK1 and the JNK/p38 pathway activated by various cytotoxic stressors. This illustration depicts the apoptosis signal-regulating kinase 1 (ASK1) signaling pathway. ASK1 is a mitogen-activated protein kinase kinase kinase (MAPKKK) family member that is activated through distinct mechanisms in response to various cytotoxic stressors, including various immune system mediators, such as tumor necrosis factor (TNF)-α or interleukin (IL)-1β, and oxidative stressors. ASK1 is situated upstream of many signal transduction pathways, such as the c-Jun N-terminal kinase (JNK) and p38 MAP kinase (MAPK) pathways, which subsequently induce not only intrinsic apoptotic signaling via mitochondria-dependent caspase activation but also inflammation or cell proliferation and differentiation. Through its signaling, ASK1 activates both the mitogen-activated protein kinase kinase 4 (MKK4)/MKK7-JNK and MKK3/MKK6-p38 pathways.

We hypothesized that ASK1 signaling can play diverse, key roles in the pathogenesis of several common but complicated human diseases, including atherosclerosis and cholestasis. Indeed, our serial *in vivo* experiments (Table 1) showed for the first time the specific expression of both mRNA and protein of phosphorylated ASK1, its activated form, in the inflammatory lesions of animal models. *ASK1*$^{-/-}$ mice displayed variable, diverse phenotypes in the acute and subacute-to-chronic phase of each inflammatory disorder, with or without the presence of apoptotic activities, in two different atherosclerotic models: (a) a model of ligation-induced injury to muscular arteries (Tasaki et al., 2013) and (b) a model of hypercholesterolemia using the aortas of *apolipoprotein E*-knockout mice (*apoE*$^{-/-}$) (Yamada et al., 2011); and in another BDL-induced cholestasis model (Noguchi et al., 2014), as shown in Table 1. We reviewed whether or not (and how) apoptosis through ASK1 signaling is involved in the initiation of the progression of not only 'so-called' atherosclerosis in a broad sense, including arteriosclerosis in a narrow sense, but also the early-to-advanced stage of cholestatic liver injury. Metabolic syndrome, representing atherosclerosis and liver injury in a background of disordered lipid/BA metabolism, is an extremely complex disease orchestrated by multiple molecular and histopathologic factors, including elevated inflammatory cytokines, apoptosis, oxidative stressors and the intestinal function (Ding et al., 2010; Wang et al., 2010; Guo et al., 2012; Nawata et al., 2016; Yamada et al., 2016). *ASK1*$^{-/-}$ mice deficient of the desired target gene might be a useful animal model for investigating the roles of apoptosis and/or inflammatory processes in the development of metabolic syndrome in the absence of any regulation mediated by ASK1 signaling. Taken together, our collective findings indicate that ASK1 is a critical factor for the pathogenesis of atherosclerosis and cholestatic liver injury.

Critical and diverse *in vivo* roles of ASK1 signaling in mouse models of atherosclerosis and liver injury

ASK1 signaling exacerbates arteriosclerotic development in ligation injury-induced vascular remodeling by enhancing apoptosis in ECs and SMCs

All *in vivo* experiments were performed in 8-week-old male C57BL/6J WT and *ASK1*-gene knockout *ASK1*$^{-/-}$ mice weighing approximately 20 to 22 g (Tobiume et al., 2001; Tasaki et al., 2013). To produce the ligation-induced vascular injury arteriosclerotic model (Sasaguri et al., 2005; Yamada et al., 2008, 2013), we ligated the left common carotid artery with a 7-0 silk suture at a site proximal to the carotid bifurcation, after a median skin incision, in 2 groups of mice under anesthesia with an intraperitoneal injection of ketamine-medetamidine. The animals were euthanized 3 weeks later by overdose with an intraperitoneal injection of ketamine-medetamidine, and the carotid arteries were excised-each bisected at both sides of the ligation site to obtain an approximately 3-mm segment. These segments were then stained with hematoxylin and eosin (H&E) or elastica van Gieson (EVG), or prepared as immunohistochemistry (IHC) preparations in sequential sections after fixation in 10% neutral buffered formalin for 24 h (Tasaki et al., 2013). We consider this ligation model to exhibit disturbed blood flow, subsequently resulting in EC injury-induced vascular remodeling via inside-out signaling, according to the 'Response to Injury Hypothesis' (Ross et al., 1999). The current arteriosclerotic model shows SMC-rich neointimal hyperplasia without definite evidence of necrotic lipid cores in the center of the intima, likely reminiscent of human restenosis after angioplasty or shoulder lesions of vulnerable atheroma, potentially representing acute-to-subacute inflammatory disease of 'so-called' atherosclerosis in a broad sense.

As shown in Table 1, compared to the WT mice, the *ASK1*$^{-/-}$ mice showed anti-atherosclerotic profiles, including manifestations of less-SMC-predominant intimal lesions and smaller numbers of neointimal

Table 1. Summary of our serial *in vivo* studies on *ASK1*$^{-/-}$ mice (C57BL/6J strain) together with the applied model and the main quantitative results compared with WT mice.

Model	Results of *ASK1*$^{-/-}$ mice	Reference
Ligation-induced Arteriosclerosis		Tasaki et al., 2013
	•Neointima↓↓ (anti-arteriosclerotic)	
	•SMC apoptosis↓	
	•Neovascularization↓	
	•Collagen synthesis↓	
	•TNF-α/NFκB/ICAM-1/PDGF-BB↓	
HcD-induced Atherosclerosis		Yamada et al., 2011
	•Atheroma↑↑ (pro-atherogenic)	
	•Mφ apoptosis↓	
	•Necrotic core↓	
	•Elastolysis↑	
	•MMPs↑	
BDL-induced liver injury (acute phase)		Noguchi et al., 2014
	•Inflammation↓ (anti-inflammatory)	
	•Hepatic and biliary apoptosis→	
	•Bile infarcts↓	
	•Proliferation↓	
	•TNF-α/IL-1β/ICAM-1/iNOS/PCNA↓	
BDL-induced liver injury (subacute to chronic phase)		Noguchi et al ., 2014
	• Peribiliary fibrosis↓ (anti-fibrogenic)	
	• Hepatic and biliary apoptosis→	
	• Repair process↓	
	• Collagen synthesis↓	
	• TGF -β1↓	

HcD, high-cholesterol diet; BDL, bile duct ligation; SMC, smooth muscle cell; Mφ, macrophage; TNF, tumor necrosis factor; NF, nuclear factor; ICAM, intercellular adhesion molecule; PDGF, platelet-derived growth factor; MMP, matrix metalloproteinase; IL, interleukin; iNOS, inducible nitric oxide synthase; PCNA, proliferating cell nuclear; TGF, transforming

microvessels, which was associated with the repression of apoptosis in SMCs and ECs (Tasaki et al., 2013). We performed terminal deoxynucleotidyl transferase end-labeling (TUNEL) staining and confirmed the presence of significantly fewer apoptotic SMCs in both the smaller neointima and media in the injured *ASK1*$^{-/-}$ mice than in the WT mice, which was associated with the decreased expression of the pro-apoptotic gene Bax and caspase-3. In addition, our *in vitro* examination showed that immune system (especially TNF-α)-mediated apoptosis was markedly attenuated in cultured aortic SMCs obtained from *ASK1*$^{-/-}$ mice (Tasaki et al., 2013).

In the present animal model, *ASK1* deficiency results in amelioration of neointimal hyperplasia together with (i) suppressed migration of not only SMCs but inflammatory cells into the neointima via decreased neovascularization, as well as repressed apoptosis of ECs and/or both medial and intimal SMCs; and (ii) reduced synthesis of the collagen-rich matrix via the suppression of SMC dedifferentiation, as shown in an ultrastructural analysis of the thickened neointima in ligated arteries. A diagram depicting those key, crucial roles of ASK1 is presented in Fig. 2A.

These data gathered through our studies therefore support the hypothesis that increased SMC apoptosis enhances the development of neointimal hyperplasia in the early stage of atherosclerosis and exerts detrimental effects in injury-induced vascular remodeling, representing acute-to-subacute inflammatory disorders. Furthermore, ASK1 signaling exacerbates arteriosclerotic development in ligation injury-induced neointimal thickening by enhancing the apoptosis of ECs and SMCs, at least in part. In this context, a specific blocker of ASK1/apoptosis signaling might offer a therapeutic strategy against early atherosclerosis progression (e.g. restenosis after angioplasty) by suppressing SMC migration and EC neovascularization.

ASK1 signaling conversely protects against atherosclerotic progression in hypercholesterolemia-induced atheromatous formation by enhancing the apoptosis of Mφs

To model hypercholesterolemia-induced atherosclerosis, we generated *ASK1* and *apoE* double-knockout mice (*ASK1*$^{-/-}$/*apoE*$^{-/-}$) by crossing the *apoE*$^{-/-}$ mice (Tobiume et al., 2001; Yamada et al., 2011). In marked contrast to lipid metabolism in humans, the lipoprotein profile in mice is high-density-lipoprotein (HDL)-dominant; as such, WT mice are essentially resistant to high-cholesterol-diet (HcD)-induced atheromatous plaque formation (Getz and Reardon, 2012). *ApoE*$^{-/-}$ and *ASK1*$^{-/-}$/*apoE*$^{-/-}$ mice were fed an HcD (the control diet [54.4% carbohydrate, 23.6% protein, 5.3% fat, 2.9% fiber and 6.1% ash] supplemented with 1.25% cholesterol, 15.0% lard, and 0.5% sodium cholic acid [CA]) and sacrificed 12 weeks later by overdose of intraperitoneal injection of ketamine and medetomidine (Yamada et al., 2011).

In contrast to the aforementioned vascular injury-induced arteriosclerotic model, this murine model of hyperlipidemia-induced atheroma shows the initiation of

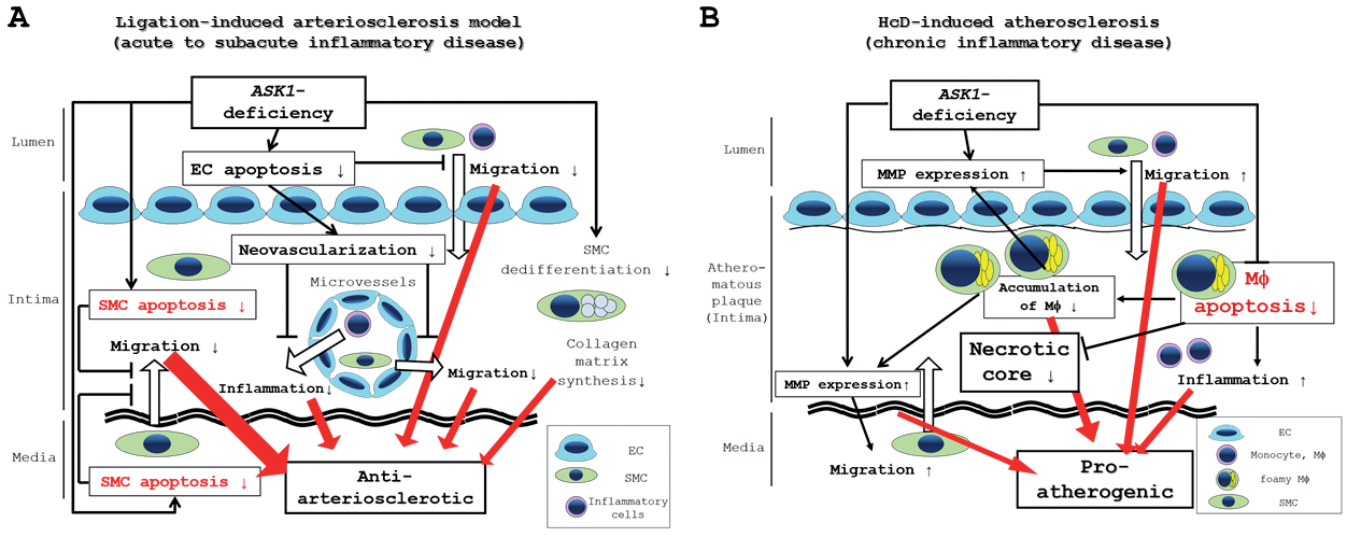

Fig. 2. Schematic illustration of the critical and diverse roles of ASK1 in animal models of atherosclerosis. These diagrams depict the diverse key roles of ASK1 deficiency in a ligation-injury-induced arteriosclerotic model (i.e. representing acute-to-subacute inflammatory disease) **(A)** and a high-cholesterol diet (HcD)-induced hyperlipidemic atherosclerosis model (i.e. representing chronic inflammatory disease) **(B)**. The finding that ASK1-knockout status results in anti-atherogenic (anti-inflammatory, anti-apoptotic and/or anti-fibrogenic) features in model A suggests ASK1-knockout would induce protective, beneficial effects against acute-to-subacute inflammatory disorders. However, ASK1 deficiency conversely results in pro-atherogenic (pro-inflammatory but anti-apoptotic) features in model B, suggesting that ASK1-knockout would induce harmful effects under conditions of chronic inflammatory disorder. EC, endothelial cell; SMC, smooth muscle cell; Mϕ, macrophage; MMP, matrix metalloproteinase.

the progression of Mϕ (foam cell)-rich neointimal lesions with central necrotic lipid cores and fibrous cap formation, resembling human somewhat 'true' atherosclerosis and manifesting as chronic inflammatory disease (Sasaguri et al., 2005; Yamada et al., 2008, 2015; Wang et al., 2011). Intriguingly, as shown in Table 1 and Fig. 2B, compared to the *apoE*$^{-/-}$ mice, the *ASK1*$^{-/-}$/*apoE*$^{-/-}$ mice show pro-atherogenic profiles, including the manifestations of more Mϕ-rich intimal lesions, smaller necrotic cores, and faster atherosclerosis progression, in addition to the suppression of TUNEL-positive apoptosis in Mϕs (Yamada et al., 2011). Therefore, the rapid progression of Mϕ-rich and necrotic-core-poor atherosclerosis in these mice may be exclusively attributed to the suppression of apoptosis in *ASK1*-deficient Mϕs.

At the molecular level, we confirmed that the inhibition of Mϕ apoptosis was due to a significant decrease in the activated (i.e. fragmented) form of caspase-3 with an increase in the anti-apoptotic bcl-2 expression (Yamada et al., 2011). Furthermore, the present atheroma model showed significant up-regulation of various matrix metalloproteinases (MMPs), namely MMP-2, MMP-9, MMP-12 and MMP-13, and prominent elastolysis of the basal membrane and internal elastic lamina in the *ASK1*$^{-/-}$/*apoE*$^{-/-}$ mice, suggesting the importance of the functions of MMPs in this model. The development of Mϕ-rich atherosclerosis in *ASK1*$^{-/-}$/*apoE*$^{-/-}$ mice may also be due, at least in part, to the enhanced migration and recruitment of Mϕs/monocytes through the actions of MMPs. Similarly, the expression of several other key inflammatory factors, such as toll-like receptors (TLRs) 3 and 4, signal transduction and activator of transcription (STAT) 1, TNF-α, adhesion molecules like intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), has been shown to differ markedly between *ASK1*$^{-/-}$/*apoE*$^{-/-}$ and *apoE*$^{-/-}$ mice (Yamada et al., 2011). These observations coincide with the findings from our previous animal study, where we found that elastolysis by MMP-12 plays crucial roles in the migration of Mϕs and the progression of hyperlipidemia-induced atherosclerosis in MMP-12 transgenic rabbits (Yamada et al., 2008).

A diagram depicting the diverse, critical roles of *ASK1*-deficiency and ASK1/apoptosis signaling is summarized in Fig. 2B. ASK1 signaling attenuates Mϕ-rich atherosclerosis but promotes necrotic core formation via the stimulation of Mϕ apoptosis, which might simultaneously cause plaque vulnerability. These data gathered through our studies therefore support the hypothesis that increased Mϕ apoptosis protects against the development of atheromatous formation in the intermediate-to-advanced stage of atherosclerosis and exerts beneficial effects on HcD-induced atherosclerosis, which represents chronic inflammatory disorders. A specific activator of ASK1/apoptosis signaling might be useful as a therapeutic agent against atherosclerotic progression by accelerating Mϕ apoptosis and suppressing Mϕ/SMC migration and MMPs up-regulation, at least to some degree.

In conclusion, our results suggest that ASK1 expression and its signaling together with apoptotic activities, especially in SMCs and Mϕs, might be not only critically but diversely responsible for various potentially pro-atherosclerotic and anti-atherosclerotic effects, depending on the predominant cell types in the thickened intima and disease stage/phase, from acute to chronic status. Ultimately, we emphasize that the fundamental mechanisms responsible for atherosclerosis in a broad sense differ completely between injured arteriosclerotic arteries and the atheromatous plaques in the aorta. Nevertheless, since atherosclerosis is an extremely complex disease orchestrated by multiple factors, our ongoing and future studies will examine several intriguing issues between ASK1 signaling and oxidative stress or antioxidant properties, such as peroxiredoxin (PRDX) family members (Yamada et al., 2012), potentially related to lipid/BA metabolism in further detail after accumulating experimental data in a multidirectional manner.

ASK1 signaling accelerates bile-duct-ligation-induced cholestatic liver injury by enhancing necro-inflammation, but not apoptosis, and by subsequently activating peribiliary fibrogenesis/fibrosis

To produce a bile-duct-ligation (BDL)-induced cholestatic liver injury model (Noguchi et al., 2014), the peritoneal cavity was opened after a midline upper-abdominal incision, and the common bile duct in parallel with the hepatic artery was double-ligated with sterile surgical 7-0 silk sutures and cut between the ligatures in 2 groups (WT and *ASK1*$^{-/-}$) of male mice at 8 weeks of age under anesthesia, as shown in Fig. 3A. Sham-operated mice, as controls, underwent laparotomy with exposure but without ligation of the common bile duct. The fascia and skin of the midline abdominal incision were closed with sterile surgical 6-0 silk sutures. After a defined period of BDL or sham-operation at 3 or 14 days, the animals were euthanized by exsanguination under re-anesthetization with an intraperitoneal injection of ketamine-medetamidine, as follows: the peritoneal cavity was re-opened, and blood samples were taken from the inferior vena cava, followed by immediate cannulation of the suprahepatic vena cava (Noguchi et al., 2014). In all animals, after the blood had been flushed out of the liver via the suprahepatic vena cava catheter, the livers were excised and cut into small pieces and used for various experiments, including histological analyses, IHC, immunofluorescence, TUNEL staining, real-time reverse transcriptase-polymerase chain reaction (RT-PCR), microarray studies, Western blot analyses, and electron microscopy.

It had been previously proposed that hepatocytes undergoing apoptosis might provide a critical hit to drive progression from the acute or subacute inflammatory phase to cirrhosis (i.e. the chronic inflammatory phase)

in cases of cholestatic liver injury in humans, such as with primary biliary cirrhosis, primary sclerosing cholangitis, biliary atresia and chronic cholelithiasis (Miyoshi et al., 1999; Canbay et al., 2003). In striking contrast, several other groups have recently suggested using standard morphologic criteria that there is no clear evidence of hepatocellular or biliary apoptosis in or around BA-induced necrotic foci, i.e. bile infarcts, in rodent models of BDL (Gujral et al., 2004; Nalapareddy et al., 2009; Mitchell et al., 2011), as summarized in Fig. 3B. They also concluded that BDL-induced cholestatic oncotic necrosis but not apoptosis of hepatocytes is closely correlated with the severity of the inflammatory response and subsequent peribiliary fibrogensis/fibrosis. Furthermore, although cholangiocytes are a minor component of liver cells, comprising merely 3%-4% of the rodent liver, the cells lining the large bile ducts (i.e. large cholangiocytes) in the portal areas are the main target in not only animal models of cholestasis but intractable human cholestatic diseases (Yahagi et al.,

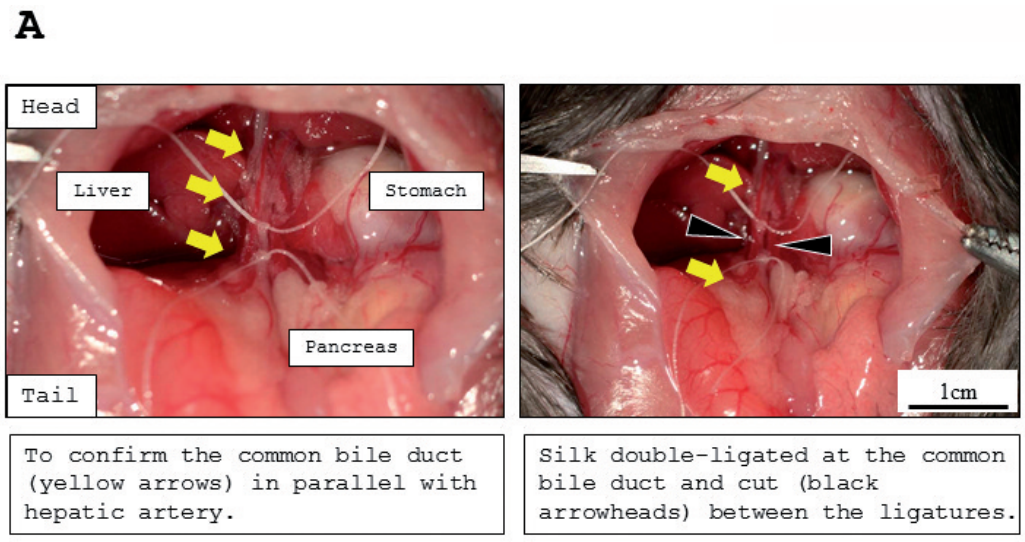

Fig. 3. Representative photographs of BDL-induced cholestatic liver injury models, and a schematic illustration of a hypothetical mechanism for BDL. **A.** The procedure for developing a mouse model of bile-duct-ligation (BDL)-induced cholestasis to evaluate the critical roles of ASK1 signaling with and without enhanced apoptotic activities in wild-type (WT) and ASK1-/- mice livers. **B.** This illustration shows the hypothetical mechanism for bile acid (BA)-induced hepatocellular and biliary injury in the present murine cholestasis model, clarifying whether or not (and how) apoptosis of hepatocytes and cholangiocytes plays a role in the subsequent fibrogenesis associated with BDL. A, P and B indicate the hepatic artery, portal vein and biliary duct, respectively.

1998; Lazaridis et al., 2004), as shown in Fig. 3B. We therefore focused on the roles and function of not only hepatocytes but also cholangiocytes in the present models of BDL-induced cholestatic liver injury in a multidirectional manner.

Detailed analyses of this mouse model of BDL between 8 h and 6 weeks after operation (Georgiev et al., 2008) have shown that the presence of multiple foci of hepatocellular necrosis (i.e. biliary infarcts) represents the acute phase of cholestatic liver injury, peaking at around day 3, along with a predominant inflammatory cell type of neutrophils, corresponding to the early few weeks of human cholestatic diseases, such as primary biliary cirrhosis, primary sclerosing cholangitis, biliary atresia and cholelithiasis (Fig. 4). Following the acute phase, BDL mice show hepatocellular proliferation (i.e. repair) and proliferating responses of small bile ducts until day 7, corresponding to several months of disease progression in human cholestasis of subacute phase (Fig. 4). Finally, the subacute to chronic phase in the mouse model of BDL-induced liver injury is characterized by peribiliary fibrogenesis/fibrosis and the accumulation of collagen and continuous infiltration of lymphocytes and Mϕs (Kupffer cells) from days 14 and 21 to 6 weeks later, corresponding to several years of disease progression in human cholestatic liver injury (Fig. 4). Fig. 4 summarizes this time course for specific phases of acute-to-chronic liver injury both in mice and humans.

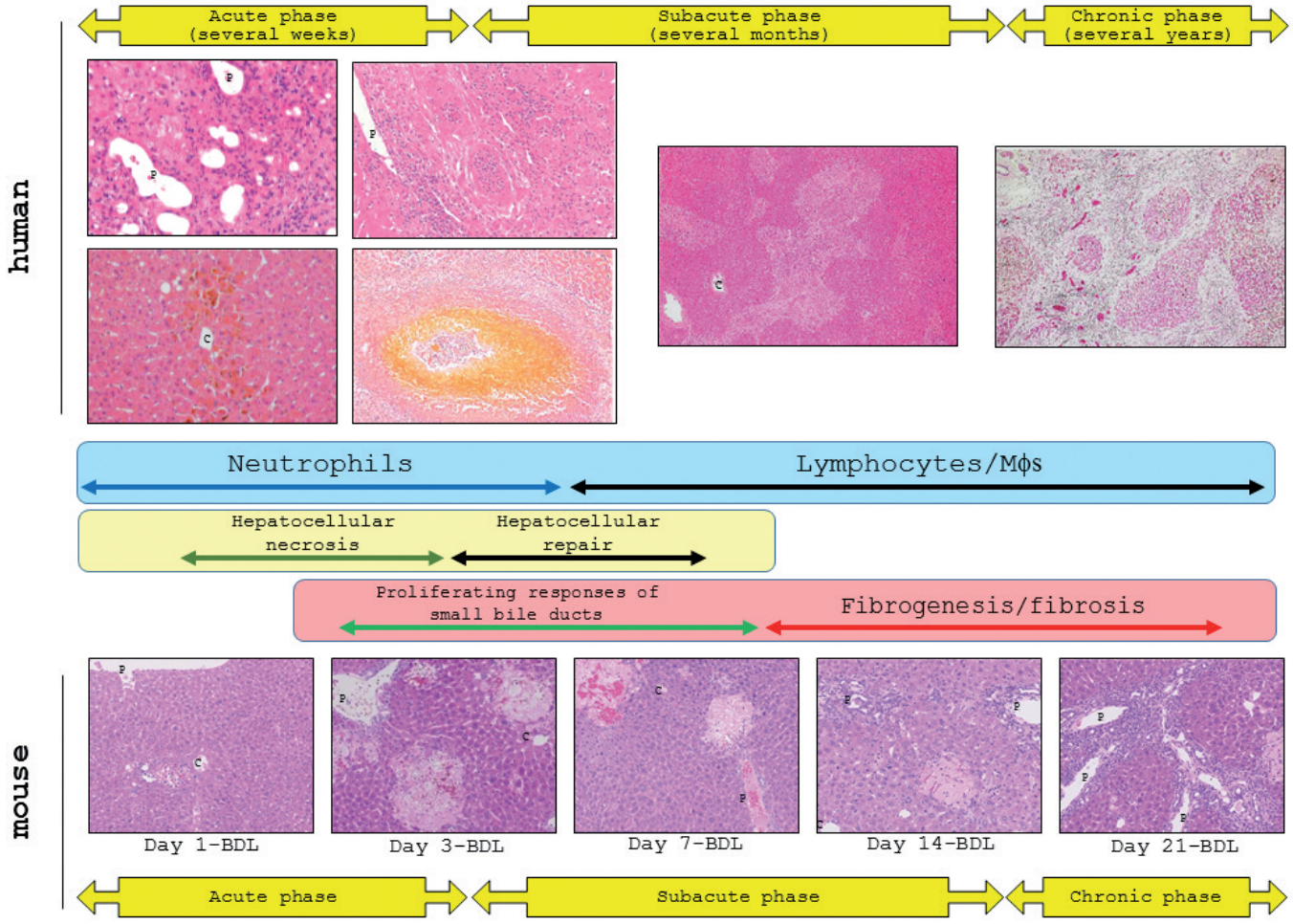

Fig. 4. Time course for the acute-to-chronic inflammatory phase both in human cholestatic liver diseases and a murine BDL model, together with representative photographs. Multiple foci of hepatocellular necrosis (i.e. biliary infarcts) in BDL mice represent the acute phase of cholestatic liver injury, peaking at around day 3, along with a predominant inflammatory cell type of neutrophils, corresponding to the early few weeks of human cholestatic diseases, such as primary biliary cirrhosis, primary sclerosing cholangitis, biliary atresia and cholelithiasis. Following the acute phase, BDL mice show hepatocellular proliferation (i.e. repair) and proliferating responses of small bile ducts until day 7 (subacute phase), corresponding to several months of disease progression in human cholestasis of subacute phase. Finally, the chronic phase in the mouse model of BDL-induced liver injury is characterized by peribiliary fibrogenesis/fibrosis and the accumulation of collagen and continuous infiltration of lymphocytes and Mϕs (Kupffer cells) from days 14 and 21 to 6 weeks later, corresponding to several years of disease progression in human cholestatic liver injury. C and P indicate the central and portal veins, respectively.

We herein review the critical and diverse roles of ASK1 in BDL-induced cholestatic liver injury using mice genetically deficient of *ASK1* (*ASK1*$^{-/-}$) with the ultimate aim of determining the net effects and key roles of ASK1 in the progression of liver necro-inflammation (i.e. acute inflammatory phase) to subsequent fibrogenesis/fibrosis (i.e. subacute-to-chronic inflammatory phase).

Compared with WT mice that expressed elevated levels of phosphorylated ASK1, *ASK1*$^{-/-}$ mice showed potential anti-inflammatory profiles, especially in the acute phase (around day 3) of BDL-induced liver injury, including manifestations of reduced hepatocellular necrotic foci, suppressed extensive inflammatory reactions in lobules and portal areas and decreased proliferation of hepatocytes and large cholangiocytes, as summarized in Table 1 and Fig. 5. These features are in agreement with the decreased expression of several pro-inflammatory and inflammatory-related molecules, including TNF-α, IL-1β, ICAM-1, inducible nitric oxide synthase (iNOS) and proliferating cell nuclear antigen (PCNA), in the liver of BDL *ASK1*$^{-/-}$ mice around day 3. The morphological characteristics of apoptosis were not completely identified in the TUNEL-positive hepatocytes and cholangiocytes during the BDL experiments (Figs. 5, 6). The numbers of apoptotic liver cells after BDL were negligible based on the histological morphology, such as cell shrinkage, chromatin condensation and margination and formation of apoptotic bodies, and intriguingly, these numbers were not markedly increased compared to sham-operated controls (Noguchi et al., 2014). In line with these findings, there were no significant differences in the caspase-3 activities and caspase-3 processing or Bax expression between the respective groups (WT vs. *ASK1*$^{-/-}$ and BDL vs. sham). Next, as depicted in the schematic illustration of Fig. 6, the above effects of *ASK1* deficiency in the acute phase also improved the subsequent peribiliary fibrosis/fibrogenesis in *ASK1*$^{-/-}$ mice with anti-fibrogenic profiles in the subacute-to-chronic phase at day 14 post-BDL injury (Noguchi et al., 2014). Accordingly, *ASK1*$^{-/-}$ mice showed significantly suppressed activation of peribiliary fibrogenic cells, decreased biliary ductules and reduction of collagen

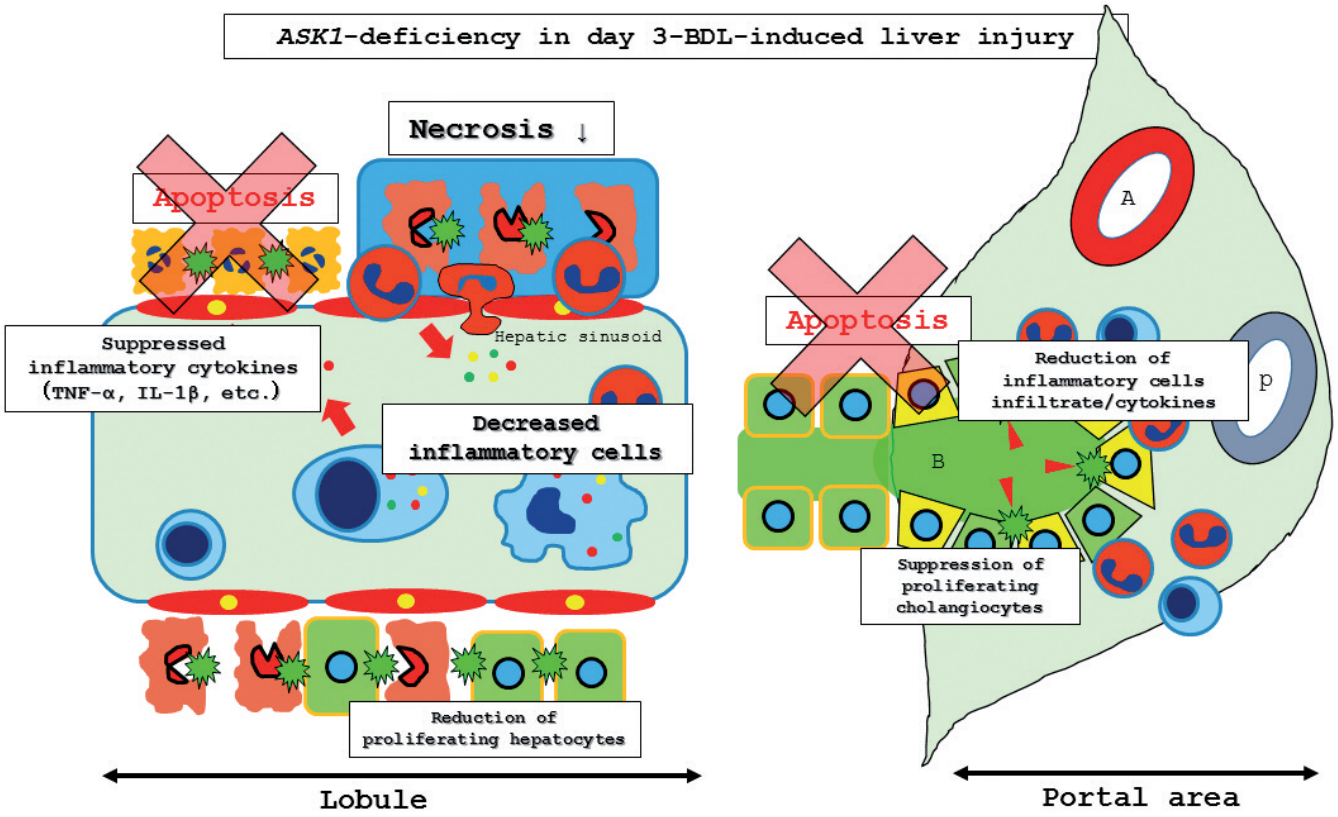

Fig. 5. Schematic illustration of the critical and diverse roles of ASK1 in a model of BDL-induced liver injury (day 3). This schematic illustration shows the critical and diverse roles of ASK1 deficiency, correlated closely with anti-inflammatory, anti-necrotic and anti-proliferative phenotypes in the acute inflammatory phase in a murine BDL model. Taken together, these findings suggest that ASK1 deficiency will result in significant beneficial effects against acute inflammatory disorders. There is no definite evidence of apoptotic cells in the cholestatic livers. A, P and B indicate the hepatic artery, portal vein and biliary duct, respectively.

matrix synthesis, all of which are associated with the reduced expression of transforming growth factor β1 (TGF-β1).

Despite the fact that a number of limitations are associated with this *in vivo* study, based on the findings in our animal model using *ASK1*$^{-/-}$ mice, we conclude that liver cells undergoing apoptosis cannot exclusively play a pivotal role in BDL-induced cholestatic liver injury. By contrast, ASK1 signaling tightly controls the development of not only early necro-inflammation but also subsequent peribiliary fibrosis/fibrogenesis under conditions that induce acute inflammatory processes and the subacute-to-chronic fibrogenic response. Taken together, these findings suggest that a specific ASK1 pathway blocker may be useful as a therapeutic strategy against the progression of human cholestatic liver disease. However, *ASK1*$^{-/-}$ mice also exhibited delayed healing (i.e. a delayed repair process) in day 14 BDL liver lobules, influenced by the suppressed proliferation of hepatocytes and cholangiocytes, as shown in Figs. 5, 6. As such, we should consider that the deletion of *ASK1* might result in a somewhat detrimental profile in the subacute-to-chronic phase of BDL (Fig. 6).

Concluding remarks and future perspective

In conclusion, our serial *in vivo* studies of the initiation of the progression of atherosclerosis and cholestatic liver injury provide new evidence of potential mechanisms by which ASK1 signaling can critically but diversely affect the severity of those inflammatory disease processes. For example, *ASK1* deficiency results in anti-atherogenic (anti-inflammatory, anti-apoptotic and/or anti-fibrogenic) features in mechanical injury (carotid artery ligation)-induced vascular remodeling, representing acute-to-subacute inflammatory disease (atherosclerosis). Furthermore, the deletion of *ASK1* results in anti-inflammatory and anti-fibrogenic profiles in the acute phase (around day 3) of BDL-induced liver injury. Thus, *ASK1* deficiency can induce significant protective, beneficial effects against acute-to-subacute inflammatory disorders. In addition, *ASK1*-knockout

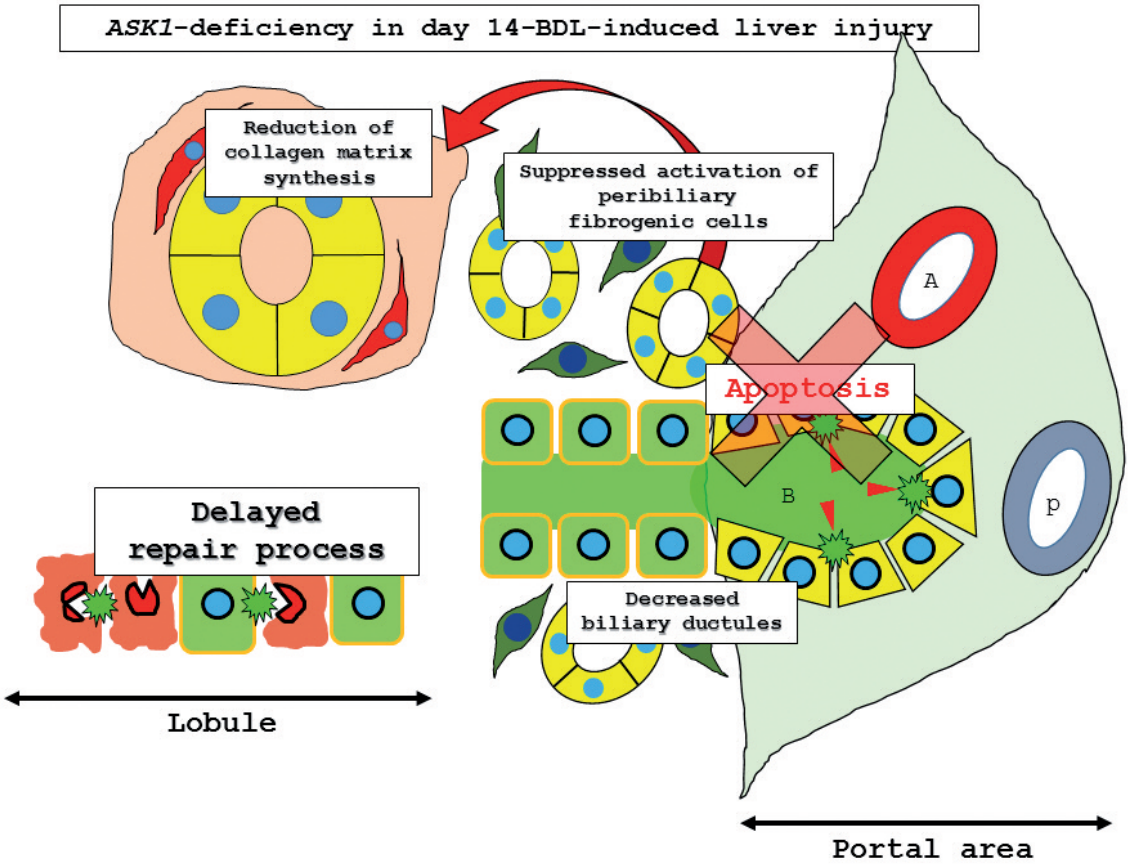

Fig. 6. Schematic illustration of the critical and diverse roles of ASK1 in a model of BDL-induced liver injury (day 14). This schematic illustration shows the critical and diverse roles of ASK1 deficiency, correlated closely with anti-fibrogenic and anti-proliferative phenotypes, leading to some degree of protective and beneficial effects, in the subacute-to-chronic inflammatory phase of a murine BDL model. In contrast, to the acute phase, ASK1 deficiency results in a detrimental profile of delayed repair processes in BDL *ASK1*$^{-/-}$ livers (day 14). There is no definite evidence of apoptotic cells in the cholestatic livers. A, P and B indicate the hepatic artery, portal vein and biliary duct, respectively.

status resulted in pro-atherogenic (pro-inflammatory but anti-apoptotic) features in our model of hyperlipidemia-induced atherosclerosis, very similarly to human atheromatous plaques, representing a chronic inflammatory disease. Correspondingly, *ASK1*-knockout animals exhibited a detrimental, anti-proliferative profile of delayed repair processes in the subacute-to-chronic phase (day 14) of BDL. In this scenario, *ASK1* deficiency conversely exerts significant harmful effects under conditions of chronic inflammatory disorder. Taken together, these findings show that stimulated ASK1 signaling critically and diversely regulates the initiation of progression within each disease process, depending on not only the animal model, influenced by each target organ, but also the phase, from acute to chronic stages. Finally, the relevance of apoptotic activities relies on the characteristics of each murine model of human disease, as well.

Metabolic syndrome is an extremely complicated and multifactorial disease, orchestrated variably by inflammatory cell types and cytokine levels, apoptotic activities, oxidative stressors, insulin sensitivity in various organs, diet affecting lipid/BA metabolism, translocation of either intestinal bacteria or microbial cell components, the background of the hepatic/intestinal function, and other factors (Guo et al., 2012; Nabeshima et al., 2013; Nawata et al., 2016; Yamada et al., 2016). Nevertheless, the potential presence of innate links between ASK1 signaling and metabolic syndrome, which are strongly involved in not only inflammatory but also fibrogenic responses, at least in part, is noteworthy. Therefore, ASK1 blockers or stimulators might become useful in the treatment of metabolic syndrome diseases. *ASK1*$^{-/-}$ mice will be a useful animal model for studying the associations of ASK1 signaling with inflammatory disorders and may also be a promising, novel animal model of human metabolic syndrome.

Our laboratories recently reported that PRDX4 (Ding et al., 2010; Guo et al., 2012; Nabeshima et al., 2013; Nawata et al., 2016), the only known secretory member of the PRDX antioxidant family, protects against the progression of atherosclerosis, liver injury via steatosis and/or insulin resistance in human PRDX4 transgenic mice fed a high-cholesterol (Guo et al., 2012), high-fructose diet after the injection of streptozotocin (Nabeshima et al., 2013) or consumption of a methionine- and choline-deficient high-fat diet (Nawata et al., 2016). Our experiments using these transgenic mice provide the first evidence of the beneficial effects of PRDX4 on not only the atherosclerotic progression, but also the hepatic/intestinal function in the reduction of the severity of metabolic syndrome by ameliorating oxidative stress-induced local and systemic injury and suppressing the inflammatory reaction and apoptotic activities (Ding et al., 2010; Guo et al., 2012; Nabeshima et al., 2013; Nawata et al., 2016). Our ongoing and future studies will examine several intriguing issues between ASK1 signaling and oxidative stress or antioxidant properties, such as PRDX4, related closely to anti-inflammatory and anti-apoptotic features in more detail, after accumulating further experimental data. Furthermore, we recently established a novel animal model for studies of hyperlipidemia-induced atherosclerosis and non-alcoholic fatty liver disease using the world's smallest Microminipigs™ (μMPs; Fuji Micra Inc., Shizuoka, Japan) fed a HcD (Kawaguchi et al., 2014). Swine represent a more promising and potentially useful experimental animal model than mice or rabbits, since their lipoprotein metabolism as well as their anatomy, physiology and feeding and sleeping habits are quite similar to those of humans. Thus, one of our future aims is to clarify the details of the pathogenic and molecular mechanisms underlying ASK1 signaling during the development of metabolic syndrome in μMPs.

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References

Canbay A., Taimr P., Torok N., Higuchi H., Friedman S. and Gores G.J. (2003). Apoptotic body engulfment by a human stellate cell line is profibrogenic. Lab. Invest. 83, 655-663.

Ding Y., Yamada S., Wang K.Y., Shimajiri S., Guo X., Tanimoto A., Murata Y., Kitajima S., Watanabe T., Izumi H., Kohno K. and Sasaguri Y. (2010). Overexpression of peroxiredoxin 4 protects against high-dose streptozotocin-induced diabetes by suppressing oxidative stress and cytokines in transgenic mice. Antioxid. Redox Signal. 13, 1477-1490.

Elmore S. (2007). Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495-516.

Georgiev P., Jochum W., Heinrich S., Jang J.H., Nocito A., Dahm F. and Clavien P.A. (2008). Characterization of time-related changes after experimental bile duct ligation. Br. J. Surg. 95, 646-656.

Getz G.S. and Reardon C.A. (2012). Animal models of atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 32, 1104-1115.

Glaser S., Gaudio E. Rao A., Pierce L.M., Onori P., Franchitto A., Francis H.L., Dostal D.E. Venter J.K., DeMorrow S., Mancinelli R., Carpino G., Alvaro D., Kopriva S.E. Savage J.M. and Alpini G. (2009). Morphological and functional heterogeneity of the mouse intrahepatic biliary epithelium. Lab. Invest. 89, 456-469.

Gujral J.S., Liu J., Farhood A. and Jaeschke H. (2004). Reduced oncotic necrosis in Fas receptor-deficient C57BL/6J-lpr mice after bile duct ligation. Hepatology 40, 998-1007.

Guo X., Yamada S., Tanimoto A., Ding Y., Wang K.Y., Shimajiri S., Murata Y., Kimura S., Tasaki T., Nabeshima A., Watanabe T., Kohno K. and Sasaguri Y. (2012). Overexpression of peroxiredoxin 4 attenuates atherosclerosis in apolipoprotein E knockout mice. Antioxid. Redox Signal. 17, 1362-1375.

Harada C., Nakamura K., Namekata K., Okumura A., Mitamura Y., Iizuka Y., Kashiwagi K., Yoshida K., Ohno S., Matsuzawa A., Tanaka K., Ichijo H. and Harada T. (2006). Role of apoptosis signal-regulating kinase 1 in stress-induced neural cell apoptosis *in vivo*. Am. J. Pathol. 168, 261-269.

Ichijo H., Nishida E. Irie K., Digike P.T., Saitoh M., Moriguchi T., Takagi M., Matsumoto K., Miyazono K. and Gotoh Y. (1997). Induction of apoptosis by ASK1, mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 275, 90-94.

Izumiya Y., Kim S., Izumi Y., Yoshida K., Yoshiyama M., Matsuzawa A., Ichijo H. and Iwao H. (2003). Apoptosis signal-regulating kinase 1 plays a pivotal role in angiotensin II-induced cardiac hypertrophy and remodeling. Circ. Res. 93, 874-883.

Kawaguchi H., Yamada T., Miura N., Ayaori M., Uto-Kondo H., Ikegawa M., Noguchi M., Wang K.Y., Izumi H. and Tanimoto A. (2014). Rapid development of atherosclerosis in the world's smallest Microminipig fed a high-fat/high-cholesterol diet. J. Atheroscler. Thromb. 21, 186-203.

Lazaridis K.N., Strazzabosco M. and Larusso N.F. (2004). The cholangiopathies: disorders of biliary epithelia. Gastroenterology 127, 1565-1577.

Liu Q., Sargent M.A., York A.J. and Molkentin J.D. (2009). ASK1 regulates cardiomyocyte death but not hypertrophy in transgenic mice. Circ. Res. 105, 1110-1117.

Lusis A.J. (2000). Atherosclerosis. Nature 407, 233-241.

Makena P.S., Gorantla V.K., Ghosh M.C., Bezawada L., Kandasamy K., Balazs L., Luellen C.L., Thompson K.E. Parthasarathi K., Ichijo H., Waters C.M. and Sinclair S.E. (2012). Deletion of apoptosis signal-regulating kinase-1 prevents ventilator-induced lung injury in mice. Am. J. Respir. Cell Mol. Biol. 46, 461-469.

Mitchell C., Mahrouf-Yorgov M., Mayeuf A., Robin M.A., Mansouri A., Fromenty B. and Gilgenkrantz H. (2011). Overexpression of Bcl-2 in hepatocytes protects against injury but does not attenuate fibrosis in a mouse model of chronic cholestatic liver disease. Lab. Invest. 91, 273-282.

Miyoshi H., Rust C., Roberts P.J., Burgart L.J. and Gores G.J. (1999). Hepatocyte apoptosis after bile duct ligation in the mouse involves Fas. Gastroenterology 117, 669-677.

Nabeshima A., Yamada S., Guo X., Tanimoto A., Wang K.Y., Shimajiri S., Kimura S., Tasaki T., Noguchi H., Kitada S., Watanabe T., Fujii J., Kohno K. and Sasaguri Y. (2013). Peroxiredoxin 4 protects against nonalcoholic steatohepatitis and type 2 diabetes in a nongenetic mouse model. Antioxid. Redox Signal. 19, 1983-1998.

Nalapareddy P.D., Schüngel S., Hong J.Y., Manns M.P., Jaeschke H. and Vogel A. (2009). The BH3-only protein bid does not mediate death-receptor-induced liver injury in obstructive cholestasis. Am. J. Pathol. 175, 1077-1085.

Nawata A., Noguchi H., Mazaki Y., Kurahashi T., Izumi H., Wang K.Y., Guo X., Uramoto H., Kohno K., Taniguchi H., Tanaka Y., Fujii J., Sasaguri Y., Tanimoto A., Nakayama T. and Yamada S. (2016). Overexpression of peroxiredoxin 4 affects intestinal function in a dietary mouse model of nonalcoholic fatty liver disease. PLoS One 11, e0152549.

Noguchi H., Yamada S., Nabeshima A., Guo X., Tanimoto A., Wang K.Y., Kitada S., Tasaki T., Takama T., Shimajiri S., Horlad H., Komohara Y., Izumi H., Kohno K., Ichijo H. and Sasaguri Y. (2014). Depletion of apoptosis signal-regulating kinase 1 prevents bile duct ligation-induced necro-inflammation and subsequent peribiliary fibrosis. Am. J. Pathol. 184, 644-661.

Ross R. (1999). Atherosclerosis - an inflammatory disease. N. Engl. J. Med. 340, 115-126.

Sasaguri Y., Wang K.Y., Tanimoto A., Tsutsui M., Ueno H., Murata Y., Kohno Y., Yamada S. and Ohtsu H. (2005). Role of histamine produced by bone marrow-derived vascular cells in pathogenesis of atherosclerosis. Circ. Res. 96, 974-981.

Tasaki T., Yamada S., Guo X., Tanimoto A., Wang K.Y., Nabeshima A., Kitada S., Noguchi H., Kimura S., Shimajiri S., Kohno K., Ichijo H. and Sasaguri Y. (2013). Apoptosis signal-regulating kinase 1 deficiency attenuates vascular injury-induced neointimal hyperplasia by suppressing apoptosis in smooth muscle cells. Am. J. Pathol. 182, 597-609.

Tobiume K., Matsuzawa A., Takahashi T., Nishitoh H., Morita K., Takeda K., Minowa O., Miyazono K., Noda T. and Ichijo H. (2001). ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. EMBO Rep. 2, 222-228.

Wang K.Y., Tanimoto A., Yamada S., Guo X., Ding Y., Watanabe T., Watanabe T., Kohno K., Hirano K., Tsukada H. and Sasaguri Y. (2010). Histamine regulation in glucose and lipid metabolism via histamine receptors: model for nonalcoholic steatohepatitis in mice. Am. J. Pathol. 177, 713-723.

Wang K.Y., Tanimoto A., Guo X., Yamada S., Shimajiri S., Murata Y., Ding Y., Tsutsui M., Kato S., Watanabe T., Ohtsu H., Hirano K., Kohno K. and Sasaguri Y. (2011). Histamine deficiency decreases atherosclerosis and inflammatory response in apolipoprotein E knockout mice independently of serum cholesterol level. Arterioscler. Thromb. Vasc. Biol. 31, 800-807.

Watanabe T., Otsu K., Takeda T., Yamaguchi O., Hikoso S., Kashiwase K., Higuchi Y., Taniike M., Nakai A., Matsumura Y., Nishida K., Ichijo H. and Hori M. (2005). Apoptosis signal-regulating kinase 1 is involved not only in apoptosis but also in non-apoptotic cardiomyocyte death. Biochem. Biophys. Res. Commun. 333, 562-567.

Yahagi K., Ishii M., Kobayashi K., Ueno Y., Mano Y., Niitsuma H., Igarashi T. and Toyota T. (1998). Primary culture of cholangiocytes from normal mouse liver. In Vitro Cell Dev. Biol. Anim, 34, 512-514.

Yamada S., Wang K.Y., Tanimoto A., Fan J., Shimajiri S., Kitajima S., Morimoto M., Tsutsui M., Watanabe T., Yasumoto K. and Sasaguri Y. (2008). Matrix metalloproteinase 12 accelerates the initiation of atherosclerosis and stimulates the progression of fatty streaks to fibrous plaques in transgenic rabbits. Am. J. Pathol. 172, 1419-1429.

Yamada S., Ding Y., Tanimoto A., Wang K.Y., Guo X., Li Z., Tasaki T., Nabesima A., Murata Y., Shimajiri S., Kohno K., Ichijo H. and Sasaguri Y. (2011). Apoptosis signal-regulating kinase 1 deficiency

accelerates hyperlipidemia-induced atheromatous plaques via suppression of macrophage apoptosis. Arterioscler. Thromb. Vasc. Biol. 31, 1555-1564.

Yamada S., Ding Y. and Sasaguri Y. (2012). Peroxiredoxin 4: Critical roles in inflammatory diseases. J. UOEH 34, 27-39.

Yamada S., Wang K.Y., Tanimoto A., Guo X., Nabeshima A., Watanabe T. and Sasaguri Y. (2013). Histamine receptors expressed in circulating progenitor cells have reciprocal actions in ligation-induced arteriosclerosis. Pathol. Int. 63, 435-447.

Yamada S., Wang K.Y., Tanimoto A. and Sasaguri Y. (2015). Novel function of histamine signaling in hyperlipidemia-induced atherosclerosis: Histamine H1 receptors protect and H2 receptors accelerate atherosclerosis. Pathol. Int. 65, 67-80.

Yamada S., Guo X., Wang K.Y., Tanimoto A. and Sasaguri Y. (2016). Novel function of histamine signaling via histamine receptors in cholesterol and bile acid metabolism: Histamine H2 receptor protects against nonalcoholic fatty liver disease. Pathol. Int. 66, 376-385.

Yamaguchi O., Higuchi Y., Hirotani S., Kashiwase K., Nakayama H., Hikoso S., Takeda T., Watanabe T., Asahi M., Taniike M., Matsumura Y., Tsujimoto I., Hongo K., Kusakari Y., Kurihara S., Nishida K., Ichijo H., Hori M. and Otsu K. (2003). Targeted deletion of apoptosis signal-regulating kinase 1 attenuates left ventricular remodeling. Proc. Natl. Acad. Sci. USA 100, 15883-15888.

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