The Caspase Inhibitor IDN-6556 Attenuates Hepatic Injury and Fibrosis in the Bile Duct Ligated Mouse

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ABSTRACT

Liver injury is characterized by hepatocyte apoptosis and collagen-producing activated hepatic stellate cells (HSC). Hepatocyte apoptosis promotes liver injury and fibrosis, whereas activated HSC apoptosis limits hepatic fibrosis. Pharmacological inhibition of liver cell apoptosis may potentially attenuate liver injury and fibrosis by blocking hepatocyte apoptosis or promote fibrosis by permitting accumulation of activated HSCs. To ascertain the net effect of inhibiting liver cell apoptosis on liver injury, inflammation, and hepatic fibrogenesis, we examined the effect of a pan-caspase inhibitor IDN-6556 on these parameters in the bile duct ligated (BDL) mouse. Hepatocyte apoptosis was assessed by the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay and immunofluorescence for active caspases 3/7, and liver injury by histopathology and serum alanine aminotransferase (ALT) determinations. Real-time polymerase chain reaction was used to measure mRNA transcripts for markers of hepatic inflammation, HSC activation, and fibrosis. Immunohistochemistry for α-smooth muscle actin was performed to identify HSC activation. Collagen deposition was quantitated by Sirius red staining and digital imaging techniques. Hepatocyte apoptosis and liver injury (bile infarcts and serum ALT values) were reduced in IDN-6556-treated versus saline-treated 3-day BDL mice. Markers for liver inflammation [chemokine (C-X-C) ligand 1 and macrophage inflammatory protein-2 chemokine expression] and hepatic fibrogenesis (transforming growth factor-β and collagen I expression) were also attenuated. Consistent with these data, HSC activation as assessed by α-smooth muscle actin mRNA expression and immunohistochemistry was markedly reduced in both 3- and 10-day BDL animals. Collectively, these data suggest hepatocyte apoptosis initiates cascades culminating in liver injury and fibrosis. The pan-caspase inhibitor IDN-6556 is a promising agent for cholestatic liver injury.

Apoptosis and fibrosis are both ubiquitous features of chronic liver injury (Canbay et al., 2002; Yoon and Gores, 2002). Over time, the maladaptive fibrogenic response of the liver to injury culminates in cirrhosis, a pathological condition characterized by an interconnecting web of dense fibrous strands comprised of collagen. Cirrhosis, when advanced, leads to several deleterious sequela of chronic liver disease, liver failure, and premature death or need for liver transplantation (Kim et al., 2002). Collagen I, the principal collagen responsible for cirrhosis, is generated within the liver by activated hepatic stellate cells (HSCs), a resident perisinusoidal cell of the liver. These cells are normally quiescent fat-storing cells but undergo activation to a myofibrolast-like phenotype during liver injury, generating collagen I (Friedman, 2000). Apoptosis has been linked to fibrosis by two opposing processes. Hepatocyte apoptosis by death receptors seems to be profibrogenic. For example, genetic or small interfering RNA ablation of Fas expression, a death receptor richly expressed in the liver, attenuates fibrosis in murine models of liver injury (Canbay et al., 2002; Song et al., 2003). In contrast, HSC apoptosis is thought to be essential for the resolution phase of fibrosis (Iredale et al., 1998; Iredale, 2001; Issa et al., 2001). Activated HSCs seem to undergo an activation-associated cell death terminating their life span and limiting hepatic fibrogenesis. Inhibition of HSC apoptosis would be expected to be profibrogenic by permitting the accumulation of these activated cells within the liver. Therefore, the net effect of broadly inhibiting liver cell apoptosis is unclear; it may be antifibrogenic by blocking hepatocyte apoptosis or profibrogenic by inhibiting HSC apoptosis. Furthermore, different drugs might have different cellular specificity; however, the net effect of the drug’s administration on hepatic fibrosis is probably the most important. This issue needs to be resolved by preclinical trials before embarking on human trials with apoptosis inhibitors. Although inhibition of Fas death receptor-mediated apo-

ABBREVIATIONS: HSC, hepatic stellate cell; BDL, bile duct ligated/ligatured; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; ALT, alanine aminotransferase; TGF, transforming growth factor; PCR, polymerase chain reaction; KC, chemokine (C-X-C) ligand 1; MIP, macrophage inflammatory protein; α-SMA, alpha smooth muscle actin.
ptosis reduces hepatic fibrosis, studies cannot be equated with broad inhibition of apoptosis. Death receptors signal through several intracellular cascades, many of which are proinflammatory rather than death-producing. For example, activation of nuclear factor-κB by death receptors is proinflammatory (Jaeschke et al., 1998). Consistent with this concept, Fas-activation in the liver is proinflammatory and triggers the production of several inflammatory chemokines (Faouzi et al., 2001). The resulting inflammation from Fas activation may result in HSC activation and fibrosis (Maher, 2001). In this context, blocking Fas stimulation may prevent hepatic fibrosis by blocking inflammation, not apoptosis. Therefore, the elegant studies examining the relationship between Fas expression and hepatic fibrosis do not directly address the question as to whether broad-based antiapoptotic therapy would be pro- or antifibrogenic. Given the complexity of the relationships between apoptosis, inflammation, and fibrosis, it is important that the in toto effects are evaluated experimentally and then clinically.

Apoptosis is executed by a family of intracellular proteases, referred to as caspases (Thornberry, 1998; Thornberry and Lazebnik, 1998). Although 13 mammalian caspases have been cloned and differ structurally, all caspases cleave on the carboxyl side of aspartate residues. Furthermore, these proteases, which are synthesized as zymogens, are themselves activated by cleavage at aspartate sites (Shi, 2002). Thus, caspase activation requires caspase activity. Because these common characteristics are shared by all caspases, it is possible to synthesize broad-spectrum caspase inhibitors that are very effective in blocking cell death (Hoglen et al., 2001; Natori et al., 2003; Valentino et al., 2003). The use of a pan-caspase pharmacological inhibitor could, therefore, be used to ascertain whether blocking liver cell apoptosis is pro- or antifibrogenic.

The aims of the current study were to examine the effects of the pan-caspase inhibitor IDN-6556 on liver injury and fibrogenesis. This drug is the first pan-caspase inhibitor of apoptosis to enter clinical trials (Valentino et al., 2003). The bile duct ligated (BDL) mouse was used for these studies because it duplicates the hepatocyte apoptosis, HSC activation, and liver fibrosis observed in human liver diseases (Miyoshi et al., 1999; Canbay et al., 2002). To address our aim, several questions were formulated. Specifically, we asked does the pan-caspase inhibitor 1) reduce hepatocyte apoptosis? 2) reduce hepatic inflammation? 3) promote or inhibit HSC activation? and 4) attenuate hepatic fibrosis? The results demonstrate that the pan-caspase inhibitor IDN-6556 reduces hepatocyte apoptosis, liver injury, hepatic inflammation, HSC activation, and hepatic fibrosis; this drug would seem to have potential for the treatment of human liver diseases.

Materials and Methods

Extrahepatic Cholestasis by Ligation of the Common Hepatic Duct. The use and the care of the animals were reviewed and approved by the Institutional Animal Care and Use Committee at the Mayo Clinic. C57/BL6 mice 6 to 8 weeks of age were used for these studies. Common BDL was performed as described by us in detail previously (Miyoshi et al., 1999). Sham-operated mice, used as controls, underwent a laparotomy with exposure but not ligation of the common bile duct. In selected experiments, mice were treated with the pan-caspase inhibitor. This drug binds irreversibly to activated caspasas with a K_i value in the low to subnanomolar concentrations. The binding is selective, because it does not bind to other cysteine or serine enzymes at nanomolar or even low micromolar concentrations (>10 μM). IDN-6556 (10 mg/kg) in sterile H_2O mannitol phosphate buffer was given i.p. 3 h after the BDL, and twice a day thereafter. The dosing was based on preliminary data demonstrating that IDN-6556 at doses of 10 mg/kg was required to prevent liver injury in this model. The agent was obtained from Idun Pharmaceuticals, Inc. (San Diego, CA).

Histology and the TUNEL Assay, and Immunofluorescence for Active Caspases 3 and 7. The liver sections were fixed in 4% paraformaldehyde for 48 h and then embedded in paraffin (Curtin Matheson Scientific Inc., Houston, TX). Tissue sections (4 μm) were prepared using a microtome (Reichert Scientific Instruments, Buffalo, NY) and placed on glass slides. Hematoxylin/eosin staining was performed following standard techniques. TUNEL assay was performed using a commercially available kit, following the manufacturer's instructions (In Situ Cell Death Detection kit; Roche Diagnostics, Indianapolis, IN). Hepatocyte apoptosis in liver sections was quantitated by counting the number of TUNEL-positive cells in 30 random microscopic low-power fields (630×) as described previously (Miyoshi et al., 1999). Immunofluorescence for active caspases 3 and 7 was performed as described previously (Natori et al., 2001). Briefly, tissue sections were deparaffinized in xylene, rehydrated with ethanol series, and washed three times with phosphate-buffered saline for 3 min. The tissue sections were blocked for 60 min in 37°C in blocking buffer (5% goat serum, 5% glycerol, and 0.004% sodium azide). The specimens were next incubated for 2 h with a rabbit polyclonal anti-active caspase 37 antibody (1:50) recognizing a common neoepitope shared by activated caspases 3 and 7 (CM1; BD Pharmingen, San Diego, CA). The sections were washed in phosphate-buffered saline three times for 10 min and were incubated with the secondary antibody (1:50 fluorescein isothiocyanate-conjugated swine anti-rabbit antibody immunoglobulins (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 37°C for 45 min. The slides were mounted with ProLong antifade kit as described in manufacturer's instructions (Molecular Probes, Eugene, OR).

Determination of Serum ALT Determination. Serum alanine aminotransferase (ALT) determinations were performed using commercially available assay kits following the manufacturer's instructions (Sigma Diagnostics kit no. 505; Sigma-Aldrich, St. Louis, MO).

Real-Time-Polymerase Chain Reaction (PCR). Total RNA was obtained from whole liver using the TRIzol Reagent (Invitrogen, Carlsbad, CA). For each RNA sample, a 10-μg aliquot was reverse transcribed into cDNA using oligo-dT random primers and Moloney Murine Leukemia virus reverse transcriptase. Real-time PCR was performed using Taq polymerase (Invitrogen) and primers for α-smooth muscle actin (α-SMA), collagen I (COL1A1), transforming growth factor (TGF)-β1, chemokine (C-X-C) ligand 1 (KC), and macrophage inflammatory protein (MIP)-2 as described by us previously (Canbay et al., 2002, 2003b). 18S primers (Ambion, Austin TX) were used as a control for RNA isolation and integrity. All PCR products were confirmed by gel electrophoresis.

Real-time PCR was performed using the LightCycler (Roche Diagnostics, Mannheim, Germany) and SYBR green as the fluorophore (Molecular Probes). The results were expressed as a ratio of product copies per milliliter to copies per milliliter of housekeeping gene 18S from the same RNA (respective cDNA) sample and PCR run.

Determination of Liver Fibrosis. Liver fibrosis was quantified using Sirius red. Direct red 80 and fast green FCF (color index 752) for Sirius red (chemokine (C-X-C) ligand 1 (KC), and macrophage inflammatory protein (MIP)-2 as described by us previously (Canbay et al., 2002, 2003b). 18S primers (Ambion, Austin TX) were used as a control for RNA isolation and integrity. All PCR products were confirmed by gel electrophoresis.

Real-time PCR was performed using the LightCycler (Roche Diagnostics, Mannheim, Germany) and SYBR green as the fluorophore (Molecular Probes). The results were expressed as a ratio of product copies per milliliter to copies per milliliter of housekeeping gene 18S from the same RNA (respective cDNA) sample and PCR run.

Immunohistochemistry for α-SMA. The sections (4 μm in thickness) were stained for α-SMA using a mouse monoclonal antibody (NeoMarkers, Fremont, CA), which is prelabeled by the manufacturer for staining formalin-fixed paraffin-embedded tissues. The
sections were incubated with the antibody overnight at 4°C. Negative control slides were incubated with nonimmune immunoglobulin under the same conditions. Secondary reagents were obtained from the LSAB2 kit (DAKO, Carpinteria, CA), and 3,3’-diaminobezidine tetrahydrochloride (Sigma-Aldrich) was used for visualization. Finally, the tissue was counterstained with hematoxylin.

**Statistics.** All data represent at least four independent experiments and are expressed as the mean ± S.E.M. unless otherwise indicated. Differences between groups were compared using analysis of variance for repeated measures and a post hoc Bonferroni test to correct for multiple comparisons. A p value less than 0.05 was considered to be statistically significant.

**Results**

*Is Hepatocyte Apoptosis Attenuated in IDN-6556-Treated BDL Mice?* We first examined the effects of the caspase inhibitor IDN-6556 on liver cell apoptosis assessed 3 days after BDL. Apoptosis was quantitated using the TUNEL assay as described under Materials and Methods. TUNEL-positive cells (low-power field) were significantly reduced in IDN-6556-treated mice compared with saline-treated mice (Fig. 1, A and B). Immunohistochemistry for activated caspases 3/7 was also performed to biochemically confirm the occurrence of apoptosis and to determine the efficiency of the pan-caspase inhibitor IDN-6556. The number of caspase 3/7-positive cells increased after BDL and quantitatively were similar to that obtained with the TUNEL assay (Fig. 1C). Likewise, in BDL animals receiving IDN-6556, the number of caspase 3/7-positive cells were significantly reduced (Fig. 1C). These data demonstrate that IDN-6556 does attenuate hepatocyte apoptosis in the 3-day BDL mouse.

*Is Liver Injury Reduced in IDN-6556-Treated BDL Mice?* To examine the effects of caspases in mediating liver injury, liver histology and serum ALT values were examined in BDL mice treated with saline or IDN-6556. Histopathologic examination of liver specimens demonstrated bile ductular proliferation, portal edema, and mild portal infiltrates in saline-treated mice and in IDN-6556-treated mice, indicating similarly ductular response of the liver to large bile duct obstruction in all groups (Fig. 2A). Confluent foci of hepatocyte feathery degeneration due to bile acid cytotoxicity (bile infarcts) were reduced in IDN-6556-treated BDL animals versus saline-treated (23 ± 4.9 versus 4 ± 1 per low power field; p < 0.01) (Fig. 2A). Serum ALT values, an index of hepatocellular injury, were also significantly lower in BDL IDN-6556-treated compared with saline-treated BDL mice (Fig. 2B). These data implicate a pathogenic role for caspase-mediated hepatocyte apoptosis in liver damage due to cholestasis.

*Hepatic Inflammation Is Reduced in IDN-6556-Treated BDL Mouse.* Hepatocyte apoptosis can be a proinflammatory process in the liver that promotes chemokine
generation and neutrophil infiltration (Jaeschke et al., 1998; Lawson et al., 1998; Faouzi et al., 2001; Canbay et al., 2003b). To examine the effects of caspase-dependent hepatocyte apoptosis in mediating liver inflammation, mice were BDL for 3 days. Messenger RNA transcripts of KC and MIP-2, potent neutrophil chemoattractants, and markers of hepatic inflammation were quantitated using real-time PCR technology (Luster, 1998). In saline-treated animals BDL for 3 days, the mRNA for these two chemokines was significantly increased compared with IDN-6556-treated BDL animals. (Fig. 3, A and B). These data suggest inflammation occurs in cholestasis and is coupled to caspase-dependent liver injury.

**Are Liver Fibrogenesis and HSC Activation Reduced in IDN-6556-Treated BDL Mice?** To investigate the role of caspase-dependent liver injury in liver fibrogenesis, mRNA was extracted from the livers of 3-day saline-treated and IDN-6556-treated BDL mice. α-SMA messenger RNA transcripts, an established marker for HSC activation, were quantitated using real-time PCR technology, as described under Materials and Methods. In saline-treated 3-day BDL animals, α-SMA mRNA transcripts were significantly increased compared with sham-operated controls, indicating HSC activation in cholestasis (Fig. 4A). In contrast, the transcripts for α-SMA were significantly reduced in IDN-6556-treated BDL animals compared with saline-treated BDL mice (Fig. 4A). These results were confirmed at the protein and cellular level by performing α-SMA immunohistochemistry in 10-day BDL animals. This duration of BDL was selected to permit the potential accumulation of activated HSC in animals receiving the active drug. Consistent with the

**Fig. 4.** Markers for HSC activation are increased in untreated compared with IDN-6556-treated BDL mice. Three days after the surgical procedure, liver tissue was procured and total hepatic RNA was isolated as described under Materials and Methods. α-SMA, TGF-β1, and collagen 1α (I) (COL1A1) were quantitated by real-time PCR. The expression was normalized as a ratio using 18S mRNA as a housekeeping RNA. A value of one for this ratio was arbitrarily assigned to the data obtained from sham operated saline-treated mice. A, α-SMA mRNA expression in saline-treated BDL mice was significantly greater in IDN-6556-treated BDL mice (p < 0.001, n = 4 for each group). B, immunoreactivity for α-SMA was increased in saline-treated compared with IDN-6556-treated 10-day BDL mice. The immunoreactivity was sinusoidal in location consistent with staining of HSC or myofibroblasts. Indeed, liver sections from saline-treated and IDN-6556-treated sham operated mice displayed little to no α-SMA immunoreactivity (original magnification, 20×). C, expression of TGF-β1 mRNA was greater in saline-treated than IDN-6556-treated BDL mice (p < 0.005, n = 4 for each group). D, expression of collagen 1α (I) mRNA was also significantly elevated in saline-treated BDL mice and attenuated in IDN-6556-treated BDL mice (p < 0.001, n = 4 for each group).
mRNA data, the number of α-SMA-positive cells was actually reduced in drug-treated BDL mice compared with saline-treated BDL animals (Fig. 4B). To ascertain whether other markers for HSC activation were also reduced in drug-treated BDL mice, transcripts for molecules implicated in fibrogenesis were quantitated in TGF-β-treated BDL mice, transcripts for molecules implicated in fibrogenesis were also reduced in IDN-6556-treated BDL animals (Fig. 4B). To ascertain whether other markers for HSC activation were also reduced in drug-treated BDL mice compared with saline-treated BDL animals (original magnification, 20×/H11003).

The current studies relate caspase inhibition with hepatocyte apoptosis and fibrogenesis in cholestasis. The observations demonstrate that, in the bile duct ligated animal, pharmacological inhibition of caspases reduces 1) hepatocyte apoptosis, serum ALT values, and histological evidence of liver injury; 2) hepatic inflammation; 3) mRNA expression for markers of HSC activation; and 4) collagen expression and deposition. Together, these observations suggest a critical role for hepatocyte apoptosis in the initiation of HSC activation and hepatic fibrogenesis during cholestatic liver injury. Each of these observations is discussed in greater detail below.

Hepatocyte apoptosis after BDL in the mouse is mediated, in part, by the death receptors Fas and death receptor 5/tumor necrosis factor-related apoptosis inducing ligand-receptor 2 (Miyoshi et al., 1999; Higuchi et al., 2002). Apoptosis by death receptors is caspase-dependent (Ashkenazi and Dixit, 1998; Hengartner, 2000). Therefore, our current observations demonstrating that IDN-6556 inhibits hepatocyte apoptosis in the BDL mouse is consistent with these prior observations. Also, as observed in this study, prior publications have linked hepatocyte apoptosis with liver injury (e.g., bile infarcts) and elevated ALT values, and a reduction in these parameters by inhibiting apoptosis. However, the current observations demonstrate that a pan-caspase inhibitor currently in clinical trials for the treatment of liver disease has a salutary benefit in a cholestatic disease model. Based on these collective data, this class of drugs merits further study in human cholestatic liver disease.

Our study suggests that apoptosis not only is associated with liver injury but is also proinflammatory in the liver during cholestasis. In this study, inhibition of apoptosis with the pan-caspase inhibitor reduced hepatic expression of chemokines. Apoptosis may induce inflammation by several mechanisms. First, dysregulated apoptosis in pathological conditions can disrupt hepatocyte integrity. For example, after experimental induction of apoptosis with Fas-agonists mice develop fulminant hepatic failure, with massive necrosis and inflammation (Ogasawara et al., 1993). Second, death receptor-mediated apoptosis may contribute to liver inflammation, possibly by initiating proinflammatory signaling cascades (Chen et al., 1998; Jaeschke et al., 1998; Kurosaka et al., 2001). For example, Fas agonists induce chemokine expression (Faouzi et al., 2001), and hepatocyte apoptosis is a potent stimulus to neutrophil extravasation (Lawson et al., 1998). Consistent with this concept, a role for neutrophils in liver injury after BDL has recently been reported highlighting a potential relationship between apoptosis and inflammation (Gujral et al., 2003). The disposition of apoptotic bodies may also link apoptosis to inflammation in the liver. For example, engulfment of neutrophil apoptotic bodies by macrophages and/or Kupffer cells can induce expression of death ligands, especially Fas ligand (Kiener et al., 1997; Kurosaka et al., 2001; Geske et al., 2002; Canbay et al., 2003a), thereby accelerating apoptosis. Finally, caspases may directly contribute to liver inflammation. Caspases 1, 4, 5, and 11 have all been implicated in proinflammatory cascades (Thornberry and Lazebnik, 1998). A pan-caspase inhibitor such as IDN-6556 may also block liver inflammation...
by inhibiting these caspases, in addition to reducing cellular apoptosis. Thus, likely by a combination of mechanisms, broad spectrum caspase inhibition is salutary against liver inflammation during cholestasis.

Rather than promoting accumulation of activated HSC, the caspase inhibitor actually blocked their activation. The results suggest apoptosis plays an instrumental if not a pivotal role in HSC activation and hepatic fibrogenesis. Without significant HSC activation, the fear that a caspase inhibitor would block apoptosis of activated HSC, thereby promoting fibrosis, is not a concern. The mechanism by which hepatocyte apoptosis results in HSC activation may be direct or indirect. A direct pathway linking hepatocyte apoptosis to HSC activation is stellate cell engulfment of hepatocyte apoptotic bodies as has been documented in vitro (Canbay et al., 2003c). Phagocytosis of apoptotic bodies by HSC results in their activation and promotes collagen expression. An indirect mechanism coupling hepatocyte apoptosis to HSC activation is via a secondary inflammatory response. As described above, hepatocyte apoptosis elicits an inflammatory response associated with chemokine expression and neutrophil infiltration (Maher et al., 1997; Lawson et al., 1998; Miwa et al., 1998; Jaeschke, 2002; Canbay et al., 2003b). This inflammatory response has been well established to cause HSC activation (Maher, 2001). In either model, hepatocyte apoptosis is an apical event resulting in liver injury, HSC activation and liver scarring.

In summary, our findings suggest that during extrahepatic cholestasis in the mouse, both liver injury, inflammation, markers of HSC activation, and elevation of indices of hepatic fibrogenesis are, in part, hepatocyte apoptosis-dependent. Inhibition of hepatocyte apoptosis with a selective caspase inhibitor seems to be a viable therapeutic option for cholestatic liver injury, inflammation and fibrosis. These data also implicate a mechanistic link between hepatocyte apoptosis or the inflammatory response to apoptosis and HSC activation. These preclinical studies support the employment of caspase inhibitors in the treatment of cholestatic and potentially other liver diseases. However, the potentially proneoplastic consequences of prolonged and potent apoptosis inhibition will need to be considered and thorough monitoring processes put into place.

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References


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