Transcriptional Profiling After Bile Duct Ligation Identifies PAI-1 as a Contributor to Cholestatic Injury in Mice

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Extrahepatic cholestasis leads to complex injury and repair processes that result in bile infarct formation, neutrophil infiltration, cholangiocyte and hepatocyte proliferation, extracellular matrix remodeling, and fibrosis. To identify early molecular mechanisms of injury and repair after bile duct obstruction, microarray analysis was performed on liver tissue 24 hours after bile duct ligation (BDL) or sham surgery. The most upregulated gene identified encodes plasminogen activator inhibitor 1 (PAI-1, Serpine 1), a protease inhibitor that blocks urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) activity. Because PAI-1, uPA, and tPA influence growth factor and cytokine processing as well as extracellular matrix remodeling, we evaluated the role of PAI-1 in cholestatic liver injury by comparing the injury and repair processes in wild-type (WT) and PAI-1–deficient (PAI-1$^{-/-}$) mice after BDL. PAI-1$^{-/-}$ mice had fewer and smaller bile infarcts, less neutrophil infiltration, and higher levels of cholangiocyte and hepatocyte proliferation than WT animals after BDL. Furthermore, PAI-1$^{-/-}$ mice had higher levels of tPA activation and mature hepatocyte growth factor (HGF) after BDL than WT mice, suggesting that PAI-1 effects on HGF activation critically influence cholestatic liver injury. This was further supported by elevated levels of c-Met and Akt phosphorylation in PAI-1$^{-/-}$ mice after BDL. In conclusion, PAI-1 deficiency reduces liver injury after BDL in mice. These data suggest that inhibiting PAI-1 might attenuate liver injury in cholestatic liver diseases. (HEPATOLOGY **2005;42:1099-1108.**)

Biliary tract obstruction is a common mechanism of hepatic injury in a variety of clinical settings, including obstructing neoplasms, post-operative bile duct injury, biliary atresia, sclerosing cholangitis, and primary biliary cirrhosis. For this reason, understanding mechanisms that control the liver's response to cholestatic injury and determine the extent and rate of repair is a necessary first step toward the development of new therapeutic interventions.

Extrahepatic cholestasis can be modeled by surgical bile duct ligation (BDL) in rodents. This method reliably induces stereotypical histopathological changes, including hepatocellular necrosis, neutrophil infiltration, cholangiocyte and hepatocyte proliferation, stellate cell activation, and progressive fibrosis. BDL also causes early mortality in mice and mimics human cholestatic liver disease. To identify proteins that influence liver injury during biliary tract obstruction, we performed microarray analysis on liver tissue obtained from mice 24 hours after BDL or sham surgery. Although the expression of many genes changed significantly, the most dramatic change was for PAI-1. PAI-1 inhibits tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA), the enzymes that convert plasminogen to the active protease plasmin.[1] Because tPA, uPA, and plasmin activate many growth factors and cytokines that influence liver injury,[2] including hepatocyte growth factor (HGF),

Abbreviations: BDL, bile duct ligation; tPA, tissue-type plasminogen activator; uPA, urokinase plasminogen activator; HGF, hepatocyte growth factor; PAI-1, plasminogen activator inhibitor-1; WT, wild type; qRT-PCR, quantitative real time polymerase chain reaction; HRP, horseradish peroxidase; GGT, gamma-glutamyltransferase; BrdU, bromodeoxyuridine; Cxcl1, chemokine (C-X-C motif) ligand 1; Cxcl2, chemokine (C-X-C motif) ligand 2.

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Table 1. Primers for qRT-PCR

Accession Number	Gene Symbol	Unigene Title		Sequences*
M33960	PAI-1	Plasminogen activator inhibitor 1	f	ACTGCAAAAGGTCAGGATCG
			r	ACAAAGGCTGTGGAGGAAGA
M17922	uPA	Urokinase-type plasminogen activator	f	CCCCACTTGATCCTTGCTTA
			r	TTCTGACAGCCACAGTTTGC
J03520	tPA	Tissue-type plasminogen activator	f	GATGAAGGTCTGGCTTTGGA
			r	TATGGAAGGTTGGCATCTCC
J04766	plasmgn	Plasminogen	f	ACCCCTCATAGGCACAACAG
			r	CCGGCAGTAGTTCTCTTCCA
NM_010427	HGF	Hepatocyte growth factor	f	ACGCGGATGGTTTATTACGA
			r	TGATGGTGCTGACTGCATTT
NM_008176	CXCL1	chemokine (C-X-C motif) ligand 1	f	TGGGATTCACCTCAAGAACA
			r	TGGGGACACCTTAGCATC
NM_009140	CXCL2	chemokine (C-X-C motif) ligand 2	f	CCACCAACCACCAGGCTAC
			r	GCTTCAGGGTCAAGGGCAAA

*5′ to 3′ sequences; f = forward primer; r = reverse-strand primer.

it seemed likely that plasminogen activator inhibitor-1 (PAI-1) would critically alter the liver's response to cholestatic injury.[3-11] The role of PAI-1 has not, however, been studied in biliary obstruction.

In this study, we demonstrate that PAI-$1^{-/-}$ mice have fewer and smaller bile infarcts, less neutrophil infiltration, and more cholangiocyte and hepatocyte proliferation than wild-type (WT) mice after BDL. This altered response to cholestasis correlates with elevated hepatic tPA activity, increased mature HGF, and increased Met receptor activation in PAI-$1^{-/-}$ compared with WT mice after BDL. Reduced levels of the mRNA for neutrophil chemoattractants Cxcl1 and Cxcl2 are seen in PAI-$1^{-/-}$ compared with WT mice. These data suggest that blocking PAI-1 activity could provide a novel strategy for treating cholestatic liver disease.

Materials and Methods

Mouse Model of Extrahepatic Cholestasis. Eight- to 10-week-old (22-26 g) male C57BL/6J WT (#0664) and homozygous C57BL/6J PAI-$1^{-/-}$ mice (#2507) were obtained from the Jackson Laboratory (Bar Harbor, ME). The common bile duct was double ligated below the bifurcation, single ligated above the pancreas, and transected between the ligatures under methoxyflurane (Schering-Plough Co, Union, NJ) anesthesia. Sham operated mouse controls underwent a similar laparotomy without bile duct ligation. The use and care of mice were accredited and approved by the Washington University Animal Care Committee.

DNA Microarray Analysis. Total RNA isolated using TRI Reagent (Sigma, St. Louis, MO) was purified with an RNeasy mini kit (Qiagen, Valencia, CA). Twenty micrograms cRNA was hybridized to a mouse GeneChip (U74Av2, Affymetrix, Santa Clara, CA) at the Siteman Cancer Center GeneChip facility as described by the manufacturer. Analyses used one mouse per chip. Gene expression changes were analyzed using Affymetrix MicroArray Suite 4.0 and GeneChip 3.1 Expression Analysis and Statistical Algorithms (Affymetrix). The complete methodology and full data sets for all 6 analyzed chips are available at http://bioinformatics.wustl.edu.

Quantitative Real-Time PCR (qRT-PCR). mRNA isolated using Micro-FastTrack 2.0 mRNA isolation kit (Invitrogen, Carlsbad, CA) was reverse transcribed using SMART polymerase chain reaction (PCR) cDNA synthesis kit (Clontech Inc, Palo Alto, CA). Quantitative real time polymerase chain reaction (qRT-PCR) was performed in duplicate using SYBR green PCR Master mix (Applied Biosystems, Foster City, CA) and an iCycler iQ (Bio-Rad, Hercules, CA). Primers are shown in Table 1. RNA content was normalized based on amplification of glyceraldehyde-3-phosphate dehydrogenase.

Bile Infarcts Analysis. Four-micron paraffin sections were stained with hematoxylin-eosin. Bile infarct number was counted in 10 randomly selected 1-mm^2 regions per mouse. Bile infarct area was determined using NIH imageJ 1.30 (NIH, Bethesda, MD) in 10 randomly selected 0.63-mm^2 regions. The percentage of the liver section occupied by bile infarcts = total area of bile infarcts divided by (total area of the field − vascular lumen area) × 100%. All microscopic evaluations were performed in a blinded fashion, and the same region of the liver was studied to minimize potential effects of regional variability.

Neutrophil Analysis. Livers sections were stained for myeloperoxidase (MPO), a specific neutrophil marker, using a rabbit polyclonal antibody (1:1000, NeoMarkers, Fremont, CA) and horseradish peroxidase (HRP) coupled detection system (Accurate Chemical & Scientific Corp,

Westbury, NY). Tissues were counterstained with hematoxylin. Neutrophils in sinusoids (not extravasated) and extravasated into parenchymal tissues were counted in 20 randomly selected 0.25-mm^2 regions.

Serum Biochemistry. Serum total and direct bilirubin, gamma-glutamyl transpeptidase (GGT), and serum alanine aminotransferase were measured using commercial assay kits and the manufacturer's instructions (kits #2692, #960, #0930 Stanbio, Boerne, TX).

Quantification of Hepatocyte and Cholangiocyte Proliferation. Liver sections from mice injected with bromodeoxyuridine (BrdU, Sigma) (100 μg/g body weight) 1 hour before sacrifice were evaluated after immunostaining with a BrdU antibody (Accurate Chemical & Scientific Corp) and an HRP coupled detection method. Sections were hematoxylin counterstained. Ten randomly selected 62.5 μm^2 regions were evaluated for BrdU-positive hepatocytes (>1,000 hepatocytes per mouse) and cholangiocytes (>500 cholangiocytes per mouse). Bile ducts were categorized based on the number of circumferential cholangiocytes (small, $\leq$ 10 cells; 10 cells $<$ medium $\leq$ 20 cells; and large $>$ 20 cells).[12] Ductular reaction was assessed after immunohistochemistry using a rabbit polyclonal antibody against bovine cytokeratin (1:200, DakoCytomation, Carpinteria, CA), a specific cholangiocyte marker,[13] and an HRP-coupled detection system. Tissues were counterstained with hematoxylin. Area occupied by cholangiocytes was determined in 10 randomly selected non-overlapping 0.63-mm^2 fields/mouse using NIH imageJ 1.30.

Hepatocyte Growth Factor, Akt, and Phospho-Akt Immunoblotting. Liver (100 mg) was homogenized in 1 mL buffer A (100 mmol/L Tris, 0.5% Triton X-100 [pH 7.6]) plus protease inhibitor cocktail (Complete Mini, Roche, Penzberg, Germany) and centrifuged (15,000*g*, 15 minutes) before analyzing the supernatant. One hundred micrograms protein/sample was separated by electrophoresis before transfer to Hybond nitrocellulose membranes (Amersham, Buckinghamshire, UK). Equal loading was confirmed by Ponceau S membrane staining. Membranes were blocked with 5% nonfat dry milk (1 hour) in TBST buffer (100 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% Tween-20, pH 7.8), and then incubated with goat anti-human α-HGF antibody (1:100, N-17, Santa Cruz),[14] anti-AKT or anti-phospho-AKT (Ser473) antibodies at 4°C overnight (1:1,000, Cell Signaling, Beverly, MA). HRP-conjugated secondary antibody (2 hours, 25°C), enhanced chemiluminescence reagent, and Hyperfilm (Amersham) were used to visualize immunoreactive proteins. Band optical density was determined using NIH ImageJ 1.30.

Met Phosphorylation Analysis. c-Met was immunoprecipitated from 3 mg of liver protein homogenate in 1 mL lysis buffer (Tris 50 mmol/L, pH = 7.6, NaCl 150 mmol/L, EDTA 5 mmol/L, EGTA 1 mmol/L, sodium dodecyl sulfate [SDS] 1%, Na_3VO_4 1 mmol/L, NaF 0.1 mol/L, β-glycerophosphate 1 mmol/L, and protease inhibitor cocktail [Roche]) by incubating with anti–c-Met antibody (3 μg, 4°C, 1 hour, SP260, Santa Cruz) and then precipitating with protein G-PLUS-agarose (20 μL, 4°C, overnight, Santa Cruz). Beads were washed 3 times in the lysis buffer, and bound protein was subjected to immunoblot analysis using anti-phosphotyrosine antibody (1:1,000, clone 4G10, Upstate, Waltham, MA) or anti–c-Met antibody (1:100).

Analysis of tPA, uPA, and Plasmin Activity. One hundred milligrams liver homogenized in 1 mL buffer A without protease inhibitors was centrifuged (15,000*g*, 15 min), and supernatant was analyzed. Fifty micrograms protein was electrophoresed on a 10% SDS-polyacrylamide gel containing 1 mg/mL α-casein and 10 μg/mL plasminogen (Sigma) in a non-reducing Tris-glycine SDS running buffer. Gels were washed (30 minutes, 25°C) in renaturing buffer (50 mmol/L Tris [pH 7.6], 2.5% Triton X-100), incubated in zymogram developing buffer (Novex; 37°C, 42 hours), then stained with 0.1% Coomassie Brilliant blue G250/45% methanol/10% acetic acid (1 hour) and de-stained (45% methanol/10% acetic acid) until negative bands indicating protease activity appeared. Gels were scanned (Epson Expression 800 scanner, Nagano, Japan) and bands quantified with NIH imageJ 1.30. Plasminogen activator–specific bands were verified by their absence in an identical gel that lacked plasminogen. Hepatic plasmin activity was also measured using a plasmin-specific fluorogenic substrate (0.2 mmol/L N-Succinyl-Ala-Phe-Lys 7-amido-4-methylcoumarin acetate (AFK-AMC, S0763, Sigma) by comparing the change in fluorescence between 5 and 10 minutes (Fluotomark Microplate fluorometer [Bio-Rad]; excitation 355 nm; emission 460 nm) to a standard curve made with human plasmin (P1867, Sigma).

Statistical Analysis. For every experiment, data were derived from 3 to 6 bile duct–ligated (BDL) or sham operated mice of each genotype. All results are reported as mean $\pm$ SEM. Student *t* test or ANOVA methods were used. $P < .05$ was considered significant.

Results

DNA Microarray Analysis Revealed Significant Increases in PAI-1 After BDL. To gain new insight into molecular mechanisms that influence injury and repair in cholestatic liver disease, we analyzed gene expression in

mouse livers 24 hours after BDL or sham surgery using the Affymetrix U74Av2 arrays (12,488 probe sets). Genes corresponding to 45 probe sets were expressed at higher levels after BDL than after sham surgery. Eight probe sets were expressed at lower levels after BDL than after sham operations. PAI-1 was the most dramatically upregulated gene identified. Real-time PCR confirmed a 20-fold increase in hepatic PAI-1 mRNA 24 hours after BDL compared with sham surgery (Fig. 1A). Because PAI-1 and the plasmin protease system have been implicated in both extracellular matrix remodeling and growth factor processing,[1-8,11,15,16] further analyses focused on PAI-1 function after BDL.

PAI-1, tPA, and uPA mRNA Levels Increase After BDL. To determine how the mRNA levels for PAI-1 and other plasmin protease system components change after BDL, qRT-PCR was performed. Six hours after sham surgery, PAI-1 mRNA levels increased 20-fold (Fig. 1A) but returned to near baseline within 24 hours. In contrast, PAI-1 mRNA levels remained markedly elevated after BDL. Similarly, uPA and tPA mRNA levels gradually increased, reaching 10-fold above baseline within 1 week after BDL, but remained low after sham surgery (Fig. 1B-C). Plasminogen mRNA levels did not change after either BDL or sham surgery. These dynamic changes suggested that the plasmin protease system might critically influence liver injury and repair after bile duct obstruction.

PAI-1$^{-/-}$ Mice Have Fewer and Smaller Bile Infarcts After BDL. During the first week after BDL, predictable changes occur in liver architecture, including neutrophil infiltration, bile infarct formation, and then cholangiocyte and hepatocyte proliferation. To determine whether PAI-1 influences the liver's response to biliary tract obstruction, BDL was performed on PAI-1$^{-/-}$ and WT animals. Serum studies showed comparable bilirubin levels in both groups of mice after BDL (*e.g.,* 1 week after BDL, WT: 9.9 ± 2.2 mg/dL; PAI-1$^{-/-}$: 10.1 ± 1.7 mg/dL, $P = .9$), but considerably lower GGT levels in PAI-1$^{-/-}$ mice compared with WT controls (*e.g.,* 72 hours after BDL, WT: 86 ± 10 U/L; PAI-1$^{-/-}$: 39 ± 4 U/L, $P = .003$). Mean alanine aminotransferase levels were also lower in PAI-1$^{-/-}$ mice, but this was not statistical significant (*e.g.,* 72 hours after BDL, WT: 1,044 ± 364 U/L; PAI-1$^{-/-}$: 572 ± 85 U/L, $P = .4$). To determine whether the reduced GGT levels in PAI-1$^{-/-}$ animals correlated with reduced hepatic injury, bile infarct number and total bile infarct area were evaluated. PAI-1$^{-/-}$ mice had significantly fewer and smaller bile infarcts than WT mice after BDL (Fig. 2A-C) suggesting that PAI-1 deficiency either reduced liver cell injury, encouraged liver repair, or both.

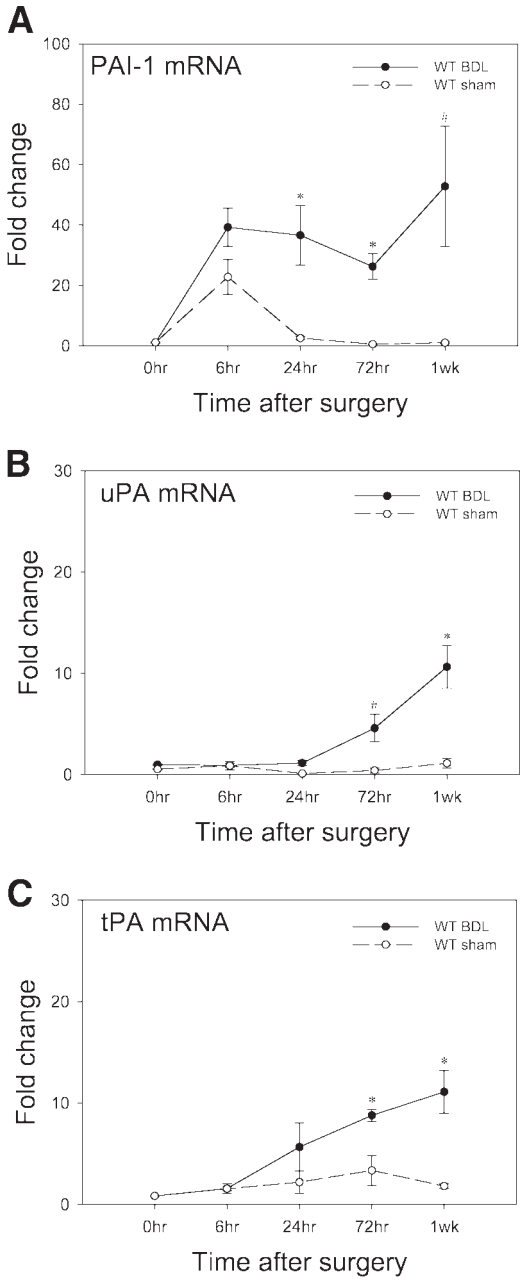

Fig. 1. PAI-1, uPA, and tPA mRNA levels increase after BDL. (A-C) Relative hepatic mRNA levels (compared with glyceraldehyde-3-phosphate dehydrogenase) for PAI-1, uPA, and tPA after BDL or sham surgery were determined by qRT-PCR. To simplify interpretation, data are represented as "fold change" with the assumption that a one-cycle change in crossing threshold corresponds to a 2-fold change in mRNA abundance. *$P < .003$ and #$P < .01$ vs. sham. PAI-1, plasminogen activator inhibitor-1; uPA, urokinase plasminogen activator; tPA, tissue-type plasminogen activator; BDL, bile duct ligation; qRT-PCR, quantitative real time polymerase chain reaction.

PAI-1 Deficiency Reduces Hepatic Inflammation After BDL. Neutrophil infiltration into the liver parenchyma is a significant contributor to liver injury after BDL.[17-19] To determine whether neutrophil infiltration was reduced in PAI-1$^{-/-}$ mice, liver sections obtained 72

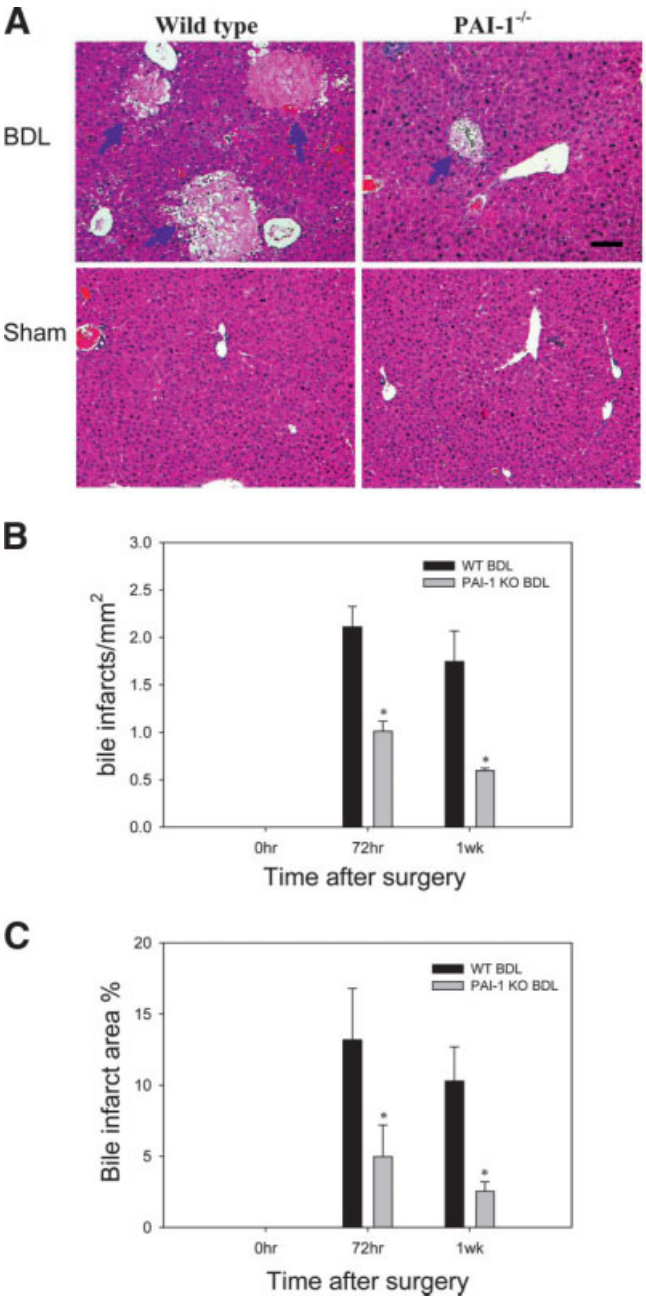

Fig. 2. Bile infarcts are reduced in PAI-$1^{-/-}$ compared with WT mice after BDL. (A) Representative hematoxylin-eosin–stained liver sections obtained 72 hours after BDL show increased size and number of bile infarcts (arrows) in WT compared with PAI-$1^{-/-}$ mice. Scale bar = 100 μm. (B) Bile infarct number and (C) area were reduced in PAI-$1^{-/-}$ vs. WT mice (*$P < .01$ vs. WT).

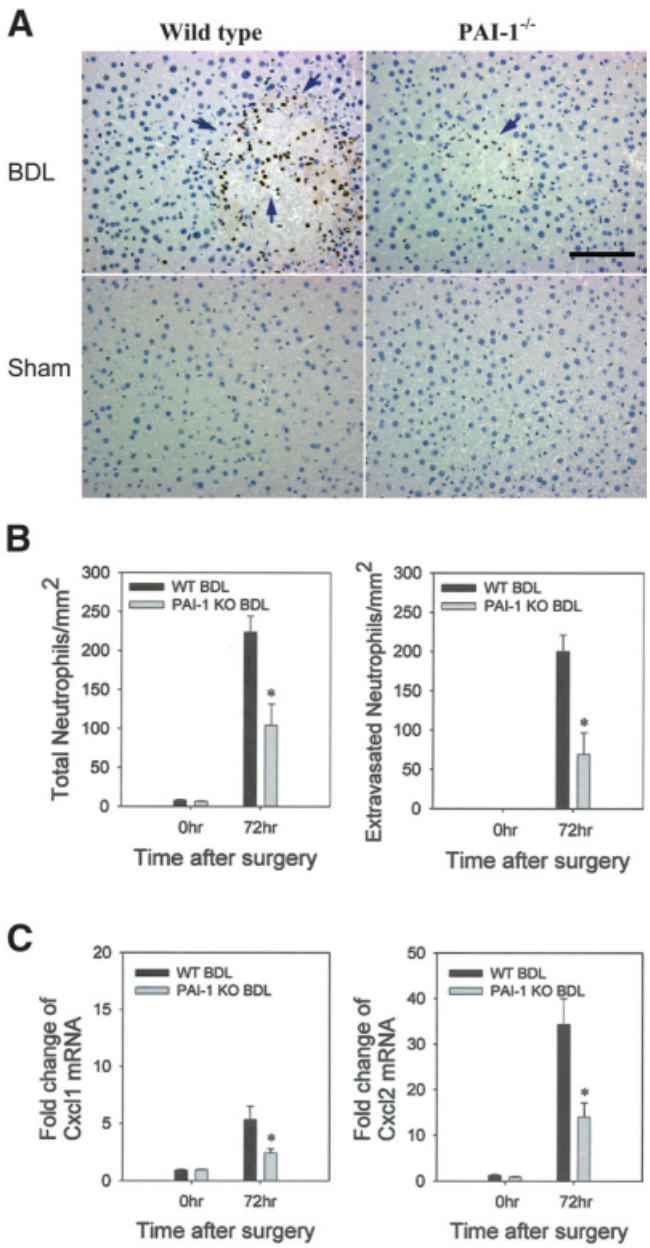

Fig. 3. Neutrophil accumulation and extravasation are reduced in PAI-$1^{-/-}$ mice after BDL. (A) Neutrophils visualized by myeloperoxidase immunohistochemistry are less abundant in PAI-$1^{-/-}$ mice and less frequently extravasated into liver parenchyma. Scale bar = 100 μm. (B) Quantitative analysis of total and extravasated neutrophils 72 hours after BDL confirms reduced neutrophil infiltration in PAI-$1^{-/-}$ mice (*$P < .01$ vs. WT). (C) Cxcl1 and Cxcl2 mRNA levels increase in both WT and PAI-$1^{-/-}$ mice after BDL, but they are less abundant in PAI-$1^{-/-}$ than in WT mice (*$P < .02$ vs. WT). PAI-1, plasminogen activator inhibitor-1; BDL, bile duct ligation; WT, wild-type.

hours after BDL were stained for myeloperoxidase. Sham-operated WT and PAI-$1^{-/-}$ mice each had approximately 7 neutrophils/mm^2, and neutrophils were not extravasated into the hepatic parenchyma (Fig. 3A). After BDL, the total hepatic neutrophil number increased 28-fold in WT mice, and 89% were extravasated (Fig. 3B). In contrast, total hepatic neutrophils increased only 15-fold in PAI-$1^{-/-}$ mice after BDL, and 66% had extravasated. Thus, the number of extravasated neutrophils was reduced by 65% in PAI-$1^{-/-}$ compared with WT mice.

PAI-$1^{-/-}$ Mice Had Reduced Neutrophil Chemoattractant mRNA Levels After BDL. PAI-1 deficiency might reduce hepatic neutrophil infiltration by several potential mechanisms (see Discussion). One possibility is

that reduced hepatocellular injury leads to reduced production of neutrophil chemoattractants in PAI-$1^{-/-}$ mice. To test this hypothesis, mRNA levels for Cxcl1 (KC) and Cxcl2 (MIP-2) were determined by qRT-PCR 72 hours after BDL. Although Cxcl1 and Cxcl2 levels increase after BDL, PAI-$1^{-/-}$ mice accumulated significantly less Cxcl1 or Cxcl2 mRNA than WT animals (Fig. 3C).

PAI-1 Deficiency Increased Hepatocyte and Cholangiocyte Proliferation After BDL. During biliary tract obstruction, significant cholangiocyte proliferation and small increases in hepatocyte proliferation occur. To determine whether PAI-1 deficiency alters cell proliferation after BDL, we measured BrdU incorporation into hepatocytes and cholangiocytes. These studies demonstrated significantly increased proliferation of cholangiocytes in small and medium bile ducts at 72 hours and 1 week after BDL in PAI-$1^{-/-}$ mice (Fig. 4B-C). This correlated with significantly increased ductular reaction after BDL in PAI-$1^{-/-}$ compared with WT animals (Fig. 4A,D). In addition, increased hepatocyte proliferation occurred 1 week after BDL in PAI-$1^{-/-}$ mice (Fig. 4E). Interestingly, cholangiocyte proliferation in large bile ducts was equivalent in WT and PAI-$1^{-/-}$ mice (data not shown).

Overall, these data suggest that PAI-1 deficiency attenuates liver injury, reduces neutrophil infiltration, and enhances proliferative repair processes after BDL. Although several potential mechanisms exist for these observations, the simplest explanation is that these changes in the liver's response to cholestatic injury result from altered activity of tPA, uPA, or plasmin.

PAI-1 Deficiency Causes Increased tPA Activity, But Does Not Alter uPA or Plasmin Activity After BDL. Although it might be anticipated that tPA, uPA, and plasmin activity would all be elevated in PAI-$1^{-/-}$ mice compared with WT after BDL, several recent studies demonstrated that PAI-1 deficiency does not necessarily alter uPA or plasmin activity.[15,20,21] To directly determine the activity of tPA and uPA after BDL in WT and PAI-$1^{-/-}$ mice, we performed casein/plasminogen gel zymography. These analyses showed that tPA activity was markedly elevated, but uPA levels were comparable, in PAI-$1^{-/-}$ and WT mice (Fig. 5A-B). Similarly, plasmin activity was equivalent in WT and PAI-$1^{-/-}$ mice after BDL as measured by casein gel zymography or using a fluorogenic plasmin substrate (data not shown).

PAI-$1^{-/-}$ Mice Had Elevated Active HGF Levels, Increased c-Met Phosphorylation, and Increased Akt Activation. Because tPA activates HGF,[2] we hypothesized that PAI-$1^{-/-}$ mice would have more HGF activation than WT animals after BDL. This hypothesis

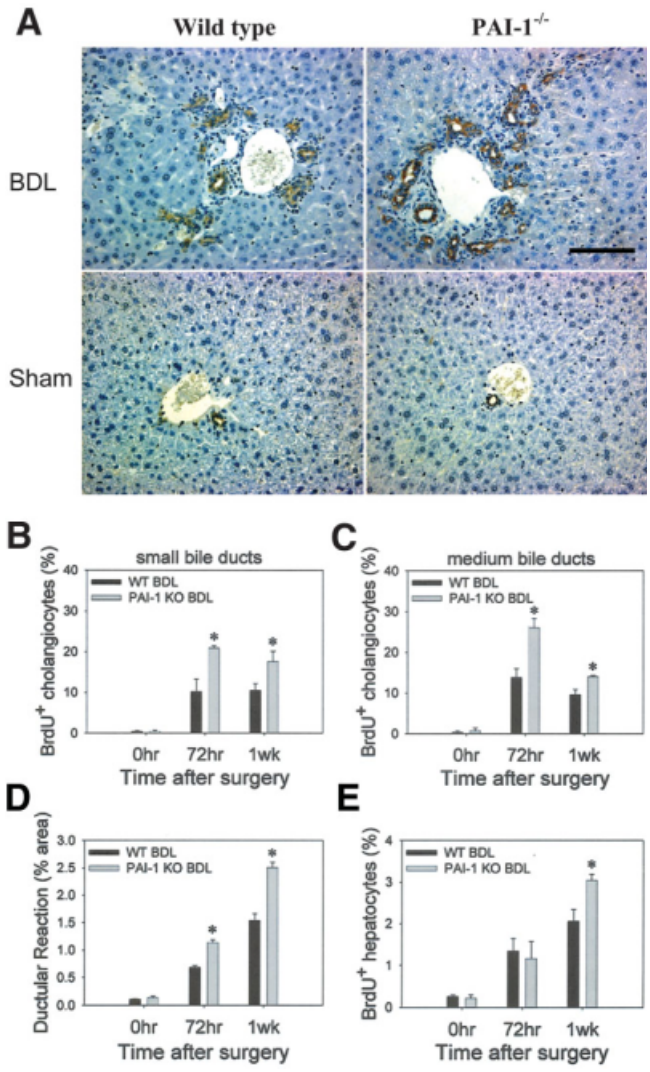

Fig. 4. Increased hepatocyte and cholangiocyte proliferation in PAI-$1^{-/-}$ mice after BDL. (A-D) Cholangiocyte proliferation in small (B) and medium (C) bile ducts is elevated in PAI-$1^{-/-}$ compared with WT mice after BDL (*$P < .02$ vs. WT). (A, D) This increase in cholangiocyte proliferation correlated with an increase in ductular reaction in PAI-$1^{-/-}$ mice after BDL compared with WT animals (*$P < .002$ vs. WT). (A) Shows liver histology with cytokeratin immunohistochemistry 1 week after BDL or sham surgery. Scale bar = 100 μm. (E) Hepatocyte proliferation rates are elevated in PAI-$1^{-/-}$ compared with WT mice 1 week after BDL (*$P < .01$ vs. WT). PAI-1, plasminogen activator inhibitor-1; WT, wild-type; BDL, bile duct ligation.

was appealing because HGF is a survival factor[22-24] and mitogen[25-27] for hepatocytes and cholangiocytes[28] and because other hepatocyte mitogens are not known to be influenced by tPA. Protein immunoblotting confirmed that levels of mature α-HGF at 72 hours and 1 week after BDL are significantly elevated in PAI-$1^{-/-}$ compared with WT mice (Fig. 6). This increase in active HGF occurred despite comparable levels of HGF mRNA (data not shown) and pro-HGF protein at these points, suggesting increased processing of pro-HGF to mature α-HGF in PAI-$1^{-/-}$ mice after BDL.

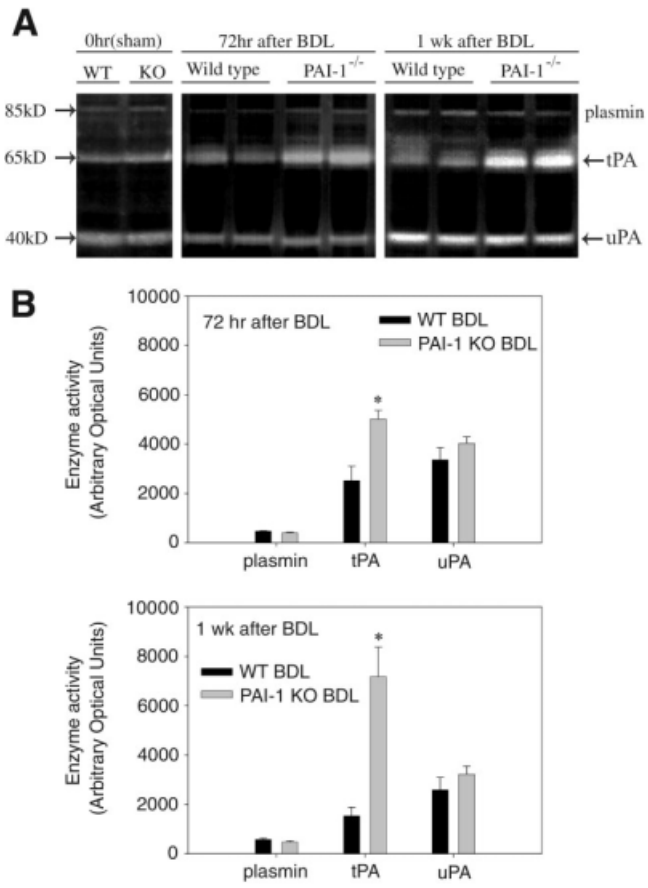

Fig. 5. Casein/plasminogen gel zymography analysis of tPA, uPA, and plasmin activity in WT and PAI-$1^{-/-}$ mice. (A) Gel zymography demonstrates levels of active uPA, tPA, and plasmin in the liver after BDL. (B) Quantitative image analysis demonstrated increased tPA activity in PAI-$1^{-/-}$ vs. WT animals after BDL, but no significant differences in uPA or plasmin activity (*$P < .02$ vs. WT).

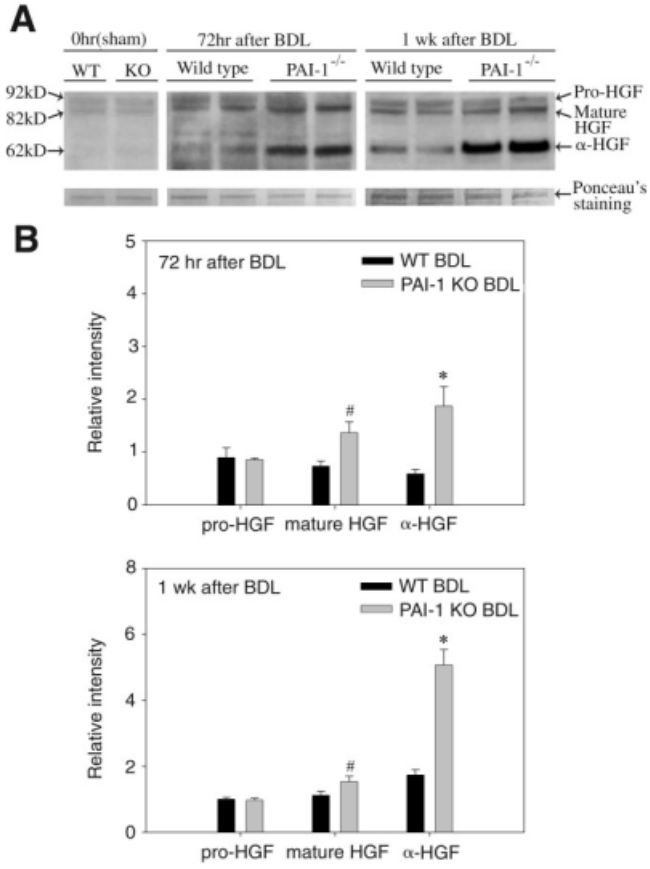

Fig. 6. Protein immunoblot analysis demonstrated increased mature and α-HGF in PAI-$1^{-/-}$ liver after BDL compared with WT mice. (A) Bands representing pro-HGF, nonreduced α,β-chain mature HGF, and α-chain HGF (reduced mature HGF) are indicated. (B) Quantitative analysis of HGF demonstrated increased mature and α-HGF in PAI-$1^{-/-}$ mice (*$P < .01$ and #$P < 0.05$ vs. WT). HGF, hepatocyte growth factor; PAI-1, plasminogen activator inhibitor-1; BDL, bile duct ligation; WT, wild-type.

The survival effects of HGF on hepatocytes are mediated by c-Met activation, which in turn activates PI-3 kinase and Akt.[29-31] To confirm that the increase in mature α-HGF demonstrated by protein immunoblotting stimulated this hepatocyte survival pathway, c-Met phosphorylation in WT and PAI-$1^{-/-}$ mouse liver was evaluated 72 hours and 1 week after BDL (Fig. 7A). This analysis demonstrated significantly increased c-Met phosphorylation in PAI-$1^{-/-}$ mice compared with WT animals at both times. Analysis of Akt phosphorylation also demonstrated elevated levels of phospho-Akt in PAI-$1^{-/-}$ mice compared with WT animals at 72 hours and 1 week after BDL (Fig. 7B). These data suggest that, in the setting of PAI-1 deficiency, increased HGF activation reduces hepatocellular injury.

Discussion

The early pathogenic processes in cholestatic liver injury, including bile infarct formation, neutrophil infiltration, cell proliferation, and tissue remodeling, are interrelated and many genes have been identified that are important in each process. To identify additional proteins that critically influence early liver injury and repair during biliary tract obstruction, microarray analysis was performed on mouse liver tissue 24 hours after BDL. These studies demonstrated many changes in gene expression and led to the identification of PAI-1 as an important mediator of hepatic injury and repair.

PAI-1, tPA, uPA, and Plasmin in Cholestatic Injury and Repair. The most dramatic change in gene expression after BDL was for PAI-1, which inhibits the plasminogen activators uPA and tPA. This elevation in PAI-1 mRNA levels after BDL is consistent with the observed increase in PAI-1 in a variety of other types of tissue injury and the reported ability of a large number of mediators,[1] including transforming growth factor beta-1,[32] HGF,[33,34] and angiotensin II,[35,36] to increase PAI-1 ex-

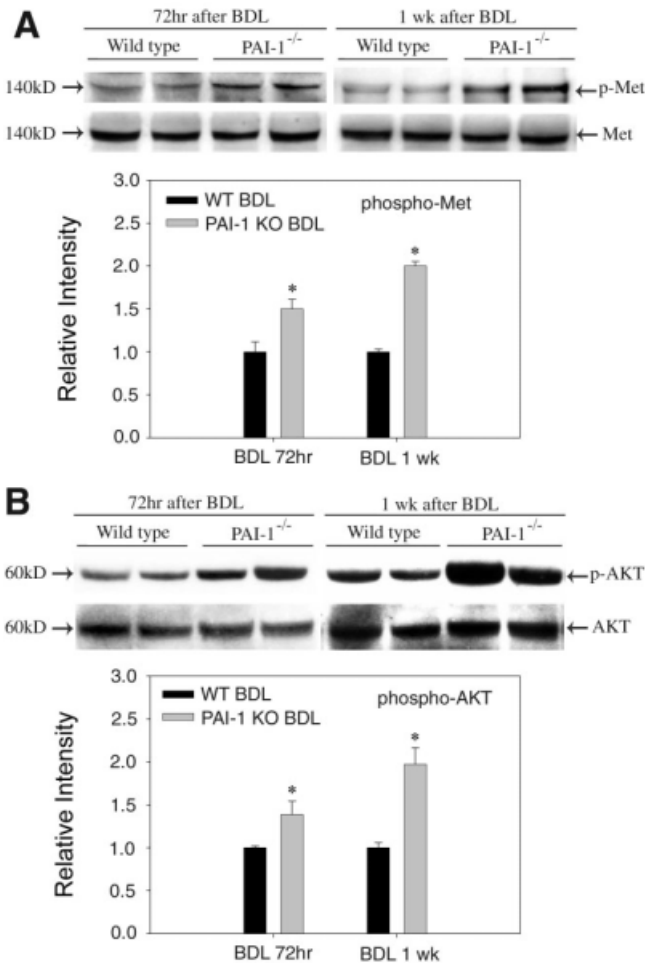

Fig. 7. PAI-1 deficient mice have increased phosphorylation of c-Met and Akt after BDL compared with WT mice. (A) c-Met was immunoprecipitated from liver at 72 hours or 1 week after BDL. Met phosphorylation was determined by anti-phosphotyrosine immunoblotting. Total Met protein was evaluated using a c-Met antibody. Quantitative analysis demonstrated increased c-Met phosphorylation in PAI-$1^{-/-}$ mice compared with WT ($P < .02$). Total c-Met levels were similar in WT and PAI-$1^{-/-}$ mice. (B) Immunoblotting demonstrated that phospho-Akt was elevated in PAI-$1^{-/-}$ mice compared with WT animals ($P < .02$), but total Akt levels were equivalent in WT and PAI-$1^{-/-}$ mice. PAI-1, plasminogen activator inhibitor-1; BDL, bile duct ligation; WT, wild-type.

pression. Because PAI-1 inhibits the tPA and uPA, which in turn activate HGF, we initially hypothesized that PAI-1 might exacerbate liver injury after BDL. To test this hypothesis, we performed BDL on PAI-1–deficient mice. These studies demonstrated that eliminating PAI-1 reduces bile infarct size and hepatic neutrophil infiltration while enhancing cholangiocyte and hepatocyte proliferation. Although several hypotheses might be advanced to explain these observations, we directly demonstrate that PAI-1 deficiency results in increased accumulation of active HGF, increased c-Met phosphorylation, increased Akt phosphorylation, and reduced accumulation of mRNA for the neutrophil chemoattractants Cxcl1 and Cxcl2. We further demonstrate that tPA activity is elevated in PAI-$1^{-/-}$ mice after BDL. Because tPA is known to activate HGF,[2] and because HGF is mitogen[25,37] for hepatocytes and cholangiocytes[12,28,38] as well as a hepatocyte survival factor,[22-24] at least one explanation for the reduced injury in PAI-$1^{-/-}$ mice after BDL is likely to be the increase in active HGF in these animals.

Neutrophils in Cholestatic Liver Injury. Neutrophils accumulate in the liver and contribute to hepatic injury in many model systems,[39-42] including after BDL.[18,43] Although mechanisms that cause hepatic neutrophil accumulation and extravasation after BDL are not well established, Cxcl1 and Cxcl2 have been implicated as neutrophil chemoattractants in some[42,44,45] but not all[46] models of liver injury, and their expression is induced in the liver after BDL.[47] Furthermore, extravasated neutrophils contribute to liver injury after BDL,[18,19] creating a positive feedback loop in which increased injury leads to increased neutrophil infiltration, and the infiltrating neutrophils cause increased injury. The current studies demonstrate that PAI-1 deficiency both reduces neutrophil extravasation into the liver and reduces the size of bile infarcts after BDL. One potential explanation for these observations is that the protective effect of increased HGF in the liver of PAI-$1^{-/-}$ mice reduces the initial cell death and this in turn leads to reduced production of neutrophil chemoattractants. This would be consistent with the observed reduction in Cxcl1 and Cxcl2 mRNA in PAI-$1^{-/-}$ mouse liver after BDL. An alternative hypothesis is that PAI-1 or other components of the plasmin protease system directly influence neutrophil migration or extravasation. This could occur because the uPA receptor uPAR is important for chemotaxin-induced neutrophil migration.[48] The potential direct effects of PAI-1 on cell migration and extravasation are complex[49] and depend on the specific cell type and extracellular environment studied. This is because PAI-1's ability to inhibit uPAR and integrin interactions with vitronectin at the leading edge of the migrating cell should inhibit cell migration, whereas PAI-1's ability to increase uPAR/uPA internalization at the trailing edge of the cell could facilitate detachment of the trailing edge and encourage migration. Similarly, potential effects of PAI-1 on fibrin and vitronectin degradation also may influence neutrophil migration and reduce neutrophil extravasation in the PAI-$1^{-/-}$ mouse. The complexity of these effects is illustrated by the observation that PAI-1 inhibits cell migration for some cell types but not others.[50] Nonetheless, the observation that PAI-$1^{-/-}$ mice have smaller bile infarcts, fewer neutrophils extravasated into the liver, and reduced production of neutrophil chemoattractants suggests that PAI-1 is essential for

the positive feedback loop that allows neutrophils to infiltrate the liver and increases cholestatic liver injury.

PAI-1, tPA, uPA, and Plasminogen in Other Liver Injury Models. Our new data demonstrating a role for PAI-1 in cholestatic liver injury are consistent with observed effects of PAI-1, tPA, uPA, and plasminogen in other liver injury models. For example, accelerated liver regeneration also occurs in PAI-$1^{-/-}$ mice after Fas-mediated hepatic necrosis.[7] Mechanisms by which the plasmin protease system components influence liver injury and repair, however, appear to differ depending on the nature of the injury. For example, in the BDL model, PAI-$1^{-/-}$ mice have elevated tPA activity, but not elevated uPA or plasmin activity. Similar observations were made in PAI-$1^{-/-}$ mice in both a renal injury model[15] and an arthritis model,[21] suggesting that factors other than PAI-1 critically regulate plasmin and uPA at least in some settings. Interestingly, both uPA and plasminogen also influence liver injury and repair. Specifically, hepatocyte proliferation was delayed in $uPA^{-/-}$ and $plasminogen^{-/-}$ mice after partial hepatectomy[11] and in $uPA^{-/-}$ mice after Fas-induced hepatic necrosis.[7] In contrast, although carbon tetrachloride (CCl_4)-induced liver injury was more gradually repaired in $uPA^{-/-}$, $uPA^{-/-}/tPA^{-/-}$, and $plasminogen^{-/-}$ mice[4,10] than in WT animals, hepatocyte proliferation was similar in $uPA^{-/-}$, $tPA^{-/-}$, $plasminogen^{-/-}$, and WT mice. Collectively these data suggest that PAI-1, tPA, uPA, and plasminogen are all important mediators of liver injury and repair processes, but that the influence of each of these proteins depends on the specific type of liver injury. These analyses suggest the possibility that inhibiting PAI-1 may be beneficial in the setting of acute cholestatic injury.

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