The Antifibrinolytic Drug Tranexamic Acid Reduces Liver Injury and Fibrosis in a Mouse Model of Chronic Bile Duct Injury

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ABSTRACT

Hepatic fibrin deposition has been shown to inhibit hepatocellular injury in mice exposed to the bile duct toxicant α -naphthylisothiocyanate (ANIT). Degradation of fibrin clots by fibrinolysis controls the duration and extent of tissue fibrin deposition. Thus, we sought to determine the effect of treatment with the antifibrinolytic drug tranexamic acid (TA) and plasminogen activator inhibitor-1 (PAI-1) deficiency on ANIT-induced liver injury and fibrosis in mice. Plasmin-dependent lysis of fibrin clots was impaired in plasma from mice treated with TA (1200 mg/kg i.p., administered twice daily). Prophylactic TA administration reduced hepatic inflammation and hepatocellular necrosis in mice fed a diet containing 0.025% ANIT for 2 weeks. Hepatic type 1 collagen mRNA expression and deposition increased markedly in

livers of mice fed ANIT diet for 4 weeks. To determine whether TA treatment could inhibit this progression of liver fibrosis, mice were fed ANIT diet for 4 weeks and treated with TA for the last 2 weeks. Interestingly, TA treatment largely prevented increased deposition of type 1 collagen in livers of mice fed ANIT diet for 4 weeks. In contrast, biliary hyperplasia/inflammation and liver fibrosis were significantly increased in PAI-1^{-/-} mice fed ANIT diet for 4 weeks. Overall, the results indicate that fibrinolytic activity contributes to ANIT diet-induced liver injury and fibrosis in mice. In addition, these proof-of-principle studies suggest the possibility that therapeutic intervention with an antifibrinolytic drug could form a novel strategy to prevent or reduce liver injury and fibrosis in patients with liver disease.

Introduction

Chronic injury to bile duct epithelial cells (BDECs) occurs in humans with cholestatic liver disease and is modeled in mice by exposure to the toxicant α -naphthylisothiocyanate (ANIT). Transport of ANIT into the bile by hepatocytes injures intrahepatic BDECs (Dietrich et al., 2001), and exposure of mice to ANIT in the diet causes compensatory biliary hyperplasia, elevation in serum bile acids, portal lymphocytic inflammation, and mild to moderate hepatocellular injury (Tjandra et al., 2000; Xu et al., 2004; Sullivan et al., 2010; Golbar et al., 2013). Prolonged ANIT exposure in mice models progressive liver fibrosis characterized by exaggerated peribiliary collagen deposition (Tjandra et al., 2000; Xu et al., 2004; Sullivan et al., 2010; Golbar et al., 2013). BDEC injury in this model is associated with tissue factor-dependent activation of the blood coagulation cascade and increased plasma levels of the coagulation protease thrombin (Sullivan et al., 2010). This increase in thrombin activity is associated with the deposition

of insoluble fibrin clots in livers of mice fed ANIT diet (Luyendyk et al., 2011). Notably, increased plasma thrombin levels and hepatic fibrin deposition are also features of chronic cholestatic liver disease in humans (Segal et al., 1997; Luyendyk et al., 2011).

The formation and degradation of fibrin clots are a balanced and highly regulated process. The coagulation protease thrombin cleaves soluble fibrin(ogen) to fibrin monomers and activates coagulation factor XIII, which cross-links fibrin monomers to form insoluble fibrin clots. Fibrin is degraded in a process termed fibrinolysis, largely by the enzyme plasmin, which is generated from plasminogen (Lesage et al., 2001). Although fibrin deposition is an inevitable consequence of tissue injury, its regulation and impact in liver disease are not completely understood (Tripodi and Mannucci, 2007). Previously, we found that fibrin(ogen) deficiency increased hepatocellular necrosis in mice fed ANIT diet (Luyendyk et al., 2011). This suggests a protective role for hepatic fibrin deposition in this model of cholestatic liver injury and that modulating fibrin degradation (i.e., inhibiting fibrinolysis) could prevent the progression of ANIT-induced liver injury and fibrosis.

Complete plasminogen deficiency impaired liver remodeling and regeneration in a model of carbon tetrachloride hepatotoxicity in a fibrin-independent manner (Bezerra et al., 1999; Bezerra et al., 2001; Pohl et al., 2001). In contrast, acetaminophen hepatotoxicity was reduced in plasminogen-null mice

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ABBREVIATIONS: ALT, alanine aminotransferase; ANIT, α -naphthylisothiocyanate; BDEC, bile duct epithelial cell; BDL, bile duct ligation; CK19, cytokeratin 19; COL1A1, type 1 collagen; ITGB6, integrin β 6; PAI-1, plasminogen activator inhibitor-1; PCR, polymerase chain reaction; α -SMA, α -smooth muscle actin; TA, tranexamic acid; TGF- β , transforming growth factor β ; TIMP1, tissue inhibitor of metalloproteinase1; tPA, tissue plasminogen activator; WT, wild-type.

(Sullivan et al., 2012b), suggesting that the role of plasmin is injury/model-dependent. Plasminogen activator inhibitor-1 (PAI-1) is a primary physiological inhibitor of fibrinolysis, via inhibition of the urokinase and tissue plasminogen activators uPA and tPA, respectively (Iwaki et al., 2012). Plasmin activity can be reduced pharmacologically by administration of tranexamic acid (TA), a drug that inhibits conversion of plasminogen to plasmin (Iwamoto, 1975). TA is a Food and Drug Administration–approved hemostatic agent for the treatment of traumatic bleeding and is also available over-the-counter elsewhere for indications such as heavy menstrual bleeding (Dunn and Goa, 1999; McCormack, 2012). As a hemostatic agent, TA is reported to have a favorable safety profile for existing indications (Lukes et al., 2011; Muse et al., 2011) and has been demonstrated as an inexpensive and effective treatment for traumatic bleeding, as suggested recently by the multicenter Clinical Randomization of an Antifibrinolytic in Significant Hemorrhage-2 trial (Roberts et al., 2011). In agreement with our findings in plasminogen-null mice, administration of TA also attenuated acetaminophen hepatotoxicity in mice, albeit to a lesser extent (Sullivan et al., 2012b). However, the impact of TA treatment on chronic liver injury has not been broadly investigated in humans or animal models.

We determined the impact of TA treatment on ANIT diet–induced liver injury and the progression of ANIT diet–induced liver fibrosis in mice and compared this to the effect of PAI-1 deficiency. Based on our previous observation that fibrin(ogen) deficiency increased ANIT diet–induced liver injury, we hypothesized that TA administration would inhibit ANIT diet–induced liver injury and fibrosis in mice.

Materials and Methods

Mice. PAI-1^{−/−} mice (Stock no. 002507) and wild-type (WT) mice on an identical C57Bl/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained by homozygous breeding. Age-matched male mice between the ages of 8 and 14 weeks were used for these studies. Mice were housed at an ambient temperature of approximately 22°C with alternating 12 hour light/dark cycles and provided water and rodent chow ad libitum prior to study initiation. Mice were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facility at Michigan State University. All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee.

ANIT Diet Model and Pharmacological Intervention. Custom diets were prepared by Dyets, Inc. (Bethlehem, PA). The ANIT diet was an AIN-93M-purified diet containing 0.025% ANIT (Sigma-Aldrich, St. Louis, MO). The control diet was the purified AIN-93M diet. Groups of mice were fed each diet for a total of 2 weeks (Experiment 1) or 4 weeks (Experiment 2) ad libitum. For Experiment 1, TA (1200 mg/kg i.p., twice a day; United States Pharmacopeia Grade) (Spectrum Chemical Company, New Brunswick, NJ) or its vehicle (sterile endotoxin-free water) was administered for the study duration. This dose was selected as approximating the range of TA doses used in previous mouse studies (Hattori et al., 2000; Sullivan et al., 2012b) and was well tolerated by the mice. The dose required in mice is higher than the recommended human dose (4 g per day), because of the rapid elimination of TA in the kidney by glomerular filtration (Eriksson et al., 1974). For Experiment 2, mice were fed a control diet or ANIT diet for 4 weeks, and TA or its vehicle were administered only in weeks 3 and 4. Wild-type and PAI-1^{−/−} mice were fed ANIT diet for 4 weeks. Mice were anesthetized with isoflurane, and blood was collected from the caudal vena cava into sodium citrate (final, 0.38%) or an empty syringe for the collection

of plasma and serum, respectively. The liver was removed and washed with saline. The left medial lobe of the liver was affixed to a cork with optimal cutting temperature compound (VWR Scientific, Radnor, PA) and frozen for 3 minutes in liquid nitrogen-chilled isopentane. Sections of the left lateral lobe were fixed in neutral-buffered formalin for 48 hours before routine processing. The remaining liver was cut into approximately 100-mg sections and flash-frozen in liquid nitrogen.

Histopathology and Clinical Chemistry. Formalin-fixed liver sections were cut at 5 μ m and stained with hematoxylin and eosin or Sirius red by the Michigan State University Investigative Histopathology Laboratory for analysis of liver histopathology by light microscopy. At least two sections of liver from the left lateral lobe of each animal were evaluated in their entirety by a Board-certified veterinary pathologist (K.J.W.). Sections were assigned a score of mild (1), moderate (2), or severe (3) for multifocal hepatocellular necrosis and bile duct hyperplasia. Total bile acids in serum were determined using a colorimetric assay (Bio-Quant, San Diego, CA). The serum activities of alanine aminotransferase (ALT) and alkaline phosphatase were determined using commercially available reagents (Thermo Fisher Scientific, Waltham, MA; Pointe Scientific, Canton, MI).

Clot Turbidity Assay. Formation and lysis of fibrin clots were assessed by clot turbidity, as described previously (Machlus et al., 2011), with slight modification. A sample of 100- μ l normal pooled citrated-plasma (90% human plasma [George Kind Biomedical, Overland Park, KS]/10% pooled normal mouse plasma) was recalcified (16 mM final) and clotted with human α -thrombin (1 U/ml final) in the presence of 250 ng/ml human tissue plasminogen activator (tPA) (Molecular Innovations, Novi, MI). Clot turbidity was assessed by determining the absorbance at 405 nm over time. For select experiments, plasma from TA-treated mice and vehicle-treated mice was substituted for pooled normal mouse plasma. To approximate the TA concentration observed in TA-treated mice, the time to 50% clot lysis was determined for each sample and compared to a standard curve generated by spiking the pooled plasma with various concentrations of TA prior to clot formation.

Immunofluorescent Staining of Mouse Tissues and Quantification. Fibrin, type 1 collagen, and cytokeratin-19 (CK19) immunostaining and quantification were performed as described previously (Sullivan et al., 2010). In brief, approximately 10 low-power images (100 $\times$) for each tissue section were captured in a random and masked fashion and were analyzed using ImageJ (rsbweb.nih.gov/ij/). The percentage of positive pixels was expressed as a fold change relative to mice fed a control diet and treated with vehicle.

RNA Isolation, cDNA Synthesis, and Real-Time Polymerase Chain Reaction. Total RNA was isolated from approximately 50 mg of snap-frozen liver using TRI Reagent (Molecular Research Center, Cincinnati, OH). cDNA synthesis used 1 μ g of total RNA and was accomplished using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and a C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Hepatic levels of mRNAs encoding the profibrogenic genes type 1 collagen (COL1A1), integrin β 6 (ITGB6), transforming growth factor- β 1 (TGF β 1) and - β 2 (TGF β 2), α -smooth muscle actin (α -SMA), and tissue inhibitor of metalloproteinase1 (TIMP1) were determined using SYBR Green PCR, iTaq (Bio-Rad Laboratories) and a CFX Connect thermal cycler (Bio-Rad Laboratories). Primers were purchased from IDT (Coralville, IA). The expression of each gene was adjusted to the geometric mean C_t of two individual housekeeper genes, HPRT and 18S RNA, as described (Vandesompele et al., 2002), and the relative levels of each gene were evaluated using the $\Delta\Delta C_t$ method. Mouse COL1A1 primer sequences were 5'-GAGCGAGAGTACTGGATCG-3' (forward primer), 5'-GCTTCTTTTCCTTGGGGTTC-3' (reverse primer). Mouse TIMP1 primer sequences were 5'-GAGACACACCAGAGCAGATACC-3' (forward primer), 5'-CCAGGTCCGAGTTGCAGAAG-3' (reverse primer). Mouse ITGB6 primer sequences were 5'-CTCACGGGTACAGTAACGCA-3' (forward primer), 5'-AAATGAGCTCTCAGGCAGGC-3' (reverse primer). Mouse TGF β 1 primer sequences were 5'-CTCCCGTGGCTTCTAGTGC-3' (forward primer), 5'-GCCTTAGTTTGGACAGGATCTG-3' (reverse

primer). Mouse *TGF β 2* primer sequences were 5'-CCCCGAGGTGA-TTTCATC-3' (forward primer), 5'-GATGGCATTTCGGAGGGGA-3' (reverse primer). α -SMA primer sequences were 5'-GGA CGT ACA ACT GGT ATT GTG C-3' (forward primer), 5'-CGG CAG TAG TCA CGA AGG AAT-3' (reverse primer).

Statistics. Comparison of two groups was performed using Student's *t* test. Comparison of three or more groups was performed using one- or two-way analysis of variance, as appropriate, and Student-Newman-Keuls' post hoc test. The criterion for statistical significance was $P < 0.05$.

Results

Assessment of Hepatic Fibrin Deposition and TA Inhibition of Fibrinolytic Activity in Mice Fed ANIT Diet. Compared to WT mice fed a control diet for 4 weeks, marked hepatic fibrin deposition occurred in ANIT-treated WT mice (Fig. 1A). Similar hepatic fibrin deposition has been reported previously in mice fed ANIT diet for 2 weeks (Luyendyk et al., 2011). It is noteworthy that we did not observe a statistically significant increase in hepatic fibrin deposits in TA-treated mice (data not shown). The effect of TA treatment on fibrin clot lysis was analyzed ex vivo utilizing a fibrin clot turbidity assay. Compared to fibrin clots generated with plasma from vehicle-treated mice fed ANIT diet, fibrin clot lysis time was markedly prolonged when clots were generated with plasma from TA-treated mice fed ANIT diet (Fig. 1B). The time to reduce peak fibrin clot turbidity by 50% was determined and compared to a standard curve generated by spiking normal pooled plasma with TA (data not shown). This analysis yielded a plasma concentration of approximately 0.2 mg/l TA, roughly 16 hours after the last dose of drug. This is similar to levels observed at this time

after administration of a therapeutic dose of TA in humans (Eriksson et al., 1974; Pilbrant et al., 1981).

Effect of TA Treatment on Liver Histopathology in Mice Fed ANIT Diet for 2 Weeks. No lesions were identified in livers of mice fed a control diet for 2 weeks, irrespective of TA treatment (Fig. 2A). Vehicle-treated mice fed ANIT diet for 2 weeks developed liver injury characterized by multifocal acute hepatocellular coagulative necrosis and inflammation (six of seven mice), mild peribiliary fibrosis, and moderate lymphocytic inflammation/bile duct epithelial hyperplasia (seven of seven mice) (Fig. 2, A and B), in agreement with previous studies (Tjandra et al., 2000; Lesage et al., 2001; Sullivan et al., 2010). Treatment with TA reduced liver necrosis and inflammation in mice fed ANIT diet (Fig. 2, A and B), as indicated by a reduction in the number of mice with evidence of necrosis and inflammation (three of six mice) and a reduction in the average necrosis severity score from 1.8 in vehicle-treated mice to 0.5 in TA-treated mice. This corresponded to an approximately 50% reduction in necrotic area in TA-treated mice. Serum ALT activity and bile acid concentration increased in vehicle-treated mice fed ANIT diet (Fig. 2, C and D). Serum alkaline phosphatase activity did not increase in ANIT-treated mice (data not shown). Treatment with TA tended to reduce serum ALT activity, although this did not achieve statistical significance ($P = 0.1$; Fig. 2C). Treatment with TA did not affect serum bile acid concentration in mice fed ANIT diet (Fig. 2D).

Effect of TA Treatment on Hepatic Profibrogenic Gene Induction and Type 1 Collagen Deposition in Mice Fed ANIT Diet for 2 Weeks. Peribiliary fibrosis and induction of several profibrogenic genes are evident in livers of mice fed ANIT for 2 weeks (Sullivan et al., 2010). We chose to evaluate the expression of mRNAs encoding gene products

Fig. 1. Hepatic fibrin deposition and antifibrinolytic activity of TA in mice fed ANIT diet. Male, WT C57Bl/6J mice were fed a control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. (A) Representative photomicrographs (100 $\times$) show liver sections stained for fibrin (red) and counterstained with 4',6-diamidino-2-phenylindole to visualize nuclei (blue). (B) Comparison of clot formation and lysis by turbidity (absorbance [A] 450 nm) in ANIT diet-fed mice treated with vehicle ($n = 5$ mice) or TA (1200 mg/kg i.p.) ($n = 5$ mice) twice daily for 2 weeks. Recalcified human pooled plasma was spiked with 10% mouse plasma from each group and clotting was initiated with thrombin (1 U/ml final) in the presence of 250 ng/ml human tPA. Data are expressed as mean $\pm$ S.E.M. An increase in absorbance implies fibrin clot formation and a decrease indicates lysis.

Fig. 2. Effect of TA on liver injury in mice fed ANIT diet for 2 weeks. Male, WT C57Bl/6J mice were fed a control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 2 weeks. Mice were treated with vehicle (sterile water) or TA twice daily (1200 mg/kg i.p.) for the duration of ANIT exposure. (A) and (B) Representative photomicrographs showing (A) low magnification (40 $\times$) and (B) high magnification (200 $\times$) hematoxylin and eosin-stained liver sections. Arrow indicates area of coagulative necrosis. Asterisk indicates area of biliary hyperplasia and portal inflammation. (C) Serum ALT activity and (D) bile acid concentration were determined as described under *Materials and Methods*. Data are expressed as mean $\pm$ S.E.M.; $n = 3$ mice per group for control diet and 6 or 7 mice per group for mice fed ANIT diet. * $P < 0.05$; significantly different from respective treatment fed a control diet.

that are known to participate in liver fibrosis accompanying cholestasis (e.g., TIMP1, ITGB6, TGF β) as an initial screen to identify profibrogenic pathways controlled by plasmin. Although it reduced liver injury, TA treatment did not significantly inhibit the induction of TIMP1, ITGB6, TGF β 1, or TGF β 2 mRNAs in livers of mice fed ANIT diet for 2 weeks (Fig. 3, A–D). Moreover, the induction of COL1A1 mRNA (Fig. 3E) and deposition of type 1 collagen protein (Fig. 3F) were not significantly reduced by TA treatment. This suggests that the profibrogenic and necrotic processes in mice fed ANIT diet are not interdependent at this time point.

Intervention with TA Treatment Reduces Necrosis and Biliary Hyperplasia in Livers of Mice Fed ANIT Diet for 4 Weeks. Next, we determined the effect of TA treatment on the progression of ANIT diet-induced liver injury and fibrosis. Mice were fed ANIT diet for 4 weeks and given either TA or vehicle beginning in week 3. Liver histology was unremarkable in vehicle-treated mice fed a control diet for 4 weeks. Tranexamic acid treatment in mice fed a control diet tended to increase hepatocellular vacuolization, suggestive of glycogen or lipid droplets (Fig. 4A). Coagulative necrosis and inflammation were observed in livers of vehicle-treated mice fed ANIT diet for 4 weeks (Fig. 4, A and B). However, the frequency (~50% mice with necrosis) and severity [average score 1 (mild)] were reduced compared to mice fed ANIT diet for 2 weeks. Nonetheless, intervention with TA treatment beginning in week 3 tended to reduce necrosis and peribiliary inflammation in mice fed ANIT diet (Fig. 4, A and B). Analysis of liver histopathology revealed moderate biliary hyperplasia and portal inflammation in vehicle-treated mice

fed ANIT diet (average score 2), whereas these changes were noted to be mild in TA-treated mice fed ANIT diet (average score 1.1) (Fig. 4B). Serum ALT activity increased significantly in vehicle-treated mice fed ANIT diet. TA treatment tended to reduce serum ALT activity in mice fed ANIT diet (Fig. 4C), but this did not achieve statistical significance ($P = 0.2$; Fig. 4C). TA treatment did not impact serum bile acid concentration in mice fed ANIT diet (Fig. 4D). BDEC hyperplasia was evaluated by immunofluorescent staining of cytokeratin 19, a biomarker of BDECs in mouse liver (Fig. 5, A and B). As determined by morphometry, the area of CK19 staining increased approximately 2.5- and 4.5-fold in livers of mice fed ANIT diet for 2 weeks and 4 weeks, respectively (Fig. 5, C and D). Treatment with TA significantly reduced CK19 staining in livers of mice fed ANIT diet (Fig. 5C).

TA Treatment Inhibits Induction of Select Profibrogenic Genes and Prevents the Progression of Collagen Deposition in Livers of Mice Fed ANIT Diet for 4 Weeks. Compared to mice fed a control diet, expression of ITGB6, TGF β 1, TGF β 2, and TIMP1 mRNAs was increased in livers of mice fed ANIT diet for 4 weeks (Fig. 6, A–D). The expression of each mRNA was notably higher than the increase observed after 2 weeks of ANIT diet (Fig. 3). TA treatment did not impact the expression of ITGB6 or TGF β 1 mRNAs in mice fed ANIT diet (Fig. 6, A and B). In contrast, TA treatment reduced TGF β 2 ($P = 0.08$) and TIMP1 ($P < 0.05$) mRNA expression in livers of mice fed ANIT diet (Fig. 6, C and D). Type 1 collagen mRNA expression increased approximately 7-fold in mice fed ANIT diet for 4 weeks

Fig. 3. Effect of TA on early hepatic profibrogenic changes in mice fed ANIT diet for 2 weeks. Male, WT C57Bl/6J mice were fed a control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 2 weeks. Mice were treated with vehicle (sterile water) or TA twice daily (1200 mg/kg i.p.) for the duration of ANIT exposure. Hepatic levels of mRNAs encoding (A) TIMP1, (B) ITGB6, (C) TGFβ1, (D) TGFβ2, and (E) COL1A1 were determined using real-time quantitative PCR. (F) Deposition of type 1 collagen protein in liver was quantified as described under *Materials and Methods*. Data are expressed as mean ± S.E.M.; $n = 3$ mice per group for control diet and six or seven mice per group for mice fed ANIT diet. * $P < 0.05$; significantly different from respective treatment fed a control diet. # $P < 0.05$; significantly different from vehicle-treated mice fed the same diet.

compared to mice fed a control diet (Fig. 7C). This was approximately doubled compared to mice fed ANIT diet for 2 weeks. In agreement, type 1 collagen protein deposition increased significantly in livers of mice fed ANIT diet for 4 weeks, as indicated by type 1 collagen immunofluorescence and Sirius red staining (Fig. 7, A and B). Administration of TA significantly reduced COL1A1 mRNA and protein levels in mice fed ANIT diet (Fig. 7, A–C). Collagen deposition was reduced by TA treatment to a level similar to mice fed ANIT diet for 2 weeks (Fig. 7, A and B).

PAI-1 Deficiency Increases Peribiliary Fibrosis in Mice Fed ANIT Diet for 4 Weeks. To complement our TA experiments, we examined the role of PAI-1 in ANIT-induced liver injury and fibrosis. PAI-1^{-/-} mice are viable and do not have evidence of liver pathology (Carmeliet et al., 1993). Compared to ANIT-treated WT mice, histological changes including biliary hyperplasia and peribiliary inflammation were increased in ANIT-treated PAI-1^{-/-} mice (Fig. 8, A and B). PAI-1 deficiency did not dramatically increase liver necrosis in ANIT-treated mice, as indicated by liver histopathology (Fig. 8, A and B) and serum ALT activity (Fig. 8C). Hepatic COL1A1 and α-SMA mRNA levels increased approximately 2-fold in ANIT-treated PAI-1^{-/-} mice compared to

ANIT-treated WT mice, but this did not achieve statistical significance (Fig. 8D). However, peribiliary type 1 collagen protein deposition increased significantly (~3-fold) in livers of ANIT-treated PAI-1^{-/-} mice compared with ANIT-treated WT mice (Fig. 8E).

Discussion

Hepatic fibrin deposition is evident in patients with chronic cholestatic liver disease (Luyendyk et al., 2011) and in rodent models of chronic cholestasis (Wang et al., 2007c; Luyendyk et al., 2011). Genetically imposed loss of fibrin(ogen) increases hepatic inflammation and injury in mice fed ANIT diet (Luyendyk et al., 2011). A parallel extension would suggest that uncontrolled fibrinolysis could contribute to the progression of liver disease. Clinical studies suggest exaggerated fibrinolysis in patients with liver disease, as indicated by elevated D-dimer levels, a product of fibrin degradation (Paramo et al., 1991; Pernambuco et al., 1993; Violl et al., 1996). Moreover, reductions in the primary inhibitors of fibrinolysis: thrombin-activatable fibrinolysis inhibitor and α2-antiplasmin, have been noted in patients with liver disease (Marongiu et al., 1985; Van Thiel et al., 2001; Colucci

Fig. 4. Effect of TA treatment on liver injury in mice fed ANIT diet for 4 weeks. Male, WT C57Bl/6J mice were fed a control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. Mice were treated with vehicle (sterile water) or TA twice daily (1200 mg/kg i.p.) beginning in week 3. Representative photomicrographs show low magnification (40 $\times$) (A) and high magnification (200 $\times$) (B) hematoxylin and eosin-stained liver sections. Arrow indicates area of coagulative necrosis. Asterisk indicates area of biliary hyperplasia and portal inflammation. Serum ALT activity (C) and bile acid concentration (D) were determined as described under *Materials and Methods*. Data are expressed as mean $\pm$ S.E.M.; $n = 5$ mice per group for control diet and 10 mice per group for mice fed ANIT diet. * $P < 0.05$; significantly different from respective treatment fed a control diet.

et al., 2003; Gresele et al., 2008). Preserving residual hepatic fibrin deposition could form a potential strategy to reduce the progression of chronic liver disease. Our proof-of-principle study suggests that a relatively short duration of TA treatment, at plasma levels approximating those observed in humans given TA, reduces multiple aspects of cholestatic liver injury in mice. Indeed, more prolonged treatment with TA could yield more robust protection. Because TA is already approved in the United States by the Food and Drug Administration for treatment of traumatic bleeding and is available over-the-counter internationally for other indications, it may be a potential candidate to repurpose for the treatment of liver disease.

Our previous studies showed that fibrin(ogen) protects the liver from chronic ANIT diet-induced cholestatic liver injury. Thus, one mechanism whereby TA treatment could reduce ANIT-induced liver injury is by inhibiting plasmin-mediated fibrinolysis, thereby stabilizing hepatic fibrin deposits. Our results indicate that plasma TA levels in TA-treated mice sufficiently prolonged clot lysis *ex vivo*, consistent with inhibition of plasmin, and were similar to those observed in TA-treated humans (Eriksson et al., 1974; Pilbrant et al., 1981). Although TA treatment probably sustained hepatic fibrin deposition in ANIT-treated mice, we were unable to detect a significant increase in hepatic fibrin deposition in ANIT-treated mice given TA. One reason is potentially because the extent of hepatic fibrin deposition represents the net balance between thrombin-driven fibrin formation and plasmin-mediated degradation. In TA-treated mice, there was a probable reduction in injury-driven coagulation and

deposition of fibrin in necrotic areas, complicating analysis of overall fibrin deposition. Nonetheless, additional studies are required to determine if fibrin is required for the protective effects of TA in chronic ANIT-induced liver injury.

It is possible that fibrin(ogen)-independent effects of plasmin contribute to liver pathology in this model. For example, plasmin has been shown to promote the activation of inflammatory cells, including neutrophils (Syrovets et al., 2012), which contribute to liver necrosis induced by cholestasis (Gujral et al., 2003). Of interest, at a 2-week time point where it significantly reduced liver injury, TA treatment did not affect hepatic expression of mRNAs encoding numerous chemokines, including MCP-1, MIP-2, and KC (data not shown). Even so, we cannot exclude the possibility that direct activation of inflammatory cells by plasmin promotes liver injury in mice fed ANIT diet. Determining the exact mechanism whereby plasmin and fibrin(ogen) modulate ANIT-induced liver pathology is the focus of ongoing investigation in the laboratory.

TA treatment significantly reduced hepatocyte necrosis without impacting early profibrogenic changes in mice fed ANIT diet for 2 weeks. Previous studies suggest that in the ANIT diet model, the mechanisms responsible for hepatocellular injury and fibrosis are distinct at early time points (2 weeks). For example, deficiency in the thrombin receptor protease-activated receptor-1 reduced fibrosis without reducing hepatocellular necrosis in mice fed ANIT diet (Sullivan et al., 2010). Early growth response-1-null mice develop markedly increased ANIT diet-induced liver fibrosis without a corresponding exacerbation of hepatocyte injury (Sullivan

Fig. 5. Effect of TA treatment on biliary hyperplasia in mice fed ANIT diet for 4 weeks. Male, WT C57Bl/6J mice were fed a control diet (AIN-93M) or an identical diet containing 0.025% ANIT for up to 4 weeks. Mice were treated with vehicle (sterile water) or TA twice daily (1200 mg/kg i.p.) beginning in week 3. (A) and (B) Representative photomicrographs showing CK19 staining in liver sections of mice fed a control and ANIT diet fed mice for 2 weeks (A) and 4 weeks (B). Images were converted to grayscale and inverted such that CK19 staining is dark. (C) Quantification of CK19 staining area as described under *Materials and Methods*. Data are expressed as mean $\pm$ S.E.M.; $n = 3-5$ mice per group for control diet and 7–10 mice per group for mice fed ANIT diet. * $P < 0.05$; significantly different from respective treatment fed a control diet. # $P < 0.05$, significantly different from vehicle-treated mice fed the same diet.

et al., 2012a). Moreover, analogous to our findings with TA treatment, fibrin(ogen) deficiency increased hepatocyte injury at this time without affecting hepatic collagen expression (Luyendyk et al., 2011). This dichotomy probably stems from BDECs being the primary cellular target of ANIT. These cells

undergo a compensatory expansion in mice exposed to ANIT (Lesage et al., 2001). The expression of integrin $\alpha_V\beta_6$ is increased on proliferating BDECs in vitro and in mice fed ANIT diet (Patsenker et al., 2008; Sullivan et al., 2010). Previous studies have demonstrated that activation of latent-TGF β 1

Fig. 6. Effect of TA treatment on profibrogenic gene induction in livers of mice fed ANIT diet for 4 weeks. Male, WT C57Bl/6J mice were fed a control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. Mice were treated with vehicle (sterile water) or TA twice daily (1200 mg/kg i.p.) beginning in week 3. Hepatic expression of mRNAs encoding ITGB6 (A), TGF β 1 (B), TGF β 2 (C), and TIMP1 (D) were determined using real-time quantitative PCR. Data are expressed as mean $\pm$ S.E.M.; $n = 5-10$ mice per group. * $P < 0.05$; significantly different from respective treatment fed a control diet. # $P < 0.05$; significantly different from vehicle-treated mice fed the same diet.

Fig. 7. Effect of TA treatment on type 1 collagen expression and deposition in livers of mice fed ANIT diet for 4 weeks. Male, WT C57Bl/6J mice were fed a control diet (AIN-93M) or an identical diet containing 0.025% ANIT for 4 weeks. Mice were treated with vehicle (sterile water) or TA twice daily (1200 mg/kg i.p.) beginning in week 3. (A) Representative photomicrographs showing type 1 collagen (100 $\times$) and Sirius red-stained (200 $\times$) liver sections. Type 1 collagen images were converted to grayscale and inverted such that collagen staining is dark. (B) Collagen staining was quantified as described under *Materials and Methods*. (C) Hepatic mRNA expression levels of COL1A1 were determined using real-time quantitative PCR. Data are expressed as mean $\pm$ S.E.M.; $n = 5$ –10 mice per group. * $P < 0.05$; significantly different from respective treatment fed a control diet. ** $P < 0.05$; significantly different from vehicle-treated mice fed the same diet.

by $\alpha_v\beta_6$ integrin contributes to peribiliary fibrosis in models of cholestatic liver injury (Wang et al., 2007a; Patsenker et al., 2008; Sullivan et al., 2010). Peribiliary collagen deposits probably represent early activation of portal fibroblasts by TGF β 1 (Dranoff and Wells, 2010; Lee and Friedman, 2011). Overall, the results suggest that plasmin is not a critical mediator of the initial, peribiliary profibrogenic changes in mice fed ANIT diet.

Although TA treatment did not inhibit the early profibrogenic response to ANIT, it largely prevented the progression of liver fibrosis in mice fed ANIT diet for 4 weeks. A failure to resolve persistent liver damage in mice fed ANIT diet could result in more extensive collagen deposition. Moreover, the reduction in biliary hyperplasia by TA treatment could be the basis for reduced fibrosis. Proliferating BDECs are known to produce a number of profibrogenic mediators that coordinate matrix production by surrounding portal fibroblasts (Sedlacek et al., 2001).

Although these resident fibroblasts likely contribute to liver fibrosis in this model, engagement of accumulating bone marrow-derived fibrocytes cannot be excluded (Kisseleva et al., 2006). In our initial screen of profibrogenic pathways affected by plasmin, we found that TIMP1 expression was dramatically reduced, suggesting enhanced clearance of matrix. In contrast, TA treatment did not inhibit induction of ITGB6 mRNA, a component of the $\alpha_v\beta_6$ integrin that activates latent TGF β 1. Although TA treatment did not affect expression of TGF β 1 mRNA levels, plasmin has been shown to enzymatically activate latent TGF β 1 (Lyons et al., 1990; Khalil et al., 1996), and plasmin-catalyzed TGF β 1 activation has been implicated in other models of fibrosis (Lyons et al., 1990; Zhang et al., 2007). Collectively, additional studies investigating plasmin-driven profibrogenic protein expression and function are required, as these studies suggest multiple mechanisms whereby TA could inhibit liver fibrosis.

Fig. 8. Effect of PAI-1 deficiency on liver injury and fibrosis in mice fed ANIT diet for 4 weeks. Male, WT and PAI-1^{-/-} mice were fed a diet containing 0.025% ANIT for 4 weeks. Representative photomicrographs showing low magnification (40×) (A) and high magnification (100×) (B) hematoxylin and eosin-stained liver sections. Asterisks mark areas of biliary hyperplasia and lymphocytic inflammation. Arrowheads mark necrotic foci (bile infarcts). (C) Serum ALT activity was determined as described under *Materials and Methods*. (D) Hepatic mRNA expression levels of COL1A1 and α-SMA were determined using real-time quantitative PCR. Data are expressed as mean ± S.E.M.; *n* = 5 mice per group. (E) Representative photomicrographs showing type I collagen staining (red) on 4',6-diamidino-2-phenylindole-counterstained (blue) liver sections of mice fed ANIT diet for 4 weeks. Collagen protein levels were quantified as described under *Materials and Methods*. Data are expressed as mean ± S.E.M.; *n* = 5 mice per group. **P* < 0.05 vs. WT mice fed the same diet.

The role of PAI-1 in models of liver injury and disease has been extensively studied. For example, PAI-1 deficiency increases liver injury in models of acetaminophen and carbon tetrachloride hepatotoxicity (von Montfort et al., 2010; Sullivan et al., 2012b). In agreement with our finding that inhibition of fibrinolysis with TA reduces ANIT-mediated liver injury and fibrosis, we found that PAI-1 deficiency increased ANIT diet-induced liver injury and fibrosis. In contrast, PAI-1^{-/-} mice were protected from alcohol-induced liver damage (Bergheim et al., 2006b). Moreover, PAI-1 deficiency reduced liver injury and fibrosis in mice subjected to bile duct ligation (BDL), a model of extrahepatic obstructive cholestasis (Wang et al., 2005; Bergheim et al., 2006a). However, the role of fibrin(ogen) in BDL-induced liver injury and fibrosis has not been described, with the reduction in hepatic fibrin in PAI-1^{-/-} mice after BDL (Wang et al., 2005) likely a reflection of reduced liver injury. Indeed, PAI-1 deficiency did not affect plasmin activity in BDL mice (Wang

et al., 2007b). Moreover, PAI-1 deficiency was shown to protect BDL mice by enhancing tPA-mediated activation of hepatocyte growth factor, a process not directly impacting fibrinolysis (Wang et al., 2007c). Additional studies are required to clarify the mechanisms underlying the different functions of PAI-1 in BDL and ANIT diet models of cholestatic liver injury.

Taken together, the results indicate that administration of the antifibrinolytic TA reduces liver injury and fibrosis in a mouse model of chronic xenobiotic-induced biliary injury. The protection from liver injury after treatment with an antifibrinolytic drug is in agreement with our finding that PAI-1 deficiency increases ANIT-induced liver injury and fibrosis and supports our previous observation that fibrin(ogen) deficiency worsens liver injury in this model. Collectively, this study provides important proof-of-principle findings on which additional studies of TA as a novel treatment for liver disease could be based.

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Authorship Contributions

Participated in research design: Joshi, Kopec, Luyendyk.
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Performed data analysis: Joshi, Kopec, Towery, Williams, Luyendyk.
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