

# Fibrinogen- $\gamma$ Proteolysis and Solubility Dynamics During Apoptotic Mouse Liver Injury: Heparin Prevents and Treats Liver Damage

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Fas ligand (FasL)-mediated hepatocyte apoptosis occurs in the context of acute liver injury that can be accompanied by intravascular coagulation (IC). We tested the hypothesis that analysis of selected protein fractions from livers undergoing apoptosis will shed light on mechanisms that are involved in liver injury that might be amenable to intervention. Proteomic analysis of the major insoluble liver proteins after FasL exposure for 4–5 hours identified fibrinogen- $\gamma$  (FIB- $\gamma$ ) dimers and FIB- $\gamma$ -containing high molecular mass complexes among the major insoluble proteins visible via Coomassie blue staining. Presence of the FIB- $\gamma$ -containing products was confirmed using FIB- $\gamma$ -specific antibodies. The FIB- $\gamma$ -containing products partition selectively and quantitatively into the liver parenchyma after inducing apoptosis. Similar formation of FIB- $\gamma$  products occurs after acetaminophen administration. The observed intrahepatic IC raised the possibility that heparin therapy may ameliorate FasL-mediated liver injury. Notably, heparin administration in mice 4 hours before or up to 2 hours after FasL injection resulted in a dramatic reduction of liver injury—including liver hemorrhage, serum alanine aminotransferase, caspase activation, and liver apoptosis—compared with heparin-untreated mice. Heparin did not directly interfere with FasL-induced apoptosis in isolated hepatocytes, and heparin-treated mice survived the FasL-induced liver injury longer compared with heparin-untreated animals. There was a sharp, near-simultaneous rise in FasL-induced intrahepatic apoptosis and coagulation, with IC remaining stable while apoptosis continued to increase. *Conclusion:* Formation of FIB- $\gamma$  dimers and their high molecular mass products are readily detectable within the liver during mouse apoptotic liver injury. Heparin provides a potential therapeutic modality, because it not only prevents extensive FasL-related liver injury but also limits the extent of injury if given at early stages of injury exposure. (HEPATOLOGY 2011;53:1323–1332)

**A**poptosis occurs in the context of acute and chronic injury, which provides an important target for intervention and amelioration of the injury.<sup>1,2</sup> A major mechanism that leads to hepatocyte apoptosis is the interaction of a cell surface death receptor such as Fas with its ligand (the Fas ligand [FasL]). Similarly, tumor necrosis factor  $\alpha$  can interact

with its cell surface receptor to lead to apoptosis. Both tumor necrosis factor  $\alpha$  and FasL interactions with their respective receptors lead to downstream activation of caspases, which function as the executioners of cell death. The activated caspases, in the context of pathological conditions, cleave key cellular proteins, thereby causing cells to undergo apoptosis. Numerous caspase

*Abbreviations:* ALF, acute liver failure; ALT, alanine aminotransferase; FasL, Fas ligand; FIB- $\gamma$ , fibrinogen- $\gamma$ ; H&E, hematoxylin and eosin; HMW, high molecular weight; HSE, high salt extraction; IC, intravascular coagulation; K18, keratin polypeptide 18; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLL, total liver lysates; TX-100, Triton X-100.

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Received August 26, 2010; accepted January 9, 2011.

Supported by National Institutes of Health Grant DK47918 and the Department of Veterans Affairs (M.B.O.).

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DOI 10.1002/hep.24203

Potential conflict of interest: Nothing to report.

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substrates have been identified, including cytoplasmic proteins such as keratins<sup>3,4</sup> and nuclear proteins such as lamins.<sup>5</sup> Mice that are exposed to the functional anti-Fas receptor antibody, Jo2, which serves as a FasL, develop fulminant liver failure and die within hours after administration of the antibody,<sup>6,7</sup> thereby mimicking the cell death that occurs in the context of a variety of acute and chronic liver diseases.<sup>8</sup>

Acute liver injury is associated with several changes in the hemostatic system that may lead to intrahepatic or intravascular coagulation (IC) and changes that promote both bleeding and thrombosis.<sup>9</sup> Fibrinogen, a major blood protein that consists of three pairs of polypeptide chains (fibrinogen A $\alpha$ , B $\beta$ , and  $\gamma$ ), is synthesized and secreted by liver parenchymal cells.<sup>10,11</sup> Apart from its essential role in blood clotting, fibrinogen- $\gamma$  (FIB- $\gamma$ ) contains binding sites for several proteins, including clotting factors, growth factors, and integrins.<sup>12,13</sup> FIB- $\gamma$  forms dimers in response to various cellular conditions through transamidation and cross-linking of FIB- $\gamma$  chains between a lysine at position 406 of one  $\gamma$ -chain and a glutamine at position 398 or 399 of a second chain.<sup>14</sup> High amounts of FIB- $\gamma$  dimers have been detected in patients with tumors, but not in control patients suffering from acute infection or inflammation. These findings suggest that the amount of cross-linked FIB- $\gamma$  dimer may correlate with tumor-associated fibrin deposition, and may be useful as a biomarker.<sup>15,16</sup> However, characterization of FIB- $\gamma$  dimers during liver damage has not been studied.

Depending on the context, hemostatic imbalance in acute liver failure (ALF) may contribute to cell injury or may have a protective function.<sup>9</sup> The therapeutic effect of the anticoagulant antithrombin-III, a protease inhibitor of thrombin, has been evaluated in dimethylnitrosamine- and CCl<sub>4</sub>-induced rat liver damage.<sup>17</sup> Upon treatment with antithrombin-III, dimethylnitrosamine-intoxicated rats benefited, whereas CCl<sub>4</sub>-treated rats showed no improvement, suggesting that IC may complicate certain types of acute liver injury and contribute to its aggravation.<sup>17</sup> In addition, pretreatment with heparin decreased acetaminophen-induced liver injury in mice.<sup>18</sup> Anticoagulant treatment of human ALF was also reported in a few patients. For example, nine patients with hemorrhagic diathesis due to acute hepatic necrosis were treated with heparin, and none survived,<sup>19</sup> whereas three patients with ALF and one with severe relapse of viral hepatitis accompanied by IC were treated with heparin and fresh frozen plasma and survived.<sup>19</sup> Therefore, the efficacy of treatment with heparin in the context of apoptotic liver injury remains unclear, although the major concern is an increased risk of bleeding.<sup>9</sup>

In this study, we initially sought to identify unique proteins that are relatively insoluble and are associated with FasL-associated liver apoptosis in mice. This led us to identify FIB- $\gamma$  dimers and other FIB-related high molecular species as among the major insoluble proteins in the liver after FasL administration. Based on this finding, we hypothesized that pretreatment of mice with heparin before FasL administration or treatment of mice with heparin after FasL administration led to a protective effect. Our findings provide support for this hypothesis and raise the possibility that targeted anticoagulation may have a beneficial effect in some forms of ALF.

## Materials and Methods

**Toxin and Heparin Administration, Liver Tissue, and Blood Analysis.** FasL (Jo2 clone, BD Pharmingen) was injected intraperitoneally into age/sex-matched (10–12 weeks old/female) FVB/N mice (0.15  $\mu$ g/g) to induce liver apoptosis. Heparin (AAP Pharmaceuticals) was administered (dorsal midline, level of scapula) through subcutaneous injection (20 U per mouse, with mice weighing  $\approx$ 25 g). Mice were sacrificed by way of CO<sub>2</sub> inhalation at the indicated time points, or survival time was monitored for the lethality experiments. Livers were isolated and divided into pieces that were either stored in liquid nitrogen for biochemical analysis or fixed in 10% formalin for hematoxylin and eosin (H&E) staining and histological analysis. Blood samples were collected from the sacrificed mice by way of intracardiac puncture and stored (4°C overnight) before analysis.

Serum alanine aminotransferase (ALT) levels were determined using Vetscan-vs2 (ABAXIS) employing the comprehensive diagnostic profile. Serum fibrinogen levels were determined using a mouse fibrinogen enzyme-linked immunosorbent assay kit (GenWay).

**Sample Preparation and Biochemical Analysis.** High salt extraction (HSE) was performed as described.<sup>20</sup> Supernatants (Triton X-100 [TX-100] and high salt fractions) were saved where necessary and used after mixing with 2 $\times$  sodium dodecyl sulfate (SDS) sample buffer. Total liver lysates (TLL) were prepared from liver tissues by way of homogenization using 2 $\times$  SDS sample buffer. Serum, plasma, and clot fractions were also mixed (serum and plasma) or homogenized (clot) using 2 $\times$  SDS sample buffer. Proteins were separated by way of SDS-polyacrylamide gel electrophoresis (SDS-PAGE), then stained with Coomassie blue or transferred to polyvinylidene fluoride membranes followed by blotting with antibodies to FIB- $\gamma$  (Abcam), tubulin, and actin (Neomarkers),

active caspases 3 or 7 (Cell Signaling), tissue factor and plasminogen activator inhibitor-1 (R&D Systems), and an antibody to keratin polypeptide 18 (K18) p29 apoptotic fragment.<sup>21</sup>

**Determination of Percent Apoptotic Cells and Extent of Hemorrhage.** Apoptotic cells were detected using a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay kit (Roche). The percentage of apoptotic cells in the liver was determined by counting the total number of nucleated cells (4',6-diamidino-2-phenylindole-stained) and the number of apoptotic cells (TUNEL-stained) in five random high-power fields. The extent of hemorrhage replacing normal architecture was scored qualitatively by a pathologist (D. S. M.) in a blinded fashion as follows: 0, no hemorrhage; 1, 1%-10% hemorrhage (mild); 2, 11%-20% (mild-moderate); 3, 20%-30% (moderate); 4, 30%-40% (moderate-severe); and 5, >40% (severe). The scores were then used for statistical analysis.

**Statistical Analysis.** All statistical analyses were performed using a one-way analysis of variance or log-rank (Mantel-Cox) test (for the lethality experiments) using GraphPad Prism 5 statistical software.

**Mass Spectrometry and N-terminal Sequencing.** Mass spectrometry was performed using standard protocols by the Michigan Proteome Consortium (University of Michigan). N-terminal sequencing was performed by the Molecular Structure Facility (University of California, Davis).

## Results

**Comparison of the Liver Insoluble Protein Fractions Before and After Fas-Mediated Apoptosis.** We used intraperitoneal injection of the FasL (Jo2), which is known to induce significant liver injury manifested as apoptosis and intrahepatic hemorrhage (Fig. 1A).<sup>6,7</sup> We compared the insoluble fractions of livers obtained from control and FasL-injected mice using HSE that removes nonionic detergent-soluble and high salt buffer-soluble proteins. Notably, three major proteins became clearly prominent in the livers of the FasL-treated mice (Fig. 1B). Proteolysis followed by mass spectrometry identified bands 1-3 as FIB- $\alpha/\gamma$ , FIB- $\gamma$ , and actin, respectively. For band 2, the peptides that were predicted by mass spectrometry are displayed in bold lettering in Fig. 2A. N-terminal sequencing of band 2 identified five amino acids (Fig. 2A) of FIB- $\gamma$ , which indicates that band 2 (100-kDa) is a cleaved dimer of FIB- $\gamma$ . It is already known that FIB- $\gamma$  undergoes cleavage and dimerization during the coagulation cascade.<sup>14,22</sup>

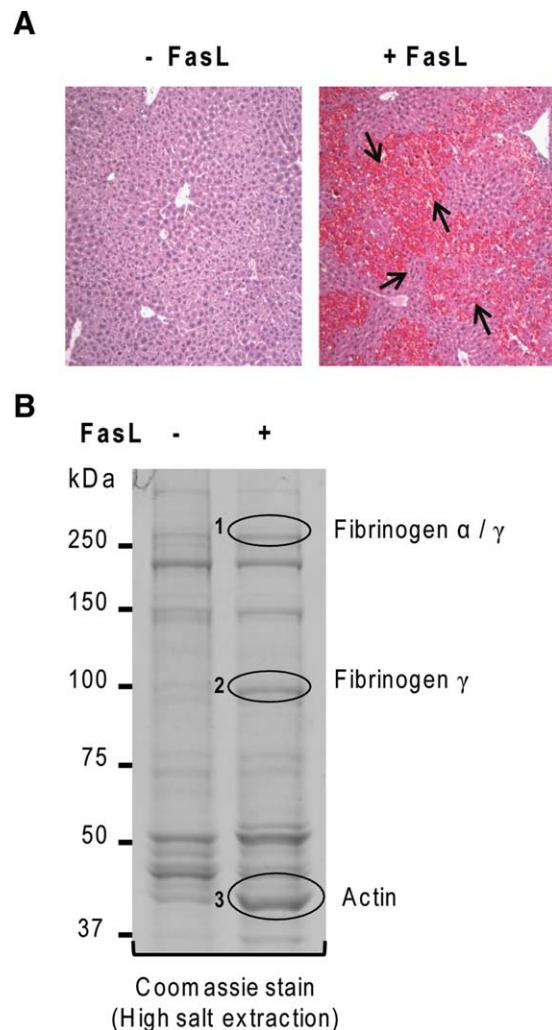


Fig. 1. Characterization of the insoluble liver proteins after FasL-induced apoptosis. (A) FasL was administered to mice. After 4.5 hours, mice were sacrificed, followed by H&E staining. Note the dramatic hemorrhage (arrows) in FasL-treated (+ FasL) compared with untreated (- FasL) mice. (B) Insoluble protein fractions of livers from FasL-treated and untreated mice were isolated using HSE followed by SDS-PAGE and Coomassie staining. Bands that showed increased intensity after FasL treatment (bands 1, 2, and 3) were subjected to mass spectrometric analysis which, in turn, predicted the identity of bands 1-3 as: FIB- $\alpha/\gamma$  (band 1), FIB- $\gamma$  (band 2), and actin (band 3).

To confirm the findings predicted by mass spectrometry, we used immunoblotting with antibodies specific to FIB- $\gamma$  and actin. Consistent with the mass spectrometric and N-terminal sequence analysis, the anti-FIB- $\gamma$  antibody recognized several protein species [including  $\approx$ 250 kDa and 100 kDa (Fig. 2B)] exclusively in the livers of FasL-treated mice. The 250-kDa and 100-kDa species (Fig. 2B) correspond to bands 1 and 2 in Fig. 1B, respectively. As expected, based on the predicted identity of band 3 (Fig. 1B), the actin blot demonstrated elevated levels of insoluble actin in FasL-treated livers compared with untreated control

**A**

YVATRD NCCILDERFGSFCPTTCG IADFLSSYQTDVDNDLRT  
LEDILFRAENRTEAKELIKAIQVYYNPDQPPKPGMIDSATQK  
SKKMVEEV**KYEALLLTHET**SIRYLQEIQNSNNQKITNLQKV  
QLEAQCQEPCDKDSVQIHD TTGKD CQEIAKGA**EGLYFIR**  
LKAKQQFLVCEIDGSGNGTVLQKRIDGSLDFKKNWQYK  
**EGFGHLSPTGTTEFWLGNEKIHISMQSTIPYALRIQLKDWN**  
GRTS TADYAMFRVGPGESDKYRLTYAYFIGGDAGDAFDGYDF  
GDDPSDKFFTSHNGMQFSTWDNDNDKFEGNCAEQDGSW  
WMNKCHAGHLNGVYHQGGTYSK**SSTTNGFDDGIWATWKS**  
RWYSMKETTMKIIPFNRLSIEGQQHHMGGSKQVSVDHEVEI  
EY

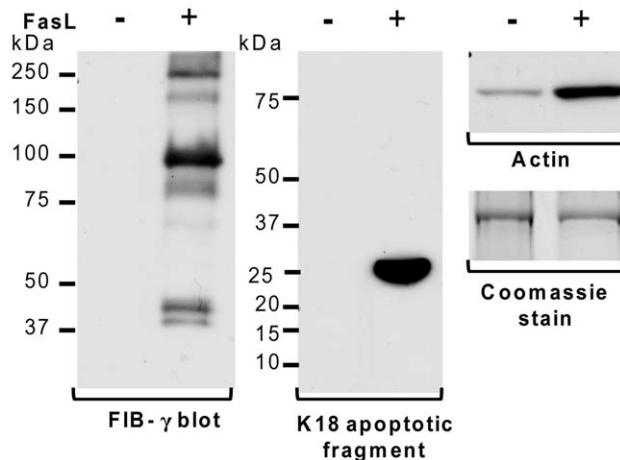
**B**

Fig. 2. FIB- $\gamma$  is the major insoluble liver protein after FasL-mediated injury. (A) Fragment sequences of FIB- $\gamma$  obtained from N-terminal sequencing (underlined) and mass spectrometry (bold) confirm the identity of band #2 shown in Fig. 1B. (B) Prediction of mass spectrometric results was verified by immunoblotting using antibodies to FIB- $\gamma$  and actin. The FIB- $\gamma$  antibody recognized several bands in the FasL-treated liver (250 kDa and 100 kDa) that were not present in untreated controls. These species correspond to bands 1 and 2 (Fig. 1B), respectively. The actin blot demonstrated elevated levels of actin protein in FasL-treated livers compared with untreated controls. Formation of the K18 apoptotic fragment confirmed the hepatocyte FasL-induced apoptosis.

(Fig. 2B). Hepatocyte apoptosis after FasL administration was also confirmed biochemically via immunoblotting using an antibody that recognized cleaved K18 after caspase digestion (Fig. 2B).

The shift in solubility of FIB- $\gamma$  upon induction of apoptosis was also tested in individual fractions of liver homogenates from mice with or without exposure to FasL. Barely detectable levels of FIB- $\gamma$  monomer ( $\approx$ 50 kDa) are present in the livers under basal conditions (Fig. 3). In contrast, the FIB- $\gamma$  blot of livers undergoing hepatocyte apoptosis showed two major bands (100 kDa and 250 kDa) that are present only in the total liver homogenate and are markedly enriched in the pellet fraction but not in the soluble TX-100 or high salt fractions (Fig. 3). Taken together, these findings indicate that FIB- $\gamma$  dimerizes and becomes insoluble upon FasL-mediated liver injury.

### FIB- $\gamma$ Partitions from Plasma to the Liver During FasL-Induced Liver Injury.

The above findings led us to hypothesize that FIB- $\gamma$  shifts its location from plasma to the liver upon apoptotic injury. We tested this hypothesis by comparing serum, plasma, and liver FIB- $\gamma$  levels before and after exposure to FasL. The FIB- $\gamma$  100 kDa dimer was detected in FasL-treated mouse serum but not in plasma, whereas this dimer and other high molecular weight (HMW) products were readily observed in the liver lysates (Fig. 4A). A

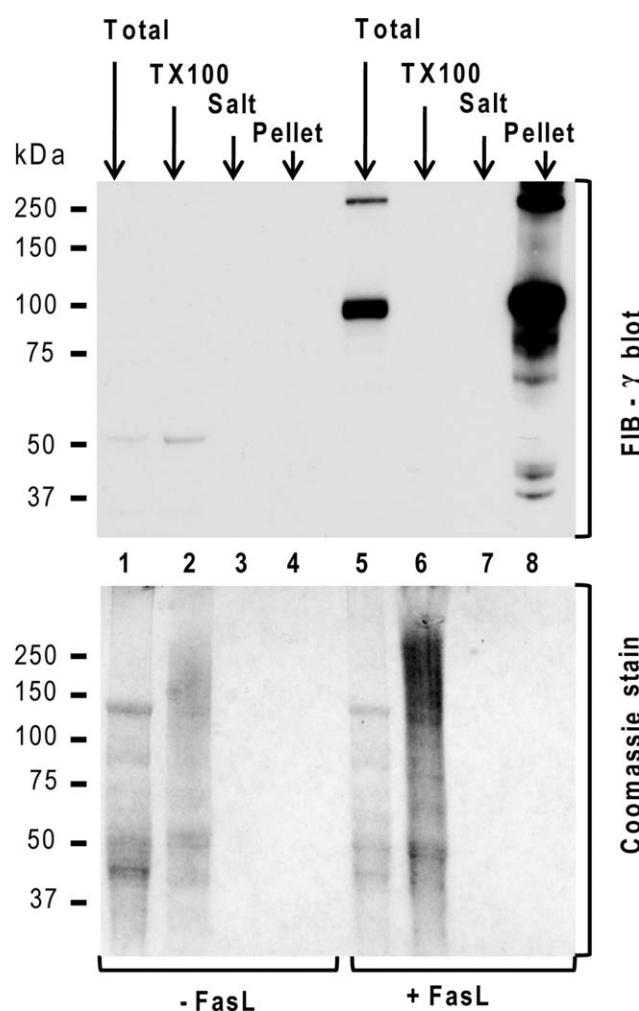


Fig. 3. Solubility dynamics of FIB- $\gamma$  during FasL-induced liver injury. Solubility dynamics of FIB- $\gamma$  during FasL-induced liver injury were analyzed biochemically by comparing the soluble TX-100 fraction, high salt wash fraction (Salt), and insoluble fraction (Pellet) from FasL-treated and untreated livers. TLL (Total) were used as controls. None of the fractions showed detectable levels of FIB- $\gamma$  dimer in untreated liver samples. However, FIB- $\gamma$  antibody faintly recognized the FIB- $\gamma$  monomer (50 kDa) in the TLL and the soluble TX-100 fraction of untreated liver. In contrast, FIB- $\gamma$  blot showed two major bands (100 kDa, 250 kDa) in FasL-treated TLL, but not in the soluble TX-100 and wash fractions. A duplicate gel to that used for immunoblotting was stained with Coomassie blue. Although the protein amounts in some of the lanes (3, 4, 7, 8) were not enough to be visible with Coomassie staining, they were readily detected by the FIB- $\gamma$  antibody (lane 8).

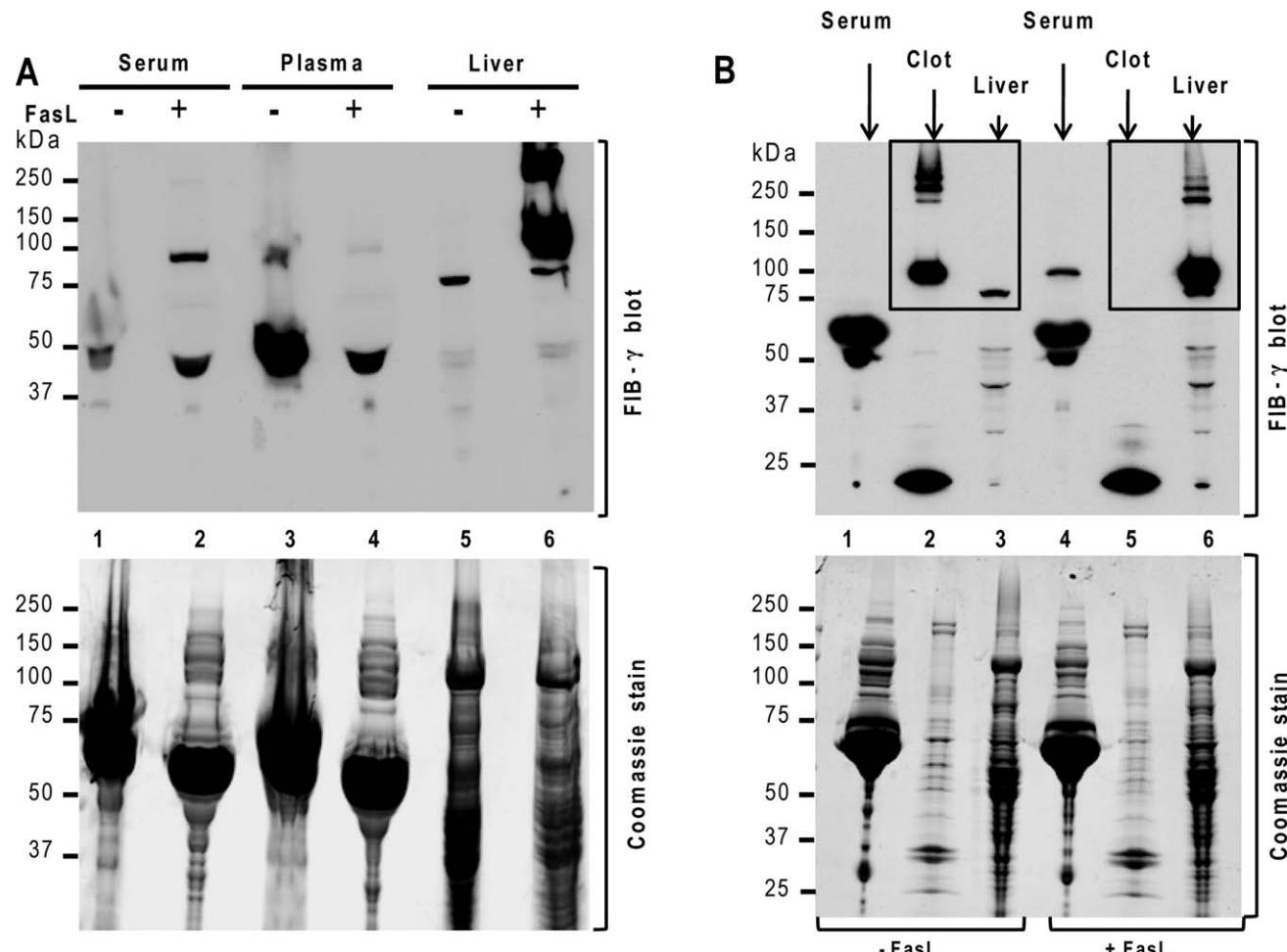


Fig. 4. Intrahepatic accumulation of FIB- $\gamma$  during FasL-induced injury. Mice were sacrificed 4.5 hours after FasL injection and blood components (serum, plasma, clot) and livers were collected. (A) FIB- $\gamma$  antibody recognized the FIB- $\gamma$  dimer (100 kDa) in FasL-treated mouse serum but not in FasL-untreated serum. FIB- $\gamma$  dimers and other HMW products (250 kDa) were readily observed in FasL-treated liver lysates. (B) FIB- $\gamma$  dimers and other HMW products were clearly observed by immunoblotting in the clot fraction of untreated mice but not in the liver, whereas the opposite occurs after FasL treatment. Coomassie staining of duplicate gels to those used for blotting are included as loading controls.

separate analysis comparing the FIB- $\gamma$  dimer and higher complexes in the clot from whole blood versus intact liver (boxed panels in Fig. 4B) shows a clear and marked shift from the clot to the liver. Therefore, the FIB- $\gamma$  dimer and its HMW complexes accumulate in the liver after FasL-mediated liver injury, which is consistent with intrahepatic IC. The intrahepatic IC is also supported by the increased levels of plasminogen activator inhibitor-1 in plasma and liver upon FasL-mediated liver injury, with concurrent increase in tissue factor levels in plasma (Supporting Fig. 1).

**Prophylactic Treatment with Heparin Reduces the Extent of FasL-Induced Apoptotic Liver Injury.** The extensive intravascular coagulation within liver parenchyma after FasL-induced hepatocyte apoptosis raised the hypothesis that anticoagulation using heparin may provide a protective effect. For this, we first defined a period whereby heparin is administered and maintains

its anticoagulant effect prior to injecting FasL. Using a dose range of 10–100 U per mouse, we found that 20 U per mouse provided anticoagulation that is similar to the higher tested doses (based on elevation of plasma fibrinogen levels without leading to significant hematoma formation, not shown). Based on this dosing regimen, heparin was administered subcutaneously followed 4 hours later by FasL administration. The extent of injury in these mice was then compared with mice given FasL alone (Fig. 5). Histological analysis of the livers showed a dramatic decrease in the extent of hemorrhage in mice that were given heparin (Fig. 5A, Supporting Fig. 2). Heparin pretreatment also resulted in a dramatic decrease in TUNEL staining (Fig. 5A). These findings were supported by significantly reduced serum ALT levels (4.8-fold) and lower liver apoptotic cell number in the heparin-pretreated mice (Fig. 5B,C). In addition, biochemical analysis showed that

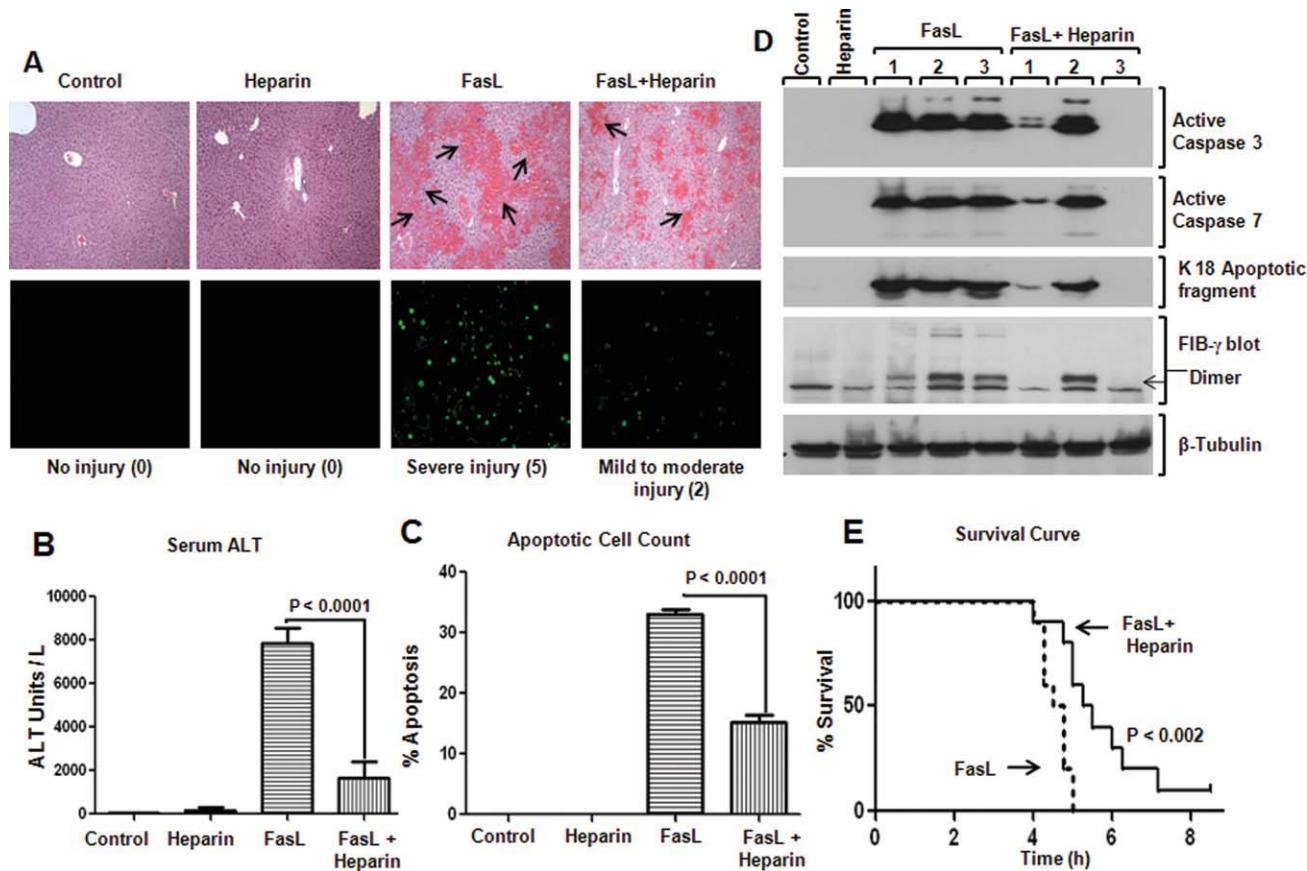


Fig. 5. Pretreatment with heparin reduces FasL-induced liver injury. (A) Heparin was administered to age/sex-matched mice followed by FasL injection (4 hours after heparin), and the extent of liver damage was assessed. H&E sections show hemorrhage formation (top row) and TUNEL staining shows apoptotic cells (bottom row). Three mice were used for each of the control and heparin-alone arms, and seven mice were used for each of the FasL and FasL+heparin arms. Numbers in parentheses reflect the injury score (see Materials and Methods and Supporting Fig. 2). (B) Serum ALT, (C) % apoptotic cell count, and (D) biochemical analysis of the livers from the indicated treatments and controls are shown. Note that all measured parameters are significantly less in mice that received heparin prior to FasL administration. In panel D, each lane represents an independent liver, and tubulin is shown as a loading control. (E) Heparin or saline were administered to mice (10–12 weeks old; n = 10 per group) followed by FasL injection (4 hours after heparin/saline), and survival time was recorded.

the activation of caspases 3 and 7 and formation of the K18 apoptotic fragment were markedly blunted in mice that received heparin (Fig. 5D). These biochemical changes also paralleled the detection of the FIB- $\gamma$  dimer (Fig. 5D). As expected, mice that received heparin showed elevated levels of serum fibrinogen (Supporting Fig. 3), thereby confirming its anticoagulative effect. Importantly, heparin-treated mice survived the FasL-induced liver injury longer compared with heparin-untreated mice (Fig. 5E). Taken together, these data indicate that prophylactic pretreatment with heparin reduces the extent of FasL-induced apoptotic liver injury in FVB/N mice.

**Therapeutic Benefit of Heparin in FasL-Induced Liver Injury.** Most cases of ALF occur in the context of an unanticipated exposure to an insult.<sup>23,24</sup> Therefore, it is important to identify potential compounds that can be used as a treatment, although prophylactic

drugs do have a role. Given the significant benefit imparted by heparin when administered before the FasL insult, we examined its effect as a therapeutic. In a preliminary experiment, we verified the rapid onset heparin action to be as early as 15 minutes after subcutaneous injection (Supporting Fig. 4). For the treatment experiment, mice were first given FasL, then heparin 1, 2, 2.5, and 3 hours after FasL injection. At 4.5 hours (i.e., the same time point used for the experiments in Fig. 1 and Fig. 5) after FasL injection, mice were sacrificed to evaluate the extent of injury using histological, serological, and biochemical means. Notably, treatment with heparin 1 hour and even 2 hours after FasL administration significantly reduced hemorrhage compared with heparin-untreated mice (Fig. 6A, Supporting Fig. 5). Serum ALT levels were markedly lower (7.3-fold) in mice that received heparin treatment 1 hour after FasL injection, but not at subsequent

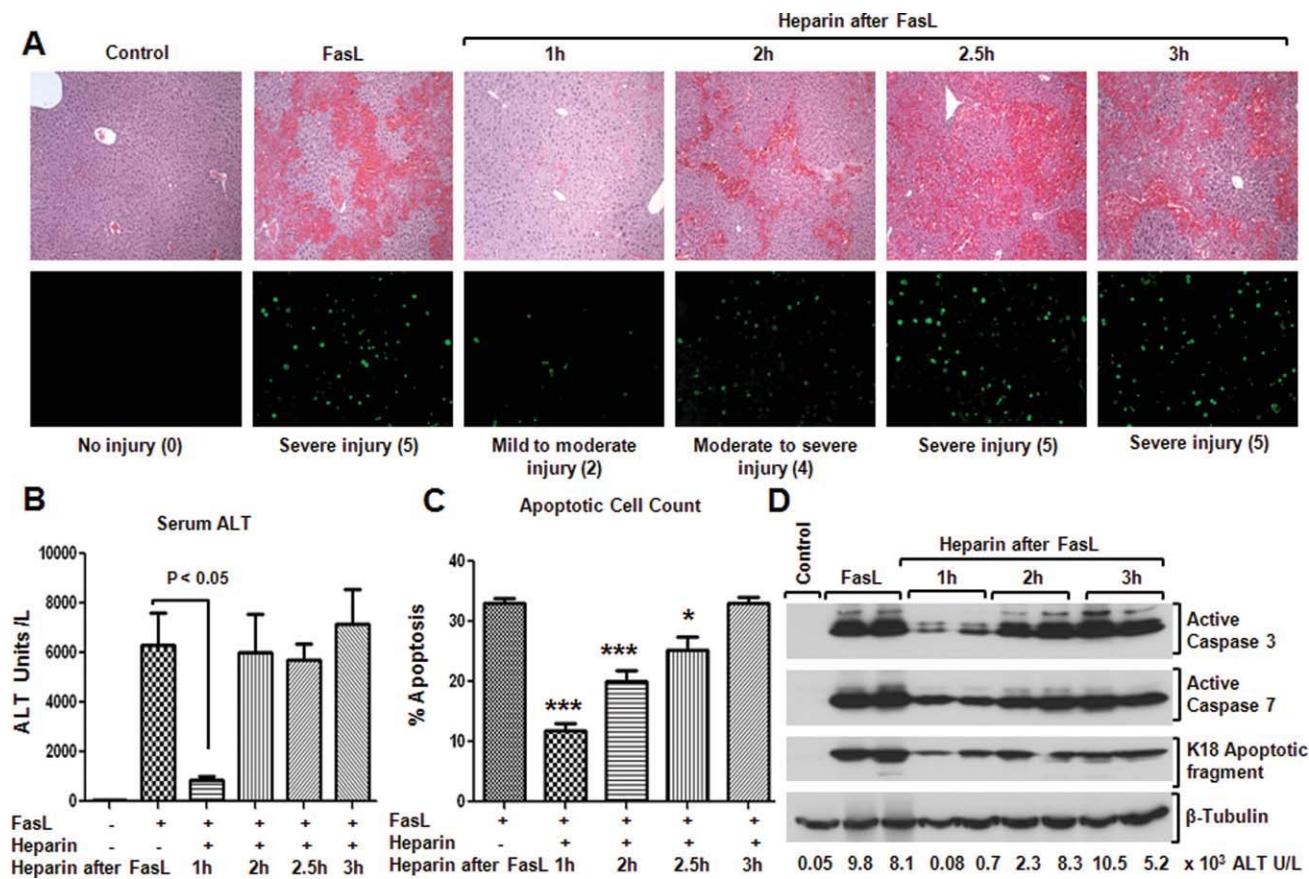


Fig. 6. Early treatment with heparin reduces FasL-induced liver injury. FasL was administered intraperitoneally followed by heparin injection 1, 2, 2.5, and 3 hours after FasL injection ( $n = 5-7$  mice per time point). After 4.5 hours of FasL injection, mice were sacrificed and liver damage was assessed. (A) H&E staining (top row) and TUNEL staining (bottom row) showed that treatment with heparin at 1 hour and 2 hours after FasL injection significantly reduced hemorrhage formation and apoptosis, indicative of only mild to moderate injury, compared with heparin-untreated mice. Numbers in parentheses reflect the injury score (see Materials and Methods and Supporting Fig. 5). (B,C) Serum ALT levels and percentage of apoptotic cells, respectively. (D) Activation of caspase 3 and 7 was markedly reduced in mice that received heparin 1 hour following FasL injection. Consistent with the decreased caspase activation, lower levels of the K18 apoptotic fragment were detected in livers of mice that received heparin treatment 1 hour following FasL injection compared with mice that received FasL alone. Serum ALT levels for each of the biochemically analyzed livers of each animal are shown. Tubulin blot is included as a loading control.

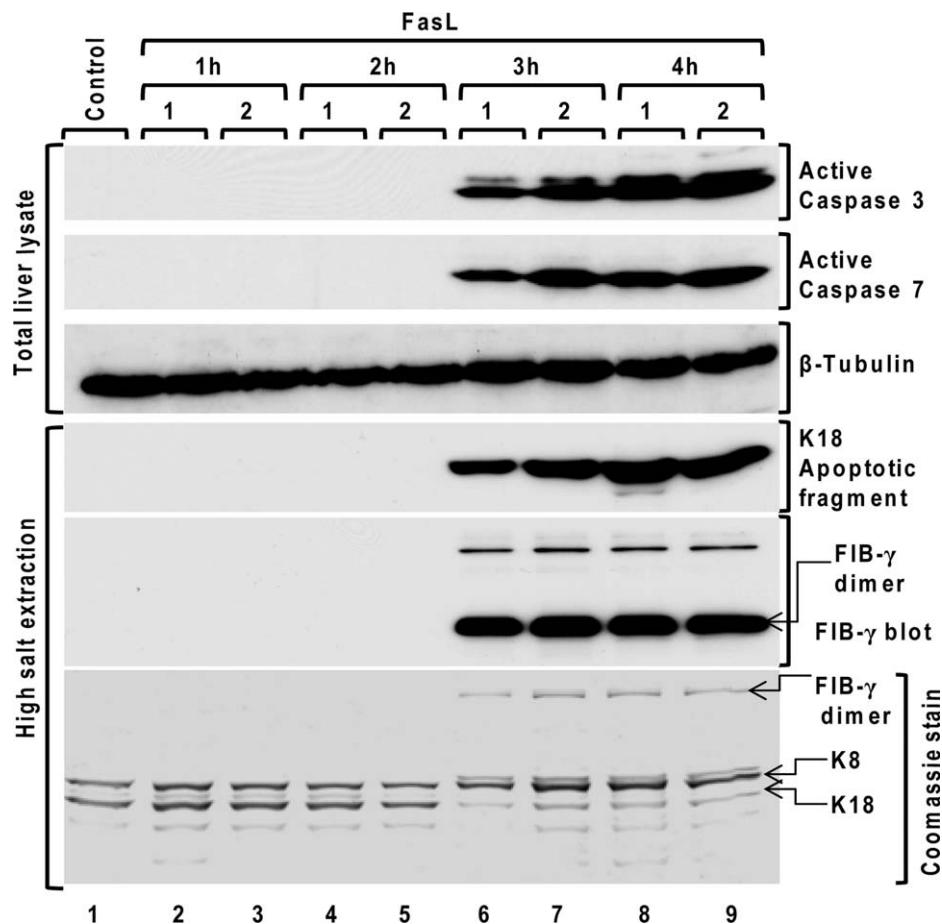
times (Fig. 6B). Quantification of the apoptotic cells showed a protective effect when heparin was given 1 hour or 2 hours after FasL administration (Fig. 6C), which is paralleled by findings using TUNEL staining (Fig. 6A). Similarly, the levels of activated caspases 3/7 and formation of the K18 apoptotic fragment were decreased, particularly at the 1-hour time point (Fig. 6D). Therefore, early treatment with heparin significantly reduces FasL-induced mouse liver injury.

**Relationship of Apoptosis to Intravascular Coagulation Within the Liver.** We addressed the time course of apoptosis progression versus IC within the liver. Administration of FasL followed by analysis of the livers at hourly intervals demonstrated that the readily detectable activation of caspases and keratin cleavage during apoptosis occur concurrently with FIB- $\gamma$  dimer formation (Fig. 7). Notably, FIB- $\gamma$  dimer formation shows a sharp rise, then remains relatively

constant as injury progresses, whereas caspase activation and keratin fragmentation also display a sharp rise but continue to increase with time (compare lanes 6 and 7 with 8 and 9). An independent experiment using analysis at 0.5-hour intervals showed similar findings (Supporting Fig. 6).

## Discussion

Our findings provide a model for FIB- $\gamma$  dynamics during mouse liver injury (Fig. 8). Upon apoptotic liver injury, plasma fibrinogen moves from plasma and is deposited within liver parenchyma as part of an intrahepatic IC that is triggered by the apoptotic cell injury. This process is accompanied by FIB- $\gamma$  cleavage, dimerization, and formation of higher molecular weight species that are detected exclusively in the liver upon FasL-induced liver injury (Fig. 4). A similar



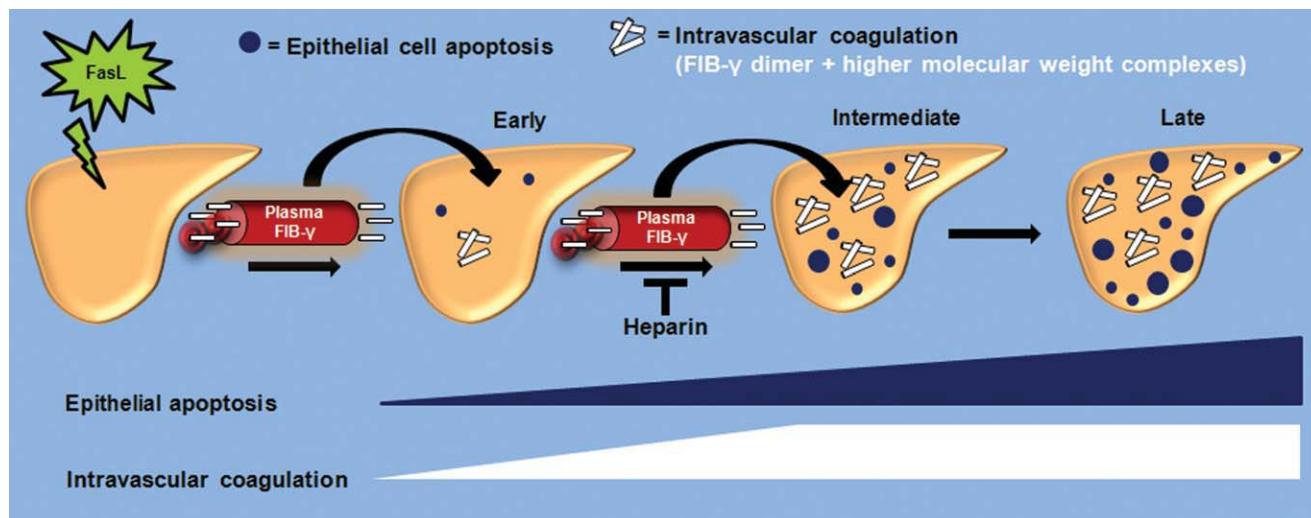
**Fig. 7.** Time course of caspase activation, K18 caspase-mediated digestion, and FIB- $\gamma$  dimer formation after FasL-induced injury. Liver apoptosis was induced by FasL injection. Mice were sacrificed 1, 2, 3, and 4 hours after FasL injection ( $n = 2$  mice per time point), followed by preparation of the total liver lysates or HSE, then immunoblotting using antibodies to the indicated antigens. A tubulin blot is included as a loading control, and a Coomassie stain of a duplicate gel of the HSE samples that were analyzed by blotting is also included.

behavior of FIB- $\gamma$  proteolysis and solubility dynamics also occurs in acetaminophen-mediated acute liver injury (Supporting Fig. 7). In contrast, under basal conditions only small amounts of fibrinogen are present in the liver, where it is synthesized in both rodents and humans, then secreted into the circulation.<sup>25</sup> The significance of the FIB- $\gamma$  dimer as a potential biomarker has been reported in cancer patients.<sup>15,16</sup> However, to our knowledge, biochemical FIB- $\gamma$  changes in the context of ALF have not been reported, although fibrin deposition in mouse liver has been observed after acetaminophen-mediated acute injury.<sup>18</sup> Notably, blood clots from mice undergoing apoptosis manifest a dramatic decrease in their 100-kDa FIB- $\gamma$  levels (Fig. 4B; compare lanes 2 and 5). Further studies will be needed to determine whether loss of FIB- $\gamma$  dimers (or other fibrinogen isoforms) in the clot of patients with ALF will serve as a potential useful marker of intrahepatic IC and disease severity.

The approach that we used to arrive at the importance of the hemostasis pathway during ALF involved a limited proteomic analysis aimed at the characterization of insoluble proteins that accumulate as a consequence of FasL-induced liver injury. Given the relative

short time from exposure to FasL to harvesting of the livers (4–5 hours), we predicted that any new protein species that either appear or disappear after FasL exposure are likely related to posttranslational modification of resident proteins or to proteins derived from infiltrating cells. The observed increase in actin (Fig. 1) is likely due to actin that is derived from infiltrating erythrocytes that accompany the observed hemorrhage, although we cannot exclude the possibility of a post-translational modification of actin that renders it insoluble.

As a result of IC, fibrin thrombi including the FIB- $\gamma$  dimer and its cleaved higher mass complexes accumulate in the liver, thereby altering normal blood flow. The consequent decreased blood flow to hepatocytes likely results in accumulation of reactive oxygen species and nitrogenous waste products in the liver, thereby perpetuating the extent of liver injury. Therefore, heparin is predicted to act by disrupting the injury cycle as injury moves from an early to an intermediate stage (Fig. 8) and preventing the deposition of fibrin thrombi, including the FIB- $\gamma$  dimer and its complexes, and facilitating adequate blood supply to liver parenchymal cells. Heparin does not appear to directly



**Fig. 8.** Model of intrahepatic vascular coagulation and epithelial cell apoptosis dynamics in response to FasL. The early stages of FasL-induced liver injury are manifested by limited hepatocyte apoptosis and intrahepatic vascular coagulation. This is followed at the intermediate stages by a near-concurrent marked increase in liver caspase activation, epithelial apoptosis, and intravascular coagulation. The IC is manifested by the formation of FIB- $\gamma$  dimers and HMW products within the liver, in association with a dramatic shift of FIB- $\gamma$  from plasma to liver. During the late stages that follow FasL exposure, IC remains relatively unchanged, whereas hepatocyte apoptosis continues. Heparin provides a therapeutic modality that not only limits the extent of FasL-related liver injury when given as prophylactic but also limits the extent of injury if given at the early stages of injury exposure.

inhibit FasL-mediated apoptosis, because heparin pretreatment of isolated mouse hepatocytes *ex vivo* did not alter the extent of FasL-induced caspase activation and K18 degradation (Supporting Fig. 8). We are not able to completely separate whether Fas and intrahepatic coagulation act simultaneously or sequentially; however, it appears that once significant coagulation occurs, it does not continue to increase, whereas caspase activation and apoptosis do continue to increase (Fig. 7, Supporting Fig. 6). This finding suggests that there may be a second wave of apoptosis/necrosis that is inhibited by heparin. In support of this notion, assessment of early time points after heparin pretreatment followed by FasL injection showed that heparin decreases caspase 3 activation, K18 caspase-mediated digestion, and formation of FIB- $\gamma$  dimers (Supporting Fig. 9). One important caveat is that heparin pretreatment delays but does not prevent animal mortality (Fig. 5E). This finding indicates that Fas–FasL interaction continues to occur despite the presence of heparin.

Another important finding herein is the beneficial effect of heparin, not only to provide prophylaxis toward apoptosis-associated liver injury but also to treat the injury, which is an important distinction in terms of potential therapeutic use. Heparin use was described to be effective in providing protection when given before administering acetaminophen, but was not tested for its effect after induction of liver injury.<sup>18</sup> In

humans, therapy for ALF is primarily supportive unless liver transplantation is available or deemed required,<sup>24,26</sup> though interventions such as the administration of *N*-acetylcysteine in non-acetaminophen-related ALF may be beneficial.<sup>27</sup> Currently anticoagulation is not used in human ALF because of the risk of increased bleeding, especially in the context of invasive procedures.<sup>28,29</sup> However, it appears that patients with ALF have complex hyper- and hypocoagulable states,<sup>9,30</sup> and patients undergoing liver transplantation with international normalized ratio values of >1.5 did well without plasma or red blood cell transfusions.<sup>31</sup> The complex antifibrinolytic and profibrinolytic milieu in patients with ALF suggests that a targeted anticoagulation approach that is patient- and disease-specific may be beneficial.

The dose of heparin used in our mice is predicted to be lower than what is currently used in patients who undergo treatment for deep venous thrombosis or other complications related to a hypercoagulable state. For example, the 20 U per 25 g mouse weight can be converted to a predicted human dose of ≈5,000 U, based on the recommended body surface area conversion,<sup>32</sup> which is lower than the typical 10,000 or more USP bolus dosing that is used in adult humans before initiating continuous infusion.<sup>33</sup> Our study provides a proof-of-principle approach that anticoagulation is effective in ameliorating FasL-induced liver injury. The use of the minimum effective dosing is of obvious

importance in order to minimize bleeding complications. For example, when we used doses of 50-100 U per mouse, hematomas of variable sizes were frequently noted proximal to the site of injection (data not shown). The use of other fibrinolytic approaches (e.g., low molecular weight heparin<sup>33</sup>) may also prove beneficial.

**Acknowledgment:** We thank Dr. Pavel Strnad for valuable comments.

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