Research Article

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Cirrhosis

Hepatocyte tissue factor contributes to the hypercoagulable state in a mouse model of chronic liver injury

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Background & Aims: Patients with chronic liver disease and cirrhosis have a dysregulated coagulation system and are prone to thrombosis. The basis for this hypercoagulable state is not completely understood. Tissue factor (TF) is the primary initiator of coagulation *in vivo*. Patients with cirrhosis have increased TF activity in white blood cells and circulating microparticles. The aim of our study was to determine the contribution of TF to the hypercoagulable state in a mouse model of chronic liver injury.

Methods: We measured levels of TF activity in the liver, white blood cells and circulating microparticles, and a marker of activation of coagulation (thrombin-antithrombin complexes (TATc)) in the plasma of mice subjected to bile duct ligation for 12 days. We used wild-type mice, mice with a global TF deficiency (low TF mice), and mice deficient for TF in either myeloid cells ($TF^{flox/flox}$,LysMCre mice) or in hepatocytes ($TF^{flox/flox}$,AlbCre).

Results: Wild-type mice with liver injury had increased levels of white blood cell, microparticle TF activity and TATc compared to sham mice. Low TF mice and mice lacking TF in hepatocytes had reduced levels of TF in the liver and in microparticles and exhibited reduced activation of coagulation without a change in liver fibrosis. In contrast, mice lacking TF in myeloid cells had reduced white blood cell TF but no change in microparticle TF activity or TATc.

Conclusions: Hepatocyte TF activates coagulation in a mouse model of chronic liver injury. TF may contribute to the hypercoagulable state associated with chronic liver diseases in patients.

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Abbreviations: ALT, alanine aminotransferase; aPTT, activated partial thromboplastin time; AST, aspartate aminotransferase; BDL, bile duct ligation; F, factor; IL-6, interleukin 6; LysM, lysozyme; MP, microparticle; PT, prothrombin time; TAT, thrombin-antithrombin complexes; TF, tissue factor; WBC, white blood cell.

Introduction

Patients with chronic liver disease, and particularly cirrhosis, have a dysregulated coagulation system [1,2]. The traditional view of coagulation disorders in patients with chronic liver disease has changed from concerns about bleeding to thrombosis [1,2]. Routine diagnostic tests of coagulation, such as the prothrombin time and the activated partial thromboplastin time, are frequently prolonged in patients with chronic liver disease suggesting that these patients would be prone to bleeding [1,2]. However, an increasing number of studies indicate that these abnormal findings in routine coagulation tests do not necessarily predict an increased bleeding tendency in patients with chronic liver disease [1–6]. Rather, recent findings indicate a thrombotic risk in these patients [7–10]. Likewise, thrombin generation tests performed in the presence of the anticoagulant thrombomodulin or snake-venom extract (Protac, Pentapharm) have shown that plasma from patients with cirrhosis generate similar, or even greater amounts of thrombin than plasmas from healthy subjects [1,3–6]. The basis for this hypercoagulable state in patients with chronic liver disease is not completely understood [1,2]. It has been suggested that it may be due, in part, to increased levels of the procoagulant factor VIII (FVIII) and von Willebrand factor and reduced levels of the anticoagulant protein C and antithrombin [1,2,11]. However, this hypercoagulable state is likely to be more complex than simply changes in a few proteins.

TF is the transmembrane receptor for FVII/VIIa and the TF: FVIIa complex functions as the primary initiator of coagulation *in vivo* [12]. It is essential for hemostasis. TF can be found in low- (also called encrypted) and high-activity (also called de-encrypted) states, which is thought to be due to differences in the conformation of TF [13]. TF is constitutively expressed by

cells within and surrounding the blood vessel wall, such as vascular smooth muscle cells, pericytes and adventitial fibroblasts [12]. In addition, TF has been implicated in thrombosis associated with a variety of diseases [12,14–16]. Several studies have shown that monocyte TF expression and circulating microparticle (MP) TF activity are increased in patients with cirrhosis [17–20]. MPs are submicron membrane vesicles derived from apoptotic and/or activated cells [21]. TF-positive MPs are highly procoagulant [21,22]. Recently, we reported that hepatocytes constitutively express TF in an inactive state, but this TF is rapidly activated during acute hepatocellular injury and activates the coagulation system [23]. The contribution of different cellular sources of TF to the activation of coagulation in chronic liver disease has not been evaluated.

In this study, we investigated the role of TF in the activation of coagulation in a mouse model of chronic liver disease. In addition, we determined the source of TF responsible for the activation of coagulation. We found that TF expression by hepatocytes activates coagulation in this model.

Material and methods

Mice

Wild-type C57BL/6J male mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Transgenic low TF male mice on a C57BL/6J background were generated as previously described [24]. These *mTF*$^{-/-}$,*hTF*$^{+/+}$ mice express no mouse TF but have low levels of human TF expressed from a minigene (~1% of levels compared with wild-type mice) in all tissues [24]. Littermate controls containing the same human transgene and expressing either 50% (*mTF*$^{+/-}$,*hTF*$^{+/+}$ mice, hereafter referred to as *TF*$^{+/-}$ mice) or 100% of levels of murine *TF* (*mTF*$^{+/+}$,*hTF*$^{+/+}$ mice, hereafter referred to as *TF*$^{+/+}$ mice) were used as controls. The generation of the *TF*$^{flox/flox}$, lysozyme (LysM) Cre recombinase mice, which deletes the TF gene in myeloid cells by ~90%, has been described [15,16]. The generation of the *TF*$^{flox/flox}$, AlbCre mice, with a deletion of the *TF* gene in hepatocytes, has been described [23]. Mice were fed a normal laboratory diet and given water *ad libitum*. All mouse studies were performed with the approval of the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee (IACUC).

Bile duct ligation (BDL)

Male mice between the ages of 9 and 17 weeks were anesthetized with 2.5% inhaled isoflurane. The abdomen was then shaved and prepared utilizing sterile technique. An upper-midline laparotomy incision was made, and the common bile duct was ligated with 6-0 silk suture (Unify® sutures, AD Surgical). The muscle layer and skin were closed with absorbable and non-absorbable suture material, respectively (Unify® sutures, AD Surgical). All steps, excluding ligation of the bile duct, were performed for sham operations. Criteria for successful BDL at the time of animal sacrifice included jaundiced soft tissues, patchy liver discoloration, and biliary tree dilation. The success rate for the different BDL surgeries was 59%, 65%, 66%, and 75% in wild-type mice, low TF mice, *TF*$^{flox/flox}$,LysMCre and *TF*$^{flox/flox}$, AlbCre mice and their respective littermate controls, respectively.

Plasma and serum preparation

Mice were sedated with 3% isoflurane and blood was collected from the inferior vena cava into syringes pre-filled with 3.8% sodium citrate (1 volume of citrate for 9 volumes of blood). Mice were then euthanized. Mouse blood was centrifuged at 4000 *g* for 15 min and then at 13,000 *g* for 2 min to prepare platelet-free plasma. Plasma was stored at −80 °C until use, while the blood pellet was processed, as stated below. The same procedure was applied using syringes without citrate to obtain serum.

Plasma levels of thrombin-antithrombin complexes (TATc)

Mouse plasma levels of TATc complexes were measured using the Enzygnost TAT micro kit (Siemens Healthcare, Marburg, Germany), according to the manufacturer's instructions.

Prothrombin time and activated partial thromboplastin time

For measuring the prothrombin time (PT) of plasma, 50 µl of PT reagent (Thromboplastin-D, Pacific Hemostasis, Middletown, VA) was added to 25 µl of plasma and the clotting time was measured using a STart4 coagulation analyzer (Diagnostica Stago, Parsippany, NJ). For measuring the activated partial thromboplastin time (aPTT) of plasma, 25 µl of aPTT reagent (TriniCLOT aPTT S, Tcoag, Wicklow, Ireland) was mixed with 25 µl of plasma and incubated. Then 25 µl of 0.02 mol/L $CaCl_2$ was added and the clotting time was measured using a STart4 coagulation analyzer.

Measurement of plasma thrombin generation

Plasma thrombin generation was measured in 6-fold diluted murine plasma by calibrated automated thrombography as described [25]. Reactions were performed in the absence and presence of rabbit thrombomodulin (20 nmol/L, Haematologic Technologies, Essex Junction, VT) to detect alterations in procoagulant and anticoagulant (protein C/S) pathways [3,6,26]. Thrombin generation parameters were calculated using Thrombinoscope software version 3.0.0.29 (Thrombinoscope BV, Maastricht, Netherlands).

MP TF activity assay

MP TF activity was measured as described [27]. Briefly, plasma MPs were pelleted at 20,000 *g* for 30 min at 4 °C, washed three times and resuspended in HBSA buffer (137 mmol/L NaCl, 5.38 mmol/L KCl, 5.55 mmol/L glucose, 10 mmol/L HEPES, 0.1% bovine serum albumin, pH 7.5). For measurement of TF-specific activity, samples were then incubated with either an inhibitory rat anti-mouse TF monoclonal antibody (1H1, 100 µg/ml, kindly provided by Dr. Daniel Kirchhofer, Genentech), or a rat IgG control (Sigma Aldrich) for 15 min at room temperature. Next, 4.88 nmol/L mouse FVIIa, 146 nmol/L human FX, and 10 mmol/L $CaCl_2$, were added to the sample and incubated for 1 h at 37 °C in a 96-well plate. FXa levels were determined using the chromogenic substrate, Pefachrome FXa 8595 (4 mmol/L; Pentapharm, Basel, Switzerland). Absorbance (at 405 nm) was determined using a SpectraMax M5 and analyzed using Softmax Pro v 5.2C software (Molecular Devices, Sunnyvale, CA). TF activity was determined from a standard curve generated with recombinant human relipidated TF (0–14 pg/ml, Innovin®, Dade Behring). TF-specific activity was determined by subtracting the activity in the presence of the blocking antibody from the activity in the presence of the IgG control.

White blood cell (WBC) TF activity

WBC were isolated from blood as described [14]. The cell pellet was resuspended in 200 µl of HBSA buffer and diluted 1:20 before adding to the TF activity assay described above for the MP TF assay and incubated for 15 min.

Liver procoagulant activity

The procoagulant activity of liver tissue lysates was measured using a 1-stage clotting assay with a Start4 coagulation analyzer as described [16]. Briefly, frozen liver tissue was homogenised in 15 mmol/L n-Octyl-β-D-glycopyranoside and 25 mmol/L HEPES buffer (10 mg tissue/100 µl buffer) for 30 s. Samples were incubated at 37 °C for 15 min. For the clotting assay, 25 µl of sample was incubated with 25 µl of pooled mouse plasma for 1 min, then 25 µl of $CaCl_2$ (20 mmol/L) was added and clotting time was measured. The procoagulant activity of the sample was calculated by reference to a standard curve generated using recombinant human relipidated TF Innovin (Dade Behring), then normalized to the total protein concentration determined using the DC protein assay (Bio-Rad, Hercules, CA). We have previously found that the anti-mouse TF antibody 1H1 (50 µg/ml) reduced the procoagulant activity by ~90% [28]. It should be noted that MP, WBC, and liver TF activity in the different mouse strains were measured over a period of 2 years with different batches of reagents and this may explain some of the observed variations between experiments.

Western blot

Liver tissue samples were homogenised in RIPA lysis buffer supplemented with proteinase and phosphatase inhibitors (Roche; Santa Cruz Biotechnologies). Thirty µg of total liver tissue protein was diluted in NuPage LDS sample buffer (Life Technologies), boiled for 5 min, and then applied to 4–20% Tris-Glycine gels

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