

The Impact of Dabigatran Treatment on Sinusoidal Protection Against Hepatic Ischemia/Reperfusion Injury in Mice

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Thrombin is a key player in the coagulation cascade, and it is attracting much attention as a promotor of cellular injured signaling. In ischemia/reperfusion injury (IRI), which is a severe complication of liver transplantation, thrombin may also promote tissue damage. The aim of this study is to reveal whether dabigatran, a direct thrombin inhibitor, can attenuate hepatic IRI with focusing on a protection of sinusoidal endothelial cells (SECs). Both clinical patients who underwent hepatectomy and in vivo mice model of 60-minute hepatic partial-warm IRI, thrombin generation was evaluated before and after IRI. In next study, IRI mice were treated with or without dabigatran. In addition, hepatic SECs and hepatocytes pretreated with or without dabigatran were incubated in hypoxia/reoxygenation (H-R) environment in vitro. Thrombin generation evaluated by thrombin–antithrombin complex (TAT) was significantly enhanced after IRI in the clinical study and in vivo study. Thrombin exacerbated lactate dehydrogenase cytotoxicity levels in a dose-dependent manner in vitro. In an IRI model of mice, dabigatran treatment significantly improved liver histological damage, induced sinusoidal protection, and provided both antiapoptotic and anti-inflammatory effects. Furthermore, dabigatran not only enhanced endogenous thrombomodulin (TM) but also reduced excessive serum high-mobility group box-1 (HMGB-1). In H-R models of SECs, not hepatocytes, pretreatment with dabigatran markedly attenuated H-R damage, enhanced TM expression in cell lysate, and decreased extracellular HMGB-1. The supernatant of SECs pretreated with dabigatran protected hepatocytes from H-R damage and cellular death. Thrombin exacerbated hepatic IRI, and excessive extracellular HMGB-1 caused severe inflammation-induced and apoptosis-induced liver damage. In this situation, dabigatran treatment improved vascular integrity via sinusoidal protection and degraded HMGB-1 by endogenous TM enhancement on SECs, greatly ameliorating hepatic IRI.

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Hepatic ischemia/reperfusion injury (IRI) is a serious problem for successful liver transplantation because IRI causes early graft failure and leads to a higher incidence of acute and chronic rejection.⁽¹⁾ However, the precise

mechanism of hepatic IRI development remains unclear. Multiple mediators and signaling pathways contribute to the pathophysiology of hepatic IRI and cause direct cellular injury as a result of inflammation and apoptosis.^(2,3) In the setting of liver damage, such as obstructive jaundice and nonalcoholic fatty liver disease, significant changes of the blood coagulation cascade develop⁽⁴⁻⁶⁾; therefore, attention has been given to the relationship between hepatic IRI and this blood coagulation cascade.

Thrombin, a pluripotent serine protease, plays a central role in the coagulation cascade and also acts as a promotor of inflammation, apoptosis, and cellular injury.⁽⁷⁾ We focused on a major high-affinity receptor of thrombin, protease-activated receptor-1 (PAR-1),⁽⁸⁻¹⁰⁾ and investigated a relationship between PAR-1 and the pathophysiology of hepatic IRI. Our previous study

Abbreviations: ALT, alanine aminotransferase; APC, activated protein C; AST, aspartate aminotransferase; CD31, cluster of differentiation 31; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; DOAC, direct oral anticoagulant; ELISA, enzyme-linked immunosorbent assay; H-R, hypoxia/reoxygenation; HMGB-1, high-mobility group box-1; IL6, interleukin-6; IQR, interquartile range; IRI, ischemia/reperfusion injury; LDH, lactate dehydrogenase; NS, not significant; PAR-1, protease-activated receptor-1; PCR, polymerase chain reaction; SEC, sinusoidal endothelial cell; TAT, thrombin–antithrombin complex; TM, thrombomodulin; TNF- α , tumor necrosis factor α ; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; VCAM-1, vascular cell adhesion molecule-1.

revealed that thrombin–PAR-1 signaling exacerbated hepatic IRI, and PAR-1 antagonism protected sinusoidal endothelial cells (SECs) by antiapoptotic effects, resulting in the attenuation of hepatic IRI.⁽¹¹⁾ Because SECs are regarded as an initial target of hepatic IRI, protecting SECs contributes to ameliorating hepatic IRI. Consequently, we hypothesized that selective inhibition of thrombin, the trigger of thrombin–PAR-1 signaling, could also protect SECs and show cytoprotective effects in hepatic IRI.

Dabigatran is a selective thrombin inhibitor that can only bind to the active site of thrombin.^(12,13) Indeed, there are a few studies that show the tissue protective effects of thrombin inhibition by dabigatran. Bogatkevich et al.⁽¹⁴⁾ showed the anti-inflammatory and antifibrotic effects of dabigatran in an interstitial lung disease model of mice. As for liver diseases, dabigatran reduced liver fibrosis in rats⁽¹⁵⁾ and attenuated liver inflammation and steatosis in

mice fed a high-fat diet.⁽⁹⁾ However, to the best of our knowledge, no studies have investigated the efficacy of thrombin inhibition by dabigatran in hepatic IRI.

The aim of the present study was to elucidate the effect of dabigatran treatment on hepatic IRI using a partial-warm IRI model of mice and a hypoxia/reoxygenation (H-R) model of hepatic SECs.

Materials and Methods

CLINICAL STUDY OF THROMBIN–ANTITHROMBIN COMPLEX CHANGE GENERATION BEFORE AND AFTER HEPATECTOMY

In patients with hepatocellular carcinoma (HCC) who underwent hepatectomy between August 2014 and April 2016 in Mie University Hospital in Japan, plasma samples for analyses were collected at the time before and 1 day after hepatectomy. The plasma concentration of thrombin–antithrombin complex (TAT) was quantified using a human enzyme-linked immunosorbent assay (ELISA) kit from Abcam (ab108907; Cambridge, MA). After excluding patients who had inadequate samples, data from the remaining 14 patients (total number of patients 45) were available for analysis. The study protocol was approved by the medical ethics committee of Mie University Hospital (No. H2020-030) and was performed in accordance with the tenets of the 1964 Declaration of Helsinki.

ANIMALS

In this study, 8-week-old to 9-week-old male C57BL/6 mice (21–25 g; Japan SLC Inc., Hamamatsu, Japan) were used. The experiments were reviewed and approved by the Animal Care and Use Committee at Mie University Graduate School of Medicine (No. 28-9) and conducted in compliance with the Guidelines for Animal Experiments of Mie University Graduate School of Medicine. All animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health.

PARTIAL HEPATIC IRI MODEL

A hepatic partial-warm IRI model was established in mice as previously reported.^(16,17) Mice were

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Daisuke Noguchi and Naohisa Kuriyama designed the study, performed the experiments and interpreted the data, and wrote the initial draft of the manuscript. Taemi Hibi, Koki Maeda, and Takehiro Fujii supported the first and corresponding authors with performing the experiments. All other authors contributed to the data analysis and critically reviewed the study design and manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Additional supporting information may be found in the online version of this article.

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Potential conflict of interest: Nothing to report.

[Correction added on January 21, 2021 after first online publication: In Fig. 4A and B(a), “Liver tissue” was changed to “Liver lysate”; In Fig. 6C(a) and Fig 7C(a), “ELISA” was changed to “Western blot.” We apologize to the author and our readers for these errors.]

anesthetized with isoflurane, and livers were exposed through a midline laparotomy. The arterial and portal venous blood supplies were interrupted to the cephalad lobes of the liver for 60 minutes using an atraumatic clip. The right hepatic and caudate lobes were perfused to prevent intestinal congestion. After 60 minutes of ischemia, the clip was removed, thereby initiating hepatic reperfusion. At 4 hours after reperfusion, the mice were euthanized to collect blood and liver tissues.

MEASUREMENT OF SERUM TRANSAMINASES

Serum aspartate transaminase (AST) and alanine transaminase (ALT) levels were measured using a commercially available kit (Transaminase Cii Test Wako Kit; Wako Pure Chemical Industries Ltd., Osaka, Japan) following the manufacturer's instructions.

DOSE DETERMINATION OF DABIGATRAN IN HEPATIC IRI STUDY OF MICE IN VIVO

A potent and highly selective thrombin inhibitor, dabigatran, was used in the present study. Dabigatran etexilate (DE), which is an orally active prodrug of dabigatran, was purchased from Chem Scene (Monmouth Junction, NJ) and administered to mice orally in vivo models. DE was dissolved in 100% dimethyl sulfoxide (DMSO), and the final DMSO concentration was 20%. The mice in our models were administered DE orally because DE must undergo chemical conversion by metabolic processes through oral intake to activate. A previous study showed that dose-dependent and time-dependent anticoagulant effects were observed with DE administered orally to conscious rats (10, 20, and 50 mg/kg) or rhesus monkeys (1, 2.5, or 5 mg/kg), with maximum effects observed between 30 and 120 minutes after administration, respectively.⁽¹⁸⁾ Following these data, to examine the effect of DE treatment in our mice hepatic IRI model based on serum AST levels at 4 hours after reperfusion, we set a dose of DE among 10, 50, 100 mg/kg, and DE was administered orally at 120 minutes before ischemia ($n = 3$ in each group). As shown in Supporting Fig. 1, a dose of 50 mg/kg had a significant effect: 4329.92 (IQR, 3887.49-4874.13) IU/L in controls versus 372.14 (IQR, 354.89-379.13) IU/L in the DE treatment group ($P = 0.05$). Accordingly, we decided to administer 50 mg/kg of DE.

EXPERIMENTAL GROUPS OF MICE

All mice were randomly allocated to 2 IRI groups ($n = 6$ in each group). The IRI + dabigatran group received an oral administration of 50 mg/kg of DE, whereas the IRI + vehicle group received an oral administration of vehicle (20% DMSO equivalent to that used to dissolve DE) at 120 minutes before ischemia. These mice underwent the surgery described previously under the same conditions.

HISTOLOGY

Liver specimens were fixed in a 10% buffered formalin solution, embedded in paraffin, and processed for hematoxylin and eosin staining as previously described.⁽¹⁹⁾ The histological severity of hepatic IRI was graded using a modified Suzuki's score.⁽¹⁴⁾ In this classification, sinusoidal congestion, hepatocyte necrosis, and ballooning degeneration were graded from 0 to 4. No necrosis, congestion, or centrilobular ballooning was given a score of 0, whereas severe congestion, ballooning degeneration, and 60% lobular necrosis were given a score of 4. The results were evaluated by averaging 10 scores in 20 high-power fields per section in a blinded manner.

IMMUNOHISTOCHEMISTRY

Liver specimens embedded in a Tissue-Tek optimal cutting temperature compound (Miles, Elkhart, IN) and snap-frozen in liquid nitrogen were used for immunostaining as previously described.⁽¹⁵⁾ Primary antibody against Ly6G (BioLegend, San Diego, CA) was used at a dilution of 1:500. The results were evaluated by averaging 10 counts of the number of Ly6G-positive cells in 20 high-power fields per section in a blinded manner.

IMMUNOFLUORESCENCE ANALYSIS

Liver specimens embedded in paraffin were deparaffinized and rehydrated. The monoclonal antibody against mouse fibrin (clone 59D8, MABS2155; EMD Millipore, Bedford, MA) at a dilution of 1:300 and the polyclonal antibody against rabbit cluster of differentiation 31 (CD31; Santa Cruz, CA) at a dilution of 1:50 were used as primary antibodies. Fluorescence signals were detected by Alexa Fluor 488 (green)-labeled and Alexa Fluor 594 (red)-

labeled secondary antibodies. Vectashield mounting media with DAPI (Vector Laboratories, Burlingame, CA) were used for nuclear staining. Slides were observed through the appropriate filter using a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE-MEDIATED DEOXYURIDINE TRIPHOSPHATE NICK-END LABELING STAINING

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining was performed to evaluate apoptotic cells in vivo study of mice ($n = 6$ in each group) and a paracrine communication model between hepatic SECs and hepatocytes in vitro ($n = 5$ in each group) using the In Situ Cell Death Detection Kit (catalog no. 11684795910; Roche Diagnostics, Temecula, CA), following the manufacturer's instructions. In an in vivo study of mice, paraffin-embedded liver tissue sections were deparaffinized and rehydrated, followed by 350 W microwave irradiation before the TUNEL reaction. Hepatocytes (0.5×10^4 /well) were seeded and cultured overnight in a collagen-coated chamber slide (Iwaki, Tokyo, Japan). After confluence, they were exposed to H-R following pretreatment with the supernatant of SECs and fixed after the TUNEL reaction. All samples were analyzed using a fluorescence microscope (BX51; Olympus). The results were evaluated by averaging 10 counts of the number of TUNEL-positive cells in 20 high-power fields per section in a blinded manner.

WESTERN BLOT ANALYSIS

Total protein from the whole-liver in vivo study or cell lysate in vitro study were extracted, and Western blot analysis were performed with equal amounts of protein loading as previously described.⁽⁹⁾ In brief, protein-transferred polyvinylidene fluoride membranes (EMD Millipore) were incubated overnight with specific primary antibodies against cleaved-caspase 9 (7237; Cell Signaling Technology, Beverly, MA), caspase 9 (9508; Cell Signaling Technology), high-mobility group box-1 (HMGB-1) (3935; Cell Signaling Technology), thrombomodulin (TM) for human (43514; Cell Signaling Technology), TM for mice (bs-20395R; Bioss Antibodies, Beijing, China), PAR-1 (bs-0828R; Biosynthesis Biotechnology

Co., Ltd, Beijing, China), and β -actin (4967; Cell Signaling Technology) at 4°C, followed by a horseradish peroxidase-linked secondary antibody for 2 hours at room temperature. After development, membranes were stripped and reblotted with the β -actin antibody. The immunoreactive bands were detected using the ImageQuant LAS 4000 mini system (GE Healthcare UK Ltd., Buckinghamshire, UK), and the intensities were then quantified using a densitometry tool (National Institutes of Health ImageJ software; <https://imagej.nih.gov/ij/>) and normalized to the internal control (β -actin protein).

RNA EXTRACTION AND REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

We evaluated mRNA levels of tumor necrosis factor (TNF)- α , interleukin (IL)-6, *bcl-2*, HMGB-1, and β -actin using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. The cDNA prepared from total RNA extracted from livers was subjected to real-time quantitative polymerase chain reaction (PCR) on a StepOne Real-Time PCR System (Applied Biosystems). The following primer/probe pairs used in this study were from Applied Biosystems: vascular cell adhesion molecule-1 (VCAM-1), Mm01320970; TNF- α , Mm00443260_g1; IL6, Mm00446190_m1; *bcl-2*, Mm00477631_m1; HMGB-1, Mm00849805_gH; and β -actin, Mm00607939_s1. β -actin was used as a normalization control.

NONISCHEMIC DAMAGE MODEL OF HEPATIC SECs

Human hepatic SECs were purchased from ScienCell Research Laboratories (San Diego, CA). In a non-ischemic damage model, SECs (0.5×10^4 /well) were seeded and cultured in an endothelial cell medium on a 24-well collagen-coated plate at 37°C with 5% CO₂ for 48 hours. Following semiconfluence, they were allocated to 2 and then cultured ($n = 4$ in each group). The sham + dabigatran group was pretreated with 0.5 μ M of dabigatran in an endothelial cell medium for 2 hours, whereas the sham + vehicle group was pretreated with vehicle (DMSO equivalent to that used to dissolve dabigatran) for 2 hours. Pretreated cells in both groups were cultured in a serum-starved medium in a nonischemic environment (37°C with 5% CO₂) for

5 hours, equivalent to the length of the present H-R models in vitro (60 minutes of hypoxia plus 4 hours of reoxygenation).

CELL CULTURE OF SECS AND HEPATOCYTES IN IN VITRO H-R MODELS

Human SECs and hepatocytes were purchased from ScienCell Research Laboratories. The H-R models were established using an anaeropack jar system (Mitsubishi Gas Chemical Co., Tokyo, Japan) as previously described.^(16,17) In brief, all cells (0.5×10^4 /well) were seeded and cultured in endothelial cell medium (ScienCell Research Laboratories) for SECs or a hepatocyte medium (ScienCell Research Laboratories) for hepatocytes on a 24-well collagen-coated plate at 37°C with 5% CO₂ for 48 hours. After semiconfluence, the cells cultured in the serum-starved medium were exposed to hypoxic conditions (<0.1% O₂) for 60 minutes, followed by reoxygenation for 4 hours.

EXPERIMENTAL GROUPS OF SECS AND HEPATOCYTES IN IN VITRO STUDY TO ELUCIDATE THE EFFECTS OF DABIGATRAN FOR H-R

In the present in vitro studies, dabigatran, which was purchased from Cayman Chemical (Ann Arbor, Michigan), was used. SECs were allocated to 2 H-R groups and then cultured ($n = 5$ in each group). The H-R + dabigatran group (SEC-1) was pretreated with 0.5 μM of dabigatran in an endothelial cell medium for 2 hours, whereas the H-R + vehicle group (SEC-2) was pretreated with vehicle (DMSO equivalent to that used to dissolve dabigatran) for 2 hours. Following the pretreatment, these SECs in both groups were exposed to 60-minute hypoxia and 4-hour reoxygenation using an anaeropack jar system in an endothelial cell medium containing 100 U/mL human alpha thrombin (HCT-0020; Haematologic Technologies, Essex Junction, VT). Hepatocytes were allocated to 2 H-R groups and then cultured ($n = 5$ in each group). The H-R + dabigatran group was pretreated with 0.5 μM of dabigatran in a hepatocyte medium for 2 hours, whereas the H-R + vehicle group was pretreated with vehicle (DMSO equivalent to that used to dissolve dabigatran) for 2 hours. Following the pretreatment, the hepatocytes

in both groups were exposed to 60-minute hypoxia and 4-hour reoxygenation using an anaeropack jar system in a hepatocyte medium containing 100 U/mL thrombin. In these in vitro H-R models, we added thrombin to the serum-starved cell culture medium to faithfully reproduce the pathological state of IRI in vivo because we demonstrated that hepatic IRI markedly upregulated thrombin generation.

PARACRINE COMMUNICATION MODEL BETWEEN HEPATIC SECS AND HEPATOCYTES

In a paracrine communication model between hepatic SECs and hepatocytes, we harvested the supernatant of SECs from the SEC-1 group and the SEC-2 group in the present H-R model in vitro to evaluate direct effect of dabigatran treatment. Subsequently, hepatocytes (0.5×10^4 /well) were seeded and cultured in a hepatocyte medium at 37°C with 5% CO₂ for 48 hours. Following semiconfluence, these cells were pretreated with the supernatant of SEC-1 (the supernatant of SECs pretreated with dabigatran) or SEC-2 (the supernatant of SECs pretreated with vehicle) for 2 hours; thereafter the treated cells were exposed to 60-minute hypoxia (<0.1% O₂) and 4-hour reoxygenation using an anaeropack jar system in a serum-starved hepatocyte medium containing 100 U/mL thrombin. In this model, hepatocytes were allocated to 2 groups ($n = 5$ in each group): the group of H-R + dabigatran using the supernatant of SECs pretreated with dabigatran from SEC-1 and the group of H-R + vehicle using the supernatant of SECs pretreated with the vehicle from SEC-2.

LACTATE DEHYDROGENASE ASSAY

Cell cytotoxicity was assessed by measuring lactate dehydrogenase (LDH) levels in the supernatant using a Cytotoxicity LDH Assay Kit-WST (Dojindo, Japan) following the manufacturer's instructions.

ENZYME-LINKED IMMUNOSORBENT ASSAY

In the present in vivo study, plasma concentrations of TAT and HMGB-1 were quantified with each of the ELISA kits: a TAT kit from Cloud-Clone Corp. (SEA831Mu; Houston, TX) and a HMGB-1 kit from Shino-Test Corp. (326054329; Kanagawa, Japan).

In the present *in vitro* study, concentrations of TM in cell culture supernatant and cell lysate were evaluated by a TM ELISA kit that was purchased from R&D Systems (DTHBD0; Minneapolis, MN). HMGB-1 levels in cell culture supernatant were quantified by a HMGB-1 ELISA kit that was purchased from Shino-Test Corp. (326054329).

DATA ANALYSIS

Data were expressed as median and interquartile range (IQR). Differences between groups were analyzed using the Mann-Whitney U test in SPSS (version 24; IBM Corp, Armonk, NY). Multiple comparisons were performed using the Kruskal-Wallis test followed by the Mann-Whitney U test with the Benjamini-Hochberg correction to control the false discovery rate at the 0.05 level using R (version 3.6.1; R Foundation for Statistical Computing, Vienna, Austria). In the clinical study of TAT, differences between 2 time points, before hepatectomy and 1 day after hepatectomy, were analyzed using the Wilcoxon signed rank test. *P* value <0.05 was considered statistically significant.

Results

CHANGE OF TAT GENERATION BEFORE AND AFTER HEPATECTOMY IN PATIENTS WITH HCC

The characteristics of 14 patients with HCC were described as shown in Supporting Table 1. Plasma TAT levels 1 day after hepatectomy were significantly high compared with before hepatectomy (3.51 [IQR, 2.39-3.83] ng/mL 1 day after hepatectomy, 2.73 [IQR, 2.63-2.84] ng/mL before hepatectomy, *P* = 0.02; Fig. 1A).

HEPATIC IRI UPREGULATED THROMBIN GENERATION AND INCREASED THROMBIN SHOWED CYTOTOXICITY ON SECs

In the hepatic IRI model *in vivo*, plasma TAT levels were significantly increased after IRI compared with the sham group (4.56 [IQR, 3.05-7.54] ng/mL after IRI and 0.01 [IQR, 0.00-0.11] ng/mL naïve, *P* = 0.004; Fig. 1B).

As shown in Fig. 1C-a, LDH cytotoxicity levels in the supernatant of SEC cultures were elevated

in a dose-dependent manner. The addition of 100 U/mL thrombin significantly increased LDH cytotoxicity compared with the groups of naïve and 10 U/mL thrombin (2.67 [IQR, 2.62-2.71] in 100 U/mL, 1.00 [IQR, 0.92-1.07] in naïve, and 1.50 [IQR, 1.42-1.54] in 10 U/mL; *P* = 0.04 between naïve and 100 U/mL, *P* = 0.04 between 10 and 100 U/mL), whereas there was no significant difference between naïve and 10 U/mL thrombin (*P* = 0.05). *In vitro* H-R models of SECs, the addition of 100 U/mL thrombin also significantly increased LDH cytotoxicity compared with vehicle (2.09 [IQR, 2.00-2.25] in 100 U/mL thrombin + H-R, 0.95 [IQR, 0.83-1.18] in vehicle + H-R, *P* = 0.009; Fig. 1C-b).

THE DIRECT THROMBIN INHIBITOR, DABIGATRAN, AMELIORATED HEPATOCELLULAR INJURY IN VIVO

IRI livers treated with vehicle were characterized by sinusoidal vascular congestion (Fig. 2A-a), which was observed diffusely from the periportal area to the pericentral area. Moreover, in the IRI + vehicle group, diffuse broken liver structures such as cell degeneration of ballooning, swelling, apoptosis, and necrosis were frequently seen, especially in the periportal area. By contrast, IRI livers treated with dabigatran showed suppressed congestion and prevented cell degeneration in the liver structure (Fig. 2A-b). The modified Suzuki's score was significantly lower in the IRI + dabigatran group than in the IRI + vehicle group (5.70 [IQR, 5.45-6.25] in IRI + vehicle, 1.80 [IQR, 1.65-2.25] in IRI + vorapaxar, *P* = 0.004; Fig. 2B). As shown in Fig. 2C,D, dabigatran treatment significantly decreased serum AST and ALT levels compared with vehicle (AST: 4874.13 [IQR, 3666.28-8466.37] IU/L in IRI + vehicle, 609.71 [IQR, 375.64-1110.64] IU/L in IRI + dabigatran, *P* = 0.01; ALT: 4470.76 [IQR, 3287.74-5301.94] IU/L in IRI + vehicle, 285.02 [IQR, 166.35-677.16] IU/L in IRI + dabigatran, *P* = 0.004).

DABIGATRAN TREATMENT PROTECTED SECs FROM HEPATIC IRI DAMAGE AND REDUCED DEPOSITION OF FIBRIN IN HEPATIC SINUSOIDS IN VIVO

Immunofluorescent analysis showed that the expression of CD31 in red, a typical marker of endothelial

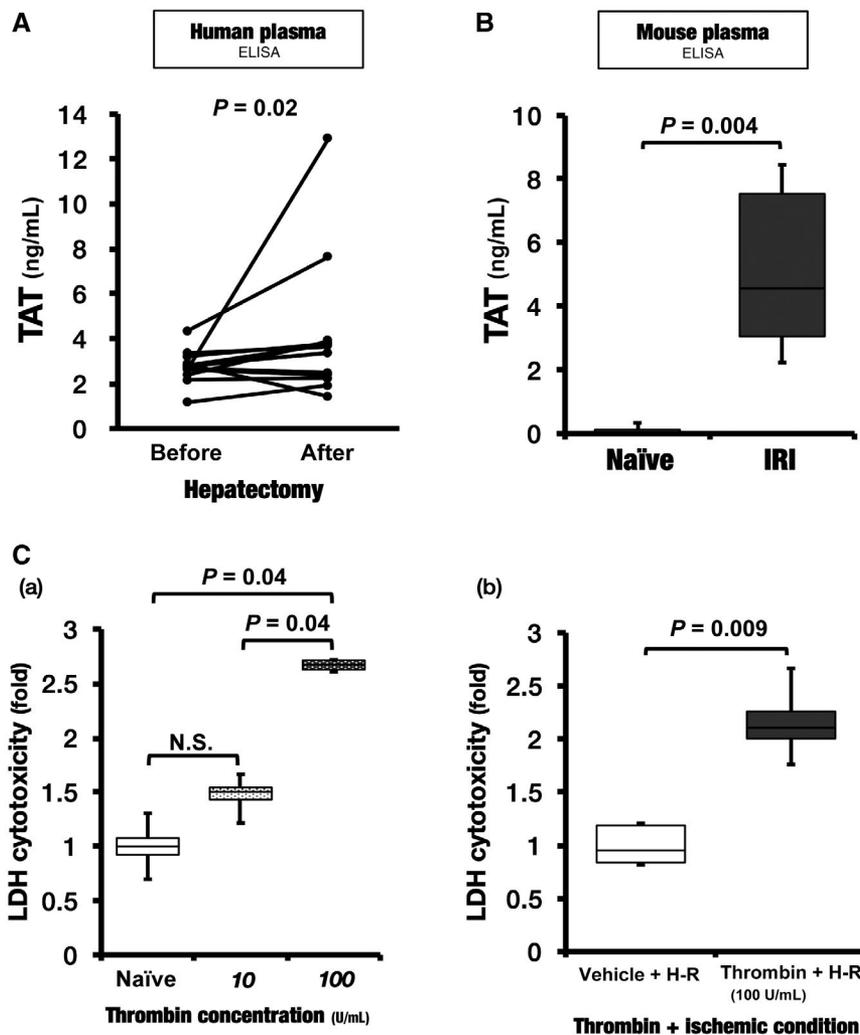


FIG. 1. Characterization of thrombin generation after hepatic IRI and cytotoxicity of thrombin for hepatic SECs. (A) In the clinical study of patients who underwent hepatectomy ($n = 14$), plasma TAT levels were plotted at 2 time points: before and 1 day after hepatectomy. TAT levels were significantly elevated after operation. (B) In in vivo hepatic IRI models of mice, plasma TAT levels measured by ELISA was significantly increased after IRI compared with the naïve group ($n = 6$ in each group). (C-a) In hepatic SECs cultured in the nonischemic condition, LDH cytotoxicity levels in the supernatant of cell cultures were elevated in a dose-dependent manner with the addition of thrombin. The addition of 100 U/mL thrombin significantly increased LDH cytotoxicity compared with both groups of naïve and 10 U/mL thrombin ($n = 4$ in each group). (C-b) In H-R models of SECs in vitro, the addition of 100 U/mL thrombin also significantly increased LDH cytotoxicity compared with vehicle ($n = 5$ in each group). H-R was established using an anaeropack jar system.

cells including SECs, was increased in the dabigatran treatment group (Fig. 2F-b) compared with vehicle (Fig. 2E-b). The expression of fibrin in green was decreased in the dabigatran treatment group (Fig. 2F-a) compared with vehicle (Fig. 2E-a). In addition, merged images showed that fibrin deposition was mainly located in sinusoids as shown in Fig. 2F-d (arrows denote both fibrin-positive and CD31-positive staining in yellow) and Fig. 2E-d (arrowheads denote only fibrin-positive staining in sinusoids after SECs dropped).

DABIGATRAN TREATMENT DEMONSTRATED BOTH ANTIAPOPTOTIC AND ANTI-INFLAMMATORY EFFECTS FOR HEPATIC IRI

Apoptosis of liver specimens after IRI was evaluated by TUNEL staining in the IRI + vehicle (Fig. 3A-a) and IRI + dabigatran groups (Fig. 3A-b). Dabigatran treatment markedly reduced the number of

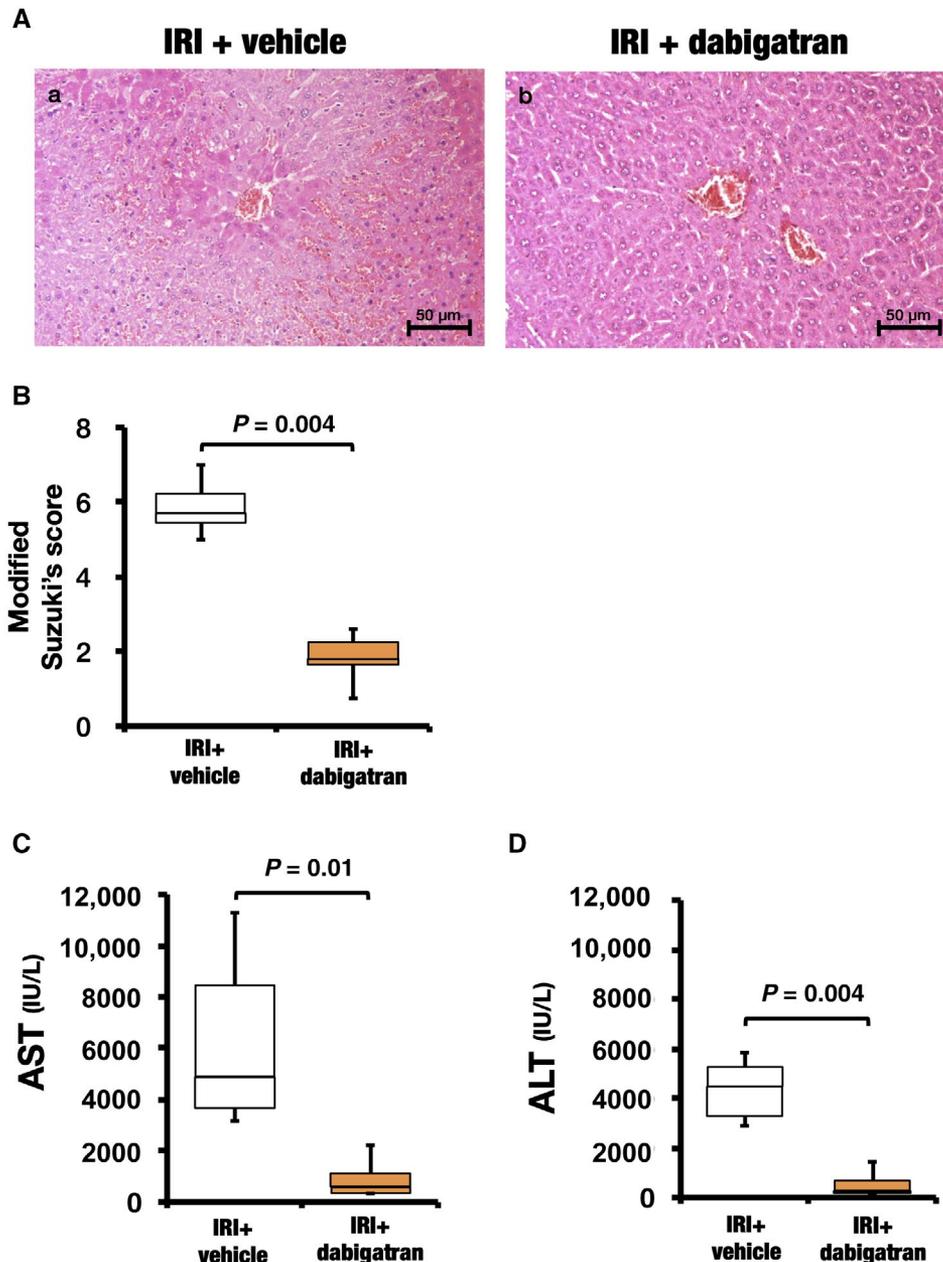


FIG. 2. Liver histology, transaminases, expression of SECs, and fibrin deposition after hepatic IRI with or without dabigatran. IRI livers treated with vehicle (A-a) were characterized by sinusoidal vascular congestion, which was observed diffusely from the periportal area to the pericentral area, and diffuse broken liver structures such as cell degeneration of ballooning, swelling, apoptosis, and necrosis were frequently seen, especially in the periportal area. By contrast, IRI livers treated with (A-b) dabigatran showed suppressed congestion and prevented cell degeneration (original magnification $\times 100$). (B) The modified Suzuki's score in the IRI + dabigatran group was significantly lower than that in the IRI + vehicle group ($n = 6$ in each group). Dabigatran treatment significantly decreased serum levels of (C) AST and (D) ALT compared with vehicle ($n = 6$ in each group). Immunofluorescent analysis in IRI livers treated with (E) vehicle and (F) dabigatran (original magnification $\times 100$): a, fibrin in green; b, CD31 in red; c, DAPI (nuclear stain) in blue; and d, merged image (arrows denote both fibrin-positive and CD31-positive staining in yellow, and arrowheads denote only fibrin-positive staining in sinusoids). Immunofluorescent analysis showed that the expression of CD31 in red was increased in the dabigatran treatment group (F-b) compared with vehicle (E-b). The expression of fibrin in green was decreased in the dabigatran group (F-a) compared with vehicle (E-a). (E-d, F-d) In addition, merged images showed that fibrin deposition was mainly located in sinusoids (arrows denote both fibrin-positive and CD31-positive staining in yellow, and arrowheads denote only fibrin-positive staining in sinusoids after SECs dropped).

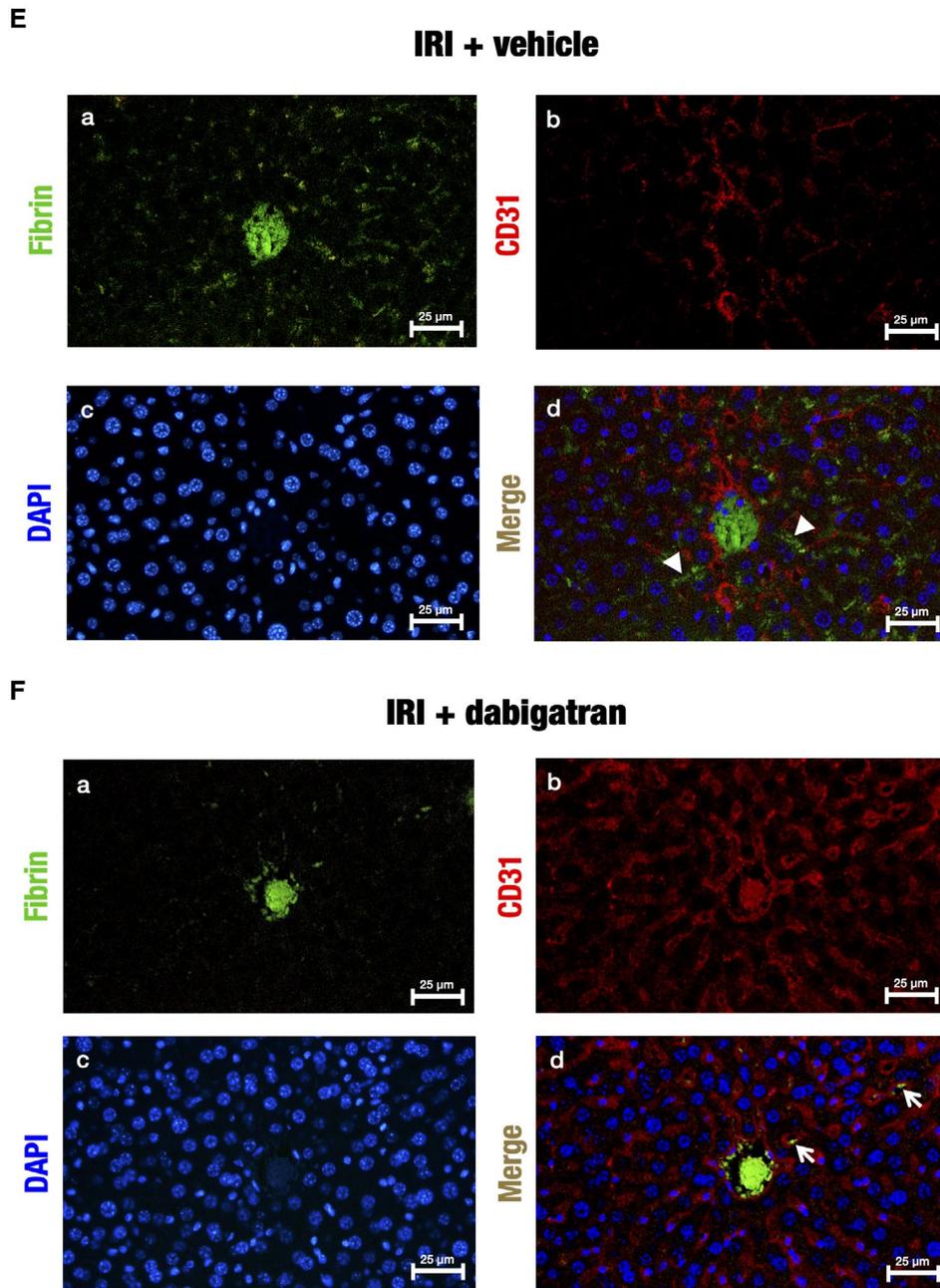


FIG. 2. Continued.

TUNEL-positive cells compared with vehicle (84.20 [IQR, 69.00-101.57] in IRI + vehicle, 5.15 [IQR, 3.85-9.67] in IRI + dabigatran, $P = 0.004$; Fig. 3B). In addition, compared with vehicle, dabigatran treatment attenuated activation of caspase 9, one of the proapoptotic mediators evaluated by Western blot analysis (cleaved-caspase 9/pro-caspase 9: 1.04

[IQR, 0.96-1.09] in IRI + vehicle, 0.69 [IQR, 0.54-0.86] in IRI + dabigatran, $P = 0.01$; Fig. 3C), and upregulated generation of *bcl-2*, an antiapoptotic gene evaluated by real-time PCR (*bcl-2*/ β -actin: 0.97 [IQR, 0.87-1.01] in IRI + vehicle, 1.61 [IQR, 1.45-1.79] in IRI + dabigatran, $P = 0.006$; Fig. 3D). Immunohistochemistry of Ly6G-positive

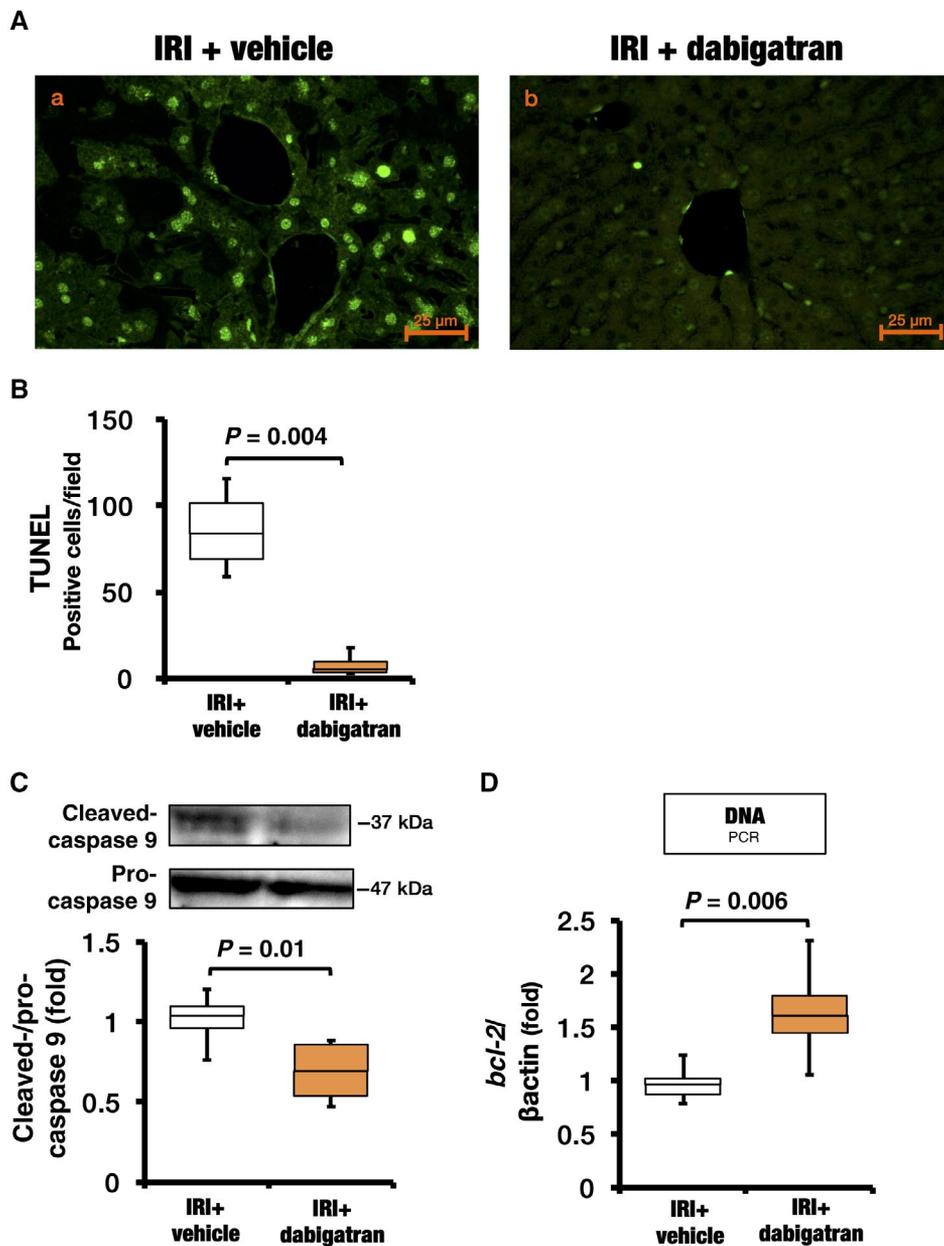


FIG. 3. Antiapoptotic and anti-inflammatory effects of dabigatran treatment in hepatic IRI. Apoptosis of liver specimens after IRI was evaluated by TUNEL staining in the (A-a) IRI + vehicle group and (A-b) IRI + dabigatran group (original magnification $\times 200$). (B) The average number of TUNEL-positive cells per field was significantly lower in the dabigatran group compared with the vehicle ($n = 6$ in each group). (C) According to Western blot analysis, dabigatran treatment reduced activation of caspase 9 compared with vehicle ($n = 6$ in each group). Quantification of cleaved-caspase 9 band intensities normalized to pro-caspase 9. (D) Real-time PCR analysis demonstrated that dabigatran treatment significantly increased generation of *bcl-2*, an antiapoptotic gene, compared with vehicle ($n = 6$ in each group). Inflammatory cell infiltration represented by neutrophils of liver specimens after IRI was evaluated by immunohistochemistry of Ly6G in the (E-a) IRI + vehicle group and (E-b) IRI + dabigatran group (original magnification $\times 100$). (F) The average number of Ly6G-positive cells per field was significantly lower in the dabigatran group compared with the vehicle ($n = 6$ in each group). Real-time PCR analysis demonstrated that dabigatran treatment significantly reduced generation of (G) VCAM-1 and inflammatory genes such as (H) TNF- α and (I) IL6 compared with vehicle ($n = 6$ in each group).

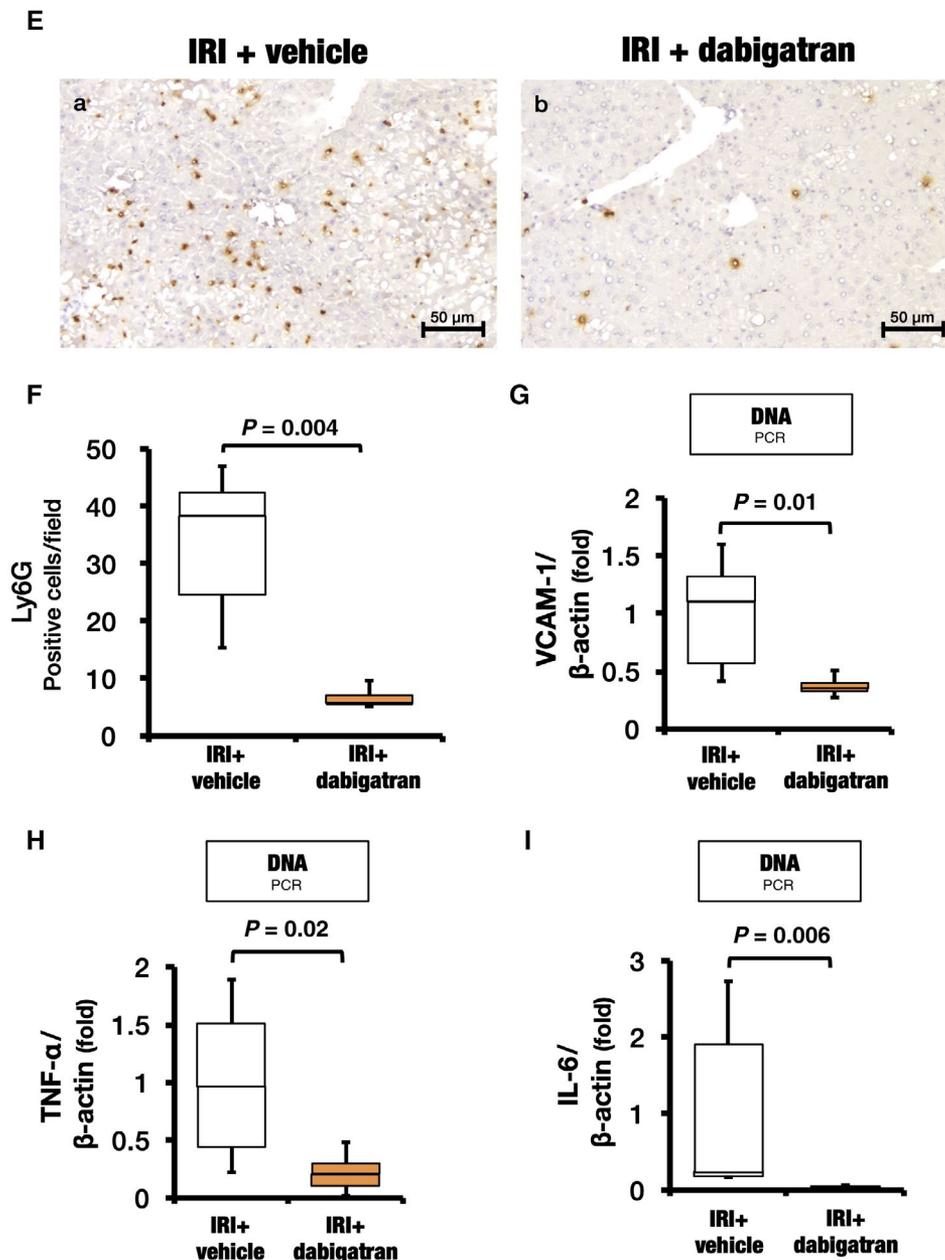


FIG. 3. Continued.

cells, which are known as neutrophil-specific markers, demonstrated diffuse neutrophil infiltration in IRI + vehicle (Fig. 3E-a), whereas the infiltration caused by IRI was attenuated in IRI + dabigatran (Fig. 3E-b). The number of Ly6G-positive cells was significantly lower in the IRI + dabigatran group than in the IRI + vehicle group (5.80 [IQR, 5.40-7.10] in IRI + dabigatran, 38.30 [IQR, 24.60-42.55] in IRI + vehicle, $P = 0.004$; Fig. 3F). Gene

expression evaluated by real-time PCR showed that dabigatran treatment significantly reduced VCAM-1 compared with vehicle (VCAM-1/β-actin: 0.35 [IQR, 0.32-0.39] in IRI + dabigatran, 1.10 [IQR, 0.56-1.32] in IRI + vehicle, $P = 0.01$; Fig. 3G). As shown in Fig. 3H,I, dabigatran treatment markedly reduced generation of inflammatory cytokines such as TNF-α and IL6 compared with vehicle (TNF-α/β-actin: 0.21 [IQR, 0.10-0.29] in IRI + dabigatran,

0.96 [IQR, 0.44-1.51] in IRI + vehicle, $P = 0.02$; IL6/ β -actin: 0.02 [IQR, 0.01-0.03] in IRI + dabigatran, 0.24 [IQR, 0.18-1.90] in IRI + vehicle, $P = 0.006$).

DABIGATRAN TREATMENT ENHANCED TM EXPRESSION IN LIVER TISSUE AND REDUCED HMGB-1 EXPRESSION IN BLOOD PLASMA AND GENE IN VIVO

As shown in Fig. 4A, Western blot analysis revealed that dabigatran treatment for hepatic IRI significantly enhanced TM expression in liver tissue compared with vehicle (3.35 [IQR, 2.69-4.34] in IRI + dabigatran, 0.89 [IQR, 0.34-1.43] in IRI + vehicle, $P = 0.01$).

Regarding the relationship between dabigatran treatment for hepatic IRI and HMGB-1 regulation, dabigatran treatment significantly decreased the blood levels of HMGB-1 measured by ELISA compared with vehicle (25.10 [IQR, 16.26-28.51] ng/mL in IRI + dabigatran, 108.01 [IQR, 80.41-124.85] ng/mL in IRI + vehicle, $P = 0.004$; Fig. 4B-b) and gene expression of HMGB-1 evaluated by PCR (HMGB-1/ β -actin: 0.76 [IQR, 0.75-0.79] in IRI + dabigatran, 0.87 [IQR, 0.81-1.10] in IRI + vehicle, $P = 0.01$; Fig. 4B-c). By contrast, there was no significant difference of HMGB-1 expression in liver tissue evaluated by Western blot between vehicle and dabigatran treatment (HMGB-1/ β -actin: 0.96 [IQR, 0.70-1.47] in IRI + vehicle, 1.66 [IQR, 1.02-1.88] in IRI + dabigatran, $P = 0.15$; Fig. 4B-a).

DABIGATRAN TREATMENT ENHANCED TM EXPRESSION ON SHAM SECs IN THE NONISCHEMIC DAMAGE MODEL

In a nonischemic damage model of hepatic SECs, TM levels in the cell lysate of sham SECs measured by ELISA were markedly higher in the group of sham + dabigatran than vehicle (103933.69 [IQR, 100321.50-107407.94] pg/mg protein in sham + dabigatran, 87448.13 [IQR, 86241.40-90311.10] pg/mg protein in sham + vehicle, $P = 0.02$; Fig. 5A), whereas pretreatment with dabigatran did not show a significant difference in TM levels in the supernatant of cell cultures measured by ELISA compared with vehicle (10.21 [IQR, 6.19-13.47] pg/mL in sham + dabigatran, 7.21

[IQR, 0.00-14.63] pg/mL in H-R + vehicle, $P = 0.77$; Fig. 5B).

DABIGATRAN TREATMENT IMPROVED H-R DAMAGE, ENHANCED TM EXPRESSION IN CELLS, AND REDUCED HMGB-1 EXPRESSION IN SUPERNATANT IN THE H-R MODEL OF PURE CULTURED SECs IN VITRO

LDH cytotoxicity levels in the supernatant of SEC cultures pretreated with dabigatran were significantly lower compared with vehicle (0.69 [IQR, 0.66-0.78] in H-R + dabigatran, 1.02 [IQR, 0.97-1.03] in H-R + vehicle, $P = 0.009$; Fig. 6A). In Fig. 6B-a,b, TM levels in the cell lysate measured by ELISA were markedly higher in the group of H-R + dabigatran than vehicle (182215.53 [IQR, 166814.27-210406.49] pg/mg protein in H-R + dabigatran, 135061.91 [IQR, 132910.23-137439.56] pg/mg protein in H-R + vehicle, $P = 0.009$), whereas preincubation of dabigatran did not show a significant difference in TM levels in the supernatant of cell cultures measured by ELISA compared with vehicle (324.60 [IQR, 318.22-325.67] pg/mL in H-R + dabigatran, 313.44 [IQR, 304.95-321.94] pg/mL in H-R + vehicle, $P = 0.25$). As shown in Fig. 6C-a,b, HMGB-1 levels in the supernatant of cell cultures measured by ELISA were markedly lower in the group of H-R + dabigatran than vehicle (12.58 [IQR, 12.54-14.95] ng/mL in H-R + dabigatran, 23.75 [IQR, 23.41-24.30] ng/mL in H-R + vehicle, $P = 0.009$), whereas preincubation of dabigatran did not show a significant difference in HMGB-1 expression on SECs evaluated by Western blot compared with vehicle (HMGB-1/ β -actin: 1.32 [IQR, 1.13-1.51] in H-R + dabigatran, 0.87 [IQR, 0.85-1.05] in H-R + vehicle, $P = 0.25$).

DABIGATRAN TREATMENT DID NOT IMPROVE H-R DAMAGE OR AFFECT EXPRESSION OF TM AND HMGB-1 IN THE H-R MODEL OF PURE CULTURED HEPATOCYTES IN VITRO

LDH cytotoxicity levels in the supernatant of SEC cultures pretreated with dabigatran did not show

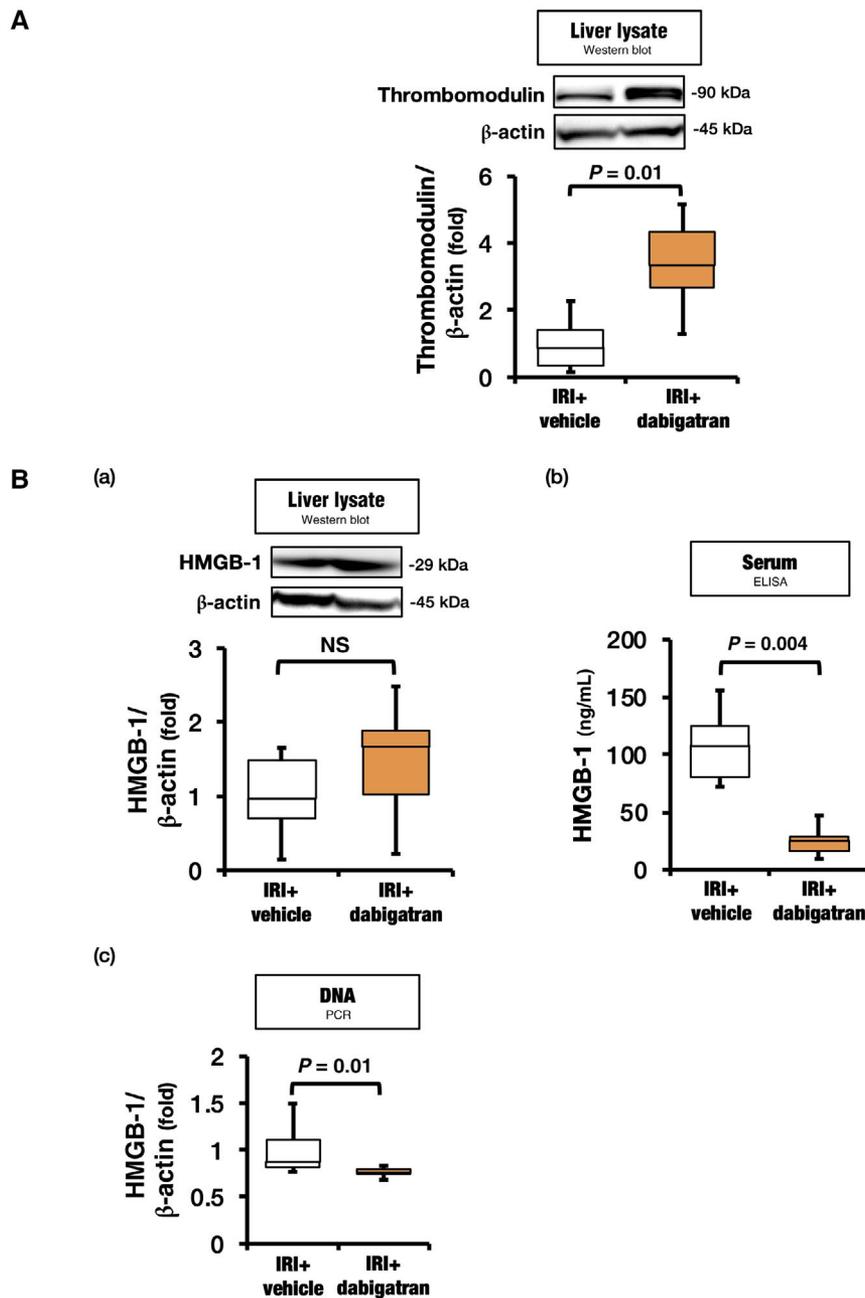


FIG. 4. The enhancement of TM expression in liver tissue and the reduction of HMGB-1 expression provided by dabigatran treatment in vivo. (A) Based on Western blot analysis, dabigatran treatment for hepatic IRI significantly enhanced TM expression in liver tissue compared with vehicle ($n = 5$ in each group). Quantification of TM band intensities normalized to β -actin. (B-a) In hepatic IRI models of mice, Western blot analysis showed that there was no significant difference in HMGB-1 expression in liver tissue between dabigatran and vehicle treatment ($n = 5$ in each group). Quantification of HMGB-1 band intensities normalized to β -actin. By contrast, dabigatran treatment significantly reduced (B-b) serum levels of HMGB-1 measured by ELISA and (B-c) gene generation evaluated by real-time PCR analysis compared with vehicle ($n = 5$ in each group).

significant differences compared with vehicle (1.17 [IQR, 1.08-1.37] in H-R + dabigatran, 1.10 [IQR, 0.74-1.21] in H-R + vehicle, $P = 0.34$; Fig. 7A). In

Fig. 7B-a,b, there was no significant difference between dabigatran treatment and vehicle treatment in both experiments of TM expression in the cell lysate measured

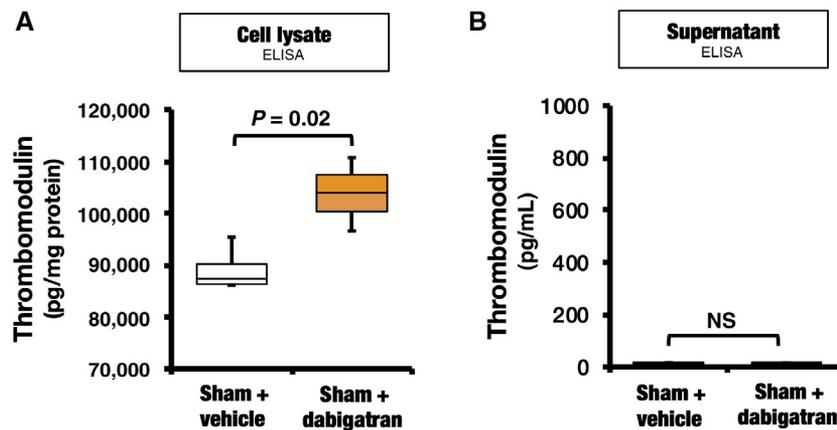


FIG. 5. Direct effect of dabigatran treatment on pure cultured hepatic SECs in a nonischemic damage model in vitro. (A) In a nonischemic damage model of hepatic SECs, TM levels in cell lysate of sham SECs measured by ELISA were markedly higher in the group of sham + dabigatran than vehicle ($n = 4$ in each group). (B) Pretreatment with dabigatran did not show significant differences of TM levels in the supernatant of cell cultures measured by ELISA compared with vehicle ($n = 4$ in each group). In a nonischemic damage model of this study, SECs were not exposed to any stress or damage such as ischemia caused by H-R and the addition of thrombin.

by ELISA (6330.75 [IQR, 5986.68-6640.93] pg/mg protein in H-R + dabigatran, 5265.77 [IQR, 5155.33-6160.43] pg/mg protein in H-R + vehicle, $P = 0.17$) and the TM levels in the supernatant of cell cultures measured by ELISA (11.40 [IQR, 10.38-11.68] pg/mL in H-R + dabigatran, 10.11 [IQR, 9.74-11.21] pg/mL in H-R + vehicle, $P = 0.46$). As shown in Fig. 7C-a,b, there was also no significant difference between dabigatran treatment and vehicle treatment in both experiments of HMGB-1 expression in the cell lysate evaluated by Western blot (HMGB-1/ β -actin: 0.81 [IQR, 0.80-1.16] in H-R + dabigatran, 0.88 [0.75-1.21] in H-R + vehicle, $P = 0.60$) and HMGB-1 levels in the supernatant of cell cultures measured by ELISA (7.53 [IQR, 7.17-9.32] ng/mL in H-R + dabigatran, 8.57 [IQR, 5.05-9.33] ng/mL in H-R + vehicle, $P = 0.91$).

SINUSOIDAL PROTECTION PROVIDED BY DABIGATRAN TREATMENT IMPROVED H-R DAMAGE AND REDUCED TUNEL-POSITIVE CELLS IN THE H-R MODEL OF PURE CULTURED HEPATOCYTES IN A PARACRINE MANNER

As shown in Fig. 8A, hepatocytes were pretreated with supernatant of SECs, which were harvested

after H-R study with or without dabigatran pretreatment followed by incubation in the H-R condition. LDH cytotoxicity levels in the supernatant of hepatocyte cultures were significantly lower in the group of H-R + the supernatant of SECs pretreated with dabigatran than in the group of H-R + the supernatant of SECs pretreated with vehicle (0.65 [IQR, 0.52-0.69] in H-R + the supernatant of SECs pretreated with dabigatran, 1.10 [IQR, 0.74-1.21] in H-R + the supernatant of SECs pretreated with vehicle, $P = 0.04$; Fig. 8B). As shown in Fig. 8C-a,b, the cellular death of hepatocytes after H-R was evaluated by TUNEL staining. The supernatant of SECs pretreated with dabigatran markedly reduced the number of TUNEL-positive hepatocytes compared with the supernatant of SECs pretreated with vehicle (8.50 [IQR, 7.20-9.20] in H-R + the supernatant of SECs pretreated with dabigatran, 17.00 [IQR, 14.90-20.90] in H-R + the supernatant of SECs pretreated with vehicle, $P = 0.009$; Fig. 8C-c).

Discussion

The present in vivo study of mice as well as the present clinical study revealed that hepatic IRI strongly enhanced TAT generation. In addition, thrombin exacerbated LDH cytotoxicity levels in a dose-dependent manner in the present in vitro study of hepatic SECs. In our hepatic IRI models of mice, dabigatran, a potent

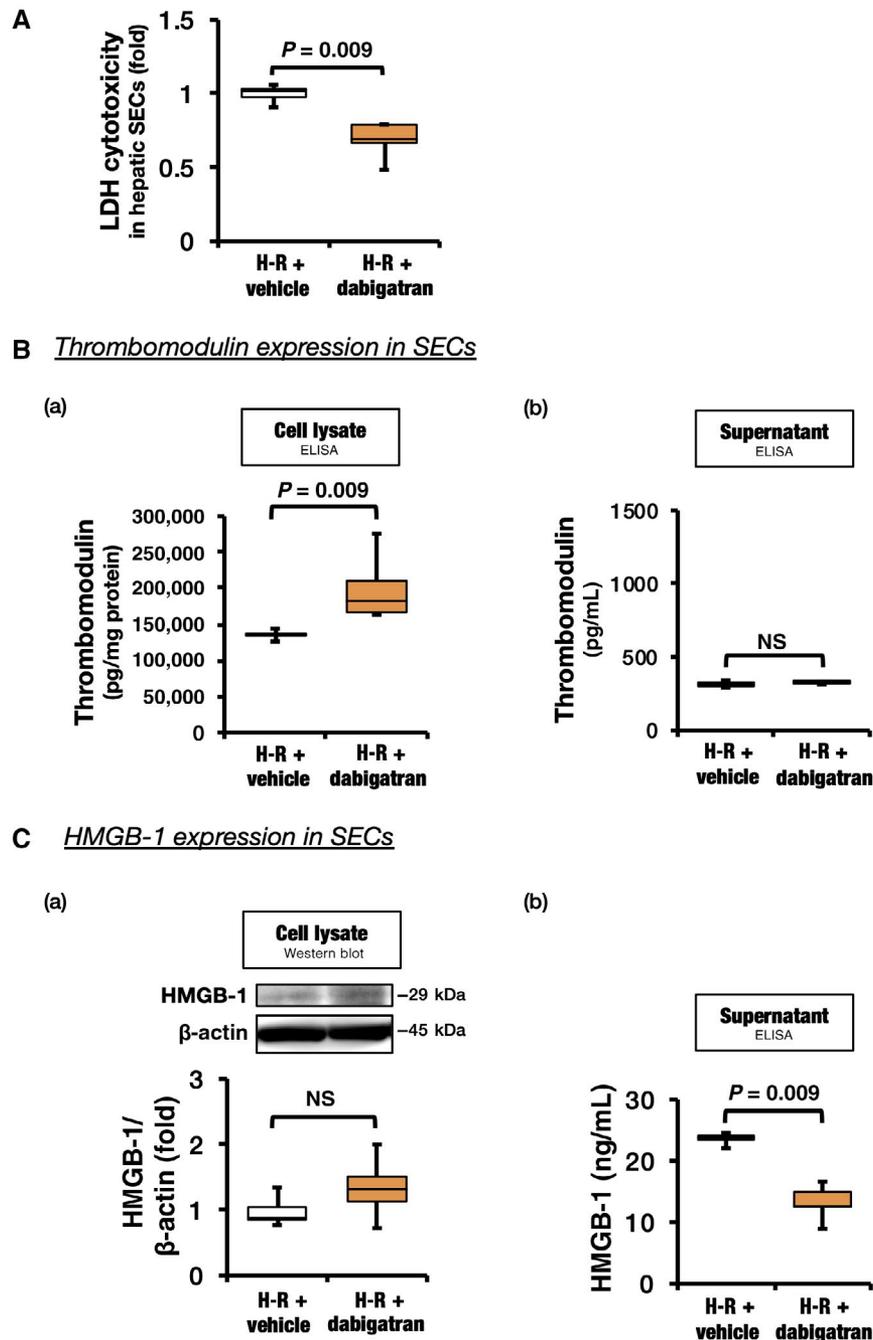


FIG. 6. Direct effect of dabigatran treatment on pure cultured hepatic SECs in an H-R model in vitro. (A) In H-R models of pure cultured hepatic SECs, dabigatran treatment significantly decreased LDH cytotoxicity levels in the supernatant compared with vehicle ($n = 5$ in each group). (B-a) Dabigatran treatment significantly increased TM expression in cell lysate measured by ELISA compared with vehicle ($n = 5$ in each group). (B-b) By contrast, in TM expression in the supernatant of cell cultures measured by ELISA, there was no significant difference between the vehicle and dabigatran groups ($n = 5$ in each group). (C-a) Based on Western blot analysis, dabigatran treatment did not significantly affect HMGB-1 expression in cell lysate compared with the vehicle group ($n = 5$ in each group). Quantification of HMGB-1 band intensities normalized to β -actin. (C-b) By contrast, HMGB-1 levels in the supernatant of cell cultures measured by ELISA were markedly reduced by dabigatran treatment compared with the vehicle ($n = 5$ in each group). In H-R models of this study, SECs were cultured in a serum-starved medium containing 100 U/mL thrombin and exposed to H-R using an anaeroback jar system.

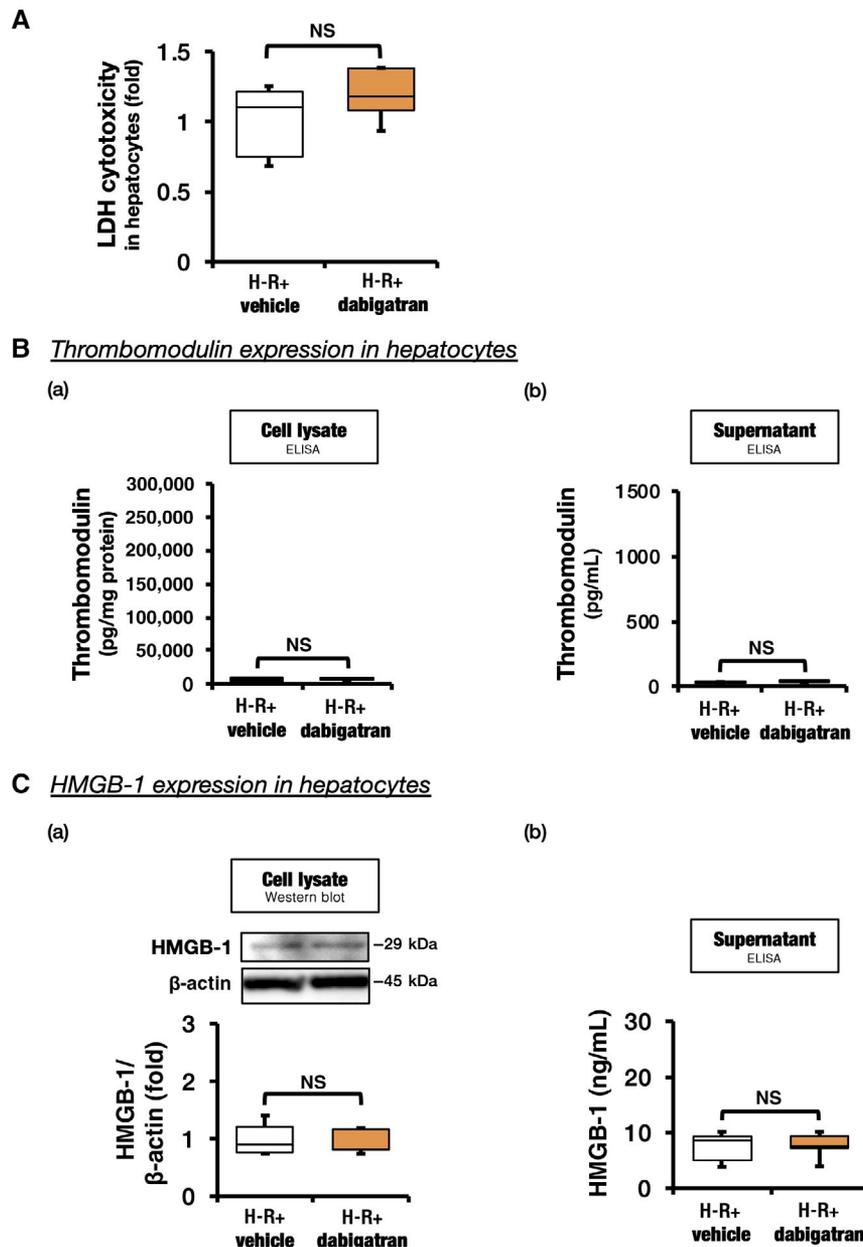


FIG. 7. Direct effect of dabigatran treatment on pure cultured hepatocytes in an H-R model in vitro. In H-R models of pure cultured hepatocytes, there was no significant difference between the H-R + vehicle group and the H-R + dabigatran group (A) in LDH cytotoxicity levels in the supernatant of cell cultures, in TM levels in cell lysate measured by ELISA (B-a), in TM levels in the supernatant of cell cultures measured by ELISA (B-b), in HMGB-1 expression in cell lysate evaluated by Western blot analysis (C-a), or in HMGB-1 levels in the supernatant of cell cultures measured by ELISA (C-b) ($n = 5$ in each group). In Western blot analysis, quantification of HMGB-1 band intensities normalized to β -actin. In H-R models of this study, cells were cultured in a serum-starved medium containing 100 U/mL thrombin and exposed to H-R using an anaeropack jar system.

and highly selective thrombin inhibitor, significantly decreased serum transaminase levels, improved liver histological damage and microthrombosis via decreasing fibrin deposition, and led to sinusoidal protection.

Furthermore, dabigatran provided both antiapoptotic effects evaluated by the reduction of apoptotic cells and caspase 9 activation and upregulation of *bcl-2* and anti-inflammatory effects evaluated by a reduction of

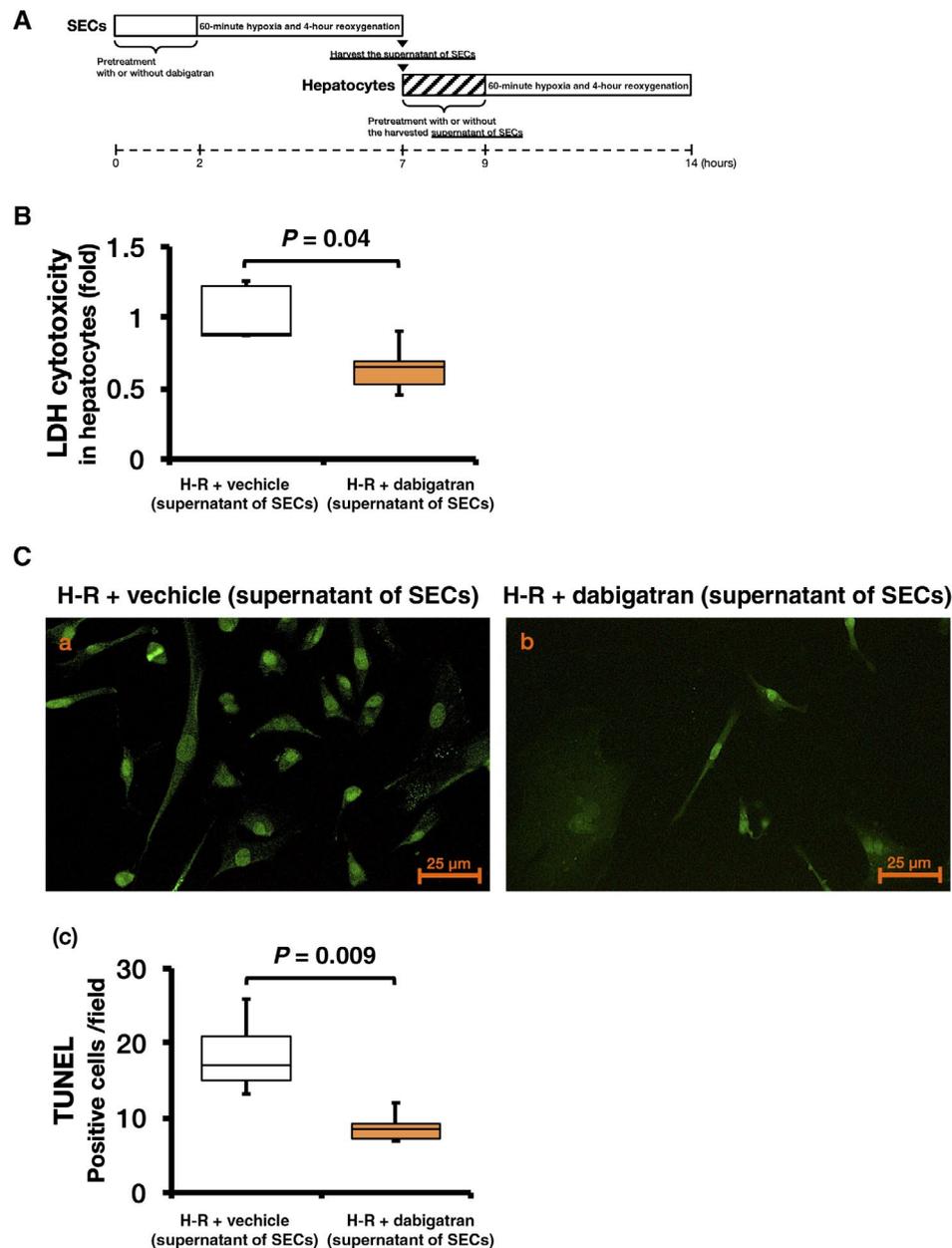
Paracrine communication model between hepatic SECs and hepatocytes

FIG. 8. Effects of dabigatran for hepatocytes in a paracrine communication model between hepatic SECs and hepatocytes. (A) A scheme of our paracrine communication model between hepatic SECs and hepatocytes. SECs were pretreated with or without dabigatran followed by incubation in a serum-starved medium in the H-R condition; thereafter the supernatant of the SECs was harvested. Hepatocytes were pretreated with the supernatant of SECs followed by incubation in a serum-starved medium in the H-R condition. (B) LDH cytotoxicity levels in the supernatant of hepatocyte cultures were significantly lower in the group of H-R + the supernatant of SECs pretreated with dabigatran than in the group of H-R + the supernatant of SECs pretreated with vehicle ($n = 5$ in each group). TUNEL staining detected cellular death of hepatocytes after H-R (C-a) in the group of H-R + the supernatant of SECs pretreated with vehicle and (C-b) in the group of H-R + the supernatant of SECs pretreated with dabigatran (original magnification $\times 200$). (C-c) The supernatant of SECs pretreated with dabigatran markedly reduced the number of TUNEL-positive hepatocytes compared with the supernatant of SECs pretreated with the vehicle ($n = 5$ in each group).

neutrophil recruitment and the downregulation of inflammatory cytokines (TNF- α and IL6) and VCAM-1. To the best of our knowledge, this is the first report to reveal cytoprotective effects of dabigatran treatment against hepatic IRI. As for a notable mechanism of dabigatran treatment, our *in vivo* study demonstrated that dabigatran increased endogenous TM generation and reduced excessive serum HMGB-1 levels. In our *in vitro* study using H-R models of pure cultured SECs, pretreatment with dabigatran also enhanced TM expression on cell surface, decreased HMGB-1 levels of cell supernatant, and improved H-R damage evaluated by LDH cytotoxicity, whereas dabigatran did not affect the expression of TM and HMGB-1 and H-R damage in pure cultured hepatocytes. In addition, in a nonischemic damage model of SECs, dabigatran also enhanced TM expression on the cell surface, and in the paracrine communication model between SECs and hepatocytes, sinusoidal protection provided by dabigatran safeguarded hepatocytes against H-R damage as reduction of LDH cytotoxicity and cell death evaluated by TUNEL in a paracrine manner.

TAT has been frequently employed as an indicator of thrombin generation and activation in many studies,⁽²⁰⁾ and we revealed that hepatic IRI strongly increased levels of plasma TAT *in vivo* as well as the clinical study as shown in a previous report.⁽²¹⁾ We considered that the enhancement of thrombin generation and activation played an important role in the pathogenesis of hepatic IRI, and then we showed the cytotoxicity of thrombin for SECs *in vitro*. Yamaguchi et al.⁽²²⁾ showed that in response to thrombin, Kupffer cells increased the cytokine-induced neutrophil chemoattachment production in a dose-dependent manner. Sarker et al.⁽²³⁾ and Donovan et al.⁽²⁴⁾ reported that in neurons and astrocytes, thrombin induced apoptosis. Accordingly, we hypothesized that direct inhibition of thrombin elicited beneficial effects for hepatic IRI.

Dabigatran, which is a highly selective, potent, and direct inhibitor of thrombin, was developed as a direct oral anticoagulant (DOAC), and it is approved and used clinically in more than 70 countries, including the United States, Europe, and Japan, for the treatment of stroke and systemic thrombosis.⁽¹²⁾ In recent years, thrombin has been well known to activate intracellular signaling via PAR-1, which is a dominant receptor of thrombin, and directly or indirectly affects cell behavior and response in a variety of diseases.⁽²⁵⁾ Indeed, our

previous study revealed that thrombin-PAR-1 signaling contributed to a pathogenesis of hepatic IRI, and PAR-1 antagonism attenuated hepatic IRI via antiapoptotic effects, whereas anti-inflammatory effects were not shown.⁽¹⁸⁾ In the present study, dabigatran treatment could markedly attenuate not only apoptosis but also inflammation, appearing in the reduction of neutrophil infiltration through down-regulating VCAM-1 and inflammatory cytokines such as TNF- α and IL6. These results suggested that a notable cytoprotective pathway other than the attenuation of thrombin-PAR-1 signaling certainly exists.

Thrombin has many roles to maintain homeostasis in molecular physiology other than the coagulation cascade, and one of them is a critical cofactor to activate TM.⁽²⁶⁾ TM is a vital endogenous anticoagulant and expressed on a surface of all vascular endothelial cells, mainly on SECs in the liver.⁽²⁷⁾ Activated TM can also act as a promotor of activated protein C (APC), resulting in APC-dependent cytoprotective signaling,⁽²⁸⁾ and is a potential suppressor of systemic inflammatory process.⁽²⁹⁾ Currently, it is known that excessive concentrations of thrombin cannot activate TM and exhibit TM-dependent functions.⁽³⁰⁾ Because dabigatran directly binds to only the catalytic site of thrombin, we considered that appropriate thrombin activity adjusted by dabigatran treatment might facilitate TM activation, resulting in a notable cytoprotective effect. The present *in vivo* study demonstrated that dabigatran treatment enhanced endogenous TM expression in liver tissue. Activated TM can act as an inflammatory suppressor through irreversible inactivation and degradation of HMGB-1, a mediator of fatal systemic disorder.^(31,32) HMGB-1 is a nuclear DNA binding protein stabilizing the nucleosomal structure⁽³¹⁾; however, once tissues are damaged by several stresses (eg, inflammation, infection, ischemia), excessive HMGB-1 is released and triggers a severe systemic organ failure such as disseminated intravascular coagulation.^(32,33) In addition, as for hepatic IRI, some previous studies reported that serum HMGB-1 levels were increased in a time-dependent manner.^(34,35) By contrast, Kimura et al.⁽³⁶⁾ revealed that there was no significant difference between sham and IRI liver, especially during the early phase. Supporting Fig. 2B also shows that only serum HMGB-1 levels not in liver tissue were markedly changed during hepatic IRI. Accordingly, it was considered that released HMGB-1 into the blood well reflected hepatic IRI damage compared

with it in liver tissue. In our present study, dabigatran treatment not only increased TM expression in liver tissue but also reduced serum levels and gene expression of HMGB-1 markedly; therefore, we hypothesized that the notable cytoprotective effect of dabigatran was provided via HMGB-1 inactivation and degradation by enhanced endogenous TM expression. Indeed, several previous studies indicated that exogenous TM administration could attenuate hepatic IRI via HMGB-1 inactivation.⁽³⁶⁻³⁹⁾

To identify the target cell of dabigatran treatment, we investigated the direct effects of dabigatran on pure cultured hepatic SECs and hepatocytes using H-R models *in vitro*. Only in SECs, but not in hepatocytes, dabigatran treatment revealed attenuation of H-R damage evaluated by LDH cytotoxicity levels, enhancement of TM expression on a cell surface, and reduction of HMGB-1 levels in supernatant as well as our *in vivo* results. Based on these findings, we concluded that the target cell of dabigatran treatment were SECs. It is well known that TM is only expressed on a surface of vascular endothelial cells, and in the liver, TM is expressed on the surface of SECs, not other cells such as hepatocytes.⁽²⁷⁾ In agreement with this, as shown in Supporting Fig. 3A, naïve hepatocyte did not contain any amount of TM compared with naïve SECs. Accordingly, we concluded that SECs were the sources of TM in dabigatran treatment. HMGB-1 is contained in almost all cells also in liver tissue.⁽³¹⁾ Indeed, we confirmed HMGB-1 expression of naïve SECs and hepatocytes (Supporting Fig. 3B); therefore, it was difficult to clarify a source of HMGB-1. However, comparing HMGB-1 expression between Figs. 6C and 7C, dabigatran could affect only SECs, not hepatocytes. Furthermore, Mullins et al.⁽³⁷⁾ demonstrated that endothelial cells were an important source of active HMGB-1 secretion in response to cellular damage. Accordingly, SECs rather than hepatocytes were a main source of HMGB-1 and deeply contributed to HMGB-1 behavior in the development of hepatic IRI. SECs are the most important stabilizer of the vascular integrity of a liver. Maintaining vascular integrity can protect liver parenchyma from infiltrations of inflammatory cells and cytokines.^(38,39) As shown in Fig. 2E-b,F-b, immunofluorescence analysis exhibited that expression of CD31, which is a typical marker of endothelial cells, was preserved in the dabigatran treatment group. This finding supported that dabigatran treatment could induce sinusoidal protection against hepatic IRI, maintaining vascular integrity.

We examined the relationship among protection of SECs, TM enhancement on the surface of SECs, and reduction of extracellular HMGB-1 levels in a mechanism of dabigatran treatment against hepatic IRI. Some previous studies showed that hepatic endothelial damage increased blood TM levels, reflecting leakage from liver tissues^(27,40,41); therefore, there was a possibility that more TM was left on cells as a result of sinusoidal protection. However, in the present H-R study, TM levels leaked from SECs into supernatant did not indicate significant difference with or without dabigatran (Fig. 6B-b). Moreover, our nonischemic damage model of hepatic SECs demonstrated that in also sham SECs, dabigatran could increase TM expression (Fig. 5). These results suggested that dabigatran directly contributed to endogenous TM enhancement. Recently, the nuclear receptor superfamily, which plays diverse roles as a transcription factor in cell differentiation, development, proliferation, and metabolism, was given consideration as a factor to upregulate TM synthesis.^(42,43) Further studies are needed to clarify a deep mechanism of TM synthesis upregulated by dabigatran. Subsequently, we examined paracrine communication between SECs and hepatocytes as shown in Fig. 8. In this model, we pretreated hepatocytes with the supernatant of SECs, which was the same supernatant as the H-R model of SECs in Fig. 6; thereafter, hepatocytes were exposed to an H-R environment. Interestingly, the supernatant of SECs following dabigatran treatment could attenuate H-R damage and cell death in hepatocytes, whereas dabigatran alone did not directly affect hepatocytes as shown in Fig. 7. This finding supported the fact that sinusoidal protection provided by dabigatran treatment led to the protection of hepatocytes against H-R damage in a paracrine manner. According to Fig. 6C, the supernatant of SECs in the vehicle group contained more HMGB-1 compared with the dabigatran group; therefore, we considered that in our paracrine communication model, the difference of H-R damage in hepatocytes might be developed depending on the HMGB-1 amount in the supernatant of SECs. In agreement with our hypothesis, previous studies have revealed that excessive extracellular HMGB-1 is deeply involved in the exacerbation of hepatic IRI.^(34,44) In particular, Tsung et al.⁽³⁵⁾ demonstrated that HMGB-1 administration immediately after reperfusion markedly exacerbated hepatic IRI in a rat model and confirmed that IRI

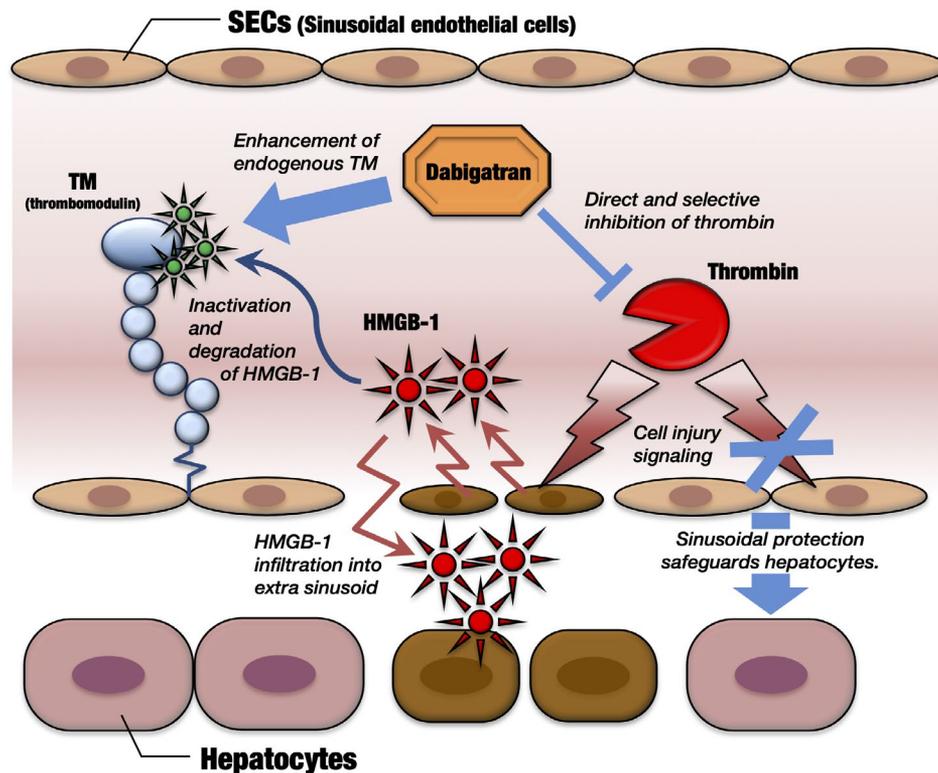


FIG. 9. A mechanism of the cytoprotective effect provided by dabigatran treatment against hepatic IRI. Dabigatran treatment protected SECs from hepatic IRI through direct and selective inhibition of thrombin-mediated cell injury signaling. The sinusoidal protection itself safeguarded hepatocytes against hepatic IRI. Enhanced endogenous TM on SECs by dabigatran treatment inactivated and degraded excessive HMGB-1, which secreted from SECs in response to hepatic IRI. Finally, the reduction of HMGB-1 infiltration into extra sinusoid led to protection of hepatocytes.

damage depended on the amount of extracellular HMGB-1. Regarding the relationship between extracellular HMGB-1 and TM, Kimura et al.⁽³⁶⁾ reported that administered exogenous TM reduced extracellular HMGB-1 without changing HMGB-1 levels in liver tissue. In other words, this study proved that TM could improve hepatic IRI through the degradation of released HMGB-1 into an extracellular space, not attenuation of HMGB-1 leakage from liver tissue. As shown in Fig. 4B, the present study also demonstrated the same results as the study by Kimura et al., importantly not using a suppressor of HMGB-1. There is nothing more well known than TM as an endogenous suppressor of HMGB-1.⁽⁴⁵⁾ Moreover, Abeyama et al.⁽⁴⁶⁾ reported that the property of TM became stronger depending on the enhancement of TM expression on a vessel wall; therefore, we considered that enhanced TM by dabigatran treatment acted as an endogenous HMGB-1

suppressor in this study. Taken together, we summarized a mechanism of the cytoprotective effect provided by dabigatran treatment in Fig. 9. Dabigatran treatment protected SECs from hepatic IRI through direct and selective inhibition of thrombin-mediated cell injury signaling. The sinusoidal protection itself safeguarded hepatocytes against hepatic IRI. Enhanced endogenous TM on SECs by dabigatran treatment inactivated and degraded excessive extracellular HMGB-1, which was secreted and infiltrated into extra sinusoid as a fatal mediator in a response to hepatic IRI. Finally, sinusoidal protection and attenuation of HMGB-1 infiltration into extra sinusoid dually ameliorated hepatic IRI.

In patients undergoing liver transplantation, portal vein thrombosis is a common complication and increases posttransplantation morbidity and mortality⁽⁴⁷⁾; therefore, an anticoagulant therapy, frequently using warfarin, is necessary for many cases

of transplantation. Currently, anticoagulant therapy with DOAC for a solid organ transplantation attracts much attention because it has a lower variability, a shorter half-life, and lower drug–drug and drug–food interactions compared with warfarin.⁽⁴²⁾ Consequently, in the clinical setting of liver transplantation, dabigatran treatment may become a prominent therapy for its dual effects; one is the prevention of portal vein thrombosis, and another is the cytoprotective effect against hepatic IRI, revealed in the present study. As shown in Fig. 2E,F, we demonstrated that hepatic IRI caused fibrin deposition in sinusoids as a result of microthrombosis. Jögi et al.⁽⁴³⁾ also showed fibrin deposition in hepatic sinusoids in a hypercoagulation model of mice. Furthermore, in the present study, dabigatran treatment reduced fibrin deposition, resulting in improving microthrombosis.

In conclusion, the activation and generation of thrombin exacerbated hepatic IRI and excessive HMGB-1 secretion caused severe inflammation-induced and apoptosis-induced liver damage. In this situation, dabigatran treatment improved vascular integrity via sinusoidal protection and microthrombosis and inactivated and degraded excessive HMGB-1 by endogenous TM enhancement on SECs, greatly ameliorating hepatic IRI.

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