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Abstract. Recombinant human soluble thrombomodulin alpha (rhTM) has been developed as an anticoagulant with anti-inflammatory activity. Notably, acetaminophen (APAP) -induced liver disease (AILI) is caused by direct metabolite-induced hepatotoxicity as well as hepatic hyper-coagulation. To evaluate the utility of anticoagulant for the treatment of AILI, rhTM was administered in a mouse AILI model and liver damage was analyzed. AILI was induced in 8-week-old mice by intraperitoneal injection of APAP. rhTM (20 mg/kg) or placebo was injected at the same time as APAP administration. Serum alanine aminotransferase, fibrin degradation products and high-mobility group box 1 levels were significantly decreased in the rhTM-treated group compared with the control group. Furthermore, rhTM reduced the necrotic area and fibrin deposition in liver sections. rhTM suppressed the mRNA expression of heme oxygenase-1, plasminogen activator inhibitor type-1, tissue factors, and inflammatory cytokines compared with the control group. rhTM did not change the hepatic GSH content at 2 h after APAP injection, but restored them at 4 h after the insult. rhTM ameliorated liver damage in mice with AILI, probably via the improvement in liver perfusion induced by it's anticoagulant acitivity, which can lead to the suppression of secondary liver damage.

Introduction

In some patients with liver injury, the liver disease proceeds to acute liver failure (ALF), a life-threatening systemic disorder characterized with severe coagulopathy and encephalopathy (1). Currently, the only effective therapy for ALF is liver transplantation (2,3). Difficulties associated with the development of effective treatments for ALF may be attributed to the incomplete understanding of the mechanisms involved in disease progression.

Intrahepatic microcirculatory disturbance is thought to play a key role in the progression of ALF (4). Hepatic microcirculatory perfusion failure is a determinant of liver dysfunction in warm ischemia-reperfusion (5). Increased fibrinogen catabolism and low platelet counts in ALF patients were consistent with the involvement of hepatic hyper-coagulation (6). Sinusoidal fibrin deposition in ALF livers in patients and experimental models may support the presence of intrahepatic coagulopathy, probably associated with disturbed sinusoidal flow (6-9). Hemodynamic study in ALF patients showed that blood inflow from portal vein was mostly excreted directly into hepatic vein, suggesting impaired parenchymal perfusion (10). Collectively, treatments to improve the hepatic hyper-coagulation may be useful to attenuate liver damage in ALF; however, suitable anticoagulant to treat ALF have not been established (11,12).

Acetaminophen (APAP) is a widely used analgesic/antipyretic drug with few side effects at therapeutic doses (13). It is well known that overdose of APAP causes liver injury via its metabolite N-acetyl-p-benzoquinone imine (NAPQI) that induces direct hepatocyte necrosis by oxidative stress and mitochondrial dysfunction (14-16). N-acetyl cysteine (NAC) is a useful antidote for APAP induced liver injury (AILI) via replenishing intracellular glutathione (GSH), however, delayed administration of NAC diminished its efficacy (17-20). In addition, hepatic hyper-coagulation seems to be involved in the pathogenesis of AILI (21). Tissue-factor (TF) dependent activation of coagulatory system, elevated concentration of PAI-1, and liver fibrin depositions suggested the disturbance in local liver perfusion (22).

Thrombomodulin (TM) is a thrombin receptor expressed on the surface of endothelial cells and plays a crucial role in regulating the coagulation cascades via anticoagulant activity by inhibiting thrombin and accelerating activated protein C (APC) activity (23-25). In addition, TM binds and neutralizes

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high-mobility group box 1 (HMGB1) released from necrotic cells, dampening the inflammatory responses (26-28). These features of TM have allowed the development of recombinant soluble human TM alpha (rhTM) as an anticoagulant with low frequency of hemorrhagic complications (29) and to treat disseminated intravascular coagulation (DIC) with inflammatory reactions, such as sepsis (30).

The features of TM tempted us to evaluate its utility to treat acute liver injury accompanied with hepatic hyper-coagulation. We administrated rhTM into a mouse model of AILI and analyzed the efficacy to suppress liver damage.

Materials and methods

Chemicals. APAP was purchased from Sigma (St. Louis, MO). rhTM was purchased from Asahi Kasei Pharma Co. Ltd. (Tokyo, Japan).

Animals. Eight-week-old male C57BL/6J mice weighing 20-25 g were obtained from Japan SLC (Shizuoka, Japan). Mice were maintained under controlled conditions with free access to standard chow and water. All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and approved by the Animal Care Committee of Kyushu University. Totally 140 mice were used in this study. All mice were fasted for 16 h before the experiments but allowed water ad libitum. The weight of the animals at the time of sacrifice was 18-22 g. APAP (200 mg/kg body weight) dissolved in phosphate-buffered solution (PBS) was injected intraperitoneally. At the same time as APAP injection, rhTM (20 mg/kg body weight) dissolved in saline was injected intraperitoneally (TM group). The dose of rh™ was chosen with reference to the previous reports (31-34) and our preliminary experiments. Control animals underwent sham injections with saline (control group). The mice in control and TM groups were sacrificed at 0, 2, 4, 24 and 48 h (n=10 at each time point/group) in this study. All amimals were euthanized by sevoflurane at concentrations of 4-5% for induction and 2-3% for maintenance, as described previously (35,36). The depth of anesthesia was confirmed by loss of the postural reaction and righting reflex (the pedal withdrawal reflex in the forelimbs and hind limbs, the tail pinch reflex, and the eyelid reflex). Blood samples were drawn from tail vein or inferior vena cava and the livers were collected. Approximately 700-1,200 μl of blood was extracted by exsanguination. A combination of lack of pulse, breathing, corneal reflex, and presence of rigor morits was used to confirm death. The blood samples were centrifuged for 15 min at 3,000 rpm (1,500 x g) at 4˚C, and serum samples were collected and stored at -80˚C. For RNA isolation, liver samples were snap-frozen in liquid nitrogen and stored at -80˚C.

To evaluate hepatic glutathione (GSH) contents, livers were excised to measure GSH contents using Total Glutathione Quantification kit (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions at 0, 2, and 4 h after APAP injection (n=10 at each time point/group).

Biochemical analyses. Blood samples (200 μl at each time point) were taken from tail vein or inferior vena cava at 24 and 48 h after the injection of APAP (n=10 in each group). Serum levels of alanine aminotransferase (AST), alanine aminotransferase (ALT), fibrin degradation products (FDP), and HMGB1 were estimated by Transaminase C-test (Wako Pure Chemical Industry, Osaka, Japan), FDP-ELISA kit (MyBioSource, San Diego, CA, USA), and HMGB1-ELISA kit (Shino-test, Japan). Serum levels of Total bilirubin (T.Bil) and lactate dehydrogenase (LDH) were measured using chemical analyzer Fuji-Drychem (Fuji Film, Tokyo, Japan). Platelet was counted using an automated hematology analyzer (Sysmex XE-5000 hematology analyzer; Sysmex, Kobe, Japan) in EDTA-anticoagulated blood samples. Prothrombin time (PT-INR) measurements were performed on venous blood sample drawn from the tail vein by a commercially available point-of-care coagulometer (CoaguChek XS; Roche, Mannheim, Germany).

Histological examinations. Liver tissue samples were collected at 24 h after APAP injection, fixed in 10% formalin, and embedded in paraffin (n=10/group). Sections were stained with hematoxylin and eosin to assess hepatic damage. Sinusoidal fibrin deposition was detected by phosphotungstic acid-hematoxylin staining. The F4/80 immunohistochemical staining assays were performed. Paraffin-embedded tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed with Proteinase K (Dako, Carpinteria, CA, USA) treatment. Endogenous peroxidase activity was blocked for 20 min with 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO, USA). After blocking with diluted serum from the secondary antibody host for 30 min, the slides were incubated overnight (4˚C) with anti-F4/80 antibody (Bio-Rad, Hercules, CA, USA; catalog no. MCA497; 1:1,000 dilution). Secondary anitibody (Histofine Simple Stain Mouse MAX-PO Rat kit; Nichirei Bioscience, Tokyo, Japan) was applied for 60 min at room temperature and stained for 1-10 min with diaminobenzidine tetrahydrochloride (Nichirei Bioscience). The sections were then counterstained with hematoxylin (Thermo Fisher Scientific, Inc., Waltham, MA, USA), dehydrated, and mounted. The sections were visualized under a Keyence BZ-X700 microscope (Keyence, Osaka, Japan) at different magnifications (x200 magnification, Fig. 1; x400 magnification, Fig. 2; and x200 magnification, Fig. 3).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from liver tissue was prepared with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was synthesized by GeneAmp RNA PCR (Applied Biosystems; Thermo Fisher Scientific, Inc.). RT-qPCR was performed using SYBR-Green on the ABI 7500 real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR reaction was carried out with a denaturation step at 95˚C for 30 sec, then 40 cycles at 95˚C for 5 sec and finally at 60˚C for 34 sec. To control for variations in the reactions, all data were normalized to GAPDH expression. Relative expression was presented using the $2^{-\Delta\Delta Cq}$ method (37). The primer sequences are listed in Table I.

Statistical analysis. Data were analyzed using JMP Pro Version 11 statistical software (SAS Institute, Inc. Cary, NC, USA). The values of serum biomarkers (AST, ALT, LDH, T.Bil, FDP and HMGB1) and PT-INR were expressed as the means and standard deviation (SD). The results of hepatic

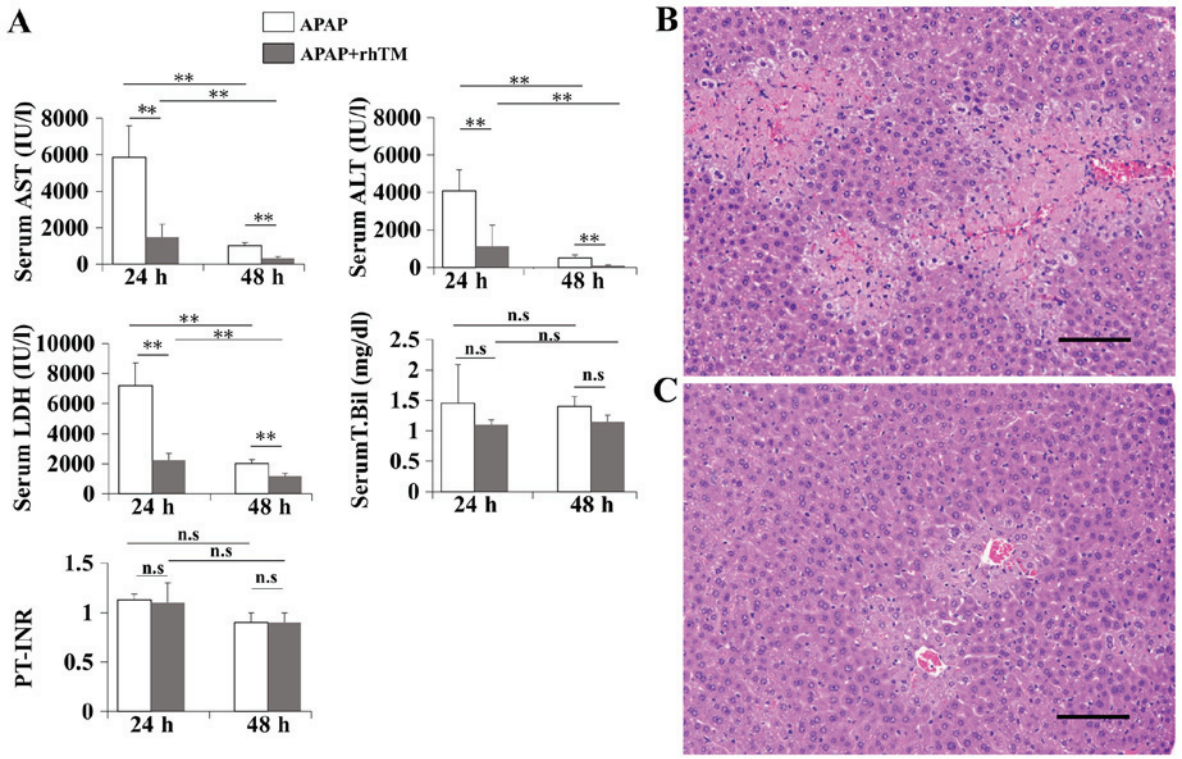

Figure 1. rhTM suppresses liver damage in a mouse AILI model. APAP was injected intraperitoneally into 8-week-old C57BL/6 mice. At the same time, rhTM (TM group) or saline (control group) was injected intraperitoneally. Biochemical examinations were performed at 24 and 48 h after APAP injection (n=10 in each group at each time point). Histological examinations (magnification, x200) were performed at 24 h after the injection (n=10 in each group). Scale bar=100 μm. (A) Serum AST, ALT, LDH, T.Bil and PT-INR levels. Data are expressed as the mean ± SD. **P<0.01. ns, non-significant. Hematoxylin and eosin staining of liver sections. (B) control group, (C) TM group. rhTM, recombinant human soluble thrombomodulin alpha; AILI, acetaminophen induced liver disease; APAP, acetaminophen; AST, alanine aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; T.Bil, total bilirubin; PT-INR, prothrombin time.

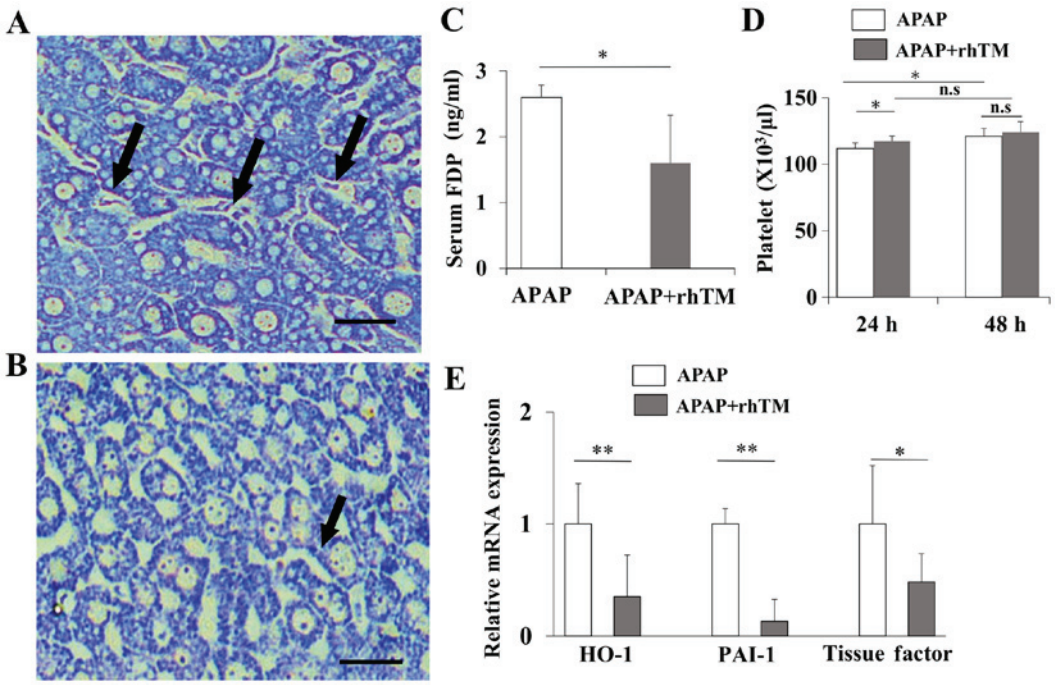

Figure 2. rhTM improves intrahepatic coagulopathy. Phosphotungstic acid-hematoxylin staining was performed to detect the sinusoidal fibrin deposition induced by sinusoidal coagulopathy (magnification, x400). (A) Control group. (B) TM group. The arrows indicate fibrin depositions in sinusoids. Scale bar=25 μm). (C) Serum FDP levels were measured at 24 h after APAP injection. (D) Platelet counts were measured at 24 and 48 h after APAP injection (n=10 in each group at each time point). Data are expressed as the mean ± SD (n=10 in each group). (E) Hepatic expression levels of HO-1, PAI-1 and TF were quantified by RT-qPCR. Data are expressed as the mean ± SEM (n=10 in each group). *P<0.05, **P<0.01. ns, non-significant. rhTM, recombinant human soluble thrombomodulin alpha; FDP, fibrin degradation products; APAP, acetaminophen; HO-1, heme oxygenase-1; PAI-1, plasminogen activator inhibitor type 1; TF, tissue factor.

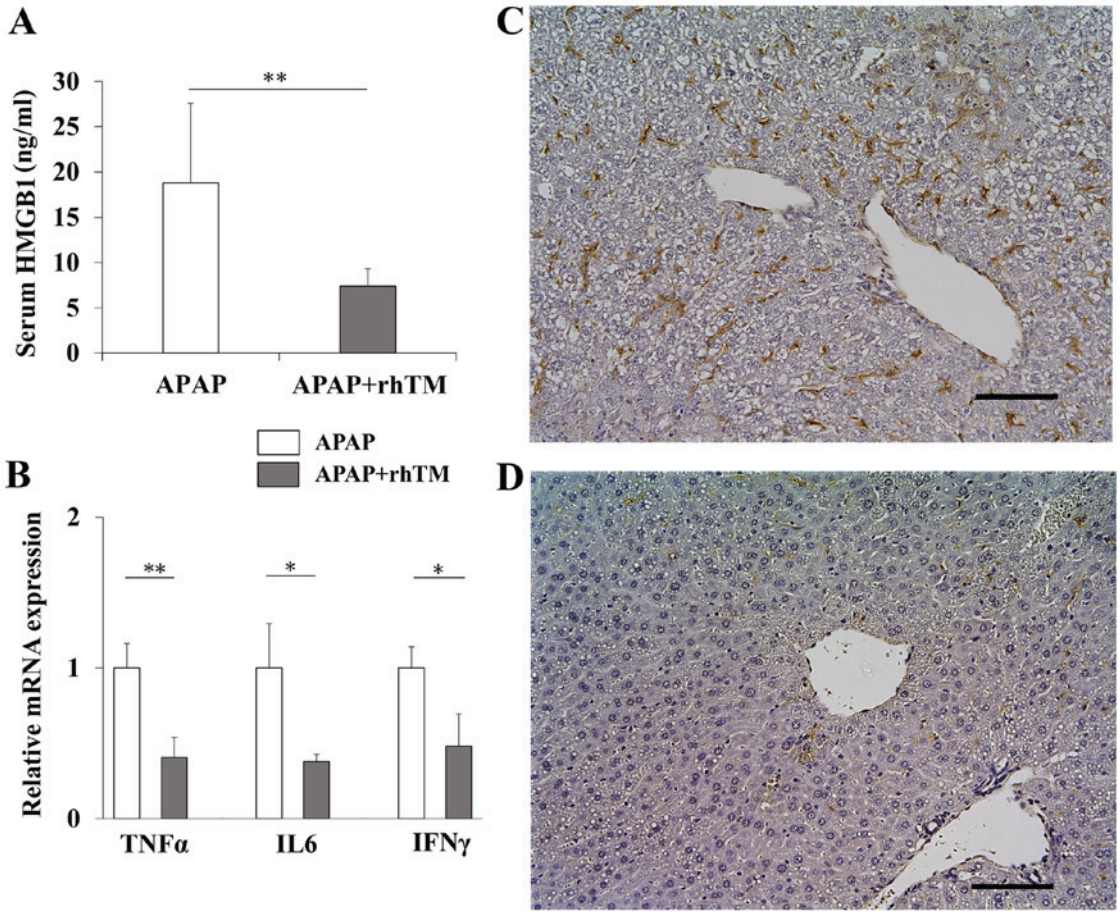

Figure 3. rhTM reduces HMGB1 and downregulates proinflammatory genes. (A) Serum HMGB1 levels were measured at 24 h after APAP injection. Data are expressed as the mean ± SD (n=10 in each group). (B) Hepatic expression levels of TNFα, IL6 and IFNγ were quantified by RT-qPCR. Data are expressed as the mean ± SEM (n=10 in each group). *P<0.05, **P<0.01 vs. control goup. Immunostaining of liver sections shows the infiltration of F4/80 positive macrophages (magnification, x200). Scale bar=100 μm. (C) Control group. (D) TM group. rhTM, recombinant human soluble thrombomodulin alpha; HMGB1, high-mobility group box 1; APAP, acetaminophen; TNFα, tumor necrosis factor α; IL6, interleukin-6; IFNγ, interferon γ.

mRNA expression were expressed as standard error of the means (SEM). Significant differences between two groups were assessed using the Mann-Whitney U-test. The differnces of means among multiple groups were analyzed by using one-way ANOVA and Tukey's post hoc test. A P-value <0.05 indicated statistical significance.

Results

rhTM attenuates APAP induced hepatotoxicity in mice. In preliminary experiment, we used 500 mg/kg of APAP to induce AILI, but rhTM did not affect liver damages (data not shown). Then we reduced APAP to 200 mg/kg and carried out the following experiments. Elevated serum liver transaminases in the control group indicated successful induction of AILI. Administration of rhTM significantly decreased serum AST, ALT, and LDH compared to those in the control group and this suppressive effect of rhTM on liver damage continued for 48 h (Fig. 1A). We also evaluated serum levels of T.Bil and PT-INR. The value remained in mostly normal range and rhTM did not significantly affect the both levels of T.Bil (control group vs TM group: 1.4±0.6 vs. 1.1±0.1 mg/dl, P=0.37) and PT-INR (control group vs. TM group: 1.2±0.1 vs. 1.1±0.2 mg/dl, P=0.82) at 24 h after APAP injection (Fig. 1A). T.Bil and PT-INR are recognized as useful markers to speculate the prognosis of liver failure patients (38). However, elevations of theses factors have not been obviously shown in APAP-induced ALF model mice (39,40), suggesting that the experimental doses of APAP may not disrupt these values. Histological examination showed extensive hepatocellular necrosis and hemorrhage with modest infiltration of inflammatory cells in the control group (Fig. 1B) and rhTM markedly reduced the necrotic area and diminished the intralobular hemorrhage at 24 h after APAP injection (Fig. 1C). Previously, rhTM has been reported to exert its anticoagulant and anti-inflammatory activity at a dose of 1-200 mg/kg (32-35). In preliminary experiments, we evaluated the effects of rhTM on APAP-induced liver injury at a dose of 10 mg/ml, however, rhTM in this dose did not show significant suppression of liver damages (data not shown). Then we tried rhTM at a dose of 20 mg/ml and significant improving effects of rhTM in this dose allowed us to continue to investigate this study. It is unclear why higher dose was required in this study, however, we speculate that the difference of animal models may affect the appropriate dose for treatment.

rhTM improves intrahepatic coagulopathy. Diffuse distribution of sinusoidal fibrin depositions was observed in control liver and rhTM treatment mostly extinguished the depositions

Table I. qPCR primer sequence.

Gene	Primer sequences (5'-3')
TNFα	F: TATGGCTCAGGGTCCAACTC
	R: CTCCCTTTGCAGAACTCAGG
IL6	F: AGTTGCCTTCTTGGGACTGA
	R: TCCACGATTTCCCAGAGAAC
IFNγ	F: ACTGGCAAAAGGATGGTGAC
	R: TGAGCTCATTGAATGCTTGG
HO-1	F: ACGCATATACCCGCTACCTG
	R: AAGGCGGTCTTAGCCTCTTC
PAI-1	F: TCTGGGAAAGGGTTCACTTTACC
	R: GACACGCCATAGGGAGAGAAG
TF	F: TGCTTCTCGACCACAGACAC
	R: TAAAAACTTTGGGGCGTTTG

TNFα, tumor necrosis factor α; IL6, interleukin6; IFNγ, interferon γ; HO-1, heme oxygenase -1; PAI-1, plasminogen activator inhibitor-1; TF, tissue factor.

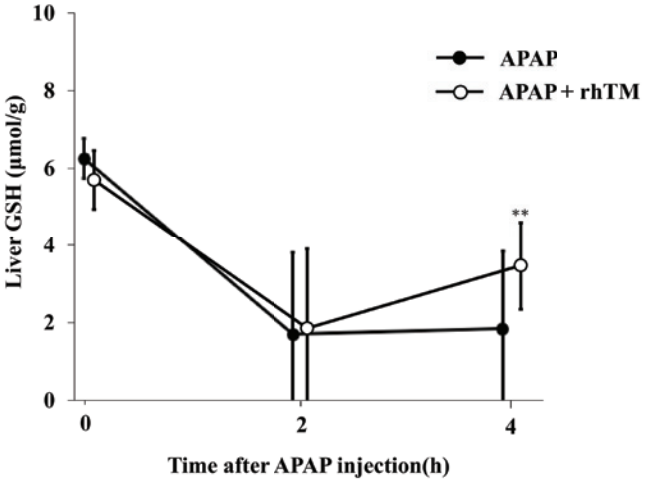

Figure 4. rhTM restores hepatic GSH contents. The liver tissue samples were collected at 0, 2, and 4 h after APAP injection and hepatic GSH contents were evaluated. GSH levels at 2 h after APAP injection were significantly decreased in both groups compared with the levels at 0 h ($P<0.01$). At 4 h after APAP injection, GSH contents in the control group were not changed but those in the TM treated group (APAP + rhTM group) significantly increased compared to those in the control group (APAP group). Data are expressed as the mean ± SD (n=10 in each group at each time point). $^{**}P<0.01$ vs. control group (APAP group). rhTM, recombinant human soluble thrombomodulin α; GSH, glutathione; APAP, acetaminophen.

(Fig. 2A and B). Then we evaluated serum FDP levels. In ALF patients, serum FDP was occasionally found elevated and considered to be useful to speculate the extent of complicated coagulopathy (8). As shown in Fig. 2C, rhTM significantly reduced serum FDP compared with control group at 24 h after APAP injection. Hemostatic alterations in liver disease with intrahepatic coagulopathy occasionally reduces the platelet counts (41). rhTM improved platelet counts at 24 h after APAP injection in TM group (117.3±4.0 vs. control group: 111.7±4.3x10^4/μl, $P<0.05$ Fig. 2D). In addition, rhTM treatment significantly reduced the hepatic expressions of heme oxygenase-1 (HO-1), plasminogen activator inhibitor type 1 (PAI-1) and tissue factor (TF), suggesting that rhTM suppressed the further exacerbation of hepatic hyper-coagulation (Fig. 2E).

rhTM treatment suppresses inflammatory reactions. As shown in Fig. 3A, serum HMGB1 in the TM group was significantly lower than those in the control group. To evaluate the induction of proinflammatory activity, we quantified mRNA expressions of TNFα, IL6, and IFNγ. rhTM treatment significantly reduced the expressions of all these genes (Fig. 3B). We also estimated hepatic macrophage accumulation by F4/80 immunostaining. In APAP hepatotoxicity, resident hepatic macrophage, Kupffer cell, and bone-marrow derived monocytes are activated to aggravate inflammation and monocyte derived macrophage (MoMF) is involved in the resolution of inflammation (42,43). As shown in Fig. 3C, F4/80 immunostaining showed the abundant hepatic infiltration of macrophages in control group, but mostly disappeared in TM group (Fig. 3D).

Temporal changes in liver GSH contents. The APAP metabolite NAPQI induces hepatic GSH depletion, resulting in hepatocyte necrosis (25). To evaluate the effect of rhTM on the direct hepatotoxicity, we evaluated hepatic GSH contents. At 2 h after APAP injection, hepatic GSH levels were equally decreased in both groups (Fig. 4). At 4 h after APAP injection, GSH contents in the TM group rose again with a significant difference compared to those in the control group.

Discussion

The mechanism of APAP induced hepatotoxity involves a oxidative stress and mitochondrial dysfunction via its metabolite NAPQI (13,15,25). In addition, recent studies indicated that APAP hepatotoxity is accompanied by inflammation response (44,45). Mediators released from necrotic hepatocyte stimulate Kupffer cell and sinusoidal endothelial cell. These mediators activate hepatic hyper-coagulation including fibrin deposition and exacerbate liver injury in AILI (22). In clinical cases, the characteristic finding in patients with AILI is the distinct elevation of serum lactate dehydrogenase similar to hypoxic hepatitis, suggesting that the liver could be in hypoxic state associated with hepatic microcirculatory perfusion failure (46).

Here, we showed that rhTM ameliorated APAP induced liver damage in mice. rhTM suppressed serum ALT elevation and reduced liver cell necrosis with decreased sinusoidal fibrin deposition, probably induced by preserved liver perfusion.

In preliminary experiment, we used 500 mg/kg of APAP to induce hepatotoxicity and rhTM did not fully suppress liver damages. Then we reduced APAP into 200 mg/kg and rhTM significantly improved liver damages. We speculate that the inefficiency of rhTM on mice injected with 500 mg/kg of APAP might be attributable to the strong direct hepatocyte necrosis proceeded independently of hepatic hyper-coagulation and inflammation. The involvement of inflammatory cell activations has been reported in AILI (43), liver injury induced by high-dose APAP (400 mg/kg) could not be attenuated in TNF/lymphotoxin-alpha knock out mice (47). The inefficiency

of blunting TNF signaling suggests that the inflammatory reaction seems to be unnecessary in cell death in high-dose APAP. Treating hepatic hyper-coagulation seems ineffective to suppress liver cell necrosis in high-dose APAP induced liver damage.

In this study, treatment with rhTM was accompanied with reduced sinusoidal fibrin deposition. Not only a result of hemostatic disturbance, fibrin deposition may be a causal player in acute liver injury (48). Fibrin could be deposited as intravascular microthrombi, leading to obstruct local liver perfusion, resulting in the secondary enhanced liver injury. Thus, anticoagulants have been tried to diminish tissue damages in various types of liver injury (11,49-51). In AILI, pretreatment of heparin significantly attenuated liver injury with diminished fibrin deposition (22). Collectively, we speculate that rhTM might suppress liver damage via preserved liver perfusion by the improvement in hepatic hyper-coagulation. The changes in liver GSH contents may support this idea. We observed that liver GSH in the TM group reduced equally at 2 h after APAP injection but rose again with a significant difference at 4 h compared to those in the control group. The reduction of GSH in the early stage suggests that rhTM could not interfere the production of harmful metabolite. Restoration of GSH contents at 4 h suggests that the preserved liver perfusion might supply GSH to injured hepatocytes, preventing further expansion of necrosis.

The role of rhTM in the prevention of secondary liver damage is also suggested by the downregulations of hepatic hyper-coagulation and inflammation related genes. HO-1 has been shown as an important component of antioxidant defense in APAP hepatotoxicity (52). PAI-1 and TF released from damaged cells exacerbate coagulopathy by inhibiting the anticoagulative cascades (53-55). Thus the downregulations of PAI-1 and TF suggest that the preservation of liver perfusion by rhTM improved the redox state in diseased liver, thereby prevented secondary coagulopathy induced by damaged tissue. In similar fashion, preserved liver perfusion by rhTM likely reduced the release of damage-associated molecular patterns (DAMPs), followed by the downregulations of proinflammatory cytokines, resulting in the prevention of secondary damages.

In the present study, the administration of rhTM effectively suppressed liver damage in the APAP-induced ALF model, probably by improving the intrahepatic coagulopathy. Because the improving effects of rhTM on liver damage could be observed in low-dose APAP intoxication, the utility of rhTM in AILI might be limited. Treatment with NAC should be considered primarily, however, rhTM might be useful to support the treatment in cases with hepatic hyper-coagulation. Also, this study has a limitation because the improving effects of rhTM on liver injury were shown when rhTM was injected at the same time of APAP intoxication, which unlikely happens in clinical situations. In addition, as an anticoagulant, rhTM is known to increase the risk of bleeding. We did not experience hemorrhagic complications in this study, however, intensive attention for such adverse effects is necessary in the cases with ALF. The involvement of anti-inflammatory activity of rhTM in the suppression of AILI is still unclear, further study would be helpful to understand the role of rhTM in the suppression of AILI.

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Availability of data and materials

The dataset used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AK, MKo, MKa and YO designed the study. AK performed experiments. HS, AY, TO, KI, MKu and YM assisted experiments and data analyses. AK wrote the initial draft of the manuscript. MKo, MKa and YO contributed to analysis and interpretation of data. MKo, MKa and YO assisted in the preparation of the manuscript and critically reviewed the manuscript. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Ethics approval and consent to participate

The present study was performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and approved by the Animal Care Committee of Kyushu University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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