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Prophylactic Effect of Recombinant Human Soluble Thrombomodulin for Hepatic Sinusoidal Obstruction Syndrome Model Mice

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Abstract. *Aim: The present study aimed to examine the effects of prophylactic administration of recombinant human soluble thrombomodulin (rTM) for the prevention of sinusoidal obstruction syndrome (SOS). Materials and Methods: Crl:CD1 mice were allocated to the rTM, placebo, and control groups. The rTM group received an intraperitoneal administration of rTM, with intraperitoneal administration of monocrotaline (MCT) 1 h later. The placebo group received PBS instead of rTM, and the control group received PBS instead of rTM and MCT. Mice were sacrificed 48 h after MCT administration, and blood and liver tissues were evaluated. Immunostaining was performed using anti-CD42b and anti-SE-1 antibodies, and AZAN staining. Levels of plasminogen activator inhibitor (PAI-1) and endothelial nitric oxide synthase (eNOS) in whole liver tissues were estimated using RT-PCR. Results: Hematoxylin-eosin staining showed that SOS-related findings were markedly attenuated in the rTM group compared to the placebo group. CD42b immunostaining showed the presence of extravasated platelet activation (EPA) in the Disse space in the placebo group, but this was less noticeable in the rTM group. PAI-1 levels were significantly lower in the rTM group than in the placebo group in RT-PCR. However, eNOS levels were significantly higher in the rTM group than in the placebo group. Conclusion: Administration of rTM may prevent SOS by protecting sinusoidal endothelial cells.*

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Key Words: Sinusoidal obstruction syndrome (SOS), SOS mouse model, recombinant human soluble thrombomodulin, liver sinusoidal endothelial cells, prophylactic effect.

Sinusoidal obstruction syndrome (SOS), also known as veno-occlusive disease (VOD), is a complication that develops after hematopoietic stem cell transplantation and digestive surgery, such as liver transplantation and combination chemotherapy using oxaliplatin (1-3). The symptoms of SOS/VOD are painful hepatomegaly, jaundice, and ascites; severe cases have an extremely high mortality rate of approximately 80% (4, 5). As the prognosis is poor, there is an urgent need to establish prevention and treatment methods against the disease.

The mechanism of injury in SOS/VOD is believed to be damage to endothelial cells in the liver followed by their necrosis (apoptosis) and extrusion into sinusoids, leading to obstruction and congestion. Hepatic stellate cells (HSCs) become activated and produce extracellular matrix and collagens (6). If the condition further progresses, portal hypertension develops and leads to thrombocytopenia (7). Liver acinus zone 3, which is the area located around the central veins of liver lobules or centrilobular zone, is the most frequent site of injury in the liver. As zone 3 contains only low concentrations of cytoprotective protein glutathione and oxygen, it is particularly susceptible to injury (8). We have previously reported that the pathogenesis of SOS/VOD is based on immunosuppressant and chemotherapeutic agents damaging mainly liver sinusoidal endothelial cells (LSECs) in zone 3, causing their detachment and destruction; as platelets migrate into the perisinusoidal space of Disse and aggregate by activated HSCs (9-11). Activated platelets release growth factors, such as thromboxane (TX) A2, serotonin, vascular endothelial growth factor (VEGF)-A, transforming growth factor (TGF)-β and plasminogen

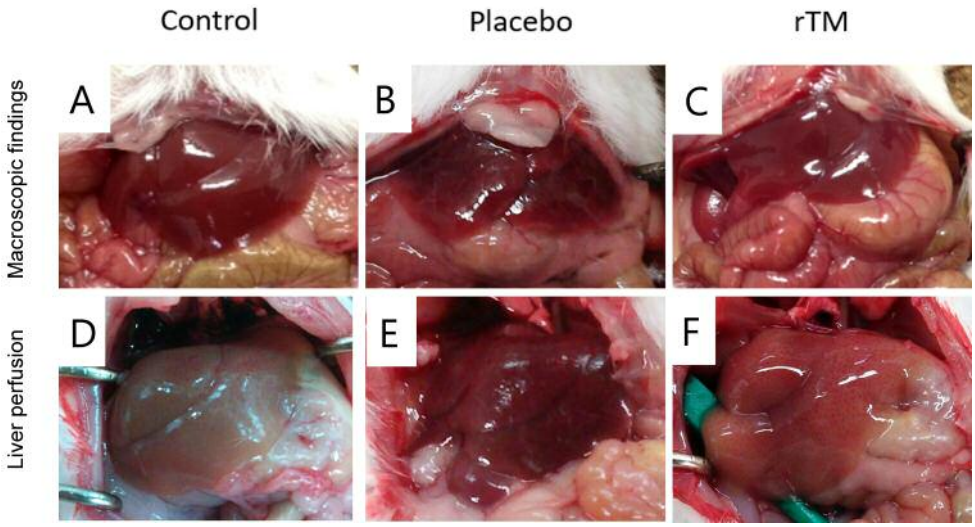

Figure 1. *Representative macroscopic images of the liver show that, in the recombinant human soluble thrombomodulin (rTM) and control groups (A, C), the liver had a reddish color and there were virtually no ascites. In contrast, liver was dark red, patchy and spotted liver surface with ascites in the placebo group (B). Blood flow reversal by portal vein cannulation revealed an even more marked group difference in congestion, suggesting an obstruction in the sinusoids in the placebo group (D-F).*

activator inhibitor (PAI)-1. TXA2 is a vasoconstrictor that increases portal venous resistance and causes portal hypertension (12). TGF-β, a major antiproliferative factor for hepatocytes, stimulates collagen synthesis through activated HSCs (13). PAI-1 is a negative regulator of the fibrinolytic system and plays a significant role in the progression to fibrosis in the tissue microenvironment (14). In this way, extravasated platelet aggregation (EPA) in the space of Disse causes portal hypertension, hepatic fibrosis and fibrinolysis abnormalities as the condition progresses toward organ damage (15). Thus, protecting LSECs by inhibiting platelet extravasation is an extremely important treatment strategy.

Although no method has yet been established for the prevention and treatment of SOS/VOD, defibrotide has been a focus of attention in other countries (16-18); however, in Japan, it cannot be used because the costs are not covered by insurance. Recently, recombinant human soluble thrombomodulin (rTM), which has been used as a therapeutic agent for the treatment of disseminated intravascular coagulation, has gained attention because of its anti-inflammatory properties and protective effect on the vascular endothelium (19).

In our previous study, we administered monocrotaline (MCT) to human vascular endothelial cells and demonstrated that rTM has an anti-apoptotic effect when administered for prophylactic purposes (20). Therefore, the administration of rTM may prevent the detachment and destruction of LSECs, which occurs in the early stage of SOS/VOD. In the present study, we assessed the protective effect of rTM on LSECs in a mouse model of SOS using whole liver tissue.

Materials and Methods

Experimental animals. The study experiments were carried out at the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center, in accordance with the guidelines of the ethics committee of Kanazawa University (approval number 153605).

SOS model. A mouse model of SOS was prepared by using monocrotaline (MCT; Wako Pure Chemical Corporation, Tokyo, Japan.), as described our previous reports (9, 10, 20). Crl:CD1 mice (females; age: 7 weeks; weight: 200-250 g; Charles River, Yokohama, Japan) were allocated into three groups, namely rTM, placebo, and control. The mice were transferred to a laboratory animal holding facility and were allowed for a period of 1 week to become acclimatized to the new environment.

The mice fasted for 12 h prior to experimental procedures. The SOS model was created by administering MCT intraperitoneally at a dose of 270 mg/kg. In the rTM group, rTM (4.0 mg/kg) was administered intraperitoneally 1 h before MCT administration. In the placebo group, phosphate-buffered saline (PBS) was administered (at an amount equivalent to that of rTM as specified above) intraperitoneally 1 h before the administration of MCT. In the control group, PBS was administered instead of rTM and MCT. The mice were sacrificed 48 h after the administration of MCT or PBS, and whole liver tissues, as well as blood samples, were collected.

Pathological assessment. The collected liver tissues were fixed in 10% formalin, embedded in paraffin, cut into 4-μm slices, and stained. Hematoxylin-eosin (H-E) staining was performed as in our previous study (10), and findings characteristic of SOS were assessed, focusing on the following three items for scoring: i) hepatocellular necrosis, ii) endothelial damage in the central vein, and iii) sinusoidal congestion (10, 21, 22). Each of these features

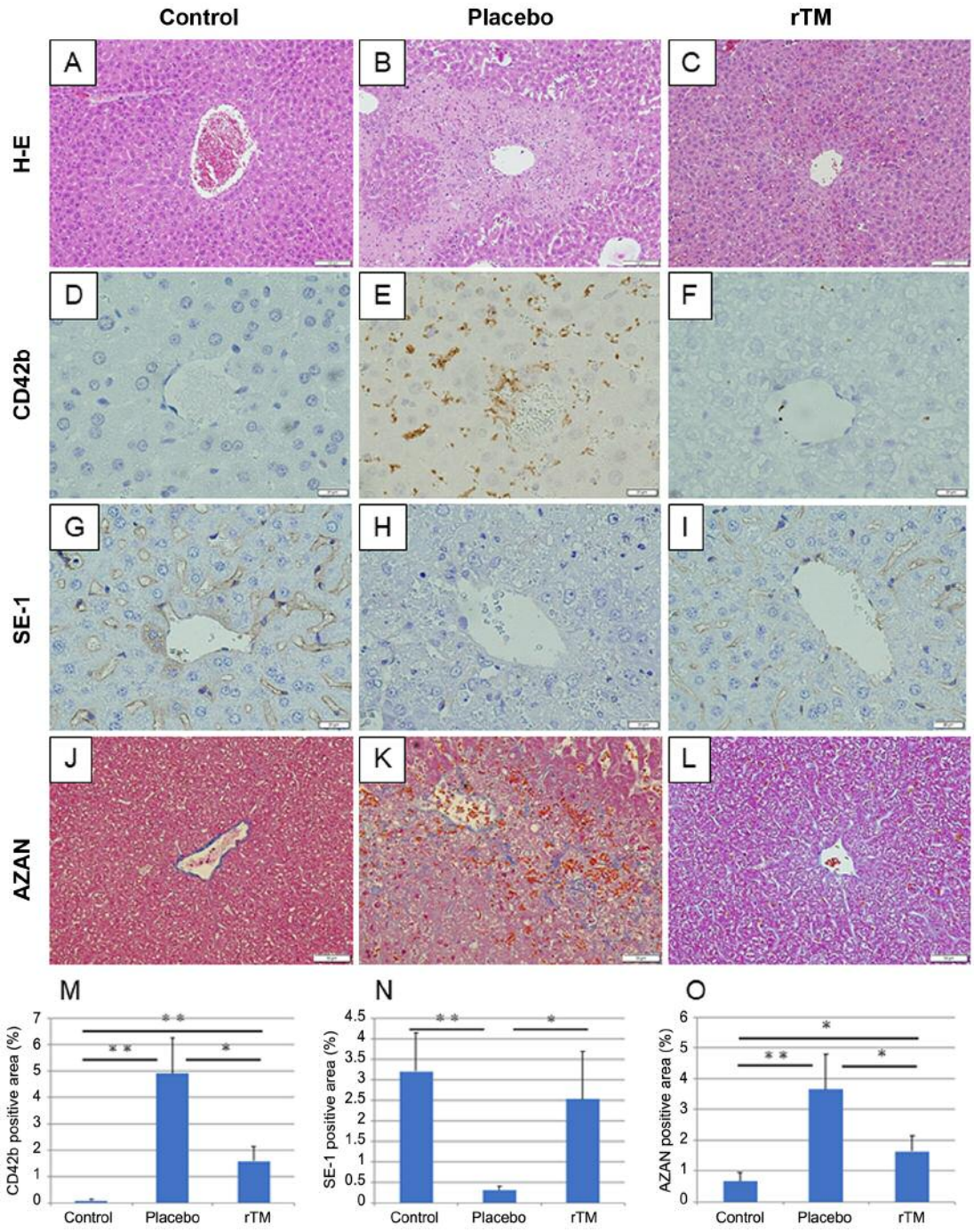

Figure 2. *Hematoxylin-eosin (H-E) staining of hepatic tissues (magnification×100) revealed markedly noticeable sinusoidal dilation, hepatocellular necrosis around zone 3, endothelial damage in the central vein, and sinusoidal congestion in the placebo group. In contrast, sinusoidal dilation was mild, and there was virtually no damage to the endothelium of the central veins and hepatocytes around zone 3 in the recombinant human soluble thrombomodulin (rTM) group (A-C). According to CD42b immunostaining (magnification×400), platelets were present in the space of Disse around zone 3 in the placebo group, while there were only a few stained areas in the space of Disse, in the rTM group (D-F). SE-1 immunostaining (magnification×400) detected the sinusoidal endothelial cells. In the control group, the sinusoidal endothelium was positively stained, whereas no staining was found in the placebo group, suggesting that there was a detachment and destruction of sinusoidal endothelial cells in the placebo group. In contrast, in the rTM group, the sinusoidal wall was stained in a similar manner to that in the control group (G-I). AZAN staining (magnification×200) revealed a blue-stained area over an extensive area outside the sinusoids, strongly suggesting liver fibrosis. In the rTM group, the blue-stained areas were limited to only the sinusoidal endothelium (J-L). Areas of CD42b, SE-1, and AZAN staining were evaluated as the average of four randomly selected images (M-O). Statistical significance was set at *p<0.05, and **p<0.01.*

Table I. *Sinusoidal obstruction syndrome (SOS) score for hematoxylin-eosin staining at 48 h sacrifice.*

Parameters	Groups		*p*-Value
	Placebo	rTM	
Hepatocellular necrosis	2.50±0.29	0.20±0.20	<0.001
Endothelial damage in the central vein	2.00±0.41	0.20±0.20	<0.001
Sinusoidal congestion	2.75±0.25	0.20±0.20	<0.001
Total	7.25±0.48	0.6±0.6	<0.001

was graded on a 4-point scale: 0, absent; 1, mild (1-30%); 2, moderate (31-60%); 3, severe (61-90%). Results are presented as mean values of four randomly selected images. The total SOS score was calculated as the sum of individual scores (10, 21, 22) (n=5 in each group).

Immunohistochemistry. The sections were immunostained with anti-CD42b antibody (ab183345; Abcam, Cambridge, UK) and with the anti-hepatic sinusoidal endothelial cells antibody (SE-1) (NB110-68095; Novus Biologicals, Centennial, Colorado, USA) as previously described (23). Briefly, deparaffinized sections were autoclaved with 10% citric acid buffer (pH 8.0) at 120˚C for 15 min. After treatment with protein block serum (Dako Cytomation, Kyoto, Japan) for 5 min, sections were incubated with a primary antibody at 4˚C overnight. The EnVision polymer solution (horseradish peroxidase; Dako Cytomation) was then applied for 1 h. Signal was developed by adding a 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution containing 0.1% H_2O_2. Sections were lightly counterstained with hematoxylin. Sections incubated with Tris (hydroxymethyl) aminomethane-buffered saline containing either non-immune mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or non-immune rabbit IgG (Santa Cruz Biotechnology) were used as negative controls. Fibrosis was evaluated by performing AZAN staining (azocarmine G, Aniline Blue/Orange G, Phosphotungstic acid solution 5%; Muto pure chemicals, Tokyo, Japan). Staining of each antibody was expressed as the mean of four randomly selected images; quantification was performed using ImageJ (National Institutes of Health, Bethesda, MD, USA) (n=5 in each group).

Blood samples. The blood samples were analyzed for white blood cell (WBC), platelets, aspartate transaminase (AST), alanine aminotransferase (ALT), hyaluronic acid (HA), and lactate dehydrogenase (LDH) (n=5 in each group).

Evaluation of mouse LSECs by qPCR. The levels of PAI-1 and endothelial nitric oxide synthase (eNOS) in whole liver tissues were estimated by performing real-time reverse transcription polymerase chain reaction (RT-PCR) (n=3 in each group). RNA was extracted from whole livers using RNeasy Micro Kits (Qiagen, Germantown, MD, USA) and qPCR was performed with the QuantiTect SYBR Green PCR Kit (Santa Clara, CA, USA).

Statistical analysis. Results are expressed as means±standard deviations (SD). Student's *t*-test was used to evaluate differences between groups. Statistical analysis was performed using the software IBM SPSS 24.0 (IBM Corp, Armonk, NY, USA). A *p*-value less than 0.05 was considered statistically significant.

Results

Macroscopic differences were observed in the livers between the groups. Macroscopically, the liver was dark red, and a patchy and spotted liver surface with ascites was observed in the placebo group. In contrast, the liver had a reddish colour with virtually no ascites in the rTM and control groups (Figure 1A-C). Blood flow reversal by portal vein cannulation revealed an even more marked group difference in congestion, suggesting an obstruction in the sinusoids in the placebo group (Figure 1D-F).

Significant differences were found in the SOS scores between placebo and rTM group. H-E staining of hepatic tissues revealed markedly noticeable sinusoidal dilation, hepatocellular necrosis around zone 3, and sinusoidal congestion in the placebo group. In contrast, sinusoidal dilation was mild, and there was virtually no damage to central veins and hepatocytes around zone 3 in the rTM group. Although there was mild sinusoidal congestion in the rTM group, the red blood cell count was lower than that in the placebo group. The overall findings in the rTM group were similar to those in the control group (Figure 2A-C). All three parameters comprising the SOS score were significantly lower in the rTM group than in the placebo group (Table I). In contrast, the SOS score items in the rTM and control groups were similar (data not shown).

Significant differences were found in CD42b and SE-1 staining between placebo and rTM group. The presence of platelets was examined using CD42b staining. CD42b expression was confirmed in the space of Disse around zone 3 in the placebo group, indicating platelet extravasation (staining area: 4.9±1.4%). In contrast, there was only low expression of CD42b in the space of Disse in the rTM group (staining area: 1.6±0.6%; p=0.0172, compared to the placebo group). CD42b expression was significantly lower in the control group (staining area: 0.1±0.1%), compared to the rTM (p=0.0075) or the placebo group (p=0.0021) (Figure 2D-F, M).

Immunohistochemical analysis of LSECs using an anti-SE-1 antibody confirmed the presence of endothelial cells along the sinus in the control group, whereas staining was significantly reduced in the placebo group (staining areas: 3.2±0.9% *vs.* 0.3±0.1%, respectively; p=0.0039). Thus, findings suggested that there was a detachment and destruction of LSECs. In the rTM group, the sinusoidal wall was stained in a similar manner to that in the control group (staining area in rTM: 2.5±1.2%, p=0.0148, compared to the placebo group), suggesting that the LSECs were preserved. There was no significant difference between control group and rTM group (p=0.3288) (Figure 2G-I, N).

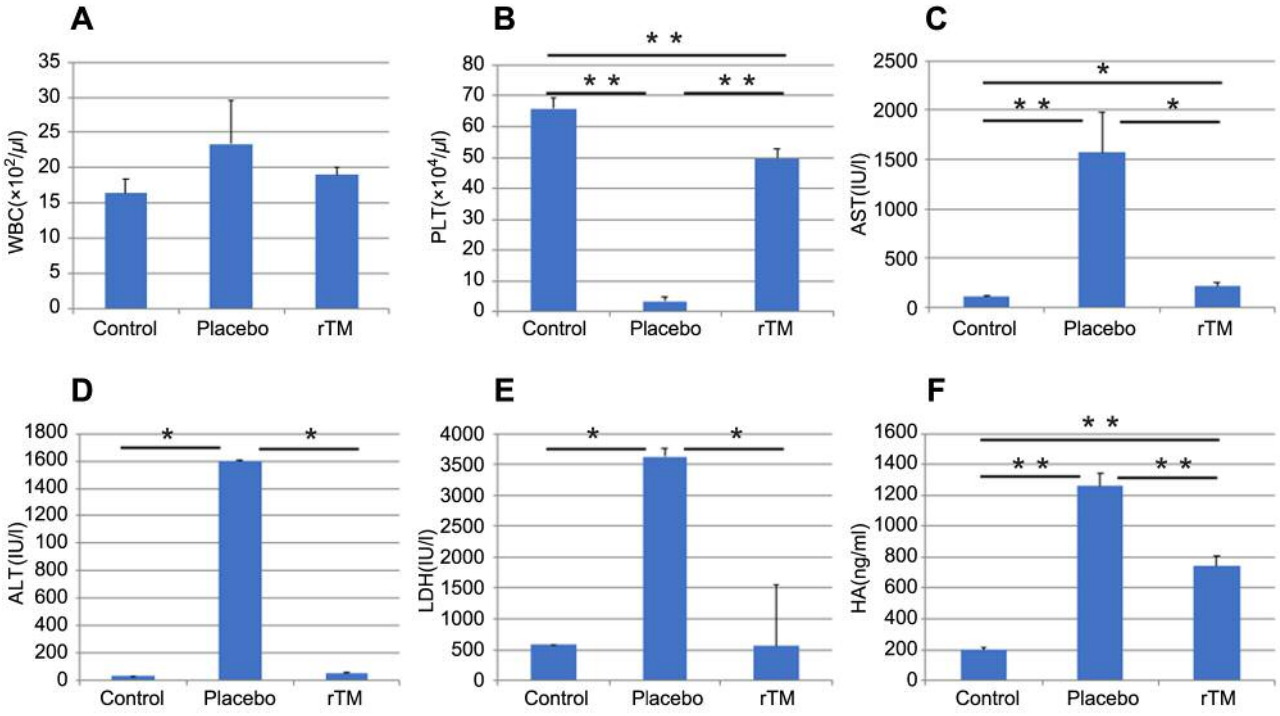

Figure 3. *Blood values were measured in all the groups. White blood cell count (WBC) did not significantly differ among the groups (p=0.504) (A). Platelet (PLT) count was significantly lower in the placebo group than in the control group, but was significantly higher in the recombinant human soluble thrombomodulin (rTM) group than in the placebo group (p=0.0001) (B). The levels of aspartate transaminase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and hyaluronic acid (HA) were all significantly higher in the placebo group than in the control group, but were all significantly lower in the rTM group than in the placebo group (p=0.0115, p=0.0331, p=0.0446, p=0.0026, respectively (C-F). Statistical significance was set at *p<0.05 and **p<0.01.*

Further evaluation of hepatic fibrosis using AZAN staining revealed that a blue-stained area depicting collagen was present along the LSECs in the control group (staining area: 0.7±0.3%). However, in the placebo group, the blue-stained areas were spread over an extensive area outside the sinusoids, strongly suggesting hepatic fibrosis (staining area: 3.7±1.1%; p=0.0064, compared to the control group). In the rTM group, the blue-stained areas were limited to only the sinusoidal endothelium, (staining area: 1.6±0.5%). In the rTM group, AZAN expression was significantly lower than in the placebo group (p=0.0132) and significantly higher compared to the control (p=0.0328) (Figure 2J-L, O).

Blood sample analysis. The platelet count (PLT) was significantly lower in the placebo group than in the control group, but it was significantly higher in the rTM group compared to the placebo group ($49.7\pm3.1\times10^4$/μl *vs*. $3.4\pm1.4\times10^4$/μl, p=0.0001). The white blood cell count (WBC) did not significantly differ among the groups ($23.4\pm6.2\times10^2$/μl in the placebo group and $19.0\pm1.0\times10^2$/μl in the rTM group, p=0.504). The levels of AST, ALT, LDH, and HA were all significantly higher in the placebo group than in the control group, but they were all significantly lower in the rTM group compared to the placebo group (AST: 217.2±33.3 IU/l *vs*. 1566.2±412.1 IU/l, p=0.0115; ALT: 54.2±8.8 IU/l *vs*. 1602.8±602.5 IU/l, p=0.0331; LDH: 561.6±90 IU/l *vs*. 3625.2±995 IU/l, p=0.0446; HA: 741.6±62.8 ng/ml *vs*. 1260±86.2 ng/ml, p=0.0026) (Figure 3).

Evaluation of LSECs by RT-PCR in the whole liver. RT-PCR results showed that PAI-1 levels were significantly higher in the placebo group than in the control group (p=0.0069), but they were significantly lower in the rTM group than in the placebo group (p=0.0074). In contrast, eNOS levels were significantly lower in the placebo group than in the control group (p=0.0001), but significantly greater in the rTM group than in the placebo group (p=0.0059) (Figure 4).

Discussion

Various factors, including the fibrinolytic inhibitors PAI-1 and protein C, contribute to the pathogenic mechanism of SOS/VOD when LSECs are damaged (24, 25). Cutler *et al*. suggested that increased levels of vascular endothelial

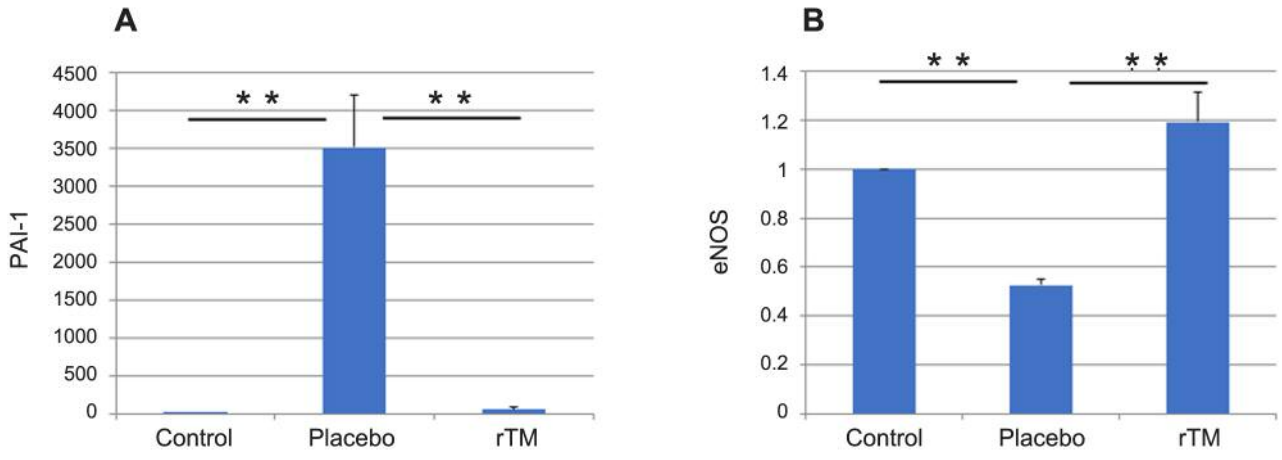

Figure 4. *Real-time reverse transcription polymerase chain reaction (RT-PCR) results showed that plasminogen activator inhibitor (PAI-1) levels were significantly higher in the placebo group than in the control group, but were significantly lower in the recombinant human soluble thrombomodulin (rTM) group than in the placebo group (A). Endothelial nitric oxide synthase (eNOS) levels were significantly lower in the placebo group than in the control group, but were significantly higher in the rTM group than in the placebo group (B). Statistical significance was set at **p<0.01.*

dysfunction markers could be predictors of SOS/VOD and that treatment of vascular endothelial dysfunction is important for treatment of SOS/VOD (17). We have previously reported that during the early stages of the pathogenetic mechanism of SOS/VOD, LSECs around the central vein are damaged by MCT (9, 10, 20). As a result, we conclude that extravasated platelet aggregation (EPA) promoted by MCT detachment of LSECs may be responsible for MCT-induced liver dysfunction (9, 10, 15, 20). We have also previously shown, using CD42b immunostaining in tissue samples from SOS/VOD cases that developed after oxaliplatin treatment, that platelets are present outside the sinusoids (1). Our study showed that platelet aggregation in the space of Disse is what causes SOS/VOD. Due to EPA in the perisinusoidal space of Disse, the platelet-derived factor TXA2 and serotonin cause vasoconstriction of the central vein, which subsequently leads to portal hypertension (11, 15) as well as septic organ failure (23, 26). Furthermore, plasmin activity is inhibited by extravascular platelet-derived PAI-1, and as a result, hepatocyte growth factor (HGF) activation is suppressed, liver regeneration is hindered, and the condition progresses toward liver failure (27, 28). In addition, liver fibrosis is believed to be induced by EPA-derived TGF-β (15). In the present study, CD42b and SEC-1 staining revealed that LSECs were damaged and activated platelets were present in the space of Disse in a mouse model of SOS/VOD treated with the placebo. However, these findings were improved in SOS/VOD mice treated with rTM; this is consistent with our laboratory's perspective on the underlying mechanism of SOS/VOD.

Various pharmacological agents are currently under investigation as potential treatments for SOS/VOD. Defibrotide, approved in many European and US, has been shown to suppress SOS/VOD by inducing TM and prostaglandin I2 in endothelial cells and by inhibiting the expression of PAI-1 (16-18). The present study focused on TM, which has recently been reported to have not only an anticoagulant effect but also a wide range of other effects, such as anti-inflammatory and angiogenic effects, and a protective effect on the vascular endothelium (29). TM has also been reported to have a prophylactic effect against SOS, and using rTM after hematopoietic cell transplantation reduces the incidence of various transplant-related complications, such as graft-*versus*-host disease and SOS/VOD (19). Furthermore, we have previously reported that rTM is effective against SOS/VOD *in vitro* (20).

TM is a single-chain glycoprotein composed of five distinct regions, and it is mainly expressed at the vascular endothelium surface. Its epidermal growth factor (EGF)-like domain can be further divided into 6 domains, among which domains 4-6 are believed to be involved in the activation of protein C (30). Recently, activated protein C has been reported to have an anticoagulant effect, as well as a protective effect on vascular endothelial cells (30). Additionally, *via* protease activated receptor 1 (PAR1), protein C is believed to exert an anti-inflammatory effect, a protective effect on the vascular endothelium, and a strengthening effect on the adhesion between endothelial cells (30, 31). Even without the mediation of activated protein C, EGF-like domain numbers 4 and 5 have protective effects on the vascular endothelium, mediated by the extracellular signal-regulated kinase intracellular signaling pathway (30).

In the present study, PAI-1 levels in hepatic tissues were decreased in the rTM group compared to those in the placebo group. PAI-1 is believed to negatively regulate liver

regeneration, and the overexpression of PAI-1 inhibits plasmin activity, leading to the suppression of the activation of ProHGF into HGF; as a result, this suppresses liver regeneration and causes liver failure (27, 32). PAI-1 has been reported to play a role in the occurrence of liver failure after excessive hepatectomy *via* accelerated maturation of pro-uPA and fibrinolytic factors (28). Ota *et al*. reported that in a rat model of 95% hepatectomy, the administration of rTM led to decreased PAI-1 expression and may have improved the survival rate (33). PAI-1 has been reported to be an independent marker of SOS/VOD and to correlate with the severity of SOS/VOD and the efficacy of treatment (34, 35). In the present study, PAI-1 expression was suppressed on RT-PCR, suggesting that rTM inhibits SOS/VOD through the protection of endothelial cells.

Nitric oxide (NO) is known to have a vasodilating effect. NO has been reported to act as a vascular endothelium-derived relaxing factor and to be produced by eNOS in vascular endothelial cells (36). Endothelial cells are associated with NO release; endothelial cells, in response to changes in blood flow early in hepatectomy, increase NO production and promote vasodilation (37, 38). In pathological conditions such as SOS, in which vascular endothelial cells are detached, it is expected that eNOS production as well as NO are decreased. We have previously conducted a sorting of sinusoidal endothelial cells from the hepatic tissues of various groups in a similar experimental model. The results showed that PAI-1 was significantly reduced and eNOS was significantly elevated, in the rTM group compared to the placebo group (20). The present study confirmed that eNOS expression in whole liver tissue samples was significantly elevated in the rTM group compared to the placebo group, suggesting that rTM could be effective against SOS/VOD through the protection of endothelial cell activity.

Conclusion

rTM may exert a prophylactic effect against the development of SOS/VOD by preventing the detachment and destruction of LSECs due to MCT, and by inhibiting the influx of platelets into the perisinusoidal space of Disse.

Conflicts of Interest

The Authors declare they have no financial or other conflicts of interest in relation to the content of this article.

Authors' Contributions

Shunsuke Kanou, Tomoharu Miyashita and Yasuhiko Yamamoto made substantial contributions to the design and coordination of the study. Shunsuke Kanou, Takada Satoshi, Nakura Makoto, Mitsuyoshi Okazaki, Yoshinao Ohbatake, Sinichi Nakanuma, Isamu Makino, Hidehiro Tajima and Sachio Fushida collected the samples and data. Shunsuke Kanou and Tomoharu Miyashita analyzed the data. Tetsuo Ohta approved the final version of the manuscript. All authors read and approved the final manuscript.

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