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Recombinant thrombomodulin prevented hepatic ischemia-reperfusion injury by inhibiting high-mobility group box 1 in rats

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ABSTRACT

Recombinant thrombomodulin (rTM) is a novel anticoagulant and anti-inflammatory agent that inhibits secretion of high-mobility group box 1 (HMGB1) from liver. We evaluated the protective effects of rTM on hepatic ischemia-reperfusion injury in rats. Ischemia was induced by clamping the portal vein and hepatic artery of left lateral and median lobes of the liver. At 30 min before ischemia and at 6 h after reperfusion, 0.3 ml of saline (IR group) or 0.3 ml of saline containing 6 mg/kg body weight of rTM (IR-rTM group) was injected into the liver through inferior vena cava or caudate vein. Blood flow was restored at 60 min of ischemia. Blood was collected 30 min prior to induction of ischemia and before restoration of blood flow, and at 6, 12, and 24 h after reperfusion. All the animals were euthanized at 24 h after reperfusion and the livers were harvested and subjected to biochemical and pathological evaluations. Serum levels of ALT, AST, and HMGB1 were significantly lower after reperfusion in the IR-rTM group compared to IR group. Marked hepatic necrosis was present in the IR group, while necrosis was almost absent in IR-rTM group. Treatment with rTM significantly reduced the expression of TNF-α and formation of 4-hydroxynonenal in the IR-rTM group compared to IR group. The results of the present study indicate that rTM could be used as a potent therapeutic agent to prevent IR-induced hepatic injury and the related adverse events.

1. Introduction

Hepatic ischemia-reperfusion (IR) injury is a major clinical problem and is associated with numerous adverse events such as hypovolemic shock (Tamura et al., 2016; van Golen et al., 2019), disseminated intravascular coagulation (DIC) (Yoshikawa et al., 1983), complications with liver transplant surgery (Konishi and Lentsch, 2017), cardiac failure and arrest, increased toxicity of alcohol, and several other pathological conditions. Although the liver may initially exhibit direct cellular damage as the result of ischemia, reperfusion further induces dysfunction and cellular injury resulting from activation of inflammatory pathways (Fondevila et al., 2003). High-mobility group box 1 (HMGB1), histones, and DNA as well as Toll-like receptor (TLR)-4 and TLR-9, have been shown to be involved in the damage and inflammation induced by hepatic IR injury (Huang et al., 2014). In particular, HMGB1 is a key molecule in liver inflammation and injury in response to IR injury (Tsung et al., 2005) and is the only relevant marker in the clinical setting (van Golen et al., 2019). HMGB1 is an evolutionarily conserved nuclear protein that binds to chromatin and involved in DNA organization and regulation during transcription (Bustin, 1999; Bianchi and Beltrame, 2000). HMGB1 is also released from necrotic cells (Scaffidi et al., 2002) and inflammatory cells such as macrophages (Wang et al., 1999). It has been reported that HMGB1 is released into the extracellular space of the liver during IR injury (Tsung et al., 2005). HMGB1 is the key endogenous ligand for TLR-4, which is responsible for the early stage of inflammatory responses in IR injury (Tsung et al., 2005, 2007).

Thrombin is a serine endopeptidase, an enzyme that catalyzes the conversion of fibrinogen into fibrin and activates procoagulant factors V, VIII, XI, and XIII (Adams and Huntington, 2006; Posma et al., 2016). Thrombin is composed of two polypeptide chains of 36 (A chain) and 259 (B chain) amino acid residues that are covalently linked through the Cys1 – Cys122 (chymotrypsinogen numbering) disulfide bond (Papaconstantinou et al., 2008). Thrombin is produced from

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prothrombin (factor II) through enzymatic cleavage at two sites by prothrombinase and is activated by clotting Factor X (Xa) (Krishnaswamy, 2013). Factor Xa is the trypsin-like proteinase of coagulation that catalyzes prothrombin activation. Prothrombin is synthesized in the liver and is a vitamin K-dependent single-chain glycoprotein of 579 amino acid residues with a molecular weight of 72 kDa, and present at approximately 100 μg/ml in normal plasma (Pontarollo et al., 2017). Thrombin catalyzes thrombus formation and a series of reactions that both positively and negatively regulate flux through the coagulation cascade (Krishnaswamy, 2013). The anticoagulant function of thrombin is under the allosteric control of the cofactor thrombomodulin (Di Cera, 2008).

Thrombomodulin (TM) or human $CD141^+$ is a 74 kDa glycoprotein and expressed on the surface of endothelial cells and serves as a cofactor for thrombin-mediated activation of protein C (Anastasiou et al., 2012; Loghmani and Conway, 2018). The protein C pathway is a major anticoagulant mechanism that prevents thrombin formation and protects from thrombosis. Recombinant thrombomodulin (rTM) is a novel anticoagulant agent composed of active extracellular domain of thrombomodulin that could function same as thrombomodulin (Mohri et al., 1999). Recently, rTM has been used widely for DIC treatment in Japan (Kadono et al., 2017; Fujii et al., 2018). It accelerates the thrombin-catalyzed conversion of protein C to activated protein C, which inhibits monocyte and macrophage activation (Esmon, 1987) and consequently suppresses the production of inflammatory cytokines (Grey et al., 1994). Moreover, rTM has an inhibitory effect on HMGB1 by its anti-inflammatory action (Nagato et al., 2009). These findings suggest that rTM may protect hepatic IR injury. It is proposed that rTM inhibits action of HMGB1 which in turn leads to reduction tumor necrosis factor-α (TNF-α) and other inflammatory cytokines. The aim of the present study was to elucidate whether rTM has any inhibitory effects on HMGB1 and inflammatory cytokines in IR-induced liver injury in rats. Furthermore, we wanted to evaluate whether the anticoagulant effect or the anti-inflammatory effect of rTM play any role to prevent hepatic IR injury.

2. Materials and methods

2.1. Animals

Wistar male rats were procured from Japan SLC (Hamamatsu, Shizuoka, Japan). The animals were housed in temperature and humidity controlled cages with 12-h light/dark cycles and allowed access to food and water ad libitum. All animal experiments were carried out with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 86–23, revised 1996). The protocol was also approved by the Animal Care and Research Committee of Kanazawa Medical University on the Ethics of Animal Experiments (#2017–91).

2.2. Experimental design

The overall experimental protocol of present study is depicted in Fig. 1. About 10 weeks old albino male rats of Wistar strain were randomly divided into four groups of 6 rats each as sham group (body weight: 319.7 ± 4.9 g), sham treated with rTM (sham-rTM group) (body weight: 316.6 ± 4.3 g), IR group (body weight: 310.4 ± 3.4 g) and IR treated with rTM (IR-rTM group) (body weight: 322.7 ± 5.1 g). All the animals were anesthetized with intraperitoneal injections of pentobarbital (4.8 mg/100 g body weight) and the abdomen was shaved and sterilized with 70% alcohol. A ventral incision was made in the upper part of abdomen without harming the internal organs and about 1.5 ml of blood was collected from inferior vena cava of all rats in all the four groups (Tamura et al., 2016). Then either 0.3 ml of normal saline (IR group) or 0.3 ml of normal saline containing 6 mg/kg body weight of rTM (Asahi Kasei Pharma, Tokyo, Japan) (IR-rTM group) were injected into the inferior vena cava. The abdominal incision was temporarily closed with partial suture and the animals were kept on a warm pad in supine under anesthesia. At 30 min after start of the procedure, abdomen was opened again, and the portal vein and hepatic artery were clamped with a vascular microclip (30 g/mm^2, Cat# AM-1-30, Bear Medic Corporation, Kuji-gun, Ibaraki-ken, Japan) to the left lateral and median lobes in order to induce hepatic ischemia. The procedure yields approximately 70% partial ischemia (Colletti et al., 1996). The right and caudate lobes (30% of liver mass) retain intact portal and arterial inflow and venous outflow, preventing intestinal congestion (Colletti et al., 1996). The abdominal incision was again partially closed with sutures. The animals were maintained on a warm pad at 37 °C under anesthesia for 60 min in supine position and were under observation.

After 60 min of ischemia, the sutures were cut open and 1.5 ml of blood was collected from the inferior vena cava. Then the blood flow was restored by unclamping the vessels. Blood supply to the ischemic lobes was restored gradually within 1–1.5 min. The abdominal incision was closed completely with surgical sutures. When the animals were awake from anesthesia, analgesics were administered orally using intragastric tubes.

At 6 h after reperfusion, the animals were anesthetized again with intraperitoneal injections of pentobarbital and 1.2 ml of blood was collected from the orbital venous plexus using capillary tube. Then 0.3 ml of normal saline (IR group) or 0.3 ml of normal saline containing 6 mg/kg body weight of rTM (IR-rTM group) was injected into the caudate vein and the animals were left for another 6 h. When the animals were recovered from anesthesia, analgesics were again administered orally. At 12 h after reperfusion, the animals were again anesthetized with intraperitoneal injections of pentobarbital and 1.2 ml of blood was collected from the orbital venous plexus using capillary tube. The animals were again administered with analgesia orally upon awake and left for another 12 h. At 24 h after reperfusion, all the animals were anesthetized, blood was collected from the right jugular vein following a deep cut with a scalpel, and euthanized. The livers were quickly collected and the median lobe cut into pieces of 3 mm thickness, and instantly fixed in 10% phosphate-buffered formalin for histopathological studies.

2.3. Measurement of serum ALT, AST, and HMGB1 levels

Blood was allowed to clot at room temperature and the serum was separated using a clinical centrifuge. Serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) were measured using a spectrophotometer (DRI-CHEM 7000, FUJIFILM, Tokyo, Japan). Serum HMGB1 level was measured by HMGB1 enzyme-linked immunosorbent assay kit II (Shino-Test Corporation, Kanagawa, Japan) according to the manufacturer's instructions.

2.4. Measurement of malondialdehyde in the liver homogenate

About 100 mg of fresh liver tissue was homogenized in 1 ml of ice-cold 50 mM Tris-HCl buffer (pH 8) containing 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100. The homogenate was centrifuged at 12,000 × *g* for 30 min at 4 °C. Then 10 μl of the supernatant was diluted to 990 μl with Milli Q water and used for protein assay. Protein content was measured using a protein assay kit (#23227; Pierce Biotechnology, Rockford, IL, USA). Another portion of the supernatant was used to measure malondialdehyde (MDA) present in the liver tissue using an assay kit (# NWK-MDA01, Northwest Life Science, Vancouver, WA) as per the manufacturer's protocol. About 200 μl of the supernatant was mixed with 600 μl of assay buffer (provided in the kit) and vortexed for 5 s. Samples were assayed in triplicate of 250 μl each and the absorbance was measured at 532 nm on a spectrophotometer. The concentration of MDA in the hepatic tissue is presented as nmol/mg protein.

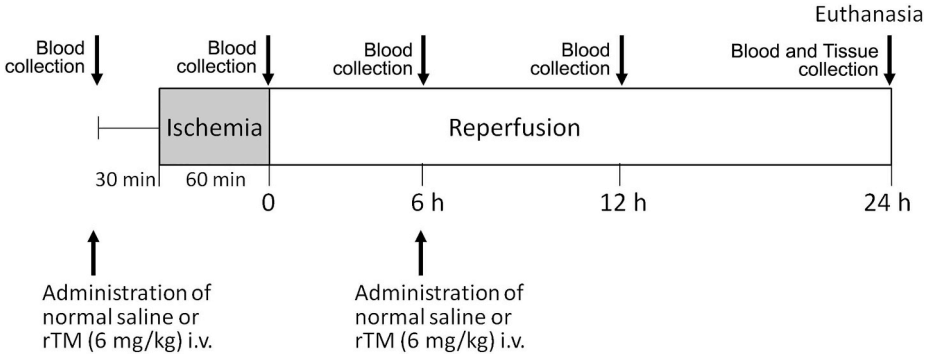

Fig. 1. Schematic illustration of the experimental design.

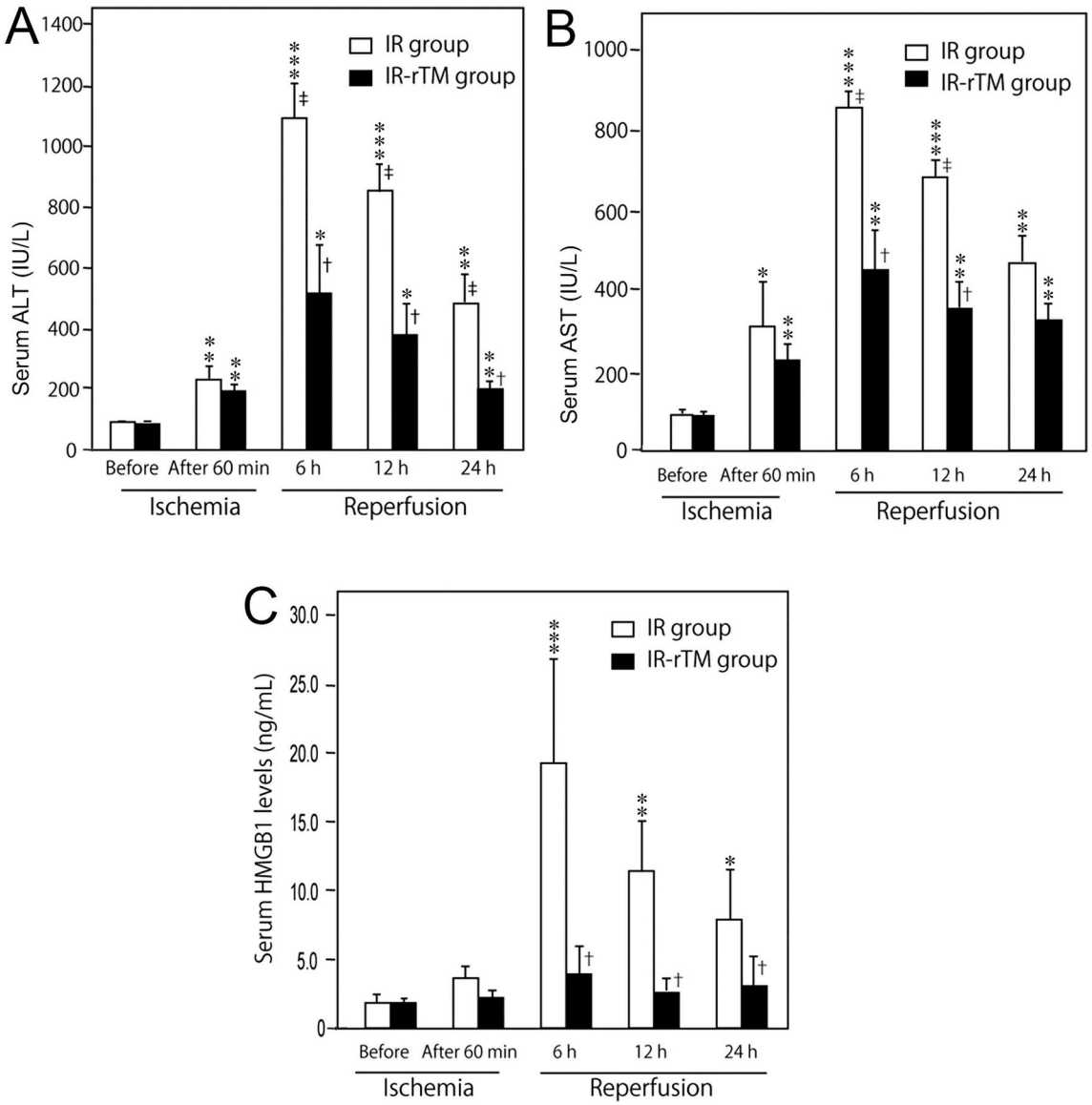

Fig. 2. Serum levels of ALT, AST, and HMGB1 before and after ischemia-reperfusion and effects of rTM treatment. (A) Serum levels of ALT. Serum ALT levels at 6, 12, and 24 h after reperfusion in the IR-rTM group were significantly lower than the respective levels in IR group. **(B) Serum levels of AST.** Serum AST levels at 6 and 12 h after reperfusion in the IR-rTM group were significantly lower than those in the IR group. **(C) Serum levels of HMGB1.** In the IR-rTM group, serum HMGB1 significantly decreased compared to the IR group at 6 and 12 h after reperfusion. Since the sham data for ALT, AST, and HMGB1 were not significantly different from control values (before ischemia), they are not shown. The values are mean ± S.E.M (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001 when compared to the respective mean values before ischemia (by Kruskal-Wallis test). †P < 0.05 when compared with IR group mean values at respective time points (Mann-Whitney U test). ‡P < 0.05, when compared with mean values in IR group after 60 min of ischemia (by Kruskal-Wallis test).

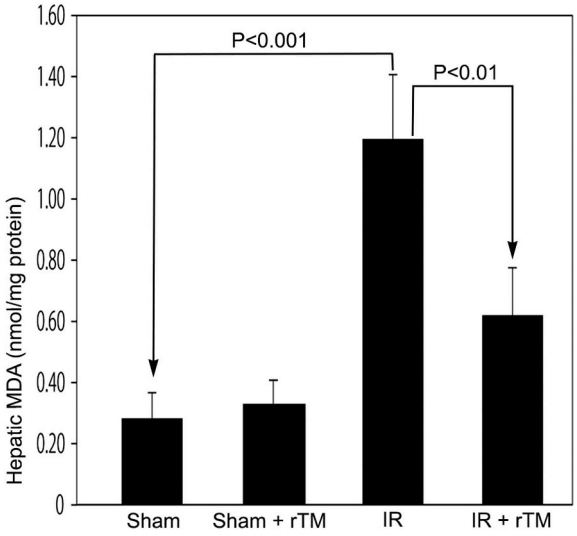

Fig. 3. Hepatic MDA levels in sham, sham-rTM, IR, and IR-rTM groups at 24 h after reperfusion. There was significant increase of MDA in the IR group compared to sham group. The mean hepatic MDA level in the IR-rTM group was significantly less compared to IR group. The values are mean ± S.E.M ($n = 6$).

2.5. Immunohistochemical staining for 4-HNE and TNF-α

The Immunohistochemical staining for 4-hydroxy-2-nonenal (4-HNE) and TNF-α were carried out on paraffin liver sections to examine the degree of inflammation and increased production of reactive oxygen species caused by IR, respectively. Reactive oxygen species are involved in cellular lipid peroxidation and membrane lipids are one of the major targets of reactive oxygen species. During the peroxidation process, a variety of aldehydes are formed and 4-HNE is an alpha, beta unsaturated aldehyde that can be formed by peroxidation of omega-6 unsaturated fatty acids such as linoleic acid and arachidonic acid. Therefore, the formation of 4-HNE is a reliable biomarker for lipid peroxidation and directly correlated with the generation of reactive oxygen species (Lee et al., 2012).

The liver sections were deparaffinized by using xylene and alcohol and hydrated to water. For antigen retrieval and exposure, the slides were autoclaved at 95 °C for 30 min in citrate buffer, pH 6 (Cat# S2031, DAKO, Bunkyo-ku, Tokyo, Japan) for TNF-α or Tris/EDTA buffer, pH 9 (Cat# S2368, DAKO, Bunkyo-ku, Tokyo, Japan) for 4-HNE and cooled to room temperature. Immunohistochemistry was performed using a broad-spectrum histostain kit (Invitrogen, Carlsbad, CA, USA). After blocking, the liver sections were incubated with TNF-α rabbit polyclonal antibody (#ab6671, abcam, Tokyo, Japan) at a concentration of 10 μg/ml (diluted to 100 fold) or 4-HNE mouse monoclonal antibody (#HNEJ-2, Nikken Seil, Shizuoka, Japan) at a concentration of 25 μg/ml (diluted to 4 fold) in a moisturized chamber (Evergreen Scientific, Los Angeles, CA, USA) at 4 °C for overnight. The slides were washed and the sections were treated with biotinylated second antibody for 2 h at room temperature. It was rinsed again and incubated with streptavidin-peroxidase for another 30 min. The intended color of the staining was produced after treating the sections with 3% 3-amino-9-ethylcarbazole (AEC) in N,N-dimethylformamide for 5–10 min. The stained sections were counterstained with Mayer's hematoxylin for progressive nuclear staining and mounted using aqueous mounting media. The slides were visualized under a microscope (Olympus BX51, Tokyo, Japan) attached with Olympus DP71 digital camera (Olympus Corporation, Tokyo, Japan) and photographed. The staining intensity of TNF-α and 4-HNE was quantified in 10 randomly selected microscopic fields using Image-pro discovery software (Media Cybernetics, Silver Spring, MD, USA). In the case of treated animals, the samples were from IR lobes. The data are presented as % square microns, where the sample with maximum staining intensity was considered as 100%.

2.6. Histopathological evaluation of the liver tissue

The formalin-fixed liver samples were processed in an automatic tissue processor optimized for liver tissue, embedded in paraffin blocks and cut into sections of 5-μm thickness. The sections were stained with hematoxylin and eosin (H&E) as per the standard protocol. The stained sections were examined under an Olympus BX51 microscope attached with a DP 71 digital camera and photographed. The degree of hepatic IR injury was determined by Suzuki's criteria based on congestion, vacuolization, and necrosis and classified into 5 grades; none (0), minimal (1+), mild (2+), moderate (3+) and severe (4+), respectively (Suzuki et al., 1993). In this study, 10 lobules in each rat liver were observed and total score of congestion, vacuolization and necrosis was calculated.

2.7. Statistical analysis

Arithmetic mean and standard error of the mean (S.E.M.) were calculated for all the data and presented as mean ± S.E.M. Kruskal-Wallis test, the non-parametric equivalent to an analysis of variance (ANOVA), was used to compare intragroup differences between various time points. Bonferroni post-hoc test was used to determine the level of significance between each time point. The difference between the IR and IR-rTM group at a given time point was assessed using Mann-Whitney *U* test. Pearson's correlation coefficient analysis was used to evaluate the correlation between ALT and histological damage score as well as between oxidative stress markers and inflammatory markers. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of rTM on serum ALT, AST, and HMGB1 during ischemia-reperfusion injury

There was no difference in the serum ALT, AST, and HMGB1 levels between sham and sham-rTM groups before ischemia and during the course of the study (data not presented). Therefore, in the present study, serum ALT, AST, and HMGB1 levels were compared between IR and IR-rTM groups only (Fig. 2). The mean serum ALT levels in IR and IR-rTM groups at 60 min after ischemia were significantly increased ($P < 0.01$) compared to the serum ALT levels before ischemia (Fig. 2A). At 6 h after reperfusion, serum ALT level in IR group markedly increased ($P < 0.001$) than serum ALT level at 60 min after ischemia. Although serum ALT level in IR-rTM group increased at 6 h after reperfusion, it was significantly lower ($P < 0.05$) compared to the respective IR group. Serum ALT level in IR group gradually decreased at 12 h and 24 h after reperfusion. On the other hand, in IR-rTM group, serum ALT level markedly decreased at 12 h and 24 h after reperfusion, which were significantly lower ($P < 0.01$ at 12 h and $P < 0.05$ at 24 h) compared to the respective levels in IR group (Fig. 2A). Serum AST levels after ischemia and reperfusion in IR and IR-rTM groups showed almost similar results as in the case of serum ALT (Fig. 2B).

Serum HMGB1 levels in ischemia and after reperfusion are presented Fig. 2C. Serum HMGB1 level in IR group at 60 min after ischemia was higher than that before ischemia. Serum HMGB1 in IR group at 6 h after reperfusion dramatically increased ($P < 0.001$) compared to the value at 60 min after ischemia, but gradually decreased at 12 h and 24 h. On the other hand, in IR-rTM group, serum HMGB1 level at 60 min after ischemia was not significantly different compared to the level before ischemia. Although serum HMGB1 level in IR-rTM group at 6 h after reperfusion increased, the value was significantly lower ($P < 0.05$) than the respective value in IR group. At 12 h and 24 h serum HMGB1 level further decreased in IR-rTM group, but the difference was not significant compared to the value at 6 h.

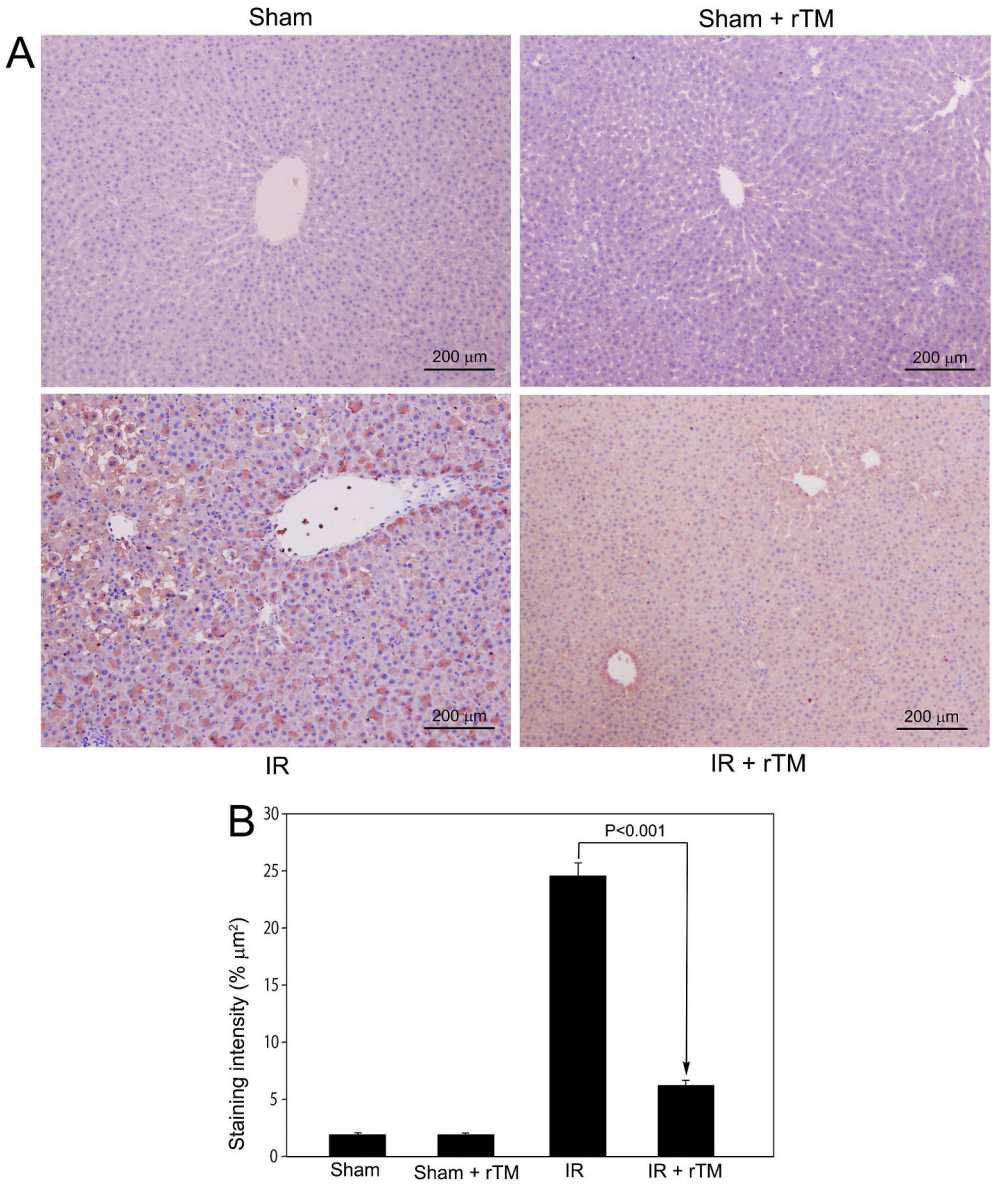

Fig. 4. Immunohistochemical staining for 4-HNE. (A) Staining for 4-HNE in the liver sections from sham, sham-rTM, IR, and IR-rTM groups at 24 h after reperfusion. Strong staining of 4-HNE was present in hepatocytes in pericentral areas and in necrotic zone in IR group, while only weak staining was present in pericentral areas in IR-rTM group (x40). **(B)** Quantification of the staining intensity of 4-HNE. The data are mean ± S.E.M (n = 6).

3.2. Treatment with rTM decreased generation of malondialdehyde

Malondialdehyde (MDA) is a marker of oxidative stress and a final product of peroxidation of polyunsaturated fatty acids in the cells. Both MDA and 4-HNE are formed by peroxidation of omega-6 unsaturated fatty acids and are cytotoxic and promote cell death (Ayala et al., 2014). Hepatic MDA levels in sham, sham-rTM, IR, and IR-rTM groups at 24 after reperfusion are presented in Fig. 3. Mean MDA level in IR group was significantly increased ($P < 0.001$) compared to the sham group. On the other hand, the mean hepatic MDA level in the IR-rTM group was significantly less ($P < 0.01$) compared to IR group. A highly significant positive correlation ($r = 0.993$, $p = 0.007$) was observed between mean hepatic MDA levels and serum HMGB1 at 24 h.

3.3. Treatment with rTM reduced production of 4-HNE

Tissue oxidative stress leads to lipid peroxidation and production of a variety of aldehydes. 4-hydroxy-2-nonenal is an α,β-unsaturated aldehyde that can be formed by peroxidation of omega-6 unsaturated fatty acids such as linoleic acid and arachidonic acid. The formation of 4-HNE is considered as one of the most reliable biomarker of lipid peroxidation in liver diseases (Poli et al., 2008). The results of the staining of 4-HNE in sham, sham-rTM, IR, and IR-rTM are presented in Fig. 4. Staining of 4-HNE was absent in the liver sections from sham and sham-rTM group (Fig. 4A). Prominent staining of 4-HNE was present in the hepatocytes in pericentral area and necrotic zones in IR group. On the other hand, only weak staining was observed in IR-rTM group in pericentral areas. Quantitative analysis using Image-pro Discovery software demonstrated that the intensity of 4-HNE staining in IR-rTM group was significantly less ($P < 0.001$) compared to the IR group (Fig. 4B). There was a strong positive correlation ($r = 0.981$, $p = 0.019$) between mean hepatic MDA levels and 4-HNE staining intensity score.

3.4. Treatment with rTM decreased production of TNF-α

Tumor necrosis factor-alpha (TNF-α) is a potent cytokine involved in systemic inflammation and plays a major role in formation of acute

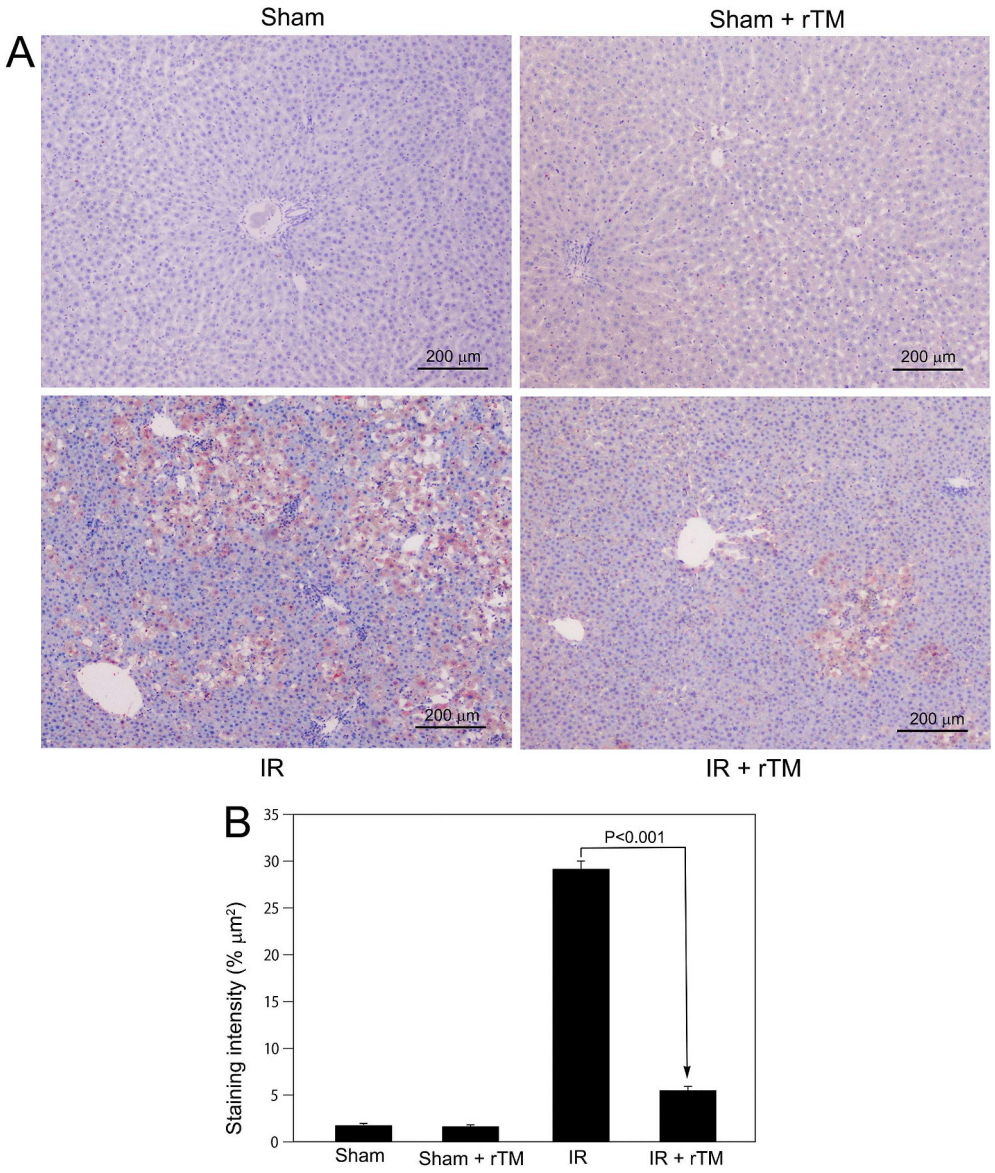

Fig. 5. Immunohistochemical staining of TNF-α after ischemia-reperfusion and effects of rTM treatment. (A) Staining for TNF-α in the liver sections from sham, sham-rTM, IR, and IR-rTM groups at 24 h after reperfusion. Staining for TNF-α was completely absent in sham and sham-rTM groups. Marked and intense staining for TNF-α was present in hepatocytes, especially in the necrotic zone in the IR group, while only mild staining was present in IR-rTM group (x40). **(B)** Quantitative analysis of the staining intensity of TNF-α. The data are mean ± S.E.M (n = 6 in all groups).

phase reaction (Salomon et al., 2018). It is mainly produced by activated macrophages during inflammation and serves a marker for the degree of hepatic inflammation. The results of the immunohistochemical staining of TNF-α in the liver tissue of rats subjected to IR and after treatment with rTM are presented in Fig. 5. Staining for TNF-α was completely absent in the liver sections from sham and sham-rTM groups (Fig. 5A). On the other hand, marked and intense staining of TNF-α was present in the hepatocytes, especially in the necrotic zone in IR group. However, few and weak staining was observed in IR-rTM group (Fig. 5A). Quantitative data of the staining intensity of TNF-α is presented in Fig. 5B. Staining intensity of TNF-α in IR-rTM group was significantly lower ($P < 0.001$) compared to IR group. Correlation analysis showed a positive correlation ($r = 0.992$, $p = 0.008$) between 4-HNE and TNF-α staining intensity. Similarly, there was a positive correlation ($r = 0.975$, $p = 0.025$) between hepatic MDA levels and TNF-α staining intensity.

3.5. Treatment with rTM prevented hepatic IR injury

The histopathological changes of liver tissue after IR injury and effects of rTM treatment are depicted in Fig. 6. There was no significant alteration in the liver tissue in sham and sham-rTM groups. Massive hepatic necrosis with infiltration of mononuclear cells, congestion, and vacuolization were present in many lobules in IR group (Fig. 6A). Severe necrosis was prominent in pericentral areas. However, only mild necrosis was present in the liver sections from IR-rTM group. Fig. 6B represents the degree of hepatic IR injury quantified by Suzuki's criteria based on congestion, vacuolization, and necrosis. The total Suzuki's score in IR-rTM group (2.0 ± 0.4) was significantly lower ($P < 0.001$) than that in IR group (6.2 ± 1.5). Correlation analysis depicted a positive correlation between ALT ($r = 0.985$, $p = 0.015$) and AST ($r = 0.962$, $p = 0.038$) levels and histological damage score.

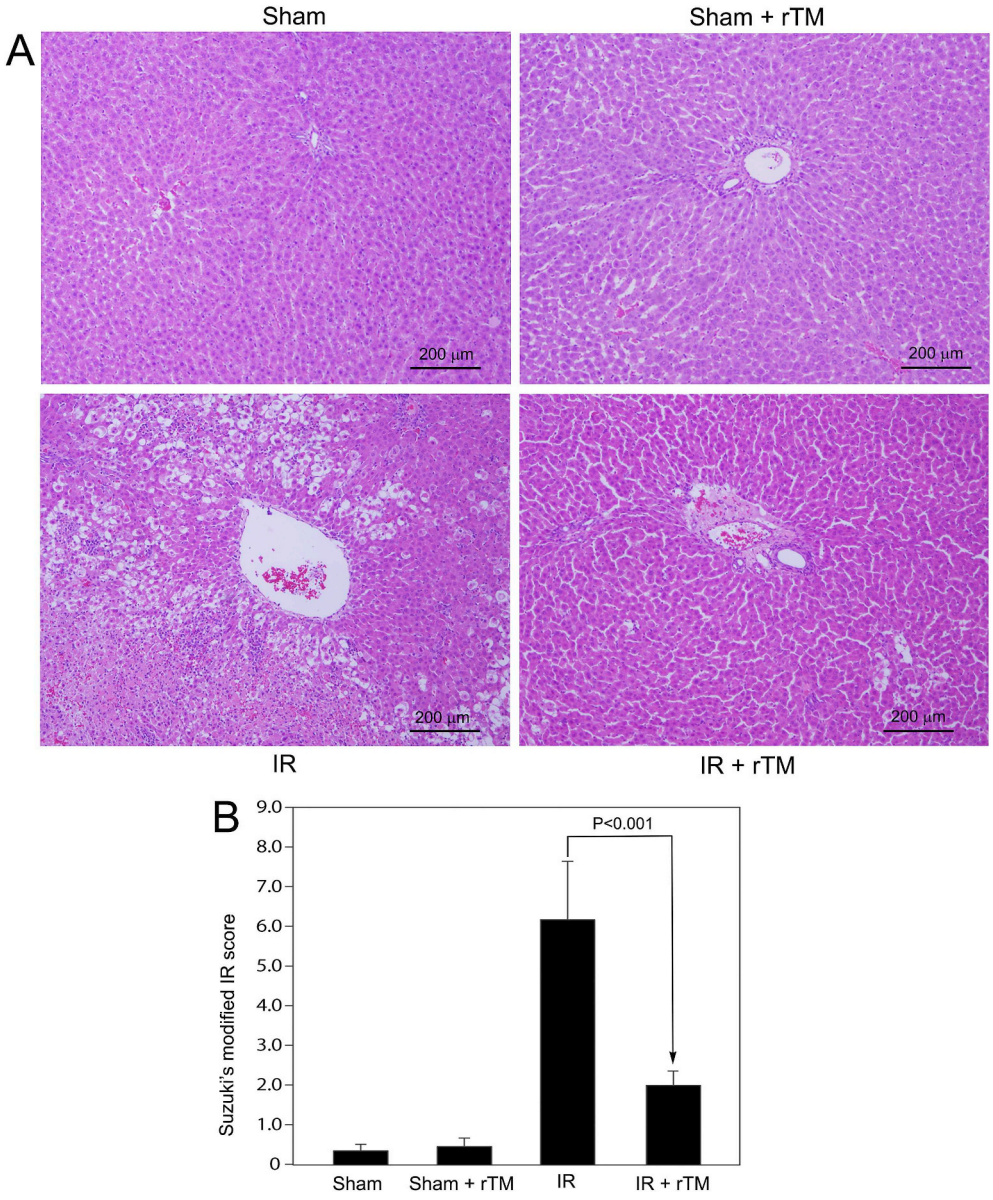

Fig. 6. Histopathological changes in the hepatic tissue after ischemia-reperfusion and effects of rTM treatment. (A) H&E staining of liver sections from sham, sham-rTM, IR, and IR-rTM groups at 24 h after reperfusion. Massive necrosis, sinusoidal congestion, and vacuolization of hepatocytes were present in IR group, which were prominent in pericentral areas. However, only mild congestion and slight necrosis were observed in rTM treated group. rTM treatment prevented intense necrosis and retained intact cellular architecture of the liver after reperfusion (x40). **(B) Suzuki's modified IR score.** The degree of hepatic IR injury was quantified by Suzuki's criteria based on congestion, vacuolization, and necrosis. The IR group score was 6.2 ± 1.5 points, whereas in IR-rTM group, it was 2.0 ± 0.4 points (n = 6).

4. Discussion

Ischemic tissue injury occurs when blood supply to part of a tissue or organ is blocked and is one of the major problems in healthcare. Ischemic tissue injury could lead to life threatening problems such as myocardial infarction, stroke, and other thrombotic process and affects transplant surgery. The restoration of blood supply or reperfusion of ischemic tissue will lead to further injury. The pathogenesis of hepatic IR injury is the result of a sequential process involving reactive oxygen species production, sterile inflammation, and hepatic necrosis (van Golen et al., 2012, 2014). Even at shorter ischemia times (30 min, 70% ischemia), livers with healthy parenchyma exhibit substantial histological damage (Kloek et al., 2012). Immunomodulation, a rather downstream event could play profound ameliorative effects on deleterious liver ischemia and reperfusion injury (Lu et al., 2016). Recombinant thrombomodulin is a novel anticoagulant agent that has also anti-inflammatory effect, which inhibits secretion of HMGB1 from liver (Nagato et al., 2009). It has been assumed that the inhibitory action of rTM on HMGB1 would lead to reduction of TNF-α and other inflammatory cytokines that play significant role in hepatic IR injury. The current study depicted that treatment with rTM could ameliorate hepatic IR injury and prevent subsequent pernicious parenchymal damage.

One of the purposes of our study was to evaluate whether the anti-inflammatory effect of rTM has any role to prevent hepatic IR injury. In both IR and IR-rTM groups, at 6 h after reperfusion, serum ALT and AST levels significantly increased compared to the levels at 60 min after ischemia. However, both serum ALT and AST levels in IR-rTM groups were significantly lower than those in IR group. The results suggest that rTM could prevent hepatic IR injury through its anti-inflammatory effect. In the present study, we injected rTM on second time at 6 h after reperfusion since the biological half-life of rTM is 7.2 h (Tsuruta et al., 2009)). Although serum levels of ALT and AST in both IR and IR-rTM groups decreased gradually at 12 h and 24 h after reperfusion, the reduction in IR-rTM group was significantly lower compared to the respective levels in IR group. Massive necrosis with infiltration of mononuclear cells, congestion and vacuolization were observed in many hepatic lobules in IR group, while only few necroses were present in livers from the IR-rTM group. These results suggest that the anti-inflammatory effect of rTM contributes to prevent hepatic IR injury.

It was reported that rTM has inhibitory effect on HMGB1 through its anti-inflammatory properties (Nagato et al., 2009). In the present study,

we have noticed that serum HMGB1 levels were significantly decreased in IR-rTM group of animals along with marked decrease of hepatic IR injury. The results of the current study are consistent with the previously published work of Kimura et al. (2018). They reported that IR injury was associated with the release of HMGB1 from hepatocytes and inhibition of HMGB1 using rTM decreased the production of inflammatory cytokines and prevented severe IR injury in rats. They observed that administration of rTM did not alter HMGB1 level in the liver tissue but remarkably reduced HMGB1 level in the serum (Kimura et al., 2018). In the current study, we did not evaluate hepatic HMGB1 levels. Treatment with rTM blocks the active domains of HMGB1, which would be the probable mechanism of the inhibitory action of rTM on HMGB1 (Kimura et al., 2018).

Thrombomodulin is normally shielded from biological action by the endothelial glycocalyx, which is present on the endothelial cells of the liver and shed following ischemia and IR (van Golen et al., 2012, 2014). As a result, ischemia itself could lead to the curtailing of ischemia and IR damage by exposure of thrombomodulin on the sinusoidal endothelial cells. The current study revealed that in-vivo thrombomodulin exposure does not confer sufficient hepatoprotection, and that systemically infused rTM was helpful to prevent post-ischemic liver damage. This observation has great translational potential and may be relevant in the clinical setting during liver transplantation surgery.

It was observed that rTM could suppress inflammatory responses by binding HMGB1 at an N-terminal lectin-like domain (Abeyama et al., 2005). Recent studies also demonstrated that rTM reduces secretion of inflammatory cytokines, such as IL-6 and TNF-α (Hagiwara et al., 2010). In the current study, we have observed significant decrease of TNF-α and 4-HNE staining in the liver sections of IR-rTM group. Furthermore, there was decrease of hepatic MDA in rTM treated animals during IR injury. These results suggest that rTM reduces secretion of inflammatory cytokines and production of reactive oxygen species through binding and inactivation of HMGB1. Thus, rTM acts in multiple ways to prevent IR-induced hepatic injury compared to other pharmacological agents that are proposed for IR-induced liver damage. Recently, GGsTop, a potent and irreversible inhibitor of γ-glutamyl transpeptidase (γ-GT) is reported as a pharmacological agent to prevent IR-induced liver injury and the related adverse events (Tamura et al., 2016). However, the effect of GGsTop is limited to the inhibition of γ-GT and the downstream action of the enzyme. In another study, it was observed that the administration of extracellular vesicles derived from bone marrow-derived mesenchymal stem cells (MSCs) ameliorates hepatic IR-injury through modulation of the inflammatory response (Haga et al., 2017). However, such a treatment is not feasible and practical during surgery and other clinical conditions. Therefore, rTM would be feasible, effective, and superior compared to the other reported pharmacological interventions to prevent IR-induced hepatic injury.

In conclusion, the results of the present study indicate that rTM treatment could decrease oxidative stress and inflammation in the liver tissue during IR injury. Furthermore, the data suggest that rTM could be used as a protective agent to prevent IR-induced hepatic injury and the related adverse events.

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Conflicts of interest

The authors do not have any conflicts of interest to declare in connection with this work.

Author contributions

M. Tsutsumi and M. Tsuchishima involved in conception and design of the study, obtained funding, and provided technical and material support. Y. Hirakawa, A. Fukumura, K. Kinoshita, N. Hayashi, and T. Saito carried out all the major experiments and collected the data. Y. Ueda carried out histopathological evaluation. J. George and N. Toshikuni analyzed and interpreted the data and wrote the manuscript.

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