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Thrombomodulin Administration Attenuates Ischemia-Reperfusion Injury of the Remnant Liver After 70% Hepatectomy in Rats: Simulated Model of Small-for-size Graft in Living Donor Liver Transplantation

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ABSTRACT

Background. Hepatic ischemia-reperfusion injury (IRI) is a serious complication affecting liver function and postoperative course after liver transplantation. Thrombomodulin (TM) has been known to have anticoagulant and anti-inflammatory activities exerting a cytoprotective effect. We evaluated the cytoprotective effect of recombinant human soluble TM (rhsTM) on the remnant liver exposed to IRI after 70% hepatectomy in rats, which was the simulated model of small-for-size graft in living donor liver transplantation.

Materials and Methods. A Wistar rat underwent 70% hepatectomy followed by 20-minute IRI for the remnant liver. rhsTM (1 mg/kg) (TM group) or saline (control group) was intravenously administered 30 minutes before operation.

Results. Alanine aminotransaminase levels were more significantly decreased during the 24 hours after operation in the TM group than in control group, especially at 6 hours. Intrahepatic infiltration of macrophages/monocytes (ED-1 immunohistochemical staining) at 6 hours was significantly decreased in the TM group compared to the control group. The number of proliferating cell nuclear antigen−positive cells at 12 hours (hepatocyte proliferation) was significantly higher in the TM group than in the control group; although liver weight 7 days after operation did not differ between the two groups. Hepatocyte apoptosis (terminal deoxynucleotidyl transferase−mediated deoxyuridine triphosphate nick-end labeling, also known as TUNEL assay) at 24 hours was more significantly diminished in the TM group than in the control group.

Conclusion. These results suggest that rshTM attenuates hepatocyte injury through its anti-inflammatory effect, and promotes hepatocyte proliferation in the reduced-size liver exposed to hepatic IRI.

HEPATIC ischemia-reperfusion injury (IRI) occurs in several surgical settings, such as liver resection, under intermittent vascular inflow occlusion, and liver transplantation. That affects liver function and postoperative course for patients who have had liver transplantation. Hepatic IRI consists of the two inter-related phases of local ischemic insult leading to the initial parenchymal cell death, and reperfusion injury inducing the profound inflammatory immune response that follows [1]. Therefore, some anti-inflammatory agents might be protective for hepatic IRI.

Among several anticoagulant agents, activated protein C (APC), thrombomodulin (TM), antithrombin, and tissue factor pathway inhibitor have been shown to have protective effects against IRI [2,3]. Regarding TM, it is an endothelial anticoagulant cofactor that plays an important role in the regulation of intravascular coagulation. In addition, TM can

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suppress the inflammatory response by binding and inhibiting high-mobility group box-1 protein (HMGB1) [4]. HMGB1 is released from necrotic or damaged cells including hepatocytes. HMGB1 is the key endogenous Toll-like receptor 4 ligand responsible for immune activation in the liver during IRI [5]. Therefore, we hypothesize that the administration of TM may prevent hepatic IRI through the dual effects of anticoagulant and anti-inflammatory potentials. The aim of this study is to evaluate the protective effect of recombinant human soluble TM (rhsTM) for hepatic IRI after partial hepatectomy in rats, which was simulated as a small-for-size graft in living donor liver transplantation (LDLT) in rats.

MATERIALS AND METHODS

Animals

Twelve-week old Male Wistar Hannover rats (CLEA Japan Inc, Tokyo, Japan) were used in all experiments. Rats were allowed free access to rat chow and water before and after surgical procedures. All experiments were conducted in compliance with the guidelines for animal experiments of the National Institutes of Health (NIH publication 86-23 revised 1985).

Experimental Model: 70% Hepatectomy Followed by Ischemia-reperfusion

Rats were anesthetized with isoflurane and livers were exposed through a midline laparotomy. They underwent 70% hepatectomy according to the method described by Higgins and Anderson [6]. After hepatectomy, complete ischemia of the remnant liver was induced by clamping the hepatoduodenal ligament for 20 minutes, followed by declamping it (reperfusion). The rats were randomly assigned to two groups: TM and control groups. In TM group, rhsTM (Asahi Chemical Industry, Tokyo, Japan; 1 mg/kg) was injected intravenously 30 minutes before the start of ischemia. In the control group, the same volume of saline was injected similarly.

To assess the survival, liver function, and liver regeneration, six animals in each group had peripheral blood drawn before ischemia (0 hours) and 6, 12, and 24 hours after the beginning of reperfusion and were sacrificed on postoperative day 7. Then, the liver was removed to measure liver regeneration rate, which was calculated from the resected liver weight at operation and the remnant liver weight on postoperative day 7 according to Selzner's manner [7]. For liver tissue analysis, six animals in each group were sacrificed at 6, 12, and 24 hours after the beginning of reperfusion, and the liver tissue samples were stored in formalin solution (10% buffered pH 7) at room temperature.

Measurement of Serum Alanine Aminotransaminase and Aspartate Aminotransaminase

Liver injury was quantified by measuring serum alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) levels using Test Wako for transaminase (Wako Pure Chemical Industries Ltd, Osaka, Japan) according to the manufacturer's instructions.

Tumor Necrosis Factor α and Interleukin 6 Protein Levels

Liver samples which were obtained 6 hours after reperfusion were homogenized in an extraction buffer (25 mmol/L Tris·HCl, pH 7.6; 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) and a protease inhibitor cocktail. The homogenates were shaken for 90 seconds and centrifuged at 3000 g and 4°C for 15 minutes. The hepatic levels of tumor necrosis factor α TNF-α and interleukin-6 (IL-6) were determined with commercially available enzyme-linked immunosorbent assays (BD Biosciences, San Diego, Calif, United States) according to the manufacturer's instructions.

Immunohistochemical Assessment

Formalin-fixed, paraffin-embedded tissue sections of liver which were obtained 6 hours after reperfusion were deparaffinized and rehydrated with phosphate-buffered saline, followed by proteinase K treatment (30 mg/mL in 100 mmol/LTris-HCl buffer, 50 mmol/L ethylenediamine tetra-acetic acid [pH 8.0]; 30 minutes at 37 1C). Next, sections were incubated in 0.3% H2O2 in methanol to block endogenous peroxidase activity. Primary antibodies against ED-1 (macrophage/monocyte; Chemicon International Inc, Temecula, Calif, United States) were added at optimal dilutions. Secondary antibodies and ABC reagent were applied according to the manufacturer's instructions (VECTASTAIN ABC kit; Vector Laboratories, Burlingame, Calif, United States). Color development was induced by incubation with 3,3-diaminobenzidine substrate for 5 to 8 minutes, and specific staining was visualized by light microscopy. The neutrophils were stained using a naphthol AS-D chloroacetate esterase staining kit (Sigma Diagnostics, St Louis, Md, United States). Neutrophils were identified by their nuclear morphology and bright red positive staining. The results were evaluated by counting labeled cells in 10 high-power (original magnification ×200) fields per section.

Evaluation of Liver Regeneration

Sections were prepared from formalin-fixed and paraffin-embedded liver samples that were obtained 12 hours after reperfusion. Hepatocyte proliferation was evaluated with anti−proliferating cell nuclear antigen (PCNA) antibody (Thermo Fisher Scientific, Calif, United States). We evaluated the results by counting labeled cells in 10 high-power (original magnification ×200) fields per section. The liver regeneration rate on postoperative day (POD) 7 was calculated as follows:

$$\text{Liver regeneration rate } (\%) = 100 \times [C - (A - B)]/A$$

where A is the estimated total liver weight at the time of partial hepatectomy, B is the excised liver weight at the time of partial hepatectomy, and C is the removed remnant liver weight on POD 7.

Detection of Apoptosis

Apoptosis was evaluated in liver samples that were obtained 24 hours after reperfusion. Apoptosis was detected with the terminal deoxynucleotidyl transferase−mediated deoxyuridine triphosphate nick-end labeling (TUNEL) method. Paraffin-embedded tissue sections were deparaffinized, and the dehydrated sections were treated with the In Situ Cell Death Detection Kit, POD (Roche Diagnostics, Temecula, Calif, United States), according to the manufacturer's instructions. The results were scored semi-quantitatively via the averaging of the number of TUNEL+ cells per field with 6 fields per tissue sample.

Statistical Analysis

Data are expressed as the mean ± standard deviation. Statistical analyses between the two groups were performed using Student *t*-test. Statistical analyses with respect to the transaminase after operation were performed using two-way repeated measures

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