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Thrombomodulin improves rat survival after extensive hepatectomy

Yohei Ota, MD, Takafumi Kumamoto, MD, PhD, Atsushi Ishibe, MD, PhD, Kazuteru Watanabe, MD, PhD, Ryutarou Mori, MD, PhD, Koichi Taniguchi, MD, PhD, Ryusei Matsuyama, MD, PhD, Hirochika Makino, MD, PhD, Michio Ueda, MD, PhD, Toru Kubota, MD, PhD, Hirotoshi Akiyama, MD, PhD, Kuniya Tanaka, MD, PhD, Yasushi Ichikawa, MD, PhD, and Itaru Endo, MD, PhD*

Department of Gastroenterological Surgery, Yokohama City University, Graduate School of Medicine, Kanazawa-ku, Yokohama, Japan

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ABSTRACT

Background: Recombinant human soluble thrombomodulin (rTM) protects against disseminated intravascular coagulopathy by inhibiting coagulation, inflammation, and apoptosis. This study tests the hypothesis that rTM is hepatoprotective after extensive hepatectomy (Hx) and investigates the mechanisms underlying this effect.

Materials and methods: Experiment 1: rats (15 per group) were injected with rTM (1.0 or 2.0 mg/kg) or saline just before 95% Hx and their 7-d survival assessed. Experiment 2: rats were assigned to either a treated (2.0 mg/kg rTM just before Hx) or control group ($n = 5$ per group). Five rats per group were euthanized immediately after surgery, and at 1, 3, 6, 12, and 24 h postoperatively; serum and liver remnant samples were collected for biochemical and histologic analysis, as well as reverse-transcription polymerase chain reaction and Western blotting.

Results: All saline-injected rats died within 52 h of Hx, whereas injection of 2.0 mg/kg rTM prolonged survival ($P = 0.003$). rTM increased the number of Ki67-positive cells and reduced the number of terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive cells. The number of myeloperoxidase-positive cells and the expression of high-mobility group box 1 protein did not differ. Reverse-transcription polymerase chain reaction revealed that rTM significantly enhanced protease-activated receptor-1 and sphingosine kinase 1 messenger RNA expression and significantly reduced plasminogen activator inhibitor-1 and Bax messenger RNA expression. Immunohistochemistry and Western blotting demonstrated that protease-activated receptor-1 expression 24 h after Hx was significantly higher in rTM-treated than in control rats.

Conclusions: rTM may improve survival after extensive Hx by inhibiting apoptosis and promoting liver regeneration.

* *Corresponding author*. Department of Gastroenterological Surgery, Yokohama City University, Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236 0004, Japan. Tel.: +81 45 787 2650; fax: +81 45 782 9161.
E-mail address: y.ota1029@gmail.com (Y. Ota).

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1. Introduction

Recent advances in surgical procedures and perioperative management have enabled aggressive and extensive hepatectomy (Hx) for the treatment of malignant liver tumors. However, such radical therapy may cause fatal liver failure. The mechanism by which extensive Hx causes liver failure was first investigated in the 1930s [1] and, more recently, several Hx rat models have been developed [2,3].

We have previously shown that 90% resection is the limit for safe Hx in rats, whereas resection of 95% of the liver is fatal [4]. Using complementary DNA (cDNA) microarrays, we analyzed gene expression in rats that had undergone 95% and 90% Hx and found that the mechanism of fatal liver failure after excessive Hx was characterized by an increase in apoptosis and a reduction in mitosis. Therefore, both inhibition of apoptosis and induction of liver regeneration are required for survival of the rats in our fatal hepatic failure model [5,6]. We also found that liver failure after extensive Hx resulted from hepatocyte apoptosis because of hypercytokinemia and a delay in the early phases of hepatic regeneration, as well as from impaired turnover of the extracellular matrix (ECM), which is regulated by the plasminogen activating system [7]. This impairment in ECM turnover was due to ECM degradation resulting from increased expression of plasminogen activator inhibitor-1 (PAI-1) [8,9]. However, neutrophil infiltration into the liver sinusoids and excessive necrosis were not observed in these rats [10].

Thrombomodulin (TM) is an endothelial anticoagulant cofactor that binds to thrombin and suppresses intravascular coagulation by reducing fibrin formation and platelet activity [11]. The anti-inflammatory and endothelial protective effects of TM occur via suppression of hypercytokinemia through inhibition of high-mobility group box-1 (HMGB-1), which has recently been shown to be an inducer of lethal cytokine storms [12]. TM also binds to thrombin on the endothelial cell protein C receptor; thrombin-TM complexes then rapidly activate the conversion of protein C into activated protein C (APC) on the endothelial cell protein C receptor [13]. The anticoagulant activity of TM results from its binding to thrombin, stimulation of the conversion of PC into APC, inactivation of the activated forms of coagulation factors V and VIII, and inhibition of thrombin generation [14].

TM-induced APC suppresses the production of inflammatory cytokines [13]. APC exerts antiapoptotic effects on endothelial cells by inducing the expression of protease-activated receptor (PAR)-1. PAR-1 inhibits the production of tissue factors and PAI-1 in vascular endothelial cells, thus inhibiting coagulation [15]. Activation of PAR-1 also has antiapoptotic effects via downregulation of p53 and Bax proteins [16], and the activation of sphingosine kinase-1 (Sphk1), which upregulates sphingosine-1 phosphate (S1P) [17].

Recombinant human soluble thrombomodulin ([rTM], Recomodulin; Asahi Kasei Pharma Corporation, Tokyo, Japan), which contains only the active extracellular domain of TM, was generated using recombinant DNA techniques for clinical use in Japan. Recently, a phase III, multicenter, double-blind, randomized, parallel-group trial found that rTM was significantly more effective than low-dose heparin for the treatment of disseminated intravascular coagulopathy [18]. In addition, APC, which is increased by TM, counteracts the impairment of the lungs [19] and liver [20] induced by lipopolysaccharides or bacteria in animal models of sepsis. We therefore hypothesized that rTM, because of its anti-inflammatory and cytoprotective properties, may prevent liver failure after excessive Hx.

2. Materials and methods

2.1. Animals

Eight-wk-old male Wistar rats, weighing 200–250 g, were purchased from CLEA Japan (Tokyo, Japan) and housed in the animal facilities of Yokohama City University with alternating 12-h dark and light cycles and with food and water available *ad libitum*. The experiment used only male rats, as was the case in previous reports of extensive Hx [4,8,10]. Regarding gender bias, according to the population pharmacokinetics of rTM [21], there are no differences in the efficacy of rTM according to gender. Additionally, there is no report indicating a gender bias in animal experiments using rTM.

All experimental protocols complied with the National Institutes of Health guidelines and were approved by the Animal Research Ethics Committee of Yokohama City University.

2.1.1. *Experiment 1: effect of rTM on survival of rats after extensive Hx*

Rats ($n = 15$ per group) were intravenously infused via the tail vein with 1.0 mL/kg of either 1.0 or 2.0 mg/mL rTM diluted in saline or with plain saline (control 1), without altering systemic blood pressure (Fig. 1). After infusion, 95% Hx was performed as described previously [4]. In brief, food was removed 12–18 h before surgery. The rats were anesthetized with sevoflurane, placed in a supine position, and a catheter (24G; NIPRO Co, Tokyo, Japan) was placed in the tail vein to allow continuous drug infusion. Branches of the hepatic artery and the portal vein were ligated, and the left, median, and right lobes and the anterior portion of the caudal lobe were resected. The rats were allowed access to a 10% glucose solution for the 24 h after surgery, and then were returned to a diet of standard food and water *ad libitum*. The rats were monitored over the next 7 d, and 7-d survival rates were compared between the groups.

2.1.2. *Experiment 2: investigation of the mechanisms underlying the hepatoprotective effect of rTM*

Rats were injected with either 2.0 mg/kg rTM ($n = 30$) or saline (control 2, $n = 30$) and then 95% Hx was performed as described. Five rats in each group were sacrificed at each of the following time points: immediately after 95% Hx, and 1, 3, 6, 12, and 24 h later. Remnant liver tissue and blood samples from the caudal vena cava were collected. Liver samples were fixed in 10% formalin or snap frozen in liquid nitrogen, with the latter samples stored at −80°C until use.

2.2. Protein assays

Serum levels of alanine aminotransferase, regarded as a biomarker of liver function, were measured using standard