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ORIGINAL ARTICLE

Recombinant human soluble thrombomodulin reduces the severity and incidence of necrotizing enterocolitis in a newborn rat model

Bo Li[1] · Ryuta Saka[1] · Yuichi Takama[1] · Takehisa Ueno[1] · Yuko Tazuke[1] · Hiroomi Okuyama[1]

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Abstract

Purpose Necrotizing enterocolitis (NEC) remains the leading cause of death in preterm infants. Recombinant human soluble thrombomodulin (rTM) has been reported to have anti-inflammatory effects as well as antithrombogenic effects. The aim of this study was to evaluate the effect of rTM in a rat NEC model.

Methods NEC was induced by enteral feeding with hyperosmolar formula, gavage administration of lipopolysaccharide and asphyxia stress. Controls were fed by their mother ad libitum. In the treatment group, rTM was administered subcutaneously twice (once each on the first and second day). All animals surviving beyond 96 h or that developed signs of distress were euthanized. The ileum was harvested for a histological evaluation and the measurement of the mRNA and protein expression.

Results The rate of NEC-like intestinal injury in the treatment group (9/25, 36%) was significantly lower than in the NEC group (25/34, 73.5%). Tissue levels of TNF-α, IL-6 and HMGB1 were significantly elevated in the NEC group, whereas those in the treatment group were decreased to similar values as in the control group.

Conclusions Our experimental study showed that rTM is able to reduce the severity and incidence of NEC. It may be an alternative option for the treatment of NEC.

Keywords Necrotizing enterocolitis · Rat · Cytokines · Inflammatory · Recombinant human soluble thrombomodulin

Introduction

Necrotizing enterocolitis (NEC) remains the leading cause of death from gastrointestinal disease in infants, especially in preterm infants, and is the most common and serious gastrointestinal neonatal disease in the neonatal intensive-care unit, with a mortality as high as 20–30%. The mortality of NEC patients who need surgical treatment is even higher [1]. In addition, the social costs for NEC survivors, especially due to long-term neurodevelopmental and intestinal effects, are enormous [2]. Although the risk factors for NEC development are widely understood, including prematurity and the administration of formula milk, its specific pathogenesis is still unclear. The current consensus indicates that the incidence of NEC is either stable or increasing, and the overall survival for patients with this disease has not changed since its initial description 30 years ago [3]. Despite therapeutic success in animal model systems, relatively few therapeutic strategies in the clinical setting have significantly improved the outcomes in infants with NEC [4].

Thrombomodulin (TM) is a type 1 membrane-bound glycoprotein that has a C-type lectin domain at its N terminus, six copies of the epidermal growth factor-like (EGF) motif and a serine/threonine-rich domain carrying a glycosoaminoglycan external to the membrane [5]. Recombinant human soluble thrombomodulin (rTM) contains only the active extracellular domain of TM [6]. In 2008, rTM was approved as a standard treatment for disseminated intravascular coagulation (DIC) in Japan. In addition to its anti-coagulant effect, the anti-inflammatory effect of rTM has been approved in studies using the septic model [7] and liver ischemia reperfusion injury model [8].

Inflammation is recognized as an important contributing factor in the progression of NEC, during which inflammatory cytokine levels increase and aggravate intestinal

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✉ Hiroomi Okuyama
okuyama@pedsurg.med.osaka-u.ac.jp

[1] Department of Pediatric Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

mucosal injury [9]. In this study, we hypothesized that the administration of rTM might have an anti-inflammatory effect and be effective in preventing the development of NEC in an experimental rat model of NEC.

Materials and methods

Animal model and study design

This study followed the guidelines approved by the Institutional Animal Care and Use Committee of Osaka University (28-051-000). Every effort was made to minimize the number of animals used and reduce their suffering. Time-mated pregnant Sprague–Dawley rats delivered spontaneously. Three groups were selected as follows: (1) control group: rats left with their mother and breast fed ad libitum with no exposure to stress; (2) NEC group: rats subjected to an NEC procedure and administered an equal volume of 0.9% saline instead of rTM subcutaneously; (3) NEC + rTM: rats subjected to an NEC procedure and treated with rTM. The dose of rTM was decided by referencing previous studies of rTM experiments [7, 8, 10, 11]. Newborn rats delivered by one mother rat were used as the control group, and those from each of three other mother rats were used as the NEC and NEC + rTM groups.

To eliminate the protective effect of mothers' milk, newborn rats in the NEC and NEC + rTM groups were isolated from their mothers immediately after birth under constant monitoring, and after being weighed, they were kept at 37 °C in a humidified incubator. The method used to induce the rat model of NEC was initially described by Barlow et al. [12] more than 40 years ago and modified by Nakame et al. [13]. Two hours after birth, newborn rats were force-fed hyperosmolar formula (Similac Advance infant formula; Abbott Nutrition, Columbus, USA and Esbilac canine milk replacer; PetAg, Hampshire, USA; at a ratio of 2:1), starting at 0.1 mL every 4 h and increasing slowly by 0.1 mL daily as tolerated, and exposed to 100% nitrogen for 10 min 3 times daily to induce NEC for 4 days. In addition, on the first and second days of life, the NEC and NEC + rTM groups were force-fed 5 mg/kg/day of *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) (Sigma-Aldrich Company, Saint Louis, USA). Pups in the NEC + rTM group were treated with rTM (Asahi Kasei Pharma, Tokyo, Japan) at a dose of 10 mg/kg/day subcutaneously on the first and second days of the NEC procedure 30 min before LPS was fed. The special rodent formula was prepared based on the method described by Feng et al. [14], and rats were force-fed using a PI catheter for neonates (24G × 30-cm type; Covidien, Shizuoka, Japan).

All rats were inspected and evaluated at each feeding point. Animals that developed distress (lethargy, abdominal distention, apnea, and intestinal perforation) or imminent death before 96 h were euthanized by cervical dislocation. After 96 h, all surviving animals were euthanized in the same manner. The number of deaths and approximate time of death were recorded daily for all groups and calculated as the survival rate. The weight of each surviving rat was also recorded daily.

Tissue harvest and the NEC evaluation

Following incision of the abdomen, the small intestine was evaluated visually for typical gross signs of NEC, such as intestinal distension, intestinal wall hemorrhaging, or necrosis. The gastrointestinal tract was carefully removed. The last 4 cm of the terminal ileum was excised. Part of each sample was formalin-fixed, paraffin-embedded, microtome-sectioned at 4 μm, and stained with hematoxylin and eosin for histological evaluations. Part of the ileum of each animal was immediately washed with phosphate-buffered saline (PBS) and frozen in liquid nitrogen for RNA isolation and Western blot analyses.

The scoring system of histological changes in the ileum was described by Dvorak et al. [15] and modified by Nakame et al. [13]. Histological changes in the ileum were scored by a blinded evaluator and graded as follows: grade 0, no damage; grade 1, slight submucosal and/or lamina propria separation without villous core separation; grade 2, moderate submucosal and/or lamina propria separation with villous core separation; grade 3, severe submucosal and/or lamina propria separation and epithelial sloughing of the villi; and grade 4, loss of villi with transmural necrosis. To determine the incidence of NEC, tissue damage with a histological injury score of grade ≥ 2 was considered positive for NEC.

Real-time polymerase chain reaction

Total RNA was extracted from frozen ileal tissue using the Total RNA Kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. RNA integrity was verified by agarose gel electrophoresis. The purity and concentration of RNA were quantified using a Nanodrop ND-1000 UV Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

RNA (500 ng) was reverse transcribed using a reverse transcription kit (Takara) under 37 °C for 15 min and 85 °C for 30 s. The gene expression of tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) was analyzed. The mRNA levels of each gene were detected in triplicate using FAST SYBR™ Green Master Mix (Applied Biosystems, Vilnius, Lithuania) by real-time quantitative polymerase chain reaction (RT-qPCR) in a volume of 10 μL with 384-well plates using an ABI Prism 7900HT (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an endogenous control. The sequences of the primer in each gene are shown in Table 1.

The amplification reaction was started at 95 °C for 20 s for initial denaturation, followed by 40 consecutive PCR cycles at 95 °C for 1.0 s, and 60 °C for 20 s.

Western blotting

RIPA buffer, which contains protease inhibitors, was used to extract protein from terminal ileal tissues, and the BCA method was used to determine the protein concentration. Equivalent proteins (80 μg) were separated by 5–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a PVDF membrane (Roche, Mannheim, Germany). The membrane was placed into 5% skimmed milk for blocking and incubated with the following antibodies: TNF-α (1:500, ab9755; Abcam, Cambridge, UK), IL-6 (1:1000, ab9324; Abcam) and HMGB1 (1:500, ab227168; Abcam). Equal loading of protein was confirmed using β-actin antibody. All experiments were repeated at least three times; representative results are presented.

Statistical analyses

Body weights were compared between the control, NEC and NEC + rTM groups using a one-way analysis of variance followed by the Newman–Keul multiple comparisons test. The survival rate was analyzed using a Kaplan–Meier analysis. The Mann–Whitney test was used to compare different pathological ileum scores between the NEC and NEC + rTM groups. The incidence of NEC-like intestinal injury was evaluated using the Chi-squared test. To analyze the results of RT-PCR, we used the $2^{-\Delta Ct}$ method. The difference in the mRNA expression of TNF-α and IL-6 among the control, NEC and NEC + rTM groups was analyzed using a one-way analysis of variance followed by the Newman–Keul multiple comparisons test. Results were considered statistically significant when $P < 0.05$.

Results

Assessment of the rat weight and survival rate

In our study, the newborn rats were force-fed using a PI catheter, which can very easily cause esophageal perforation or even tracheal perforation, especially in the first several feedings. We checked the whole intestine of mice that died immediately after feeding and noted no evidence of histological changes, indicating that they had died due to feeding failure (Supplementary Fig. 1) rather than NEC. On this basis, we excluded 5 of 39 rats in the NEC group and 8 of 33 animals in the NEC + rTM group. We ultimately assessed 11 rats in the control group, 34 in the NEC group and 25 in the NEC + rTM group.

There were no significant differences in the birth weight among the three groups. Throughout the entire experiment, the weight in the newborn rats in the control group increased, while the rats in both the NEC and NEC + rTM groups lost weight in the first 48 h, after which the weight of the surviving animals increased. The body weight between the NEC and NEC + rTM groups did not significantly differ during the study period (Fig. 1). The 96-h survival rates in the NEC and NEC + rTM groups were 23.5% (8/34) and 64% (16/25), respectively. The rats in the NEC + rTM group had a significantly higher survival rate than those in the NEC group ($P < 0.001$; Fig. 2).

Effects of rTM on the severity and incidence of NEC-like injury in neonatal rats

The ileal histological score was significantly lower in the NEC + rTM group (median 1.0) than in the NEC group (median 3.0) ($P = 0.0025$, Fig. 3a). The administration of rTM significantly decreased the rate of NEC-like intestinal injury from 73.5% (25/34) in the NEC group to 36% (9/25) in the NEC + rTM group ($P = 0.009$; Fig. 3b). Representative histological images from each group are shown in Fig. 4.

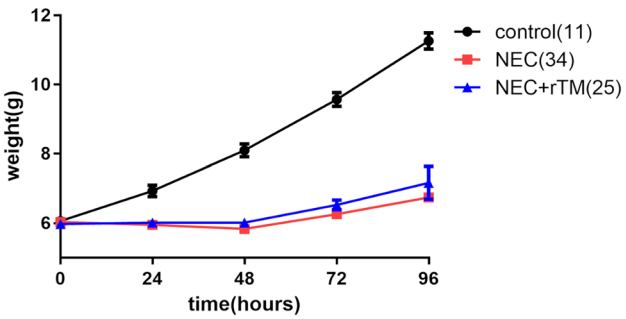

Fig. 1 Body weight in each group during the study period

Table 1 Sequences of the primer in each gene

Gene	Forward sequence	Reverse sequence
GAPDH	5′-ACAGCAACAGGGTGGTGGAC-3′	5′-TTTGAGGGTGCAGCGAACTT-3′
TNF-α	5′-ACCACGCTCTTCTGTCTACTG-3′	5′-CTTGGTGGTTTGCTACGAC-3′
IL-6	5′-CCAATTTCCAATGCTCTCCT-3′	5′-ACCACAGTGAGGAATGTCCA-3′

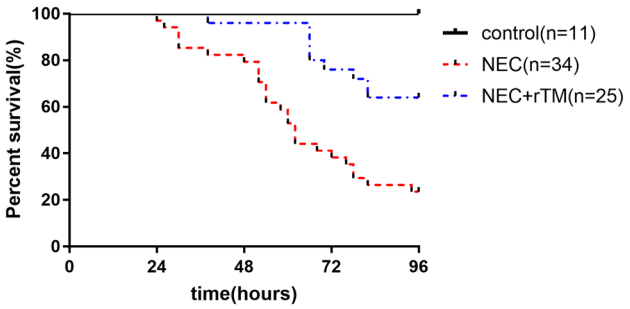

Fig. 2 Results of the Kaplan–Meier survival analysis. The rats in the NEC + rTM group had a significantly higher 96-h survival rate than those in the NEC group ($P < 0.001$)

The evaluation of the mRNA and protein expression of TNF-α, IL-6, and HMGB1 in the ileal tissues

The ileal mRNA expression of TNF-α and IL-6 increased by 6.4- and 34.4-fold, respectively, in the NEC group compared with the control group ($P < 0.05$), while the expression of TNF-α and IL-6 mRNA decreased by 3.4- and 43-fold, respectively, as a result of rTM treatment ($P < 0.05$). No significant differences in the mRNA expression of TNF-α and IL-6 were noted between the control and NEC + rTM groups (Fig. 5a). The Western blot assay confirmed the mRNA expression of TNF-α and IL-6 and also showed that HMGB1 was only elevated in the NEC group (Fig. 5b).

Discussion

The pathophysiology of NEC is generally agreed to be multifactorial, and in experimental animal models and some human epidemiologic analyses, formula feeding, intestinal hypoxic ischemic injury and abnormal bacterial colonization have been shown to be associated with an increased risk of bowel necrosis. However, our inability to adequately replicate these complex factors in animal models remains a challenge [1, 4, 16]. Although researchers now recognize that NEC development in humans is more complex than in animals and that the pathophysiology of a rat model might be different from that in humans, the model based on the induction of intestinal injury using formula feeding and hypoxia stress on premature rats is a well-established NEC model [12, 13]. In our model using formula feeding, LPS

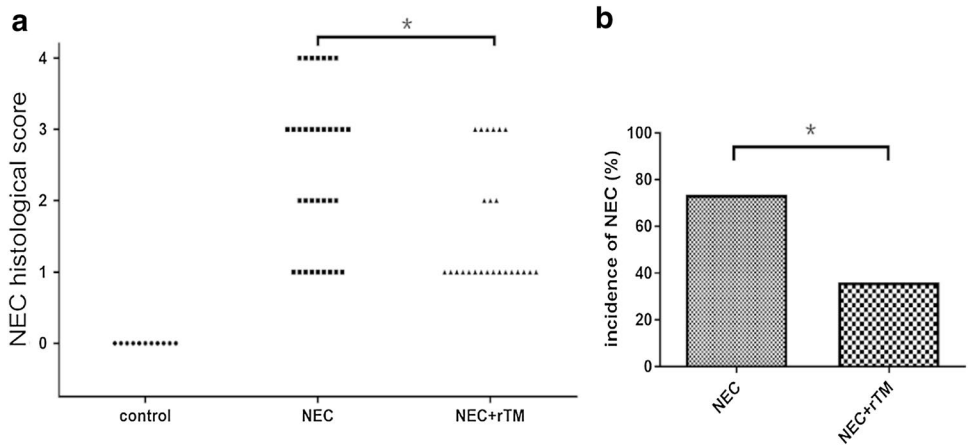

Fig. 3 **a** The NEC histological score of the ileum in each group. Histological scoring of the terminal ileum of newborn rats in the control ($n = 11$), NEC ($n = 34$) and NEC + rTM ($n = 25$) groups. NEC-like intestinal damage was defined as a score ≥ 2. **b** The NEC incidence. The rate of NEC-like intestinal injury was lower in the NEC + rTM group than in NEC group (36% vs. 73.5%). The asterisk indicates $P < 0.05$

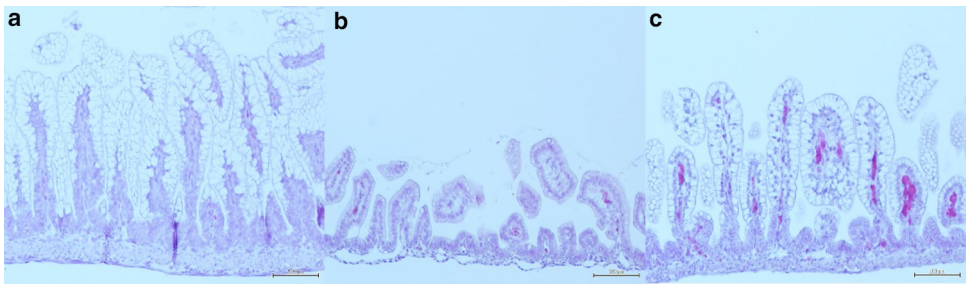

Fig. 4 Histological changes in the ileum in newborn rats. Representative images from each group are presented. **a** The structure of the intestinal mucosa was intact in the control group; **b** the structure of the ileum villus was broken and even lost in the NEC group; **c** the ileum of the newborn rats in the NEC + rTM group had less-necrotic ilea and fewer lost villi than that in the NEC group. The scale bar represents 500 μm. Original magnification: 100×

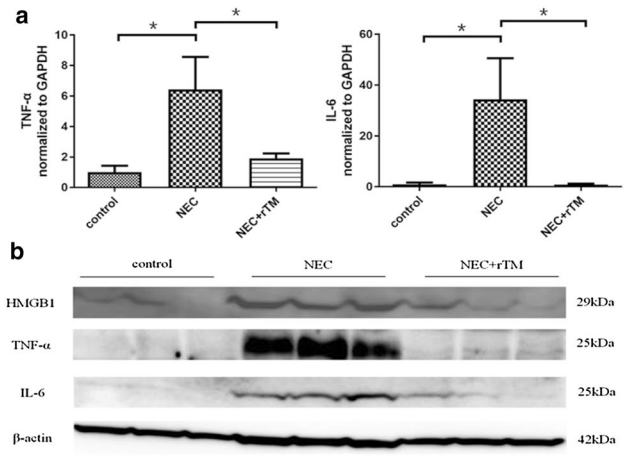

Fig. 5 **a** The mRNA expression of TNF-α and IL-6 of the ileum in the control, NEC and NEC + rTM groups. The asterisk indicates $P < 0.05$. **b** Representative Western blot bands with three animals/group

and hypoxia, intestinal perforation (Supplementary Fig. 2, the typical clinical manifestation in advanced NEC) was observed during the experiment, making it more convincing that this rat newborn NEC model can replicate the complex factors present in human NEC.

Inflammation is known to be an important contributing factor in the pathogenesis of NEC [9]. In the present study, the levels of terminal ileal intestinal TNF-α and IL-6 were increased in the NEC group compared with the control group, consistent with previous studies [13, 17]. We speculate that inhibiting the production of inflammatory cytokines reduces the severity and incidence of NEC. Previous studies have shown that rTM plays an important role in attenuating the inflammatory responses in settings such as endotoxemia, liver ischemia–reperfusion injury, glomerulonephritis and atherosclerosis [7, 8, 18, 19], which support the use of rTM in the treatment of NEC. Indeed, our study showed that the administration of rTM was able to reduce the ileal expression of TNF-α and IL-6 mRNA and protein to the same level as in the control group and to ameliorate the severity and incidence of NEC-like injury in our newborn rat model of NEC.

High-mobility-group box1 (HMGB1) is a nuclear architectural chromatin-binding protein released by necrotic or damaged cells and has been shown to be a lethal late-phase mediator [20, 21]. Extracellular HMGB1 triggers the activation of pro-inflammatory pathways [22]. rTM's lectin-like domain has been proven to play an important role in its anti-inflammatory effect through binding to HMGB1, behaving as an antagonist to HMGB1 [7, 8, 23]. In addition, there is evidence that thrombin binding to EGF-like domain degrades HMGB1 binding to lectin-like domain [20, 23]. One study showed that the pro-inflammatory protein HMGB1 is elevated in the ileal mucosa of formula-fed newborn rats exposed to hypoxia [24]. In our study, the expression of HMGB1 protein in the terminal ileum was also elevated in the NEC group, whereas this level in the NEC + rTM group was decreased to the same level as in the control group. Our data suggest that administered rTM probably prevents tissue damage by inducing the blockade of HMGB1 through both lectin-like domain and EGF-like domain, exerting its anti-inflammatory effect. Since EGF-like growth factor was proven to have the ability to reduce the incidence of NEC in several studies [15, 25]. rTM's EGF-like domain alone may also play a role in decreasing the NEC severity. In summary, the anti-inflammatory effect of rTM in this newborn rat NEC model is complicated, although the blockade of HMGB1 seems to be pivotal.

Another mechanism by which rTM prevents and decreases the severity of NEC is its anti-coagulant effect, which is related to the properties of activated protein C. rTM administered subcutaneously binds to thrombin at the inflammatory site. The complex of rTM and thrombin stops the coagulant cascade via the activation of protein C and changing thrombin's substrate specificity from pro-coagulant to anti-coagulant [5]. Impaired microcirculation is widely accepted to play an important role in the development of NEC lesions [26–28]. The anti-coagulant effect of rTM may help maintain the microcirculation of the intestine, resulting in a reduced incidence and reduced severity of NEC.

In conclusion, our study showed that the administration of rTM was able to reduce the severity and incidence of NEC and improve the survival rate, probably by inducing blockade of HMGB1 to produce an anti-inflammatory effect, thereby reducing the production of mucosal inflammatory cytokines. Further studies are needed to better clarify the mechanism by which rTM exerts its anti-inflammatory and anti-coagulant activities in the treatment of NEC.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest in association with this study.

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