Neuroprotection by Recombinant Thrombomodulin

Yuji Taoka, Kenji Okajima, Mitsuhiro Uchiba, Masayoshi Johno

From the Departments of Laboratory Medicine and Dermatology, Kumamoto University School of Medicine, Kumamoto, Japan

Key words

Thrombomodulin, spinal cord injury, activated protein C, tumor necrosis factor-α, leukocytes

Summary

We examined whether recombinant human soluble thrombomodulin (rhs-TM) reduces compression trauma-induced spinal cord injury through protein C activation in rats. Administration of rhs-TM, either before or after the induction of spinal cord injury (SCI), markedly reduced the resulting motor disturbances. However, neither rhs-TM pretreated with an anti-rhs-TM monoclonal antibody (MAb) F2H5, which inhibits thrombin binding to rhs-TM, nor those pretreated with MAb RSG12, which selectively inhibits protein C activation by rhs-TM, prevented the motor disturbances. Intramedullary hemorrhages, observed 24 h after trauma, were significantly reduced in animals given rhs-TM. The increase in the tissue levels of tumor necrosis factor-α (TNF-α), TNF-α mRNA expression, and the accumulation of leukocytes in the damaged segment of the spinal cord were significantly inhibited in animals receiving rhs-TM, but these effects were not observed following administration of rhs-TM pretreated with MAb RSG12 or MAb F2H5. Leukocytopenia and activated protein C all produced effects similar to those of rhs-TM.

These findings suggest that rhs-TM prevents compression trauma-induced SCI by inhibiting leukocyte accumulation by reducing the expression of TNF-α mRNA and such therapeutic effects of rhs-TM could be dependent on its protein C activation capacity. Findings further suggest that thrombomodulin can be implicated not only in the coagulation system but in regulation of the inflammatory response.

Introduction

Thrombomodulin (TM) is an endothelial cell membrane glycoprotein that forms a high affinity complex with thrombin (1). Thrombin bound to TM does not show an increase in clotting activity, but is capable of producing activated protein C (APC) in plasma (2). The APC thus formed exerts an anticoagulant effect by inactivating factors Va and VIIIa (3) and a profibrinolytic effect by inactivating plasminogen activator inhibitor-1 (4). APC has also been strongly implicated in the regulation of inflammatory responses involving various cytokines or activated leukocytes (5). Consistent with this hypothesis, we have demonstrated that APC prevents pulmonary vascular injury in rats given endotoxin by inhibiting production of TNF-α which potently activates neutrophils (6, 7). Recombinant human soluble-TM (rhs-TM) also prevents endotoxin-induced pulmonary vascular injury by inhibiting the activation of neutrophils through protein C activation (8, 9).

Spinal cord injury (SCI) is a serious condition which produces lifelong disabilities (10). Only limited therapeutic measures are currently available for its treatment (11). The pathophysiology of SCI includes a primary mechanical injury and a delayed secondary injury (12, 13). Therapeutic intervention in SCI should therefore be directed at reducing or alleviating this secondary injury process (11). Although the mechanisms involved in the secondary injury process are not fully understood, inflammatory responses leading to endothelial damage may be involved (14). We have recently demonstrated that tumor necrosis factor-α (TNF-α) plays an important role in compression trauma-induced SCI in rats by activating neutrophils (15, 16). We have recently reported that APC reduces the severity of compression trauma-induced SCI by inhibiting TNF-α production (16). These observations strongly suggest that rhs-TM may prevent compression trauma-induced SCI by inhibiting TNF-α production by the activation of protein C.

In the present study, we examined this possibility using a rat model of compression trauma-induced SCI. The result showed that rhs-TM prevented the SCI by inhibiting leukocyte accumulation through reducing the expression of TNF-α mRNA at the injured site. Furthermore, the therapeutic effects of rhs-TM could be dependent on its protein C activation capacity.

Materials and Methods

Materials

Recombinant human soluble-TM was kindly provided by Asahi Chemical Industry (Tokyo, Japan); the method used for preparation of rhs-TM has been described previously (17). Purified rhs-TM migrated as a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (17). Recombinant human soluble TM used in the present study does not contain chondroitin sulfate (personal communication from M. Mohri, Institute for Life Science Research, Asahi Chemical Industry, Shizuoka, Japan). Nitrogen mustard (NM) and purified myeloperoxidase (MPO) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents used were of analytical grade.

Preparation of Monoclonal Antibodies against rhs-TM

Anti-rhs-TM monoclonal antibodies (MAbs) F2H5, RSG12 and MF2 were produced by the hybridoma technique in mice using rhs-TM as the antigen (18). In brief, the hybridoma supernatants obtained from BALB/c mice immunized with rhs-TM were assayed for antibody production. The selected hybridoma clones were expanded by intraperitoneal injection of hybridomas into BALB/c mice pretreated with pristane (Wako Pure Chemical Industries, Osaka, Japan). The ascites fluid was collected 1-2 wk after the injection. The immunoglobulin G antibodies were affinity purified from mouse ascites under sterile conditions on protein A Sepharose CL-4B (Pharmacia, Uppsala, Sweden).

Treatment of rhs-TM with MAb F2H5, RSG12 and MF2

Recombinant human soluble-TM was incubated at 37°C for 30 min with an equimolar concentration of MAb F2H5, RSG12 and MF2 as previously
described. Aliquots of the solution were assayed for protein C activation capacity and fibrinogen clotting activity to determine the TM activity according to the methods as previously described (8, 9). Although MAb F2H5 inhibits thrombin binding to rhs-TM, neither MAbs R5G12 nor MF2 inhibit it (9). The protein C activation capacity of rhs-TM is almost completely inhibited by MAb R5G12, but is not inhibited by MAb MF2 (9). These activity experiments were repeated using the solutions that were administered to rats for testing (data not shown). These solutions comprised rhs-TM, various MAbs, and saline.

Preparation of Dansyl-glutamyl-glycyl-arginyl Chloromethyl Ketone-treated Factor Xa

Factor X was purified from human plasma and activated with Russell’s viper venom (19) to obtain activated factor X (factor Xa). Factor Xa was activated by incubation with a 20-fold molar excess of dansyl-glutamyl-glycyl-arginyl-chloromethyl ketone (DEGR, Calbiochem, San Diego, CA) for 30 min at 25°C, after which the mixture was subjected to extensive dialysis against a solution containing 20 mM Tris-HCl (pH 7.4) and 100 mM NaCl. DEGR-treated factor Xa (DEGR-Xa) has been shown to selectively inhibit thrombin generation by competing with intact factor Xa for prothrombinase complex formation (20).

Animal Model of Spinal Cord Injury

The protocol for this study was approved by the Kumamoto University Animal Care and Use Committee; care and handling of the animals were in accordance with the National Institutes of Health guidelines. Under pentobarbital anesthesia (45 mg/kg, ip) (Abbott Laboratories, North Chicago, IL), adult pathogen-free male Wistar rats (Nihon SLC, Hamamatsu, Japan), weighing 300-350 g, were subjected to laminectomy using a surgical airtime at the level of the 12th thoracic vertebra (T 12). SCI was induced by applying a 20-g weight extradurally to the spinal cord at T 12 for 20 min as previously described (15, 16, 21). This technique creates a model of SCI including motor disturbances of the lower extremities which persist for at least 8 weeks after compression (21, 22). Laminectomy alone was performed as a sham operation. Recombinant human soluble-TM (1 mg/kg) as intravenously administered to rats 30 min before (pretreatment group) or after (posttreatment group) the compression trauma. Recombinant human soluble-TM pretreated with MAbs, DEGR-Xa (10 mg/kg) and APC (100 µg/kg) were administered intravenously 30 min prior to trauma. The control and leukocytopenic animals received saline instead of rhs-TM or other drugs.

Grading of Motor Disturbance

The motor function of rats was assessed in a blind manner using the inclined-plane test (23) and foot print analysis (24). In the inclined-plane test, recovery from motor disturbance was assessed before, and again at 1, 7, 14, 21, 42 and 56 days after the compression. We recorded the maximum inclination of the plane on which the rats could maintain themselves for 5 sec without falling (15).

Foot print analysis was performed before and 3 weeks after the compression injury as previously described (16). The hindpaws were wetted, and the animals were made to walk on paper coated by bromophenol blue (Wako Pure Chemical Industries, Osaka, Japan) dissolved in acetone. The base of support was determined by measuring the distance between the central pads of the hindpaws. The stride lengths of the right and left hindpaws were measured in two consecutive prints.

Histologic Examination of the Spinal Cord

Rats in each group chosen randomly to be killed 24 h after compression SCI by transcardial perfusion with 10% formaldehyde in a phosphate-buffered solution. Approximately 1 cm of the spinal cord at T 12 was removed immediately and immersed overnight in the same solution. Transverse semiserial sections of 5 µm in thickness were prepared after embedding paraffin. These sections were stained with hematoxylin and eosin and assessed by a pathologist who had no knowledge of the treatment group represented.

Assay of MPO Activity

The extent of leukocyte infiltration was assessed by measurement of MPO activity using a method previously described (22). Within 1 hr of killing, segments approximately 1 cm in length were dissected from the T 12 region and placed in an ice-cold 0.9% NaCl bath. A 10% (w/v) tissue homogenate was suspended in 20 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide and sonicated for 30 sec. After centrifugation (4,500 × g at 4°C for 20 min), 0.1 mL of supernatant was added to 0.6 mL of 0.1 M phosphate buffer (pH 6.0) with 0.05% H2O2 containing 1.25 mg/mL of o-dianisidine. The assay was begun by adding 0.1 mL of sample and was stopped 5 min later adding 0.1 mL of NaN3 (1%). Absorbance at 460 nm was measured in a spectrophotometer (DU-54, Beckman, Irvine, CA). MPO activity was calculated using a standard curve prepared with purified MPO. Results are expressed as units (U) per gram of tissue.

Assay of TNF-α

The level of TNF-α in spinal cord tissue was determined according to the methods as previously described (16). Briefly, within 1 hr of killing, sections from the T 12 region measuring approximately 1 cm were dissected, removed, and placed in ice-cold 0.9% NaCl. A 20% (w/v) tissue homogenate was mixed with a 0.1 M phosphate buffer (pH 7.4), sonicated for 30 sec, and centrifuged at 4,500 × g for 20 min at 4°C. The concentration of TNF-α in the supernatant was determined using an enzyme-linked immunosorbent assay kit for rat TNF-α (Genzyme Corporation, Cambridge, MA). Results are expressed as pg TNF-α per gram of tissue.

Analysis of mRNA Levels of TNF-α and GAPDH by Reverse Transcription-PCR

Spinal cord tissues at T 12 were removed 1, 2, 3, and 4 h after compression injury or sham operation. During these procedures, the tissues were cooled by liquid nitrogen. Finally, specimens of about 1 cm in length were taken from T 12 region and placed immediately in liquid nitrogen again. Total RNA was extracted from the spinal cord tissue by the acid-guanidinium isothiocyanate-phenol-chloroform (AGPC) method (25). The amount of prepared total RNA was determined by Gene Quant II (Pharmacia, Uppsala, Sweden) and diluted

![Fig. 1](image_url) Temporal effect of rhs-TM on motor disturbances after the induction of SCI. SCI was induced by applying a 20-g weight for 20 min at T 12, the level of motor disturbances of the hindlimbs was evaluated after SCI using the inclined plane test. Recombinant human soluble-TM (1 mg/kg) and saline (as a control) were administered intravenously 30 min before compression SCI. Closed circles, Saline-treated animals; open circles, rhs-TM-treated animals. Mean ± SD of ten experiments. * p < .01 vs. traumatized animals

Downloaded from www.thrombosis-online.com on 2018-03-02 | ID: 1001066444 | IP: 54.70.40.11
For personal or educational use only. No other uses without permission. All rights reserved.
Reverse transcription-PCR was performed as described by Liu et al. (26) with some modification (27). In brief, single strand cDNA was prepared using Superscript Preamplification System (GIBCO BRL, Grand Island, NY). The cDNA was amplificated by PCR using primers for GAPDH and TNF-α/H9251. Exponentially amplifying PCR products were fractionated by 1.5 % agarose gel electrophoresis. The bands were visualized by exposing gel to 260 nm UV light and determined the fluorescence intensity by N III image software on Macintosh computer. The TNF-α/H9251 signals were normalized to the corresponding GAPDH signals from the same RNA, and expressed as TNF-α/H9251/GAPDH ratios.

Induction of Leukocytopenia by Nitrogen Mustard

Rats were made leukocytopenic by intravenous injection of nitrogen mustard (NM) (28). Nitrogen mustard (1.0 mg/kg) or 0.9% NaCl was administered intravenously to rats 2 days prior to induction of SCI as previously described (16, 21). The circulating leukocyte count on day 0 was 9,375 ± 1,365/L (n = 10) in controls and 3,350 ± 230/L in NM-treated rats (n = 10, p < .01). In differential leukocyte counts made on peripheral blood smears, the number of neutrophils counts on day 0 was 1,298 ± 428/L in controls and 733 ± 112/L in NM-treated animals (p < .01), and the number of monocytes was 539 ± 286/L in controls and 182 ± 38/L in NM-treated animals (p < .01).

Statistical Analysis

Data are presented as the mean ± SD. Circulating leukocyte counts were compared using Student’s t-test. Statistical comparisons of other data were done using the analysis of variance (ANOVA) and Scheffe’s post hoc test. A level of p < .05 was defined as statistically significant.

Results

Effects of rhs-TM and MAbs-treated rhs-TM on Motor Disturbances in Rats with SCI Induced by Compression Trauma

We have previously shown that motor disturbances as evaluated by the inclined plane test increase over the first 24 h following the

Fig. 2 Effects of rhs-TM, rhs-TM pretreated with MAbs (F2H5, RSG12 and MF2) on motor disturbances 24 h after the induction of SCI as determined by the inclined plane test. Motor disturbances of the hindlimbs were evaluated 24 h after the induction of SCI using the inclined plane test. Recombinant human soluble-TM (1 mg/kg) was administered intravenously 30 min before trauma (Pre) or 30 min after injury (Post). Recombinant human soluble-TM pretreated with MAbs (F2H5, RSG12 and MF2) was administered intravenously 30 min before injury. Mean ± SD of ten experiments. *, p < .01 vs. Trauma

Fig. 3 Effects of rhs-TM, rhs-TM pretreated with MAbs (F2H5, RSG12 and MF2) on motor disturbances 21 days after the induction of SCI as determined by the inclined plane test (A) and foot print analysis (B and C). Motor disturbances of the hindlimbs were evaluated 21 days after the induction of SCI as determined by the inclined plane test and foot print analysis. Concentrations of rhs-TM and rhs-TM pretreated with MAbs (F2H5, RSG12 and MF2) were as for Figure 2. Mean ± SD of ten experiments. *, p < .01 vs. Trauma

Compression trauma-induced SCI (15, 16, 21, 22). From 1 to 56 days after the induction of SCI, the slopes of the inclined plane were significantly higher in rats pretreated with rhs-TM than in controls (Fig. 1). Motor function had recovered to pretraumatic levels by 42 days following the insult in rats given rhs-TM pretraumatically, while they had not recovered to pretraumatic levels at 56 days after the insult in controls (Fig. 1). Although the angles of the inclined plane were significantly greater for rats pretreated with rhs-TM than for controls 24 h after the induction of SCI, rhs-TM pretreated with MAb F2H5 or MAb RSG12 did not prevent the motor disturbances (Fig. 2). MAb MF2-treated rhs-TM significantly reduced the motor dysfunction 24 h after the induction of SCI (Fig. 2). Whether evaluated by the inclined plane test (Fig. 3A) or by foot print analysis (Figs. 3B and 3C), the neurological
scores 21 days after the induction of SCI were also higher in rats given rhs-TM pretraumatically than in controls. Although rhs-TM pretreated with MAb MF2 reduced the motor disturbances 21 days after the induction of SCI, that pretreated with MAb F2H5 or MAb R5G12 did not have any effects (Fig. 3). The motor disturbances were also significantly reduced in animals given rhs-TM posttraumatically 24 h (Fig. 2) and 21 days after the induction of SCI (Fig. 3).

Histological Observations

Histological examination of the traumatized spinal cord 24 h after the induction of SCI showed the presence of intramedullary hemorrhages in control animals (Fig. 4). These hemorrhages were observed more often in the gray than the white matter. In contrast, there were markedly less hemorrhages in animals that had received rhs-TM pretraumatically (Fig. 4). These hemorrhages were not reduced in animals given rhs-TM pretreated with MAb F2H5 or MAb R5G12 24 h after the induction of SCI, but were significantly attenuated by rhs-TM pretreated with MAb MF2 (data not shown).

Effects of rhs-TM and MAbs-treated rhs-TM on Levels of TNF-α and MPO Activity in Injured Segment of Spinal Cord

The production of TNF-α in the traumatized spinal cord tissue was increased after the induction of SCI, peaking 4 h after the compression trauma (16). When we evaluated the effect of rhs-TM on tissue levels of TNF-α 4 h after the induction of SCI, rhs-TM, given pretraumatically, greatly inhibited the compression trauma-induced increases in TNF-α (Fig. 5A). However, rhs-TM pretreated with MAb F2H5 or MAb R5G12 did not prevent the trauma-induced increase in TNF-α levels. In contrast, MAb MF2-treated rhs-TM significantly inhibited these increases.

The accumulation of leukocytes in the traumatized spinal cord tissue was evaluated by measuring MPO activity 3 h after the induction of compression trauma (16, 22). The increase in MPO activity, observed in traumatized animals but not in sham-operated rats, was significantly inhibited in animals that received rhs-TM pretraumatically (Fig. 5B). Recombinant human soluble TM pretreated with MAb F2H5 or MAb R5G12 did not inhibit the accumulation of leukocytes, while that pretreated with MAb MF2 inhibited this increase (Fig. 5B).
These increases in both tissue levels of TNF-α and MPO activity were also significantly inhibited in animals given rhs-TM posttraumatically (Fig. 5).

**Discussion**

We demonstrated that rhs-TM significantly reduced the motor disturbances and spinal cord hemorrhages observed after compression trauma of spinal cord in rats. Recombinant human soluble-TM pretreated with MAb F2H5 or MAb RSG12, which selectively inhibits thrombin binding to rhs-TM and the protein C activation capacity of rhs-TM, respectively, did not reduce the severity of SCI, suggesting that rhs-TM might prevent the SCI by activating protein C at the site of thrombin generation.

Since thrombin increases vascular permeability in vivo (29, 30) and in vitro (31) and activates neutrophils which play an important role in acute SCI (15, 22), rhs-TM may have prevented the motor disturbances by simply binding thrombin. However, this seems unlikely, because MAb RSG12-treated rhs-TM could bind thrombin. Furthermore, DEGR-Xa, a selective inhibitor of thrombin generation, inhibited neither the accumulation of leukocytes in the injured spinal cord tissue nor the subsequent motor disturbances induced by spinal cord trauma (16). DEGR-Xa (10 mg/kg) prevented coagulation abnormalities to the same extent as rhs-TM (1 mg/kg) in rats given endotoxin (9).

Since thrombin bound to thrombomodulin activates not only protein C but thrombin-activatable fibrinolysis inhibitor (TAFI) to a carboxypeptidase B-like enzyme that inhibits fibrinolysis (32), TAFI might be involved in the therapeutic mechanism of rhs-TM in this animal model of SCI. However, this possibility is less likely, since MAb RSG12-treated rhs-TM that could bind thrombin did not reduce the SCI.

Taken together, these observations strongly suggested that rhs-TM reduced the severity of SCI through protein C activation. This theory is consistent with our previous observations that rhs-TM prevented endotoxin-induced pulmonary vascular injury by inhibiting TNF-α production through protein C activation, but not via its thrombin binding capacity (8, 9).

We have previously shown that in SCI the production of TNF-α in traumatized spinal cord is implicated in the secondary damage to tissue (16). Consistent with this observation is a report by Yakovlev and Faden (33) demonstrating that spinal cord impact in rats causes an elevation of TNF-α mRNA levels at the site of trauma 30 min after the injury; the level of the TNF-α mRNA was proportional to the severity of injury. Bartholdi and Schwab (34) have shown using in situ hybridization that expression of TNF-α mRNA can be detected shortly after spinal cord damage, but was downregulated 6 h following the trauma. However, these observations could not elucidate the causal relationship between TNF-α production and SCI. In the present study, we have demonstrated that both the increase in expression of TNF-α mRNA and the subsequent increases in tissue levels of TNF-α in the injured segment of spinal cord were significantly inhibited in leukocytopenic animals whose level of motor disturbances were markedly reduced. These findings indicate that an increase in the level of TNF-α at the site of injury may be a cause, rather than an effect, of the SCI induced by compression trauma.

TNF-α contributes to tissue injury by activating neutrophils (35), as well as by increasing the expression of endothelial leukocyte adhesion molecules, such as E-selectin (36). We have previously shown that the inhibition of neutrophil adhesion to the endothelial cell surface markedly reduces the severity of SCI induced by compression trauma (15). More recently, we have demonstrated that in rats inhibition of neutrophil adhesion by neutrophil elastase inhibitors reduces SCI induced by compression trauma (22). These observations suggest that
the interaction of activated neutrophils with the endothelial cell surface is important in the secondary tissue damage that occurs following compression trauma of spinal cord.

Although the precise mechanisms by which the transcriptional process of TNF-α synthesis is enhanced in the pathologic process of SCI are not fully known, ischemia/reperfusion mechanisms may at least partly explain the process (37, 38). Reactive oxygen intermediates which are produced after ischemia/reperfusion have been shown to activate nuclear factor kappa B, which leads to an increase in TNF-α production by enhancing the transcription of TNF-α mRNA in monocytes (39). Since leukocytopenia significantly inhibited both the increase in tissue levels of TNF-α mRNA and leukocyte accumulation at the damaged site, mononuclear production of the cytokine might play a role in the pathogenesis of SCI through activating neutrophils (35).

Increases in TNF-α levels, expression of TNF-α mRNA and in MPO activity in the injured section of spinal cord were significantly inhibited in animals that had received rhs-TM and APC. Recombinant human soluble TM pretreated with MAb R5G12 did not inhibit these increases, as shown in the present study. These observations strongly suggest that rhs-TM inhibited the accumulation of leukocytes by inhibiting expression of TNF-α mRNA in the injured section of spinal cord through the activation of protein C.

Although the precise mechanism(s) by which APC inhibits the production of TNF-α has not been fully known, our finding that neither DEGR-Xa, a selective inhibitor of thrombin generation, nor diisopropyl fluorophosphate-treated APC, inactivated APC, inhibited the increases in levels of TNF-α at the site of trauma (16), suggesting that APC might inhibit TNF-α production dependent on its serine proteinase activity, but not via its anticoagulant effect.

It is possible that the pretreatment of rhs-TM with any MAb adversely affects the ability of rhs-TM to reduce the severity of SCI. However, this seems unlikely because pretreatment of rhs-TM with Mab MF2, which affects neither thrombin binding nor protein C activation by rhs-TM, produced effects similar to those of rhs-TM.

We have previously reported that APC prevents the SCI in this animal model (16). Since rhs-TM prevented the SCI through protein C activation only at the site of thrombin generation (i.e. the site of tissue injury), rhs-TM would reduce the SCI more effectively and safely than APC.

Since the administration of rhs-TM after the induction of SCI was as effective in preventing the secondary effects of SCI as its administration prior to injury, rhs-TM may have a potential for clinical use not only in reduction of coagulation abnormalities but in alleviating the effects of traumatic compression injury to the spinal cord.

References

Received June 24, 1999 Accepted after resubmission November 5, 1999