Brief Report

DOI: 10.5582/bst.2016.01052

# Dual modulating functions of thrombomodulin in the alternative complement pathway

**Koichiro Tateishi, Mio Imaoka, Misao Matsushita***

*Department of Applied Biochemistry, Tokai University, Hiratsuka, Kanagawa, Japan.*

**Summary** **Thrombomodulin (TM) is a transmembrane protein expressed on vascular endothelial cells. TM has anticoagulant and anti-inflammatory properties. It has recently been reported that TM modulates complement, an immune effector system that destroys pathogens and is also involved in inflammation. TM was demonstrated to enhance the degradation of C3b into iC3b by factor I and factor H, indicating that its role is in negative regulation in the alternative pathway of the complement system. In this study, we examined the effects of recombinant human soluble TM protein composed of the extracellular domains (rTM) on the alternative pathway. The degradation of C3b into iC3b by factor I and factor H was enhanced by rTM as assessed by SDS-PAGE, confirming the previous observation. We also found that rTM enhances the cleavage of C3 into C3b as a result of activation of the alternative pathway. These results indicate that TM has both activating and inactivating functions in the alternative pathway.**

***Keywords:*** Thrombomodulin, complement, alternative pathway, modulation

## 1. Introduction

Thrombomodulin (TM) is a transmembrane protein expressed on vascular endothelial cells and is composed of a lectin-like domain, six tandemly repeated epidermal growth factor (EGF)-like domains, a Ser/Thr-rich domain, a transmembrane segment and a cytoplasmic tail (*1*). TM has an anticoagulant role by acting as a cofactor for thrombin, which activates protein C. Activated protein C in turn inactivates factor Va and factor VIIIa of the coagulation system. TM also exhibits anti-inflammatory properties.

The complement system is an immune effector mechanism, which is composed of many plasma and membrane proteins (*2*). It is activated in three ways, the classical, lectin, and alternative pathways. Once complement is activated, a chain reaction of cleavage and assembly of complement components in plasma occurs, leading to destruction of pathogens. C3a and C5a, fragments generated from C3 and C5 by their degradation during complement activation, have a role in inducing inflammation by acting as a chemotactic factor and by activating mast cells to release histamine. It has recently been reported that TM has the ability to modulate complement. The lectin domain of TM possesses a function that inhibits the activation of the classical and lectin pathways but not the alternative pathway (*3*). It also supports thrombin-mediated activation of procarboxypeptidase B (thrombin-activatable fibrinolysis inhibitor), which inactivates C3a and C5a. Delvaeye *et al*. reported that TM binds to C3b and factor H, and thereby enhances the degradation of C3b into iC3b by factor I and factor H, suggesting that it has a negative modulatory function in the alternative pathway (*4*).

Systemic inflammation results in activation of coagulation due to tissue factor-mediated thrombin generation. Disseminated intravascular coagulation (DIC) is an acquired syndrome involving systemic activation of blood coagulation, which leads to microvascular thrombosis in various organs. Based on its anticoagulant activity, TM has recently been used in clinical medicine for the treatment of DIC and also hemolytic uremic syndrome (HUS), a syndrome

Released online in J-STAGE as advance publication May 20, 2016.

**Address correspondence to:*
Dr. Misao Matsushita, Department of Applied Biochemistry, Tokai University, 4-1-1 Kitakaname, Hiratsuka, Kanagawa 259-1292, Japan.
E-mail: mmatsu@keyaki.cc.u-tokai.ac.jp

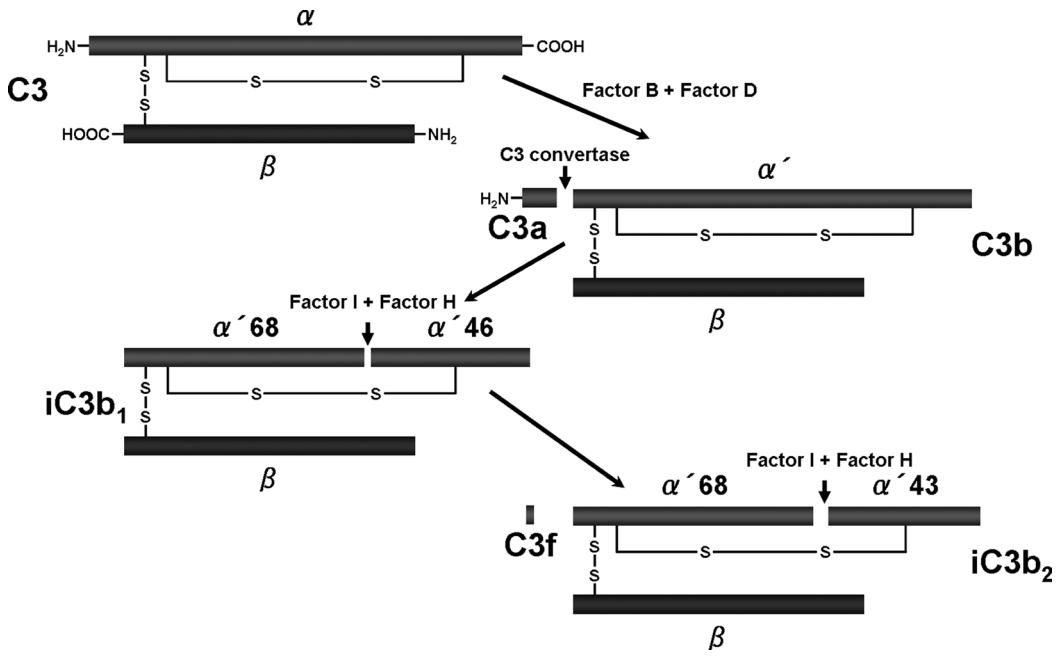


**Figure 1. The cleavage process of C3 in the alternative pathway.** The details of the cleavage process of C3 are described in the text.

characterized by microvascular thrombosis (*5-10*). Recent findings suggest that the complement-inhibitory activity of TM might also be one of the mechanisms by which it exhibits therapeutic effects against DIC and HUS.

In this study, we investigated the effects of recombinant human soluble TM protein composed of the extracellular domains (rTM) (*i.e.*, a lectin-like domain, EGF-like domains and a Ser/Thr-rich domain) on the alternative complement pathway and confirmed Delvaeye's report that TM enhances the degradation of C3b into iC3b by factor I and factor H. We also found that rTM enhances activation of the alternative pathway.

## 2. Materials and Methods

### 2.1. *Reagents*

Human complement components (C3, factor B, factor D, factor H, and factor I) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other general reagents were purchased from Wako Pure Chemicals (Osaka, Japan). rTM (thrombomodulin α) (*6*) was provided by Asahi Kasei Pharma (Tokyo, Japan).

### 2.2. *Assay for the effects of rTM on the activation of C3 by factors B and D*

C3 (50 μg/mL), factor B (8 μg/mL), factor D (1 μg/mL), and various concentrations of rTM in 20 mM Tris-HCl, pH 7.6, containing 150 mM NaCl, 2 mM $CaCl_2$, and 0.5 mM $MgCl_2$ (Tris buffer) were incubated at 37°C for 1 h. After incubation, the reaction mixture was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 8% gel) under reducing conditions. Protein bands were visualized by CBB staining and semi-quantified by a densitometer. The effect of rTM on the activation of C3 by factor B and factor D was evaluated by calculating the ratio of α to β.

### 2.3. *Assay for the effects of rTM on the degradation of C3b by factors H and I*

C3 (75 μg/mL), factor B (12 μg/mL), and factor D (1 μg/mL) in Tris buffer were incubated at 37°C for 1 h. After incubation, factor H (6 μg/mL), factor I (0.5 μg/mL), and various concentrations of rTM were added to the reaction mixture. After incubation at 37°C for 1 h, the reaction mixture was subjected to SDS-PAGE (8% gel) under reducing conditions. Protein bands were visualized by CBB staining and semi-quantified by a densitometer. The effect of rTM on the degradation of C3b by factors H and I was evaluated by calculating the ratio of α'68 to α', α'46 to α', and α'43 to α'.

### 2.4. *SDS-PAGE*

SDS-PAGE was performed using the Laemmli method (*11*). 2-Mercaptoethanol was
used as a reducing reagent.

## 3. Results and Discussion

When C3, factor B, and factor D are incubated in the fluid phase, the alternative pathway of complement is activated. C3 is composed of two polypeptide chains (α and β) linked by disulfide bonds (Figure 1).

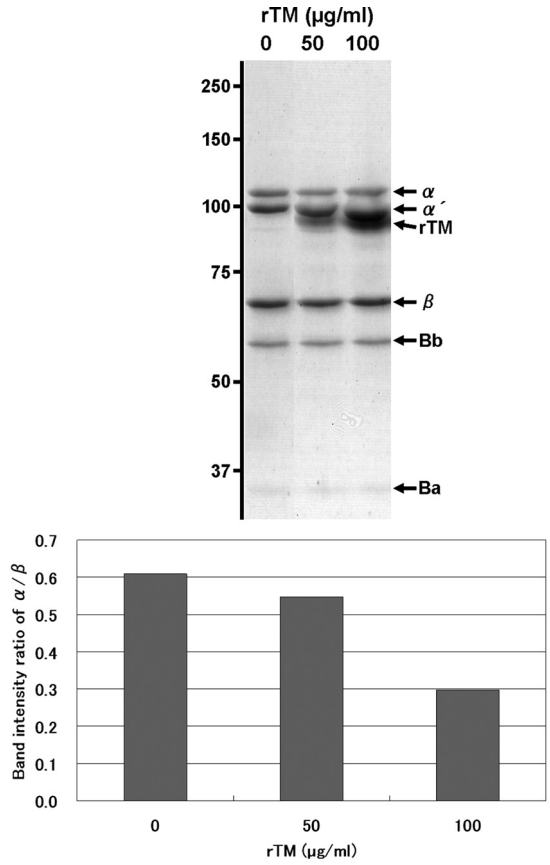


**Figure 2. SDS-PAGE analysis of the effects of rTM on the activation of C3 in the alternative pathway.** C3, factor B, and factor D were incubated in the absence or presence of various amounts of rTM. After incubation, the reaction mixtures were subjected to SDS-PAGE under reducing conditions followed by CBB staining. Positions of molecular weight markers (kDa) are indicated on the left (upper). Effects of rTM on the activation of the alternative pathway were evaluated by calculating the ratios of band intensity of α to β (lower).

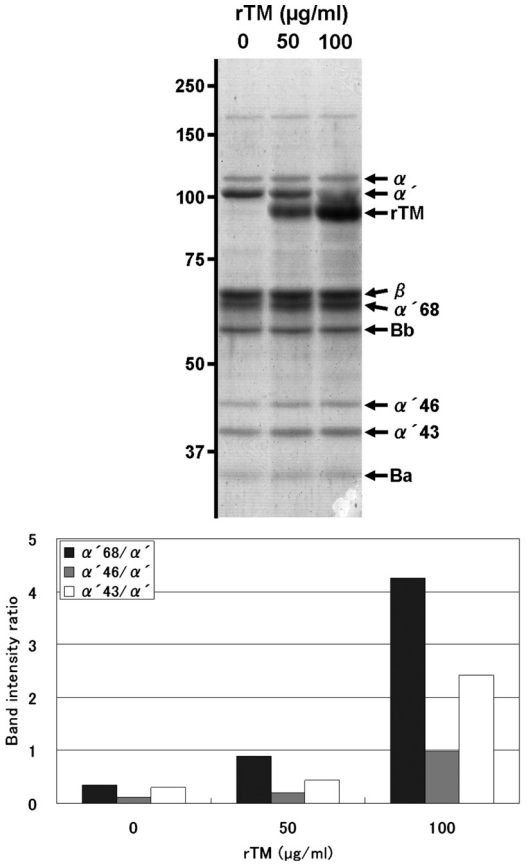


**Figure 3. SDS-PAGE analysis of the effects of rTM on the degradation of C3b by factor I and factor H.** C3b preparations which had been obtained by alternative pathway activation were incubated with factor I and factor H in the absence or presence of various amounts of rTM. After incubation, the reaction mixtures were subjected to SDS-PAGE under reducing conditions followed by CBB staining. Positions of molecular weight markers (kDa) are indicated on the left (upper). Effects of rTM on the degradation of C3b by factor I and factor H were evaluated by calculating the ratios of band intensity of α'68 to α', α'46 to α', and α'43 to α' (lower).

C3 has a thioester bond in the α chain. The thioester bond is partially hydrolyzed in the fluid phase and the hydrolyzed form of C3 is called C3($H_2O$). Factor B is a zymogen and binds to C3 ($H_2O$). The factor B molecule on C3($H_2O$) is cleaved by factor D to generate Bb. As a result, the bimolecular complex C3($H_2O$)Bb acts as the initial C3 convertase and is able to cleave C3 into C3a and C3b. C3b is composed of the α' chain and β chain. Factor B binds to C3b generated by the initial C3 convertase. Factor B on C3b is cleaved by factor D to generate Bb. The bimolecular complex C3bBb acts as the C3 convertase capable of cleaving C3 into C3a and C3b. Once C3b is generated, therefore, alternative pathway activation is amplified. Factor I is a serine protease that degrades C3b. Factor H acts as a co-factor for factor I-mediated cleavage of C3b. C3b is cleaved by factor I in association with factor H to generate $iC3b_1$ consisting of a 68 kDa-chain (α'68), a 46 kDa-chain (α'46) and β. $iC3b_1$ is further cleaved to generate $iC3b_2$ and C3f. $iC3b_2$ is composed of α'68, a 43 kDa-chain (α'43) and β. Unlike C3b, $iC3b_1$ and $iC3b_2$ are not able to activate the alternative pathway (*12,13*).

Under the condition in which C3 was partially cleaved to generate the α' chain by incubation of complement components of the alternative pathway (C3, factor B, and factor D), co-incubation of various concentrations of rTM with these complement components resulted in a decrease in the intensity of a band corresponding to the α chain (Figure 2, upper). Densitometric analysis shows that the ratio of band intensity of the α' chain to that of the β chain decreased in the presence of rTM in a dose-dependent manner (Figure 2, lower). These results indicate that rTM enhances the cleavage of C3 by factor B and factor D.

As described in Introduction, TM was reported to enhance the degradation of C3b by factor I and factor H. We, therefore, tried to confirm this modulating activity of TM. C3b was first generated by incubation of C3, factor B and factor D. C3b preparations generated in this way were then incubated with factor H and factor I in the absence or presence of various amounts of rTM. The reaction mixtures were analyzed by SDS-PAGE.

When C3, factor B and factor D were incubated, a band corresponding to the α' chain appeared, indicating a generation of C3b from C3 by alternative pathway activation. The intensity of a band corresponding to the α' chain decreased by co-incubation of rTM in a dose-dependent manner (Figure 3, upper). Densitometric analysis clearly demonstrates that all of the ratio of band intensities of α'68 to α', α'46 to α', and α'43 to α' increased with increasing concentration of rTM (Figure 3, lower). These results indicate that rTM enhances the degradation of C3b by factor I and factor H and are consistent with a report by Delvaeye *et al*. (*4*).

It has been demonstrated that compared with control Chinese hamster ovary (CHO) cells, TM expressed on CHO cells enhances the cleavage of C3b into iC3b in human serum after complement activation, indicating that TM negatively regulates the alternative pathway (*4*). The present study suggests that rTM modulates the alternative pathway by two distinct ways; rTM enhances the activation of C3 and enhances the inactivation of C3b as well. It is, therefore, possible that TM enhances the inactivation of C3b more efficiently than the activation of C3.

The mechanisms underlying the dual modulating effects of TM on the alternative pathway remain unknown at the moment. It has been shown that TM binds to C3b and its binding is increased in the presence of factor H (*4*). TM also binds to factor H alone (*4*). These facts suggest that TM bound to C3b enhances the factor I-mediated cleavage of C3b by recruiting factor H on C3b. Properdin is a positive regulatory component of the alternative pathway (*14*). It binds to C3b and enhances the activation of the alternative pathway by slowing the intrinsic decay of C3 convertase C3bBb. The modulating effect of TM on the activation of C3 in the alternative pathway seems to resemble that of propredin. It is, therefore, possible that TM enhances the activation of C3 in a similar fashion to properdin.

In conclusion, TM was found to modulate the alternative pathway by enhancing the activation of C3 and also by enhancing factor I-mediated inactivation of C3b.

**Acknowledgements**

We thank Dr. Goichi Honda for critical reading of the manuscript and Dr. Alister W. Dodds for English editing.

**References**

1. Okamoto T, Tanigami H, Suzuki K, Shimaoka M. Thrombomodulin: A bifunctional modulator of inflammation and coagulation in sepsis. Crit Care Res Pract. 2012; 2012:Article ID 614545.
2. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: A key system for immune surveillance and homeostasis. Nat Immunol. 2010; 11:785-797.
3. Van de Wouwer M, Plaisance S, De Vriese A, Waelkens E, Collen D, Persson J, Daha MR, Conway EM. The lectin-like domain of thrombomodulin interferes with complement activation and protects against arthritis. J Thromb Haemost. 2006; 4:1813-1824.
4. Delvaeye M, Noris M, De Vriese A, Esmon CT, Esmon NL, Ferrell G, Del-Favero J, Plaisance S, Claes B, Lambrechts D, Zoja C, Remuzzi G, Conway EM. Thrombomodulin mutations in atypical hemolytic–uremic syndrome. N Engl J Med. 2009; 361:345-357.
5. Noris M, Remuzzi G. Cardiovascular complications in atypical haemolytic uraemic syndrome. Nat Rev Nephrol. 2014; 10:174-180.
6. Mohri M, Sugimoto E, Sata M, Asano T. The inhibitory effect of recombinant human soluble thrombomodulin on initiation and extension of coagulation – a comparison with other anticoagulants. Thromb Haemost. 1999; 82:1687-1693.
7. Saito H, Maruyama I, Shimazaki S, Yamamoto Y, Aikawa N, Ohno R, Hirayama A, Matsuda T, Asakura H, Nakashima M, Aoki N. Efficacy and safety of recombinant human soluble thrombomodulin (ART-123) in disseminated intravascular coagulation: Results of a phase III, randomized, double-blind clinical trial. J Thromb Haemost. 2007; 5:31-41.
8. Yamakawa K, Fujimi S, Mohri T, Matsuda H, Nakamori Y, Hirose T, Tasaki O, Ogura H, Kuwagata Y, Hamasaki T, Shimazu, T. Treatment effects of recombinant human soluble thrombomodulin in patients with severe sepsis: A historical control study. Crit Care. 2011; 15:R123.
9. Aikawa N, Shimazaki S, Yamamoto Y, Saito H, Maruyama I, Ohno R, Hirayama A, Aoki Y, Aoki N. Thrombomodulin alfa in the treatment of infectious patients complicated by disseminated intravascular coagulation: Subanalysis from the phase 3 trial. Shock. 2011; 35:349-354.
10. Honda T, Ogata S, Mineo E, Nagamori Y, Nakamura S, Bando Y, Ishii M. A novel strategy for hemolytic uremic syndrome: Successful treatment with thrombomodulin α. Pediatrics. 2013; 131:e928-e933.
11. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature. 1970; 227:680-685.
12. Sahu A, Lambris JD. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. Immunol Rev. 2001; 180:35-48.
13. Harder MJ, Anliker M, Hochsmann B, Simmet T, Huber-Lang M, Schrezenmeier H, Ricklin D, Lambris JD, Barlow PN, Schmidt CQ. Comparative analysis of novel complement-targeted inhibitors, MiniFH, the natural regulators factor H and factor H-like protein 1 reveal functional determinants of complement regulation. J. Immunol. 2016; 196:866-876.
14. Lesher AM, Nilsson B, Song W-C. Properdin in complement activation and tissue injury. Mol Immunol. 2013; 56:191-198.

*(Received March 29, 2016; Revised May 8, 2016; Accepted May 15, 2016)*