Biochemical and Biophysical Research Communications 511 (2019) 312–317

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Anti-adhesive effects of human soluble thrombomodulin and its domains

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ARTICLE INFO

Article history:
Received 3 February 2019
Accepted 8 February 2019
Available online 15 February 2019

Keywords:
Thrombomodulin
β2 integrin
Vascular endothelial cell
Inflammation
Sepsis

ABSTRACT

We reported previously that leukocyte β2 integrins (LFA-1 and Mac-1) bind to the serine/threonine-rich domain of thrombomodulin (TM) expressed on vascular endothelial cells (VECs). Recombinant human soluble TM (rhsTM, TMD123) has been approved as a therapeutic drug for septic disseminated intravascular coagulation. However, the roles of TMD123 on the adhesion of leukocyte integrins to VECs remain unclear. In the current study, we have revealed that an integrin-dependent binding between human peripheral blood mononuclear cells (PBMCs) and VECs was inhibited by TMD123. Next, using mutant proteins composed of isolated TM extracellular domains, we examined the structural characteristics responsible for the anti-adhesion properties of TMD123. Namely, we investigated whether the effects of the binding of TM and leukocytes was inhibited by the administration of TMD123. In fact, we confirmed that TMD123, TMD1, and TMD3 inhibited the binding of PBMCs to the immobilized recombinant proteins TMD123 and TMD3. These results indicate that TMD123 inhibited the adhesion of leukocytes to endothelial cells via β2 integrins and endothelial TM. Moreover, since TMD1 might bind to leukocytes via other adhesion receptors than integrins, TMD1 and TMD3 appear to inhibit leukocyte binding to TM on VECs via different mechanisms. In summary, TMD123 (rhsTM), TMD1 or TMD3 is a promising treatment option for sepsis that attenuates integrin-dependent binding of leukocytes to VECs, and may inhibit the undesirable adhesion and migration of leukocytes to VECs in sepsis.

1. Introduction

In the early stage of sepsis, activated leukocytes accumulate not only at the site of inflammation, but also at distant sites. The interactions between leukocytes and vascular endothelial cells (VECs) promote excessive inflammation and trigger dysfunction in multiple organs [1]. Interestingly, organ damage can be alleviated by inhibiting the adhesion between leukocytes and the endothelium [2–4]. Thus, inhibition of excessive adhesion and migration of leukocytes to VECs presents an important treatment option for sepsis. Recent studies have reported the use of recombinant human soluble thrombomodulin (rhsTM, TMD123) to reduce the degree of acute respiratory distress syndrome (ARDS) in patients with severe sepsis [5]. ARDS is caused by excess neutrophil adhesion to VECs and migration into the lung alveoli. In addition, administration of TMD123 in a rat model of lipopolysaccharide-induced sepsis suppressed the adhesion of leukocytes to the vascular endothelium of the mesenteric microcirculation [6]. Moreover, TMD123 administration reduced the accumulation of macrophages and neutrophils in a mouse model of fatty liver disease [7]. These results suggest that administration of TMD123 attenuates the adhesion of leukocytes to VECs in inflammation, and may inhibit excessive

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https://doi.org/10.1016/j.bbrc.2019.02.041
0006-291X/

accumulation of white blood cells in subsequent stages of organ dysfunction. However, whether or not administration of TMD123 or each of its domains (TMD1, TMD2 and TMD3) with varied function affects the adhesion between leukocyte integrins and the corresponding ligands expressed on VECs remains unclear.

Thrombomodulin (TM) is a cell-surface transmembrane glycoprotein consisting of 557 amino acids that is expressed on the surface of VECs. The structure of TM consists of five domains: an N-terminal lectin-like domain (D1), an epidermal growth factor (EGF)-like domain with six cysteine residues (D2), a serine/threonine-rich domain with sugar chains (D3), a transmembrane domain (D4), and a cytoplasmic domain (D5). TMD123 (rhsTM) consists of the extracellular domains D1, D2, and D3 of TM (Fig. 1). It has become clear that TM on VECs is not only an important molecule in the anticoagulation system [8], but also acts as a cell adhesion molecule. For example, exogenous administration of TMD1 inhibits the binding of human umbilical vein endothelial cells (HUVECs) to leukocytes by binding to the Lewis Y antigen expressed on the surface of HUVECs [9]. Furthermore, TMD123 was identified as a novel ligand for the leukocyte β2 integrins LFA-1 and Mac-1 [10]. Therefore, we hypothesized that exogenous administration of TMD123 will inhibit the adhesion between leukocyte β2 integrins and TMD123 on VECs. Furthermore, we analyzed the structural characteristics by constructing mutant proteins composed of isolated TM extracellular domains such as TMD1, TMD2, and TMD3, and identified which domains depend on the anti-adhesion properties of TMD123.

In the current study, we found that the binding of Peripheral Blood Mononuclear Cells (PBMCs) or human promyelocytic leukemia (HL-60) cells to HUVECs was suppressed by exogenous administration of TMD123. In addition, we immobilized the β2 integrin novel ligands TMD123 and TMD3, and identified that the binding of PBMCs to TMD123 or TMD3 can be inhibited by soluble TMD123, TMD1, and TMD3. Our results show that TMD123 (rhsTM), which is recognized as a drug for sepsis, inhibits leukocyte integrin-mediated adhesion to VECs and might be involved in the inhibition of excessive leukocyte adhesion and migration to sites of inflammation.

2. Materials and methods

2.1. Construction of recombinant human TM domains 1, 2, and 3

A TMD123 [11] and the mutant proteins (TMD1, TMD2, TMD3) were provided by Asahi Kasei Pharma Corporation (Tokyo, Japan) (Fig. 1). In addition, to facilitate immobilization of the TM protein and maintain the physiological orientation of TM, we used TMD123-Fc (human immunoglobulin G1 Fc fragment) and TMD3-Fc as previously described [10]. A DNA fragment containing TMD123 was polymerase chain reaction (PCR)-amplified from a pSV2TMJ2 vector that contained the entire sequence of human TM [12] using KOD FX Neo reagent (Toyobo Co., Ltd., Osaka, Japan) with a forward primer containing an engineered *Hind*III restriction enzyme site (5′-CTA GCG TTT AAA CTT AAG CTT AGC CAC CAT GCT TGG GGT CCT GGT-3′) and a reverse primer with an engineered *Bam*HI site (5′-TGT GTG AGT TTT GTC GGA TCC CGA ATG CAC GAG CCC CAC-3′). The TM123 fragment was digested with the endonucleases *Hind*III and *Bam*HI, and sub-cloned into the *Hind* III/*Bam*HI restriction site in frame to the human immunoglobulin (Ig) G1 Fc fragment of the pcDNA3.1 (+) vector.

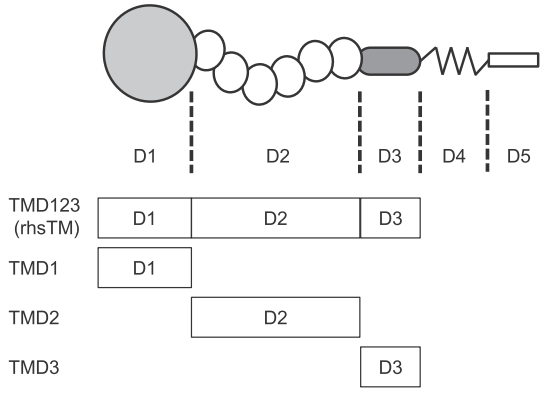

Fig. 1. Illustration of the structure of thrombomodulin. Schematic representations of the structure of wild-type and domain-deleted mutant proteins are shown. A soluble form of TM (rhsTM, TMD123) consists of the extracellular domains of thrombomodulin. D1 is a lectin-like domain of TM. D2 is an EGF-like domain with six cysteine residues. D3 is a serine/threonine-rich domain. D4 is a transmembrane domain. D5 is a cytoplasmic domain.

Domain-deleted mutants of soluble TM-Fc fusion proteins (i.e., TMD3-Fc that lacks D1 and D2) were generated by inverse PCR using a KOD Plus Mutagenesis Kit (Toyobo Co., Ltd.) with pcDNA3.1(+) containing the TMD123-Fc plasmid as a template. In addition to the universal outer primers, muTM-1 (5′-GAC TCC G GC AAG GTG GAC GGT GGC G-3′) and muTM-2 (5′-GCA CAT TAG CTG TAA GCC GAG GG-3′) were used to delete D1 and D2 of TM. The expression and purification of TMs and TMD-Fc fusion proteins were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue (CBB). To detect the TMD123-Fc protein bands, Western blot analysis was performed using polyclonal rabbit anti-TM antibody (Ab) (Sigma-Aldrich Japan K.K., Tokyo, Japan) and horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) Ab (Bio-Rad Laboratories, K.K., Tokyo, Japan). The Fc portion was detected using a peroxidase-conjugated goat anti-human IgG (Fc specific) Ab (Sigma-Aldrich Japan K.K.). Each blot was developed using an enhanced chemiluminescence kit (Nacalai Tesque, Inc.) and analyzed with an ImageQuant LAS 4000 mini digital imaging system (GE Healthcare Japan Corporation, Tokyo, Japan).

2.2. Preparation of PBMCs

PBMCs were isolated from sodium citrate-treated blood samples of healthy volunteers as described previously [13]. All blood donors submitted informed consent before blood collection. Blood samples were subjected to Percoll Plus (GE Healthcare Japan Corporation) density gradient centrifugation at 1000×*g* for 15 min. PBMCs were suspended in phosphate-buffered saline (PBS). The Institutional Review Board of Mie University Hospital approved the study protocol (approval no. 3026).

2.3. Cell adhesion assay for interactions between leukocytes and HUVECs

A cell adhesion assay was performed using 96-well V-bottom plates. Briefly, HUVECs (2×10^5 cells/mL) were included overnight in a 100-μL aliquot of endothelial cell growth medium (EGM-2; Lonza Cologne GmbH, Cologne, Germany). After the cells in the V-bottom well reached confluency, a 100-μL aliquot of PBS containing 2×10^5 2′, 7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF)-labeled PBMCs or HL-60 cells, either in the presence or absence of 1 mM $MgCl_2$ and 1 mM $CaCl_2$, was dispensed in each well. In some experiments, 2–50 μg/mL of blocking monoclonal Abs (mAbs) against integrins or control IgG were added and the plates were incubated at room temperature for 5 min, and then centrifuged at 1000 rpm for 5 min using a swinging bucket rotor (EX-125; Tomy Seiko Co., Ltd., Osaka, Japan). Accumulated non-adhered cells at the nadir of the V-bottom wells were detected

using a 2030 ARVO X-2 Multilabel Reader (PerkinElmer Japan Co. Ltd., Kanagawa, Japan). After centrifugation, crystal violet staining confirmed that the HUVECs were bound to the sides of the V-bottom wells.

2.4. Cell adhesion assay for interactions between PBMCs and integrin ligands

A cell adhesion assay using 96-well V-bottom plates was performed as described previously [14]. Briefly, a 50-μL aliquot of PBS containing 0.25 μM wild-type TMD123-Fc, TMD3-Fc fusion protein, or control human IgG1 Fc recombinant protein (Millipore-Japan) was dispensed in each well. Plates were incubated at 4 °C overnight and blocked with PBS containing 2% bovine serum albumin for 2 h at 37 °C. A 100-μL aliquot of PBS containing 2×10^5 BCECF-labeled PBMCs, either in the presence of 1 mM $MgCl_2$ and 1 mM $CaCl_2$ or 2 mM ethylenediaminetetraacetic acid, was dispensed in each well. In some experiments, 2–50 μg/mL of blocking mAbs to integrins or control IgG were added, and the plates were incubated at 37 °C for 5 min. Then, the plates were centrifuged at 1000 rpm for 5 min using a swinging bucket rotor.

2.5. Abs against integrins

The anti-integrin β2 mAb clone TS1/18 was purchased from Thermo Fisher Scientific (Waltham, MA, USA) and the mouse IgG1 isotype control Ab clone MOPC-21 was obtained from Sigma-Aldrich Japan K.K.

2.6. Statistical analyses

Statistical analyses were performed using SPSS software v.18.0 (SPSS, Inc., Chicago, IL, USA). Results are presented as the mean ± standard deviation. The Wilcoxon and Mann-Whitney tests were used for within-group comparisons and the cell-cell adhesion assay at each protein concentration (e.g., 10 μg/mL of isotype control vs. 10 μg/mL of TMD123). A probability (p) value of <0.05 was considered statistically significant.

3. Results

3.1. TMD123 inhibited the adhesion of leukocytes to endothelial cells

To confirm that TMD123 inhibits adhesion between leukocytes and VECs, we investigated whether the binding of PBMCs or HL-60 cells to HUVECs was inhibited by administration of soluble TMD123. PBMCs and HL-60 cell integrins were activated by Mg^{2+} and Ca^{2+} ions to mimic inflammatory conditions [15]. β2 integrin Ab or TMD123 were pre-incubated with leukocytes and gently placed into V-bottom wells to determine the presence or absence of cell adhesion in response to physiological shear stress. We found that TMD123 attenuated the binding of PBMCs to HUVECs (Fig. 2a and c) with a decreasing tendency in a concentration-dependent manner. In addition, TMD123 also attenuated adhesion of HL-60 cells (Fig. 2b and d). Based on these findings, TMD123 seemed to inhibit the adhesion of leukocytes to VECs, suggesting an anti-inflammatory effect of TM on leukocytes.

3.2. Domain 1 and domain 3 of TMD123 inhibit the binding of PBMCs to TMD123-Fc

Many integrin ligands are expressed on VECs. We previously reported that TMD3 is one of the integrin ligands present on the vascular endothelium [10]. To investigate the contribution of the binding of leukocytes to TM expressed on VECs, a simple experimental system was utilized to evaluate the degree of binding [16]. TMD123-Fc was immobilized in V-bottom wells to determine whether exogenous administration of TMD123, TMD1, TMD2, or TMD3 inhibited the binding of PBMCs to TMD123-Fc. As Fig. 3a shows, TMD123, TMD1, and TMD3 inhibited binding in a concentration-dependent manner, indicating that D1 and D3 play important roles in the binding of TM and PBMCs. We suggest that TMD3 binds to leukocyte β2 integrin, and that this interaction inhibits the binding of TM and leukocytes. Furthermore, we speculate that TMD1 inhibits the adhesion of leukocytes and TM by binding to already known antigen on the leukocyte surface (such as fibronectin) (Fig. 4).

3.3. D1 and D3 of TM inhibited the binding of PBMCs to TMD3-Fc

The β2 integrin was found to bind to D3 of TMD123 in an integrin-dependent manner [10]. As Fig. 3a shows, TMD1 and TMD3 are involved in cell adhesion. Therefore, we determined if TMD3 is immobilized and whether binding to PBMCs is inhibited by TMD1 or TMD3. The results showed that integrin-dependent binding of PBMCs and TMD3 was inhibited by TMD1 and TMD3 (Fig. 3b). Interestingly, the binding of TMD3 and PBMCs was also inhibited at low concentrations (0.2 μM) of TMD1 (Fig. 3b). Thus, TMD3 and TMD1 may be involved in the binding inhibition of leukocytes and VECs by different mechanisms. This led us to speculate that TMD3 has an inhibitory effect on β2 integrins, and that the lectin-like domain, TMD1, binds to the sugar chain of TMD3 and inhibits the binding between β2 integrin and TMD3 (Fig. 4).

4. Discussion

The results of the current study showed that the adhesion between leukocytes and HUVECs was suppressed by exogenous administration of TMD123 in a concentration-dependent manner (Fig. 2). Furthermore, TMD123 and TMD3, which are known as β2 integrin ligands, were immobilized, suggesting that the binding between these integrin ligand proteins and PBMCs can be inhibited by TMD123, TMD1, or TMD3. This result suggests that TMD123 (rhsTM) is useful as a therapeutic agent for leukocyte adhesion and migration to the vascular endothelium at sites of inflammation. In fact, in some animal experiments, rhsTM inhibited the accumulation of leukocytes to damaged organs [6,7]. Our findings elucidate not only a fundamental mechanism underlying the results of these animal experiments, but also a novel mechanism of TM as a promising therapeutic agent for the treatment of sepsis.

The results of our previous study demonstrated that the integrins LFA-1 and Mac-1 bind to TMD3 [10]. Therefore, one would expect the adhesion between PBMCs and TMD123-Fc to be suppressed by exogenous administration of TMD3 (Fig. 3a). Interestingly, the binding of leukocytes to TMD123 or TMD3 was suppressed with TMD1 (Fig. 3), suggesting that exogenous administration of the lectin-like domain, TMD1, binds to the sugar chain of TMD3 and inhibits binding between β2 integrin and TMD3. Research on protein - carbohydrate interactions has been increasing in recent years. The three-dimensional structure is already well known, as the interactions between sugar and protein are diverse [17]. In particular, the interaction between lectin and sugar is extremely important. Regarding oral drug delivery, lectin is considered to be a promising bio-adhesive agent, one which binds to the carbohydrate chain of intestinal mucosa [18]. TM possesses both the lectin-like domain (TMD1) and a serine-threonine rich domain with a sugar chain (TMD3). Therefore, there exists the possibility that D1 and D3 have mutual influence. In fact, it was not previously well known what kind of TM formation is shed from the

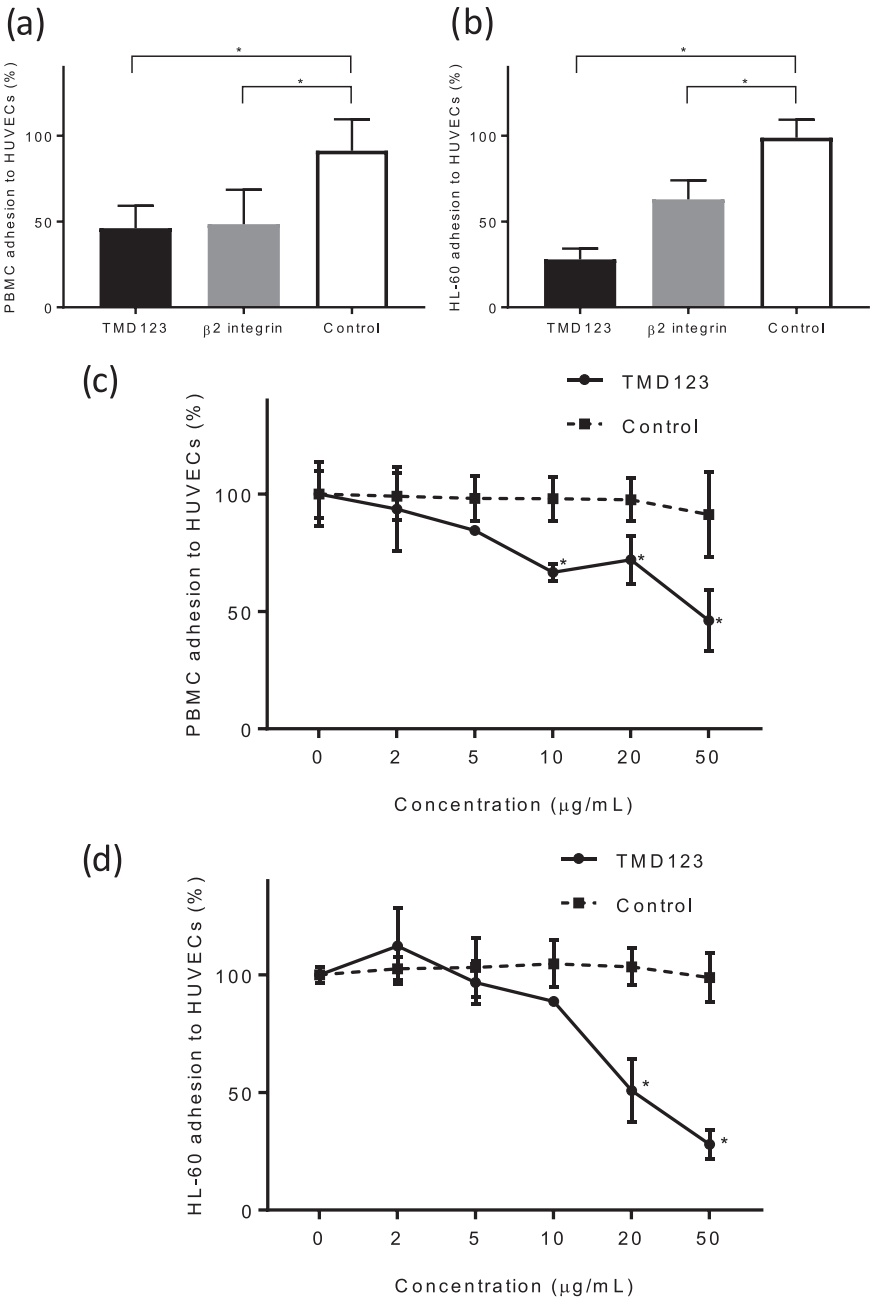

Fig. 2. The binding of leukocytes and HUVECs. (a) The binding of HUVECs and PBMCs is suppressed by 50 μg/mL TMD123 (rhsTM). (b) The binding of HUVECs and neutrophils is suppressed by 50 μg/mL TMD123 (rhsTM). The binding of PBMCs or HL-60 cells to HUVECs was studied in the presence of TMD123 and anti-β2 integrin mAb (TS1/18), or control (isotype control IgG). PBMCs or HL-60 cells were stimulated with Ca^{2+} and Mg^{2+} ions. (c) PBMC adhesion to HUVECs decreased in a TMD123 concentration manner. (d) HL60 adhesion to HUVEC decreased in a TMD123 concentration-dependent manner. Data are expressed as the mean ± SD. $^{*}p < 0.05$ vs. control (the isotype control) at each concentration.

vascular endothelium during inflammation. In the current study, our results suggest that TMD1 and TMD3 combine with each other to form a dimer or a complex in the blood.

Recently, the Sepsis Coagulopathy Asahi Recombinant LE Thrombomodulin (SCARLET) trial, the latest multinational multicenter phase III randomized controlled trial, was completed [11]. Unfortunately, the efficacy of TMD123 for sepsis was not demonstrated. As TMD123 has several functional domains within one molecule, its effects were difficult to verify. Thus, we focused on the function of each domain, created soluble proteins for each domain, and analyzed the influence of each domain on cell adhesion ability. In this way, we found that TMD1 and TMD3, among the extracellular domains of TM, have the ability to inhibit the adhesion between leukocytes and TMD123 on VECs. Taking these results into account, we will consider examining the efficacy of TMD1 or TMD3, instead of TMD 123, as well as all extracellular domains for sepsis treatment.

Several studies have investigated the binding of VECs by exogenous administration of rhsTM (TMD123) with the use of rat and mouse models [6,7]. However, since the homology of TM among rats, mice and humans is not high (~60%; aligned scores: mouse, 67%; rat, 64%), it was extremely difficult to establish an *in vivo* experimental system to evaluate the function of rhsTM. To the best of our knowledge, the present study is the first to demonstrate that integrin-dependent binding of human leukocytes and HUVECs or TM was inhibited by human TM.

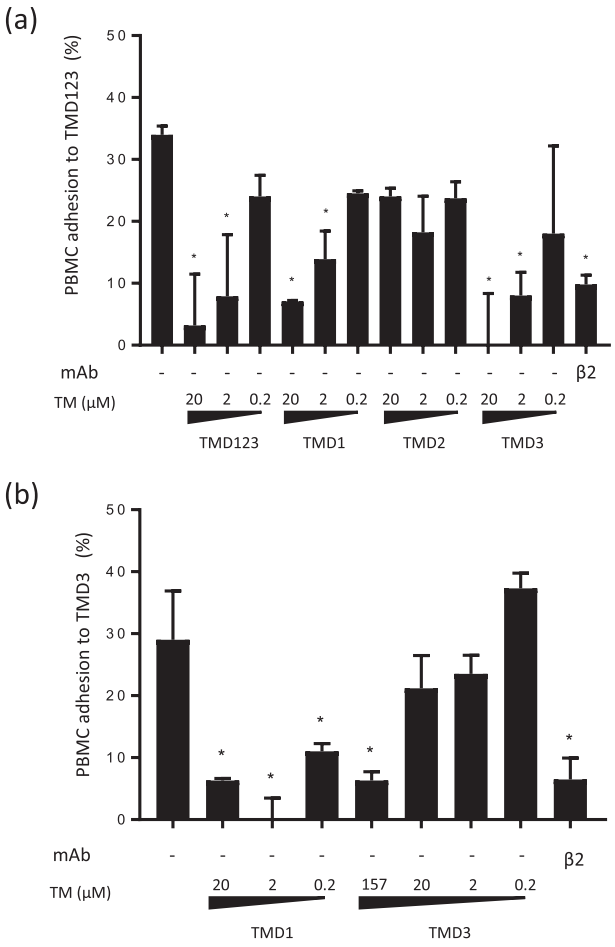

Fig. 3. The binding of leukocytes and thrombomodulin. (a) Binding of PBMCs to TMD123-Fc is suppressed by TMD123 (rhsTM), TMD1, and TMD3. (b) Binding of PBMCs to TMD3-Fc is suppressed by TMD1 as well as by TMD3. Binding of PBMCs to TMD123-Fc, TMD3-Fc, and control (Fc) was examined in the presence of TMD123 (rhsTM), TMD1, TMD2, TMD3, or β2 integrin-blocking mAb (TS1/18) at several concentrations of reagent. PBMCs were stimulated with Ca^{2+} and Mg^{2+} ions. Data show the background binding between leukocytes and control (Fc) was subtracted, and expressed as the mean ± SD. $^{*}p < 0.05$ vs. leukocyte binding to TMD123-Fc or TMD3-Fc.

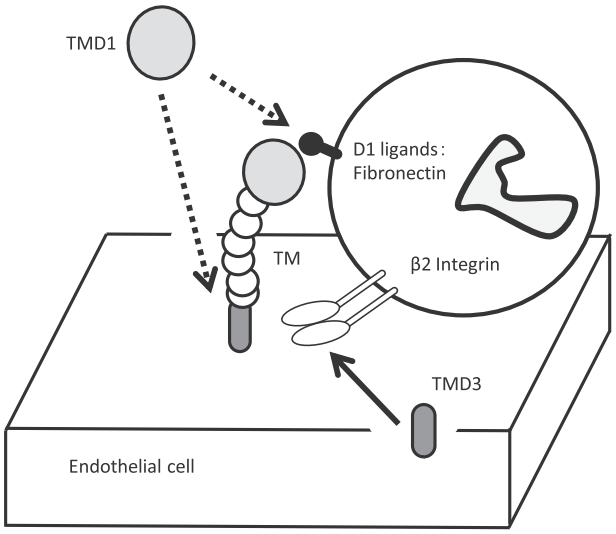

Fig. 4. Summary of results. Leukocytes and TM expressed on the VECs are shown. Activated leukocyte integrins bind to D3 of TM, which can be inhibited by exogenous administration of TMD1 or TMD3. TMD3 has an inhibitory effect by binding to integrins, while TMD1 might inhibit the binding between leukocytes and TM through adhesion to the D1 ligands (fibronectin or other molecules) of leukocytes, or adhesion to the D3 with sugar chain of TM.

A previous study reported that TMD1 bound to the Lewis Y antigen [9], which is expressed by intercellular adhesion molecule 2 (ICAM-2), a ligand of the β2 integrin [19,20]. Therefore, it is possible that TMD1 binds to ICAM-2 on HUVECs and this interaction inhibits the adhesion between leukocytes and VECs, as we demonstrated by administering leukocytes pretreated with TMD123 into V-bottom wells coated with HUVECs. In fact, the binding of leukocytes to HUVECs was strongly suppressed by the administration of TMD123 (>10 μg/mL) (Fig. 2c and d).

Interestingly, our results show that, compared to PBMCs, HL-60 cells were more strongly suppressed by TMD123 (>20 μg/mL) (Fig. 2d). A previous study showed that neutrophils more strongly adhere to the Lewis Y antigen [9]. Exogenous administration of TMD123 may be more heavily involved in the adhesion of neutrophils, rather than PBMCs (lymphocytes or monocytes), to VECs. Therefore, TM may have a marked effect of suppressing neutrophils that play an important role in inflammation.

There were several limitations to this study that should be addressed. First, this was an *in vitro* study, rather than *in vivo*. With the *in vivo* model, TM expressed on VECs binds to and reduces the pro-inflammatory effect of thrombin. As thrombin is thought to be a powerful stimulus to enhance the inflammatory response, the blood concentration of thrombin is increased and recruitment of circulating monocytes is promoted by increasing the expression levels of the integrin ligands ICAM-1 and VCAM-1 during inflammation [21]. Therefore, exogenous administration of TMD123 may further attenuate the integrin-mediated binding of leukocytes and integrin ligands, such as ICAM-1 and VCAM-1, on VECs in thrombin-presenting situations or *in vivo*. In our experimental system, only HUVECs and white blood cells were present, but not thrombin. Therefore, there is a possibility of underestimating the effect of TMD123 (rhsTM).

The next concern is that we did not mention the possibility of TMD1 binding to leukocytes through some already known binding sites (Fig. 4). Previous studies have shown that TMD1 binds to fibronectin [22], high mobility group box 1 protein [23], and the Lewis Y antigen [9,24]. Therefore, administration of TMD123 may affect TMD1 binding to leukocytes at binding sites other than integrin (Fig. 4). Future *in vivo* studies are needed that will utilize adhesion molecules, other than integrins, related to the binding of leukocytes and VECs.

In summary, adhesion between human leukocytes and endothelial cells was suppressed in a concentration-dependent manner by exogenous administration of TMD123. In addition, the binding between leukocyte integrins and TMD123-Fc or TMD3-Fc was suppressed by exogenous administration of TMD3. Furthermore, since binding was also attenuated by exogenous administration of TMD1, a novel mechanism may contribute to TMD1's ability to inhibit adhesion between TMD3 and β2 integrins. Thus, leukocytes migrate to the site of inflammation through integrin and TM, suggesting that exogenous administration of TMD1 or TMD3 is a promising option for the treatment of multiple organ dysfunction in inflammatory diseases such as sepsis.

Funding

This work was supported by JSPS KAKENHI (Grants-in-Aid for Scientific Research) [grant numbers 18K08917 and 18H02622] and a research grant from Asahi Kasei Pharma Corporation, Japan.

Acknowledgements

We gratefully acknowledge Dr. Goichi Honda (Medical Affairs Department, Asahi Kasei Pharma Corporation, Japan) for his technical assistance.

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