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Full Length Article

Thrombomodulin enhances complement regulation through strong affinity interactions with factor H and C3b-Factor H complex

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ABSTRACT

Introduction: Coagulation and complement systems are simultaneously activated at sites of tissue injury, leading to thrombin generation and opsonisation with C3b. Thrombomodulin (TM) is a cell-bound regulator of thrombin activation, but can also enhance the regulatory activity of complement factor H (FH), thus accelerating the degradation of C3b into inactive iC3b.
Objectives: This study sought to determine the biophysical interaction affinities of two recombinant TM analogs with thrombin, FH and C3b in order to analyze their ability to regulate serum complement activity.
Methods: Surface plasmon resonance (SPR) analysis was used to determine binding affinities of TM analogs with FH and C3b, and compared to thrombin as positive control. The capacity of the two recombinant TM analogs to regulate complement in serum was tested in standard complement hemolytic activity assays.
Results: SPR analysis showed that both TM analogs bind FH and C3b-Factor H with nanomolar and C3b with micromolar affinity; binding affinity for its natural ligand thrombin was several fold higher than for FH. At a physiological relevant concentration, TM inhibits complement hemolytic activity in serum via FH dependent and independent mechanisms.
Conclusions: TM exhibits significant binding affinity for complement protein FH and C3b-FH complex and its soluble form is capable at physiologically relevant concentrations of inhibiting complement activation in serum.

1. Introduction

Thrombomodulin (TM; CD141) [1,2] is an integral membrane protein while heterogeneous soluble fragments of TM (sTM) are present in blood [3] and urine [4] and act as an effective marker of endothelial cell injury. Endotoxin and inflammatory cytokines decrease membrane-bound TM expression on the endothelium and increase soluble TM in blood [5,6].

TM's role in haemostasis regulation is primarily to down-regulate thrombin generation [7]. The TM-thrombin complex converts protein C to activated protein C (APC), which in turn mediates inactivation of factors Va and VIIIa in the presence of protein S [8]. TM-thrombin complex also activates thrombin-activatable fibrinolysis inhibitor (TAFI) and hence down-regulates fibrinolysis [9]. Interestingly, TAFI inactivates C3a and C5a [10] revealing a central role for this molecule in the regulation and interface between complement and coagulation.

Complement and coagulation are both innate blood defense systems, evolutionary linked with functional similarities and many shared structural motifs. Crosstalk events between the complement and coagulation pathways have been investigated, but are as yet not comprehensively characterized [11,12].

Complement protects against pathogens – primarily bacterial infection – by rapid activation of serine proteases and generation of active products. Cell killing is achieved directly through lytic pore formation (membrane attack complex; MAC), and indirectly via anaphylatoxins (C3a, C5a) and marking targets for phagocytosis (opsonisation; C3b, iC3b). Complement is activated via three distinct pathways, triggered respectively by antibody (classical, CP), bacterial carbohydrate (mannose binding lectin, LP) or contact activation (alternative, AP). All pathways lead to enzymatic cleavage of complement C3 to the active

Abbreviations: AP, alternative pathway; CH50, the amount of serum (μl) giving 50% hemolysis of sheep erythrocytes; CFD, complement fixation diluents buffer; CP, classical pathway; DIC, disseminated intravascular coagulation; EGF, endothelial growth factor domain; FB, Factor B; FH-H50, the amount of functional FH needed to regulate lysis of 50% of antibody- sensitized sheep erythrocytes (ShEA) when incubated with 1% DBH-NHS; FH, Factor H; FI, Factor I; HUS, hemolytic uremic syndrome; IC50, the concentration of an inhibitor where the response is reduced by 50%; LP, lectin pathway; MAC, membrane attack complex; NHS, normal human serum; PPACK, Phenylalanyl-propyl-arginine chloromethyl ketone; SCR, short consensus repeat; ShEA, Amboceptor-sensitized sheep erythrocytes; SPR, surface plasmon resonance; TAFI, thrombin-activatable fibrinolysis inhibitor TM, thrombomodulin.

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fragments C3b and C3a. The opsonin C3b binds indiscriminately to pathogens and adjacent host cells. To prevent damage to self, complement is controlled by regulators, limiting complement activation by inactivating C3b, dissociating C3/C5 convertase enzymes or inhibiting MAC formation [13,14]. The regulator factor H (FH) accelerates C3 convertase dissociation and acts as a co-factor for the factor I (FI)-mediated inactivation of C3b, regulating both in the fluid phase and on cell surfaces [15].

TM appears to modulate complement activity by enhancing FH co-factor activity. TM co-precipitates with FH and C3b and both of these bind to the surface of TM-expressing HEK293 cells. In fluid phase cleavage assays, TM enhances FH/FI-mediated C3b inactivation in a dose-dependent manner [16,17], although the molecular basis by which this occurs is incompletely understood.

Complement and coagulation crosstalk in disease, and the potential therapeutic application of TM, is the subject of ongoing investigation. Recombinant human soluble TM (ART-123; Recomodulin™) has been investigated for treatment of disseminated intravascular coagulation (DIC) and sepsis-induced DIC [18,19], and approved for treatment in Japan. Recently, studies have investigated the role of soluble TM in patients with hemolytic uremic syndrome (HUS) [20,21] and atypical HUS (aHUS) [22]. aHUS has been associated with TM mutations [16], but is primarily linked to complement dysregulation caused by mutations in complement proteins [23–27] or autoantibodies [28,29].

In this study, we utilize two human recombinant TM analogs generated in different expression systems, using SPR analysis to determine binding affinity for complement C3b and FH. We further test the capacity of TM to act as a complement regulator in serum.

2. Materials and methods

2.1. Complement and coagulation components

Complement factor H (FH) and factor B (FB) were affinity-purified from EDTA-plasma of healthy volunteers on anti-FH (mAb 35H9, kind gift from Prof. Santiago Rodríguez de Cordoba, Madrid, Spain) or anti-Bb (mAb JC1, in-house) immobilized on HiTrap columns (GE Healthcare UK Limited, Amersham, UK). HRP-conjugated polyclonal anti-FH antibody was prepared in-house. Factor D (FD) and C3b were purchased from Complement Technology Inc. (Tyler, Texas, USA). Polyclonal anti-TM antibody and the two human recombinant soluble TM were from R & D Systems Europe Ltd. (Abingdon, UK) and Abcam plc (Cambridge, UK); the first was produced in a mouse myeloma cell line (NS0-derived) with sequence: Ala19-Ser515, with C-terminal 6-His-tag, TM_1. The second was from HEK293 cells, sequence: Ala19-Ala509; TM_2. Phenylalanyl-propyl-arginine chloromethyl ketone (PPACK)-inhibited thrombin was from Enzyme Research Laboratories Ltd. (Swansea, UK). Recombinant S195A thrombin [30], a catalytically inactive mutant, and TM456, a recombinant fragment of TM comprising EGF 4–6 including the thrombin binding sites EGF5-6 [31], was a kind gift from Prof. J. A. Huntington (Cambridge University, UK). Rabbit thrombomodulin was purchased from Haematologic Technologies Inc. (Vermont, USA), Complement fixation diluent (CFD) was from Oxoid Ltd. (Basingstoke, UK),sheep erythrocytes from TCS Biosciences Ltd. (Buckingham, UK) and Amboceptor from Siemens Healthcare GmbH (Erlangen, Germany). Thrombin generation assay reagents (platelet-poor-plasma reagent, fluorogenic substrate, thrombin calibration standard) were purchased from Thrombinoscope B.V. (Maastricht, Netherlands). Chromogenic substrate Biophen CS-21 for APC was from Cambridge Bioscience Ltd. (Cambridge, UK). OPD and Chondroitinase ABC from *Proteus vulgaris* was from Sigma-Aldrich Company Ltd. (Gillingham, UK). Polystyrene plates (MaxiSorp™) were from Fisher Scientific UK Ltd. (Loughborough, UK) Biacore chips and reagents from GE Healthcare UK Limited (Amersham, UK).

2.2. Generation of depleted sera for hemolysis assays

Normal human serum (NHS) isolated from pooled whole blood of three healthy volunteers was sequentially affinity-depleted (ΔBH-NHS) on anti-Bb (mAb JC1, in-house) and anti-FH (mAb 35H9) affinity columns in the presence of CFD buffer. FB, a key activator of the alternative pathway (AP), is depleted alongside FH to prevent consumption of complement due to spontaneous AP activation in the absence of FH; FB is added back immediately prior to assay activation (addition of activated ShEA).

FH was quantified in the depleted serum using an in-house developed ELISA. In ΔBH-NHS (compared to NHS), FH was undetectable at standard serum dilution (1:3200) and even at 1:100 dilution no specific signal was observed above background - residual FH was <1.5 μg/ml or <0.5% of average concentration in NHS.

2.3. Mass spectrometry analysis of thrombomodulin analogs

TM variants were subjected to mass spectrometry analysis (Applied Biosystems 4800 MALDI TOF/TOF Analyser, CBS Cardiff) to determine the molecular weights of the TM analogs.

2.4. Anticoagulant function of thrombomodulin variants

The anticoagulant function of TM in normal, platelet-poor, pooled plasma was assessed using a Fluoroskan Ascent plate reader (Thermo Lab System) by incubating 80 μL of citrated plasma with 20 μL of platelet-poor plasma reagent containing 5pM tissue factor (TF) and 4 μM phospholipids in the presence or absence of TM (5 nM). Thrombin generation was initiated by automatic dispensation of fluorogenic thrombin substrate (FluCa) and determined using a thrombin calibration standard (T-Cal). Measurements were taken at short intervals for 162 min.

Activated protein C (APC) was measured by adding varying concentrations of TM (2.5–10 nM) to a fixed concentration of thrombin (0.2 nM) and protein C (500 nM) in 10 mM Hepes, 150 mM NaCl, 3 mM $CaCl_2$, pH 7.4 then incubated at 37 °C for 2 h. Thrombin was inhibited by adding 1 μl (1 U) Hirudin for 15 min at 37 °C. APC-generation was measured by adding a chromogenic substrate (Biophen CS-21), incubated for 10 min and colorimetric output read at 405 nm absorbance.

2.5. Ligand binding assay of thrombomodulin to complement factor H

TM (1 μg/ml) or 10 μg/ml TM456 was coated onto polystyrene plates in 50 μl carbonate buffer, pH 9.6 for 2 h at room temperature, blocked with 1% gelatine in phosphate buffered saline containing 0.1% Tween (PBST) and incubated with varying concentrations of FH (1–5 μg/ml). Bound FH was detected using a HRP-conjugated polyclonal anti-FH antibody (10 μg/ml), adding OPD substrate, stopped using 10% H_2SO_4 and absorbance read at 492 nm. Background (gelatine-only coated wells) was subtracted from the binding signal as blank.

2.6. Surface plasmon resonance (SPR) binding interaction and affinity analysis

All analyses were performed on a Biacore T100 (GE Healthcare) in Hepes buffered saline (HBS), 0.01% surfactant P20, pH 7.4. Kinetics were analyzed at 20 μl/min flow rate at 25 °C. Due to its low isoelectric point, TM immobilization to a CM5 Biacore chip via standard amine coupling was not feasible. Therefore an indirect capture method was used, as described previously [32]. Briefly, polyclonal anti-TM antibody was immobilized via amine coupling to a CM5 chip at 2000RU, a density chosen to ensure that a sufficient proportion of the captured molecules exposed a binding epitope. TM analogs were captured stably on the antibody surface on adjacent flow cells. Binding stability of the TM-antibody complex was assessed over time prior to analysis and shown