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Full Length Article

Anti-inflammatory and anti-fibrinolytic effects of thrombomodulin alfa through carboxypeptidase B2 in the presence of thrombin

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ABSTRACT

Background: Thrombomodulin (TM) alfa, a recombinant human soluble TM, enhances activation of pro-carboxypeptidase B2 (pro-CPB2) by thrombin. Activated pro-CPB2 (CPB2) exerts anti-inflammatory and anti-fibrinolytic activities. Therefore, TM alfa may also have anti-inflammatory and anti-fibrinolytic effects through CPB2. However, these effects of TM alfa have not been elucidated. In the present study, we investigated the effects of TM alfa on inactivation of complement component C5a as an anti-inflammatory effect and prolongation of clot lysis time as an anti-fibrinolytic effect via CPB2 *in vitro*.

Methods: CPB2 activity and tissue factor-induced thrombin generation was examined by a chromogenic assay. C5a inactivation was evaluated by C-terminal cleavage of C5a and inhibition of C5a-induced human neutrophil migration. Clot lysis time prolongation was examined by a tissue-type plasminogen activator-induced clot lysis assay.

Results: CPB2 activity in human plasma was increased by TM alfa and thrombin in a concentration-dependent manner. TM alfa inhibited tissue factor-induced thrombin generation and enhanced pro-CPB2 activation in human plasma simultaneously. The mass spectrum of C5a treated with TM alfa, thrombin, and pro-CPB2 was decreased at 156 *m/z*, indicating that TM alfa enhanced the processing of C5a to C-terminal-cleaved C5a, an inactive form of C5a. C5a-induced human neutrophil migration was decreased after C5a treatment with TM alfa, thrombin, and pro-CPB2. TM alfa prolonged the clot lysis time in human plasma, and this effect was completely abolished by addition of a CPB2 inhibitor.

Conclusions: TM alfa exerts anti-inflammatory and anti-fibrinolytic effects through CPB2 in the presence of thrombin *in vitro*.

1. Introduction

Thrombomodulin (TM) is a membrane-bound glycoprotein on the surface of vascular endothelial cells [1]. TM contains 575 amino acids forming 4 extracellular domains; a N-terminal including signal peptide, a C-type lectin domain, 6 copies of the Epidermal Growth Factor-like domains and an O-linked domain. Additionally, there is a hydrophobic trans-membrane domain and a short cytoplasmic domain [2]. TM plays important roles in several vascular functions and homeostasis, such as anti-coagulation, anti-inflammation, and anti-fibrinolysis, by modulating the function of thrombin [2,3]. Thrombin is a serine protease that induces coagulation through conversion of fibrinogen to fibrin. However, when TM interacts with thrombin, it switches the thrombin substrate specificity from fibrinogen to protein C and enhances the activation of protein C by thrombin [4,5]. Activated protein C inactivates factors Va and VIIIa, as the underlying mechanism for the anti-coagulation effect of TM [3,5,6].

TM also enhances the activation of pro-carboxypeptidase B2 (pro-CPB2; also known as thrombin-activatable fibrinolysis inhibitor: TAFI), a plasma carboxypeptidase synthesized in the liver, and converts it into the active enzyme (CPB2 or TAFIa) with thrombin [7,8]. The catalytic efficiency of thrombin for pro-CPB2 activation is low, but is enhanced by more than 1000-fold in the presence of TM [7,9]. CPB2 inactivates several inflammatory mediators, including anaphylatoxins C3a and C5a, bradykinin, and osteopontin, through removal of the C-terminal arginine [7,9]. CPB2 also removes the C-terminal lysine from partially-degraded fibrin, thereby reducing the binding of tissue-type plasminogen activator (t-PA) and plasminogen to the clot and consequently plasmin formation [8,9]. Therefore, it is believed that TM exerts its anti-inflammatory and anti-fibrinolytic functions through CPB2.

Abbreviations: CPB2, carboxypeptidase B2; CPI, carboxypeptidase inhibitor from potato tuber; DIC, disseminated intravascular coagulation; HSA, human serum albumin; pan-CPB, carboxypeptidase B from porcine pancreas; PMN, polymorphonuclear neutrophil; pro-CPB2, pro-carboxypeptidase B2; TAFI, thrombin-activatable fibrinolysis inhibitor; TM, thrombomodulin; t-PA, tissue-type plasminogen activator.

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TM alfa (also known as ART-123) is a soluble form of recombinant human TM composed of 498 amino acid and all extracellular domains of TM. TM alfa has an anti-coagulation effect by accelerating the activation of protein C, similar to natural TM [10,11]. A clinical trial of TM alfa for disseminated intravascular coagulation (DIC) demonstrated that the DIC resolution rate was higher and the incidence of bleeding-related adverse events was lower in the TM alfa group compared with the heparin group, and TM alfa was approved for treatment of DIC in Japan [12,13]. Currently, a global phase 3 clinical trial of TM alfa in severe sepsis patients with coagulopathy is ongoing.

TM alfa was also shown to enhance the activation of pro-CPB2 following addition of thrombin to human plasma [14]. Meanwhile, a high concentration of TM isolated from rabbit lung reduced the activation of pro-CPB2 as a result of reduced thrombin generation in normal human plasma stimulated with tissue factor [15]. Therefore, the relationship between TM alfa concentration and pro-CPB2 activation in plasma stimulated with tissue factor remains unclear. Moreover, it remains to be examined whether TM alfa exerts anti-inflammatory and anti-fibrinolytic effects through CPB2. In the present study, we examined the concentration-dependent effects of TM alfa on thrombin generation and pro-CPB2 activation in tissue factor-induced thrombin generation assays. In addition, we examined the anti-inflammatory effect of TM alfa by inactivation of C5a through removal of the C-terminal arginine and inhibition of C5a-induced polymorphonuclear neutrophil (PMN) migration. The anti-fibrinolytic effect of TM alfa was investigated by prolongation of clot lysis time in human plasma.

2. Materials and methods

2.1. Materials

TM alfa was manufactured by Asahi Kasei Pharma (Tokyo, Japan). Human thrombin, phospholipid, carboxypeptidase B from porcine pancreas (pan-CPB, 188 units/mg protein), human serum albumin (HSA), t-PA, and carboxypeptidase inhibitor from potato tuber (CPI) were purchased from Sigma-Aldrich (St. Louis, MO). Purified human pro-CPB2 was obtained from Haematologic Technologies (Essex Junction, VT). Hippuryl-L-arginine was obtained from Peptide Institute (Osaka, Japan). PPACK was purchased from Calbiochem (Darmstadt, Germany). Recombinant human C5a was purchased from R&D Systems (Minneapolis, MN). Batroxobin was purchased from Pentapharma (Basel, Switzerland). Citrated pooled human plasma was obtained from George King Bio-Medical (Overland Park, KS).

2.2. Carboxypeptidase activity measurement

Carboxypeptidase activity was measured in a chromogenic assay as described previously [14]. Briefly, 40 μL of diluted human plasma (1:10 in 50 mmol/L Tris–HCl pH 8.0) was added with thrombin (0.01–1 U/mL), TM alfa (0.1–10 μg/mL), CPI (100 μg/mL), and $CaCl_2$ (1 mmol/L) to give a total volume of 50 μL and incubated at room temperature for 10 min. As the control treatment, the diluted human plasma was added to 10 μL of Tris-buffered saline (20 mmol/L Tris–HCl pH 7.4, 100 mmol/L NaCl, and 0.1% bovine serum albumin), the vehicle for thrombin and TM alfa, and incubated at room temperature for 10 min. In the purified pro-CPB2 experiment, 40 μL of purified human pro-CPB2 (4.5 μg/mL in Tris-buffered saline) was combined with thrombin (0.1 U/mL), TM alfa (0.01–1 μg/mL), and $CaCl_2$ (1 mmol/L) to give a total volume of 50 μL and incubated at room temperature for 10 min. As the control treatment, purified human pro-CPB2 was added to 10 μL of Tris-buffered saline alone and incubated at room temperature for 10 min. The reaction was stopped by addition of 5 μL of 1600 nmol/L PPACK, a thrombin inhibitor. Subsequently, 5 μL of 30 mmol/L hippuryl-L-arginine, a carboxypeptidase substrate, was added to 10 μL of reaction mixture, and substrate conversion was allowed to proceed for 45 min at 37 °C. After the incubation, 100 μL of 0.25 mol/L phosphate buffer (pH 8.3) and 75 μL of 3% cyanuric chloride in 1,4-dioxane were added and mixed well. The sample was centrifuged at 860 ×*g* for 10 min at room temperature. Next, 100 μL of the supernatant was transferred to a 96-well microtiter plate, and the absorbance was measured at 405 nm using a VersaMax Microplate Reader (Molecular Devices Japan, Tokyo, Japan). Each assay was performed 3 times independently.

2.3. Thrombin generation assay and carboxypeptidase activity measurement

The thrombin generation assay was performed as described previously [10]. Briefly, 0.5 mL of human plasma and 10 μL of 200 BU/mL batroxobin were incubated for 10 min at 37 °C. After the incubation, the mixture was defibrinated by centrifugation at 18,600 ×*g* for 10 min at 4 °C. The defibrinated plasma (160 μL) was incubated with 10 μL of TM alfa and 30 μL of tissue factor solution containing rabbit brain tissue factor (1:12,500 dilution; Sysmex, Hyogo, Japan), 20 μg/mL phospholipid vesicles composed of phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine (2:1:2), and 60 mmol/L $CaCl_2$ at 37 °C. As the control treatment, the defibrinated plasma was incubated with 10 μL of Tris-buffered saline and 30 μL of tissue factor solution at 37 °C. After 1, 3, 5, 7, 10, and 20 min of incubation, a 5 μL aliquot was isolated and incubated with 250 μL of 1 mmol/L S-2366, a chromogenic substrate for thrombin, at 37 °C for 3.5 min. To stop the chromogenic reaction, 25 μL of acetic acid was added, and the absorbance was measured at 405 nm using the VersaMax Microplate Reader. At the same time point, another 5 μL aliquot was isolated and mixed with 35 μL of 50 mmol/L Tris–HCl (pH 8.0) containing 1500 nmol/L PPACK and stored on ice until analysis. Subsequently, 5 μL of 30 mmol/L hippuryl-L-arginine and 10 μL of reaction mixture were mixed, and subjected to the same procedures for carboxypeptidase activity measurement described above. The results of the thrombin generation assay and carboxypeptidase activity measurement were expressed as the maximum thrombin concentration and maximum carboxypeptidase activity of the six measurement time points, respectively. Each assay was performed 3 times independently.

2.4. Mass spectrometry analysis

C-terminal cleavage of C5a was detected by mass spectrometry analysis. Briefly, 10 μg/mL C5a was incubated with 100 U/L pan-CPB or 1 μg/mL TM alfa, 1 U/mL thrombin, and 4.5 μg/mL pro-CPB2 for 45 min at room temperature, followed by 15 min at 37 °C. The mass of each reaction mixture was determined using an LTQ Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled to a UHPLC chromatograph (Thermo Fischer Scientific) equipped with a Meteoric Core C18 BIO column (YMC, Kyoto, Japan). Detection was performed in the *m/z* 350–1500 mass range using electrospray ionization in the positive mode. The spectra of multiply charged ions were deconvoluted using Xcalibur software (Thermo Fisher Scientific). The analysis was performed 3 times independently.

2.5. Human PMN isolation from peripheral blood

The study using human peripheral blood was performed according to protocols approved by the Ethics Committee on Human Research at the Pharmaceuticals Research Center, Asahi Kasei Pharma Corporation. We recruited healthy volunteers from the Pharmaceuticals Research Center staff and obtained written informed consent from them. Peripheral blood was obtained with heparin as an anticoagulant. PMNs were isolated from the blood by density gradient centrifugation using Mono-Poly Resolving Medium (DS Pharma Biomedical, Osaka, Japan), suspended in RPMI 1640 medium containing 0.5% HSA, and counted by an XT-2000i automated hematology analyzer (Sysmex).