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Human soluble thrombomodulin-induced blockade of peripheral HMGB1-dependent allodynia in mice requires both the lectin-like and EGF-like domains

Yusuke Hayashi [a, 1], Ryuichi Tsujita [a, b, 1], Maho Tsubota [a, 1], Haruka Saeki [a], Fumiko Sekiguchi [a], Goichi Honda [c], Atsufumi Kawabata [a, *]

[a] Laboratory of Pharmacology and Pathophysiology, Faculty of Pharmacy, Kindai University (formerly known as Kinki University), 3-4-1 Kowakae, Higashi-Osaka 577-8502, Japan
[b] Project Management Development, Asahi Kasei Pharma Corporation, 1-105 Kanda Jinbocho, Chiyoda-ku, Tokyo 101-8101, Japan
[c] Medical Affairs Development, Asahi Kasei Pharma Corporation, 1-105 Kanda Jinbocho, Chiyoda-ku, Tokyo 101-8101, Japan

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ABSTRACT

Thrombomodulin (TM), an endothelial protein with anti-coagulant activity, is composed of 5 domains, D1-D5. Recombinant human soluble TM (TMα) consisting of D1-D3, which is generated in CHO cells, suppresses inflammatory and nociceptive signals by inactivating high mobility group box 1 (HMGB1), one of damage-associated molecular patterns. TMα sequesters HMGB1 with the lectin-like D1 and promotes its degradation by thrombin binding to the EGF-like D2. We prepared TM's D123, D1 and D2 by the protein expression system of yeast, and evaluated their effects on HMGB1 degradation *in vitro* and on the allodynia caused by HMGB1 in distinct redox forms in mice *in vivo*. TMα and TM's D123, but not D1, promoted the thrombin-dependent degradation of all-thiol (at-HMGB1) and disulfide HMGB1 (ds-HMGB1), an effect mimicked by TM's D2, though to a lesser extent. Intraplantar administration of TMα and TM's D123, but not D1, D2 or D1 plus D2, strongly prevented the mechanical allodynia caused by intraplantar at-HMGB1, ds-HMGB1 or lipopolysaccharide in mice. Our data suggest that, apart from the role of D3, TMα and TM's D123 require both lectin-like D1 capable of sequestering HMGB1 and EGF-like D2 responsible for thrombin-dependent degradation of HMGB1, in abolishing the allodynia caused by exogenous or endogenous HMGB1.

1. Introduction

Thrombomodulin (TM), a transmembrane protein expressed in the vascular endothelium, activates protein C in a thrombin-dependent manner, and the activated protein C degrades coagulation factors Va and VIIIa, thereby exhibiting anticoagulant activity [1]. TM consists of five domains; the extracellular N-terminal lectin-like domain (D1), epidermal growth factor (EGF)-like domain (D2), glycosylated domain (D3), transmembrane domain (D4) and intracellular domain (D5). D2 is a thrombin-binding site and responsible for the thrombin-dependent activation of protein C. D1 is capable of sequestering high mobility group box 1 (HMGB1), one of damage-associated molecular patterns (DAMPs), known to aggravate inflammatory responses. There is evidence that thrombin binding to D2 degrades HMGB1 binding to D1, leading to irreversible inactivation of HMGB1 [2,3]. Recombinant human soluble TM, known as thrombomodulin alfa (TMα), which consists of D1, D2 and D3, is produced in the Chinese hamster ovary (CHO) cell engineering system, and retains many of biological activities of full-length TM including the activation of protein C and the sequestration of HMGB1 followed by thrombin-dependent degradation [1]. TMα is now clinically available for treatment of disseminated intravascular coagulation (DIC) in Japan.

HMGB1, a nuclear protein, is passively released from necrotic cells, and actively secreted by macrophages etc., and promotes inflammatory responses [4,5]. There are two distinct active forms of HMGB1; all-thiol HMGB1 (at-HMGB1) has three cysteine residues, C^{23}, C^{45} and C^{106}, in a thiol form, and disulfide HMGB1 (ds-HMGB1) has a disulfide bond between C^{23} and C^{45}. It has been demonstrated

* Corresponding author.
E-mail address: kawabata@phar.kindai.ac.jp (A. Kawabata).
[1] Co-first authors.

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that at-HMGB1 and ds-HMGB1 activate the receptor for advanced glycation end-products (RAGE) and Toll-like receptor-4 (TLR4), respectively, thereby playing a pro-inflammatory role [4–6]. Both at-HMGB1 and ds-HMGB1 are also considered pain mediators in the spinal cord and/or peripheral tissue, most probably by activating RAGE and TLR4, respectively [7,8]. Accumulating evidence suggests that endogenous HMGB1 is involved in the inflammatory pain, neuropathic pain induced by surgical nerve injury or chemotherapy, and visceral pain in the bladder and pancreas [9–14]. We have shown that TMα prevents or restores a variety of pain or hyperalgesia/allodynia by inactivating endogenous HMGB1 [9–12]. Given evidence that TM's D1 itself may reduce inflammatory responses [15–18], in the present study, we prepared recombinant D1, D2 and D123 proteins of TM using the yeast protein expression system, and determined their activity in suppressing the allodynia induced by HMGB1 in mice *in vivo* and in promoting thrombin-dependent degradation of HMGB1 *in vitro*.

2. Materials and methods

2.1. Major chemicals

Thrombomodulin alfa (TMα), recombinant human soluble TM consisting of TM's D1, D2 and D3 (Fig. 1A), was produced by the in-house Chinese hamster ovary (CHO) cell engineering system in Asahi Kasei Pharma Corporation (Tokyo, Japan). Recombinant D123, D1 and D2 proteins of human TM (Fig. 1A) were prepared by the protein expression system of yeast, *Pichia pastoris* strain SMD1163 (*his4pep4 prb1*) (Asahi Kasei Pharma), as described elsewhere [19]. It is to be noted that the yeast-generated D123 of TM may be structurally different from TMα in the glycosylation state of its D3 region (Fig. 1A). Recombinant human all-thiol HMGB1 (at-HMGB1) and disulfide HMGB1 (ds-HMGB1) were purchased from HMGBiotech (Milan, Italy), and lipopolysaccharide (LPS; *Escherichia coli* Serotype O26:B6), thrombin (from human plasma) and argatroban, a direct thrombin inhibitor, were from Sigma-Aldrich (St. Louis, MO, USA). TMα and TM's D123, D1 and D2 were dissolved in 0.002% Tween 80-containing saline, and all other chemicals were in saline.

2.2. Western blot analysis of the effects of yeast-generated recombinant human TM's D123, D1 or D2 on thrombin-dependent degradation of HMGB1 in vitro

As reported elsewhere [3], thrombin-induced degradation of HMGB1 was evaluated by the increase in protein levels of its primary cleaved product that lacks the N-terminal 9 amino acids of HMGB1. In the absence or presence of TMα and yeast-generated human TM's D123, D1 or D2 at 400 nM, at-HMGB1 or ds-HMGB1 at 400 nM was incubated with 2 U/mL thrombin for 15 min at 37 °C *in vitro*, and the protein levels of at-HMGB1 or ds-HMGB1 and their primary cleaved products were assessed by Western blotting followed by densitometry, as described previously [11]. A rabbit anti-HMGB1 polyclonal antibody that recognizes the C-terminal peptide of human HMGB1 (Abcam, Cambridge, UK) and horseradish peroxidase-coupled anti-rabbit IgG antibody (Cell Signaling Technology, Danvers, MA, USA) were used as the primary and secondary antibodies, respectively.

2.3. Laboratory animals used for in vivo studies

Male ddY mice (3–4 weeks old), delivered from Kiwa Laboratory Animals Co., Ltd (Wakayama, Japan), were kept in a room maintained at 22–24 °C with free access to food and water for 1–2 weeks, and then used for experiments. All procedures used for animal experiments were approved by the Kindai University's

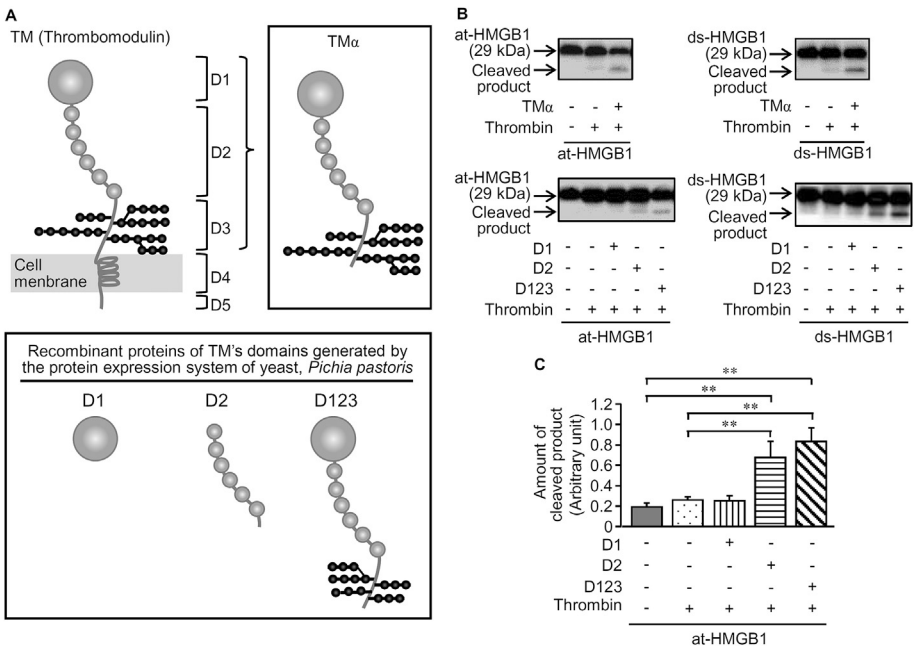

Fig. 1. Effects of TMα and yeast-generated recombinant proteins of human TM's D1, D2 and D123 on thrombin-dependent cleavage of at-HMGB1 or ds-HMGB1 *in vitro*. (A) Structure of TM, TMα and yeast-generated human TM's D1, D2 and D123. The glycosylation states in the D3 regions of yeast-generated D123 and TMα are considered structurally different. (B) Western blot analysis of the effects of TMα and TM's D1, D2 and D123 on the accumulation of thrombin-cleaved products of HMGB1 *in vitro*. In the absence or presence of TMα or TM's D1, D2 and D123, at 400 nM, at-HMGB1 or ds-HMGB1 at 400 nM was incubated with 2 U/mL thrombin for 15 min at 37 °C *in vitro*. The protein levels of at-HMGB1 or ds-HMGB1 and their primary cleaved products were assessed by Western blotting. (C) The amount of the thrombin-cleaved product of at-HMGB1 after 15-min incubation in the absence and presence of TM's D1, D2 or D123. Western blot data were quantified by densitometry. Data show the means ± S.E.M. for 5 experiments. $^{**}P < 0.01$.

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