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THROMBIN RECEPTOR AGONIST PEPTIDE DECREASES THROMBOMODULIN ACTIVITY IN CULTURED HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

Yoshikazu Maruyama[*,1], *Ikuro Maruyama*, and *Yasuko Soejima*

[*]*Third Department of Internal Medicine and Department of Clinical Laboratory Medicine, Faculty of Medicine, Kagoshima University, Kagoshima 890, Japan*

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SUMMARY: Recently, it has been shown that the N-terminal peptide from a new type of thrombin receptor exhibits thrombin receptor agonist activity. We examined the effects of this synthetic thrombin receptor against peptide (SFLLRNPNDKYEPF, TRAP) on human umbilical vein endothelial cells (HUVECs). TRAP induced rapid morphological changes in HUVECs, with marked increase in the release of prostacycline, endothelin, platelet activating factor, tissue type plasminogen activator, and plasminogen activator inhibitor-1. Incubation of cells with TRAP also induced a rapid decrease in cell-surface thrombomodulin. Thus, activation of the newly described thrombin receptor may alter their role in the hemostatic pathway.

Thrombin, a key enzyme in the hemostatic pathway, also has various effects on the function of human endothelial cells. It is known that thrombin binds thrombomodulin (TM) (1,2), cellular inhibitor, protease-Nexin (3), and a heparin-like molecule with antithrombin III (4,5) on endothelium. TM is the major thrombin receptor on vascular endothelium and forms a 1:1 complex with thrombin. This complex is responsible for the antithrombotic properties of these cells (6,7). TM plays an important role as a co-factor in the thrombin catalyzed activation of protein C (8,9), which in turn function as an anticoagulant by causing a proteolytic degradation of factors Va and VIIIa (6,10). The thrombin-TM complexes can be internalized and transported to the lysosome resulting in the degradation of thrombin and return of TM to the cell surface (11, 12). Thus, TM is a natural anticoagulant protein on the endothelium converting thrombin

[1]*To whom correspondence should be addressed at Third Department of Internal Medicine, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima City, Kagoshima 890, Japan.*

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from a procoagulant protease to an anticoagulant. We previously found that thrombin decreases the level of TM on the cell surface by inducing an endocytosis of TM (13). Internalization of TM is also induced by tumor necrosis factor (TNF) (14, 15,16), interleukin-1 (IL-1) (17) and endotoxin (18). In contrast, TM levels increase following incubation of human umbilical vein endothelial cells (HUVECs) with cAMP (19, 20, 21), forskolin or phorbol esters (20). However, the mechanism(s) regulating TM expression have not been completely determined and there is some controversy regarding the roles of internalization and transcription in down-regulating this protein. Recently, it has been proposed that activation of the thrombin receptor involves a novel mechanism in which thrombin cleaves the receptor, exposing a new N-terminus which serves as a ligand for the receptor (22). This receptor appears to belong to the seven transmembrane domain receptor super-family with a cytosolic C-terminal domain and an extracellular N-terminal segment (23). It has been demonstrated that a synthetic tetradecapeptide(SFLLRNPNDKYEPF, TRAP) identical to the new N-terminal segment exhibits thrombin receptor agonist activity. We find that TM is down-regulated in response to TRAP while the release of vasoactive substances, such as prostacyclin (PGI2), endothelin (ET), platelet activating factor (PAF), tissue-plasminogen activator (t-PA) and its inhibitor (PAI-1) are enhanced.

MATERIALS AND METHODS

Materials: Materials were obtained from the following sources: Medium 199, fetal calf serum, penicillin, streptomycin and trypsin-EDTA in phosphate-buffered saline from GIBCO Laboratories; collagenase from Sigma; COS medium from Cosmo Bio Co., Ltd; and L-glutamine from Nissui Pharmaceutical Co., Ltd; tissue culture flasks and centrifuge tubes from Corning Glass Work and Falcon Labware, cluster 48 dish wells from Coster. α-thrombin was purchased from Mochida Pharmaceutical Inc., protein C was purchased from Green Cross Co., Ltd, and hirudin was purchased from Trans Gene. s2238 was purchased from Chromogenix. Guanidium thionate, formaldehyde, etidium bromide and β-actin were purchased from Wako Pure Chemical. Synthetic and nylon membranes were purchased from Amersham and Bio Rad, respectively. Thrombin receptor agonist peptide(TRAP) was synthesized by Teijin Inc..

Cell culture: Primary culture of HUVECs was prepared as previously described (24). Cells were grown in 100 mm diameter gelatin coated tissue culture flasks for analysis of TM mRNA and PGI2, ET, t-PA, PAF and PAI-1. 48-well tissue culture plates used for analysis of TM activity were prepared using Medium 199 containing 20% fetal calf serum, 2 mM glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin in a 5% CO_2 atmosphere at 37 °C.

TM activity: Culture medium was removed from confluent monolayers of cells in 48-well tissue culture plates and monolayers were washed three times with washing buffer (20 mM Tris HCl). Monolayers were then incubated with 5 units thrombin and 0.5 μM protein C in COS medium at 37 °C. After a 30 minutes incubation, reactions were terminated by the addition of hirudin (40 units/ml) and antithrombin III (350 μg/ml). The level of protein C activating co-factor was assayed using benzoylarginine ethylester as previously described (12) and reflected TM activity.
TM mRNA: Confluent cells in 100 mm diameter tissue culture flasks were harvested with rubber policeman in serum free medium. Total cytoplasmic RNA was prepared by the guanidium thiocyanate method (25). Isolated RNA was heat denatured and fractionated on 1.2% formaldehyde agarose gels and stained with 1 mg/ml ethidium bromide for 30 minutes and washed with distilled water four times for 30 minutes. RNA was then transferred to a synthetic membrane (Hybond N, Amersham) by capillary blotting techniques and cross-linked to the nylon membrane by UV illumination. A DNA probe corresponding to the human TM coding region was used for hybridization. Prehybridization was performed at 56 °C and washes were done at 60 °C. Chemiluminescence of Northern blots were performed using Kodak X-OMAT films. Filters were washed with distilled water at 65 °C for 30 minutes prior to rehybridization with the cDNA probe for human β-actin. After densitometric analysis, TM mRNA levels were normalized to the concentration of β-actin mNRA.
PGI2, ET, t-PA, PAF and PAI-1: Analyses levels of these vasoactive proteins were measured in cultured medium of confluent HUVECs monolayers in 35 mm diameter culture flasks after incubation with 5 μM TRAP for 30 minutes. Monolayers were washed and then overlaid with COS medium for 60 minutes. The syntheses of ET, t-PA, PAF and PAI-1 were measured using enzyme immunoadsorbent assays (Biopool) and PGI2 synthesis was assayed by measuring the level of its stable metabolite, 6-keto-PGF1α in a radioimmunoassay.

RESULTS

Shape changes of HUVECs: Monolayers treated with 5 μM TRAP developed extended fibroblast-like projections 30 minutes after incubation and showed marked morphological changes after 60 minutes. These changes were reversible, with a return to their original morphology 6 hours after the removal of TRAP (Fig. 1).
Suppression of TM activity; The effect of TRAP on surface TM activity in HUVECs was determined 30 minutes after cultured endothelial cells with TRAP. There was a time-dependent decrease in TM activity after incubation with TRAP. TM activities decreased to minimal levels 2 hours after treatment but were restored within 24 hours after TRAP was removed from the medium (Fig. 2).
TM mRNA expression: Analysis of TM mRNA was performed after incubation of HUVECs with TRAP. Addition of 5 μM TRAP to cultured

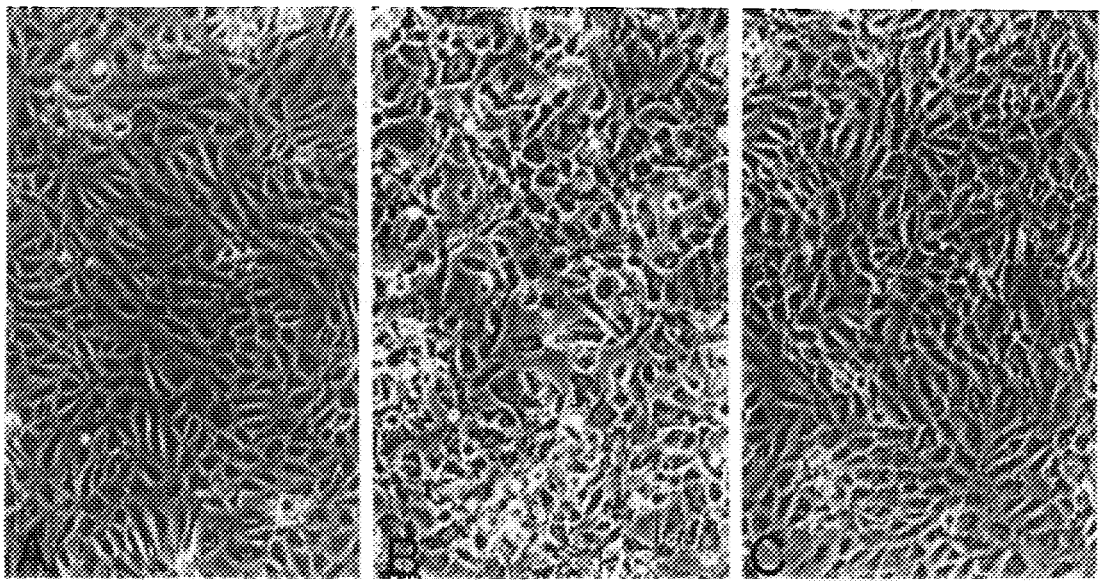

Fig.1. Morphological changes in cultured endothelial monolayer cells following incubation with TRAP (5μM) for 30 minutes. A; Original morphology. B; Marked fibroblast-like projections and gap formations formed 30 minutes after incubation with TRAP. C; Recovery to their original morphology 6 hours after the removal of TRAP.

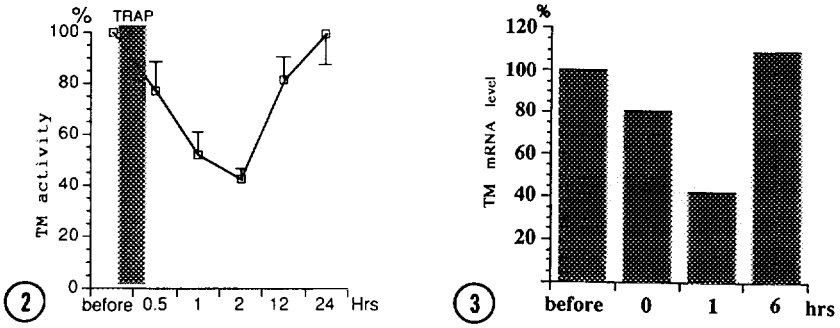

Fig.2. Effects of TRAP on surface TM activity in HUVECs. Cultured endothelial cells were treated with TRAP (5μM) at 37°C for 30 minutes. HUVEC monolayers were washed and surface TM activity was measured at indicated time points. Each value represents the average of three experiments involving three separate batches of cells.

Fig.3. Down-regulation of TM mRNA. Thirty minutes after incubation with TRAP (5μM), mRNA from HUVECs was isolated and hybridized with a TM and β-actin probe. Northern blots (normalized to the concentration of β-actin mRNA) indicate a decrease in the specific mRNA following exposure to TRAP.

HUVECs for 30 minutes resulted in a 55% reduction in TM activity 2 hours after incubation in COS medium. TM mRNA levels were minimal 1 hour after treatment and returned to pretreatment values by 6 hours (Fig. 3).

Expression of vasoactive proteins following treatment with TRAP: HUVECs monolayers, incubated with COS medium alone for 60 minutes, produced 824 pg/ml of 6 keto-PGF1α, 7.0 ng/ml of PAI-1, 132.0 ng/ml of PAF, 295.0 pg/ml of ET and 1.5 ng/ml of t-PA. The releases of these vasoactive proteins after incubation of HUVECs with TRAP were elevated as shown in Fig. 4.

DISCUSSION

Thrombin, a serine protease which plays a central role in blood coagulation, platelet activation and atherosclerosis, has a variety of effects on endothelium, including release of fibronectin (26), PGI_2 (27), PAF (28), t-PA (29) and PAI-1 (30). Activation occurs through signal transduction at the levels of the thrombin receptor. We previously showed that 50% of total thrombin are bound to TM (12), while the remaining thrombin are bound to protease-Nexin and antithrombin III. The number of different thrombin receptors present on the human endothelial cell surface has yet

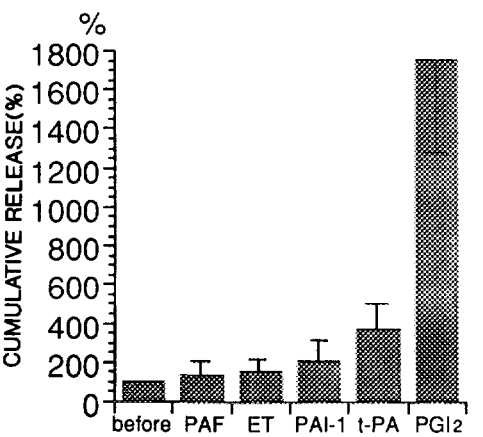

Fig.4. Effects of TRAP on the secretion of vasoactive proteins from HUVECs. Endothelial cell monolayers were incubated for 30 minutes with 5 μM TRAP at 37 °C. Cells were then incubated in COS medium for 60 minutes and the levels of PGI_2, PAF, ET, t-PA and PAI-1 in the medium were measured and obtained the following results; PAI-1 (208±66.6%, n=3), t-PA (386.4±45.7%, n=3), ET (150.5±18.1%, n=3), PAF (136.4±26.0%, n=3) and PGI_2 (1754.7±580.3%, n=3) of % control. Each value represents the average of three experiments involving three separate batches of cells.

to be determined. Recent reports have suggested that the thrombin receptor may have effects on vascular endothelial cells and platelets (22, 31). We prepared a thrombin receptor agonist peptide (TRAP), with the N-terminal sequence SFLLRNPNDKYEPF, which acts as a tethered ligand (22, 23, 32). This tetradecapeptide induces platelet aggregation and increases cytosolic free calcium and PGI2 production in vascular endothelial cells (33). We found that the addition of TRAP resulted in the reversible development of fibroblast-like projection and gap-formations in HUVEC monolayers (Fig. 1). These changes appeared 20 minutes after incubation with 5 μM TRAP and cells returned to their original morphology several hours after treatment. It has previously been shown that thrombin causes a marked retraction of confluent endothelial cells when it is coupled to the thrombin receptor, phospholipase C and A2 and Ca^{++} is released (33, 34). Our present data suggest that the morphological changes in endothelial cell monolayers induced by thrombin may be caused by the activation of the thrombin receptor. These changes may increase the permeability of endothelial cell monolayers resulting in damage to the vascular wall and formation of atherosclerotic plaques. The mechanism by which thrombin induces a decrease in TM has not been elucidated. We find that the level of TM mRNA can be down regulated by activation of the thrombin receptor with TRAP. It has been previously shown that TNF (14, 15, 16), IL-1 (17) and endotoxin (18) all down-regulate TM on vascular endothelial cells. These cytokines have been hypothesized to inhibit transcription of TM mRNA (35), thereby contributing to a decline in TM activity. However, the decrease in TM activity may also be the result of increased endocytosis and degradation of TM. We found a decreased in protein C activating co-factor activity, reflecting a decrease in TM activity, 20 minutes after TRAP treatment. This activity decreased to minimal levels 2 hours after treatment (Fig. 2). Since the half-life of biosynthetically labeled TM in hemangioma cells is 19 hours (36), TRAP may also cause a rapid decrease in TM half-life by stimulative endocytosis (half-life in this study, 2 hours). The rapid decrease in TM activity may have contributed to the morphological changes, since these also occured 20 minutes after incubation with TRAP. We hypothesize that the down-regulation of TM by thrombin receptor activation contributes to a hypercoagulable state in endothelial cells in vitro. In addition, thrombin receptor activation by TRAP accelerates release of vasoactive proteins such as t-PA, PAI-1, PAF, ET and most especially PGI2. However, the mechanisms by which thrombin mediates cellular activation have not yet to be defined. Our data suggest that thrombin receptor activation by TRAP accelerates release of these proteins. Ngaiza et al. also reported that TRAP elevated intracellular

calcium levels and stimulated PGI2 production (33). However, increase in PGI2 within 30 minutes after treatment with TRAP probably reflects the release of an available intracellular pool rather than an increase in PGI2 mRNA and protein synthesis.

In conclusion, our data suggest that thrombin has a procoagulant property which acts through the thrombin receptor to down-regulate TM mRNA and change the morphology of endothelial cells with release of vasoactive proteins.

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