International Journal of Cardiology 172 (2014) 340–349

Contents lists available at ScienceDirect

International Journal of Cardiology

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

Soluble thrombomodulin is a paracrine anti-apoptotic factor for vascular endothelial protection☆,☆☆

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ARTICLE INFO

Article history:
Received 26 April 2013
Received in revised form 11 December 2013
Accepted 8 January 2014
Available online 22 January 2014

Keywords:
Endothelium
Apoptosis
Thrombomodulin

ABSTRACT

Background: Thrombomodulin (TM) is an endothelial cell (EC) membrane-bound anticoagulant protein that has novel direct cellular effects. TM is shed from EC and becomes soluble form (sTM) in plasma. Higher sTM levels in healthy subjects are associated with lower cardiovascular risk, suggesting that sTM possesses a protective role. The purpose of the study was to evaluate the effect of sTM on vascular endothelium.

Methods and results: Apoptosis of cultured ECs was induced via serum starvation. EC-bound TM was released into the medium after serum starvation. The medium conditioned by serum-starved EC decreased apoptosis in another set of cultured EC. Direct treatment with sTM reduced EC apoptosis and decreased pro-apoptotic protein expression. TM knockdown in EC exacerbated the rate of serum starvation-induced apoptosis. Treatment of sTM activated the phosphatidylinositol 3-kinase (PI3 kinase)–protein kinase B/Akt survival pathway and suppressed the death pathway, c-Jun N-terminal kinase. We found that sTM also increased growth and reduced apoptosis of endothelial progenitor cells.

Conclusions: EC-bound TM is released during stress-induced EC damage and becomes sTM, a paracrine factor that exerts anti-apoptotic activity. Our data indicate that sTM is not only an endothelial injury biomarker but also has cytoprotective effects on vascular endothelium.

1. Introduction

The vascular endothelium is located between circulating blood cells and subendothelial tissues, and serves as a thromboresistant and non-permeable barrier. Endothelial cells (ECs) also modulate vascular tone, blood hemostasis, and inflammation within the vascular wall. EC apoptosis, which occurs in response to stressful conditions and disrupts the integrity of vascular endothelium, is an important determinant of endothelial dysfunction and a harbinger of atherosclerosis [1]. Endothelial denudation resulting from EC apoptosis enhances the accessibility of lipoproteins and leukocytes into arteries, and increases the exposure of procoagulant subendothelial tissues [2,3]. EC apoptosis has been found in a variety of stressful conditions, such as hyperglycemia, hyperlipidemia, cigarette smoking and hypoxia. It is induced through the activation of distinct signaling pathways, which converge to cleave procaspase-3 into its active form, caspase-3, and thereby promote DNA fragmentation and apoptotic cell death.

Thrombomodulin (TM) is a cell surface-expressed glycoprotein which is predominantly expressed on vascular ECs. It consists of 5 domains including a highly charged N-terminal lectin-like domain (D1), a domain with six epidermal growth factor (EGF)-like structures (D2), a serine and threonine-rich domain (D3), a transmembrane domain (D4) and a cytoplasmic tail (D5). TM acts as a thrombin receptor on the surface of vascular ECs and accelerates the rate of thrombin-catalyzed activation of protein C. Activated protein C (APC) functions as an anticoagulant by inactivating coagulation factors Va and VIIIa, thereby effectively inhibiting the propagation of the coagulant response [4]. On the other hand, APC can bind endothelial protein C receptor, activating the protease-activated receptor 1 and its downstream sphingosine-1 phosphate receptor 1 pathway to execute an anti-inflammatory effect. TM also decreases the pro-inflammatory effects of thrombin when TM binds to thrombin. Functionally, the region consisting of the fourth, fifth, and sixth EGF-like structures of the second domain (TMD2) is responsible for thrombin binding and protein C activation. TM has recently been found to have direct effects on cellular proliferation, cell–cell adhesion, and inflammation [5,6]. Previous study has shown that TM-dependent APC formation plays an important role in inhibiting

☆ All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

☆☆ This work was supported by grants NSC 100-2314-B-006-056-MY3 and 98-2314-B-006-050-MY2 from the National Science Council and grant DOH100-TD-B-111-002 from the Department of Health, Taipei, Taiwan.

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http://dx.doi.org/10.1016/j.ijcard.2014.01.009

EC and podocyte apoptosis in glomeruli [7]. Soluble forms of TM, which are shed from the EC membrane, have been found in human plasma and urine [8]. In healthy humans, an epidemiological study demonstrated that a higher baseline level of plasma soluble TM (sTM) was associated with a lower future risk of coronary heart disease, suggesting that sTM may play a protective role in vasculature [9]. In this study, we evaluated the direct effects of sTM on EC apoptosis and demonstrated that sTM is a paracrine anti-apoptotic factor with a role in endothelial protection.

2. Methods

2.1. EC culture

ECs were isolated from fresh human umbilical cords using the collagenase perfusion technique. The cell pellet was resuspended and cultured in medium 199 (Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS, Gibco, Grand Island, NY).

2.2. Recombinant TM protein

Recombinant TM protein containing domains 2 and 3 (rTMD23) was used to stimulate cells. The expression and purification of rTMD23 in the *Pichia pastoris* expression system used in our laboratory was previously described [10].

2.3. Western blot analysis

Western blot analyses were performed with loading 30 μg of total proteins from each sample into a 10% SDS-polyacrylamide gel, and separated proteins were transferred to a PVDF membrane. The membrane was blocked with 5% skim milk, followed by incubation with a 1:1000 dilution of the indicated antibody at 4 °C overnight. Antibody binding was detected with HRP-conjugated secondary antibodies and enhanced chemiluminescence (PerkinElmer, Waltham, MA), and the blots were exposed to X-ray film (Fujifilm Medical, Stamford, CT).

2.4. Apoptosis induction and detection

Apoptosis of cultured ECs was induced via serum starvation with 0.5% FBS or by adding high glucose (33 mM) and incubating for 24 hours (h). A cell death detection

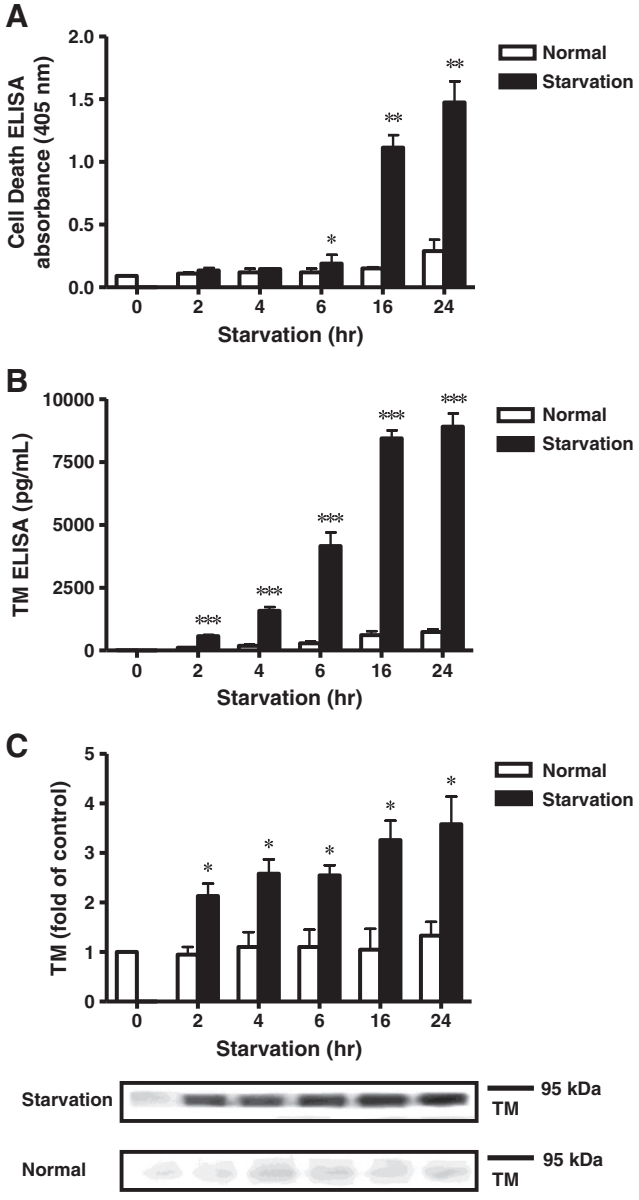

Fig. 1. Serum starvation-induced stress causes TM release from ECs. (A) Cultured ECs were exposed to medium containing 0.5% (starvation) or 20% (normal) fetal bovine serum (FBS) for 24 h. Apoptosis was determined with a cell death detection ELISA kit. The data were expressed as absorbance measured at 405 nm at different time points after starvation. (B and C) Serum starvation-conditioned medium was collected and sTM levels were determined with a TM ELISA kit (B) and Western blotting (C). The data were expressed as absolute sTM levels (B) or ratios (C) at different time points after starvation to the baseline at 0 h. All results were obtained from three independent experiments. (D) EC-bound TM in cultured ECs was detected by immunofluorescence staining. DAPI was used to stain the nuclei of ECs. TM-positive cells were counted and the ratio of TM-positive cells to total cells was measured and averaged in 3 random microscopic fields (×200). Data were shown as mean ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. cells at baseline (0 h).