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Reducing agents induce thrombomodulin shedding in human endothelial cells

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ABSTRACT

The level of thrombomodulin (TM) on cell surfaces reflects its biosynthesis, intracellular turnover, proteolytic cleavage, and release in soluble form (sTM). In the present study we examined the mechanisms mediating and regulating sTM release. Inducers of endothelial protein C receptor (EPCR) shedding, such as proinflammatory cytokines, phorbol ester, and ionomycin did not affect sTM release from human umbilical endothelial cells (HUVECs). In contrast, several natural and synthetic reducing compounds (i.e., glutathione, dihydrolipoic acid, homocysteine, N-acetyl-L-cysteine, dithiothreitol, and non-thiol cell-impermeable reductant, tris-(2-carboxyethyl)phosphine), but not oxidized glutathione or α-lipoic acid effectively up-regulated the release of sTM in endothelial cells. In addition, the direct activator of metalloproteases, 4-aminophenylmercuric acetate (APMA), was an effective inducer of TM shedding. Considerable inhibition of protein C activation was found with APMA, which is consistent with the effects of this agent on TM shedding. In addition to metalloproteases, serine proteases were shown by pharmacological inhibition studies to be involved in a similar degree in basal sTM release; however, serine proteases seem preferentially to be involved in thiol-induced TM proteolytic processing. From comparisons of non-thiol containing synthetic substrate with human recombinant TM it was demonstrated that disulfide bonds within TM are most likely modified by thiols making TM more susceptible to serine protease-mediated cleavage. In summary, the study shows that the extracellular redox state plays a crucial role in the regulation of TM shedding in HUVECs thereby offering new strategies to interfere with diminished activation of protein C during inflammatory diseases.

Introduction

Thrombomodulin (TM), a cell-surface glycoprotein, is highly expressed in endothelial cells and numerous other cell types such as keratinocytes, osteoblasts, and mononuclear phagocytes [1,2]. Both cell-associated TM and the cleaved soluble form of TM (sTM) promote specific effects on coagulation and fibrinolysis through generation of activated protein C (aPC) and thrombin-mediated activation of fibrinolysis inhibitor [3–5]. Other activities of TM include anti-inflammatory and cytoprotective properties, influences on receptor mediated cell signaling, and anti-metastatic effects in malignant tumor progression [6,7].

Increased plasma levels of sTM have been described in patients with disseminated intravascular coagulation syndrome, pulmonary thromboembolism, acute respiratory distress syndrome, and chronic renal and hepatic failure [8–10]. Furthermore, elevated levels of sTM were observed in several autoimmune disorders, including systemic lupus erythematosis, Churg–Strauss syndrome, and Wegener's granulomatosis [11,12]. For this reason, the loss of TM from endothelial cell surfaces is thought to contribute to increased risk of thrombosis associated with inflammation. Induction of sTM release has also been observed *in vitro* after exposure of endothelial cells to hydrogen peroxide, homocysteine (Hcys), prostaglandin A_2, lipopolysaccharide, and neutrophil derived proteases [13–18].

The cleavage of TM and release of its soluble fragments is thought to be mediated by neutrophil-derived proteases and accompanied by damage to cell membranes [13,19]. It was also reported that metalloproteases are implicated in TM ectodomain cleavage mediating the release of the lectin-like domain [20–22]. Apart from the above, little is known about the mechanisms underlying the induction of TM shedding or the types of proteases involved in TM cleavage.

In view of the convincing evidence that both membrane-associated and soluble forms of TM play a crucial role in the endothelial protein C

Abbreviations: ADAM, a disintegrin and metalloprotease; α-LA, α-lipoic acid; aPC, activated protein C; APMA, 4-aminophenylmercuric acetate; BSA, bovine serum albumin; CM, cytokine mixture; DHLA, dihydrolipoic acid; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EPCR, endothelial protein C receptor; FCS, fetal calf serum; GSH, reduced glutathione; GSSG, oxidized glutathione; Hcys, homocysteine; HUVECs, human umbilical vein endothelial cells; IFN-γ, interferon-γ; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; MMP, matrix metalloprotease; NAC, N-acetyl-L-cysteine; PBS, phosphate-buffered saline; PC, protein C; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; rhTM, recombinant human thrombomodulin; sEPCR, soluble endothelial protein C receptor; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TM, thrombomodulin; sTM, soluble thrombomodulin; TNF-α, tumor necrosis factor-α.

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receptor (EPCR) associated protein C pathway, the present study was carried out to assess the mechanisms regulating release of sTM in human endothelial cells and the role of redox status in this process.

Materials and Methods

Reagents and antibodies

Human plasma-derived aPC (10.8-11.6 Units/mg) was from Hematological Technologies Inc. (Cell Systems, Biotechnologie Vertrieb GmbH, St. Katharinen, Germany) and human protein C was from Enzyme Research Laboratories Ltd (South Bend, IN, USA). Recombinant human TM (rhTM) and Immubind® Thrombomodulin ELISA kits were purchased from American Diagnostica GmbH (Pfungstadt, Germany). Monoclonal mouse antibodies to the modules EGF5 (clone PBS-01) and EGF2 (clone PBS-02) of epidermal growth factor (EGF)-like domains of TM, and goat polyclonal FITC-conjugated antibody to mouse IgG were purchased from Abcam (Cambridge, UK). PMA, GM6001, TAPI-0, and ionomycin were from Calbiochem (Schwalbach, Germany). Pefabloc SC, bovine serum albumin (BSA), reduced (GSH) and oxidized glutathione (GSSG), α-lipoic acid (α-LA), dihydrolipoic acid (DHLA), N-acetyl-L-cysteine (NAC), 4-aminophenylmercuric acetate (APMA), dithiothreitol (DTT), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), trypsin from bovine pancreas and elastase from hog pancreas were purchased from Sigma-Aldrich (Deisenhofen, Germany). Recombinant human IFN-γ, IL-1β, and TNF-α were from Roche Diagnostics GmbH (Mannheim, Germany). PMA, TAPI-0, ionomycin, APMA, and GM6001 were dissolved in dimethyl sulfoxide (DMSO). The final concentrations of DMSO were 0.3% or less, and controls using DMSO alone were run in all cases. Other agents were used as aqueous solutions.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Promocell (Heidelberg, Germany) and cultivated according to the manufacturers' instructions. Briefly, the cells were maintained in endothelial cell growth medium with supplement mix containing 2% fetal calf serum (FCS) and 0.4% endothelial cell growth supplement. For all experiments, exponentially growing subconfluent cells were used at passages 5 to 8.

ELISA based quantitative determination of sTM and sEPCR

The amounts of sTM and soluble endothelial protein C receptor (sEPCR) released by endothelial cells were determined using Asserachrom sTM and sEPCR ELISA kits (Diagnostica Stago, Asnieres, France) according to the manufacturer's instructions. For this purpose, cells were grown to confluence in 96-well microplates in complete medium with FCS and supplements. Cells were then further incubated for 1 h with fresh complete medium containing inducers or inhibitors of TM and EPCR shedding. At the end of incubation, the cell medium was removed, centrifuged at 800 g for 10 min to remove the cell debris, and used for analysis to determine the levels of sTM and sEPCR released by cells. Total cell protein was determined using Bicinchoninic Acid assay kit with BSA as internal standard (Sigma-Aldrich, Deisenhofen, Germany).

Flow cytometry

Cells after incubation were trypsinized, washed in PBS and resuspended at $1 \cdot x\ 10^6$ cells/ml in FACS buffer (PBS, pH 7.4, supplemented with 1% BSA and 0.1% sodium azide) and incubated with anti-TM monoclonal antibodies (clones PBS-01 and PBS-02) for 30 min at 4 °C. Cells were then washed twice with FACS-buffer and incubated under light protected conditions for 30 min at 4 °C with anti-mouse IgG FITC-conjugated antibody produced in goat, which were used at final dilution of 1:400 of the commercially supplied stock solution. Thereafter, cells were again washed twice, fixed with 4% paraformaldehyde in PBS, and analyzed on an EPICS XL flow cytometer (Beckman Coulter GmbH, Krefeld, Germany).

Cell-free proteolysis of recombinant human thrombomodulin

To test the effect of reducing agents on proteolytic degradation of TM, the rhTM was dissolved in PBS at a final concentration of 50 ng/ml and incubated at 37° C in the presence of 10 μg/ml trypsin or 10 μg/ml elastase. In a separate panel, the incubation medium was supplemented with 0.5 mM DTT. After defined incubation time periods, the proteolysis was stopped by addition of a protease inhibitor cocktail (Sigma-Aldrich). The intensity of proteolysis was assessed by measuring the disappearance of immunochemical detectable rhTM in the incubation medium using Immubind® Thrombomodulin ELISA kit (American Diagnostica GmbH, Pfungstadt, Germany). The results were expressed as relative changes of rhTM levels as values taking the value at 0 min as 100%.

The activity of elastase towards synthetic chromogenic substrate, *N*-succinyl-$(Ala)_3$-*p*-nitroanilide (Sigma-Aldrich) was assayed using the methods recommended by the manufacturer. The reactive mixture containing 10 μg/ml of elastase and 1 mM chromogenic substrate was incubated at 37 °C in buffer solution containing 0.1 M HEPES, 0.5 M NaCl, and 10% DMSO. The reaction was followed by measuring the release of 4-nitroanilide every 5 min at 405 nm on Victor3 1420 Multilabel Counter reader (PerkinElmer LAS GmbH, Rodgau Jügesheim, Germany).

Protein C activation assay

To test the influence of TM shedding on aPC generation, HUVECs were incubated in the presence of protein C and thrombin according to the method described by Grey et al. [23]. Briefly, cells were cultured in 24-well plates to confluence, treated for 2 h with APMA as modulator of TM shedding, and thereafter washed in buffer A containing 20 mM Tris-HCl (pH 7.5), 2 mM $CaCl_2$, 150 mM NaCl, and 0.1% BSA. Washed cells were incubated for a further 1 h-period in the presence of human protein C (80 nM), thrombin (37.5 nM), and buffer A in a final volume of 200 μl/well at 37 °C and 5% CO_2. Microplates were then centrifuged at 160 x g for 5 min, 150 μl of supernatants were transferred into 96-well plates and assayed for the generation of aPC using 0.8 mM chromogenic substrate S-2366. The extinction of reaction product was measured at 405 nm on Victor3 1420 Multilabel Counter reader (PerkinElmer LAS GmbH, Rodgau Jügesheim, Germany). To prevent nonspecific cleavage of substrate by thrombin, hirudin (10 antithrombin units per well) was added to each supernatant for 5 min at room temperature before testing for aPC activity. The amounts of generated aPC were calculated using aPC standards and normalized to cell protein content.

Data analysis

Data were analyzed by one-way analysis of variance coupled with Dunnet's *post hoc* test to compare each experimental group with a nominated control group using SPSS 14.0 software. Differences were considered significant at $P<0.05$.

Results

Pharmacological regulation of sTM release in HUVEC

Exposure of HUVEC for 1 h to pro-inflammatory cytokines, IL-1β, TNF-α, and IFN-γ, either individually (data not shown) or as mixture (CM), led to a considerable inhibition of basal sTM release (Fig. 1A).