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Short Communication

Thrombomodulin regulation in human brain microvascular endothelial cells in vitro: Role of cytokines and shear stress

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ABSTRACT

Thrombomodulin (TM), an important determinant of blood vessel homeostasis, is expressed on the luminal vascular endothelial cell surface and is released into serum in response to circulatory signals. This includes the cerebrovascular endothelium, where the anti-coagulant and anti-inflammatory properties of TM are thought to be critical to the brain microcirculation and blood–brain barrier (BBB) integrity. Much is still unknown however about how circulatory stimuli may regulate TM activity within the brain microvasculature. To address this, the current short paper investigated the effects of opposing regulatory signals, namely cytokines (TNF-α, IL-6) and laminar shear stress, on the cellular levels and release of TM in cultured human brain microvascular endothelial cells (HBMvECs). Treatment of confluent HBMvECs with either TNF-α or IL-6 (100 ng/ml, 18 h) reduced TM protein levels by up to 70%, whilst inducing TM release into media by up to 4.4 and 5.5 fold, respectively. The effects of either cytokine (0–100 ng/ml) on TM protein levels (6 or 18 h) and release (0–18 h) were also found to be concentration- and time-dependent. Either cytokine (100 ng/ml, 24–72 h) also reduced TM mRNA levels by >50%. When exposed to laminar shear stress for 24 h at 8 dyn/cm^2 (SI unit equivalent = 0.8 Pa), TM protein levels were upregulated by 65% in parallel with a 2-fold increase in TM mRNA levels. Shear stress also proved to be a much more potent stimulus for TM release from HBMvECs, yielding media TM levels of 1000 pg/10^5 cells, when compared to 175 and 210 pg/10^5 cells for TNF-α and IL-6, respectively, after parallel 18 h treatments. Finally, shear-conditioned media was found to completely block thrombin-induced permeabilization of HBMvECs, confirming the functional efficacy of released TM. In summary, our data indicate that TM is differentially regulated within cultured HBMvECs by humoral and biomechanical signals.

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Introduction

Thrombomodulin (TM), an integral membrane receptor constitutively expressed on the luminal surface of vascular endothelial cells, is an important determinant of blood vessel homeostasis (for review see Martin et al., 2013). Studies have confirmed the expression of TM within the cerebrovascular endothelium, where its anti-coagulant and anti-inflammatory properties are thought to be critical to the brain microcirculation and blood–brain barrier (BBB) integrity (Tran et al., 1996; Wang et al., 1997). In this respect, soluble TM-based therapeutics have been used to treat BBB-associated neurological disorders such as stroke (Wenzel et al., 2014). Moreover, elevated TM release or "shedding" into serum invariably accompanies the cerebrovascular endothelial activation typically associated with neurodegenerative disorders (Festoff et al., 2012), stroke (Hassan et al., 2003), cerebral small vessel disease (Giwa et al., 2012) and traumatic brain injury (Yokota et al., 2002).

Much is still unknown however about how common pathological and physiological stimuli may regulate TM activity within the brain microvasculature. Proinflammatory cytokines for example, have been strongly linked to the pathological effects of stroke and other neurological diseases, imparting anti-barrier and pro-coagulant effects on the brain microvascular endothelium (Behling-Kelly et al., 2007; Tuttolomondo et al., 2008; Rochfort et al., 2014). The precise effects of cytokines, and particularly IL-6, on TM levels and release within the brain microvasculature however, are poorly documented. In contrast to proinflammatory cytokines, physiological laminar shear stress typically has a protective anti-inflammatory impact on the vascular endothelium (Traub and Berk, 1998). Moreover, the ability of shear stress to enhance BBB phenotype and to protect against cytokine-induced BBB injury has previously been reported (Krizanac-Bengez et al., 2006; Clark et al., 2011; Walsh et al., 2011). Whilst various studies have demonstrated the ability of shear stress to upregulate endothelial TM expression (for review see Martin et al., 2013), to our knowledge, there are no existing reports documenting the precise effects of shear stress on TM regulation within the brain microvasculature.

To address these knowledge gaps, the current short paper investigated the effects of opposing regulatory influences, namely cytokines (TNF-α, IL-6) and laminar shear stress, on the cellular protein/mRNA levels and release of TM in cultured HBMvECs. Both time- and

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concentration-/force-dependency studies were conducted, whilst the putative impact of TM release on thrombin-induced HBMvEC permeabilization was also investigated.

Materials and methods

Materials

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Dublin, IRL). Cytokines (TNF-α, IL-6) were purchased from Millipore (Cork, IRL). Primary antisera included mouse anti-thrombomodulin IgG ab6980 (Abcam, Cambridge, UK) and rabbit anti-GAPDH IgG (Millipore). Secondary antisera included HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Cell Signalling, MA, USA).

Cell culture

Culture of primary-derived HBMvECs has been described previously (Rochfort et al., 2014). HBMvECs, primary cultured from a single adult male donor post-mortem, were purchased from Cell Systems Corporation (WA, USA — Cat No. ACBRI 376). Cells were routinely grown in EndoGRO™ MV Basal Medium (Millipore) containing 5% foetal bovine serum, antibiotics (100 mg/ml Mycozap™), and relevant supplements. All cells (passages 5–12) were grown on tissue culture grade plasticware coated with Attachment Factor™ (Life Technologies, UK) and maintained in a humidified atmosphere of 5% CO_2/95% air at 37 °C. For experimental purposes, cells were routinely subjected to treatment with either TNF-α or IL-6 (0–100 ng/ml, 0–72 h), or to laminar shear stress (0 or 8 dyn/cm^2, 0–72 h), the latter employing an orbital rotation shear model (Walsh et al., 2011). Post-treatment, both cells and conditioned media were harvested for analysis. Total cell protein lysate preparation has been described previously (Rochfort et al., 2014). Conditioned media samples were routinely centrifuged at 700 ×g for 15 min to remove any cellular debris. Media samples were routinely assayed by ELISA prior to freezing. All samples were ultimately stored at −80 °C.

Western immunoblotting

Total cell protein lysates were resolved by 10% SDS-PAGE under reducing conditions and electroblotted as previously described (Rochfort et al., 2014). Membranes were blocked for 60 min in tris-buffered saline (TBS: 10 mM Tris pH 8.0, 150 mM NaCl) containing 5% w/v bovine serum albumin (BSA) before being incubated overnight in primary antisera with gentle agitation at 4 °C. Primary antisera were prepared in TBST (+1% BSA, +0.1% Tween-20): 1 μg/ml anti-thrombomodulin mouse monoclonal IgG and 0.2 μg/ml anti-GAPDH rabbit monoclonal IgG. Secondary antisera, applied to washed membranes for 3 h with gentle agitation at room temperature, were prepared in TBST: 1:2000 HRP-conjugated goat anti-mouse IgG (thrombomodulin) and 1:3000 HRP-conjugated goat anti-rabbit IgG (GAPDH). Membranes were developed using a Luminata™ Western HRP Kit (Millipore). Scanning densitometry of Western blots was routinely performed using NIH ImageJ software, with GAPDH routinely employed as a loading control to facilitate densitometric normalization of bands.

Quantitative real-time PCR

Following experiments, endothelial cells were harvested for extraction of total RNA and analysis of TM mRNA expression as previously described (Guinan et al., 2013). Ribosomal subunit S18 was routinely used for normalization purposes. Primer pairs were screened for correct product size by 1% agarose gel electrophoresis and underwent melt-curve analysis for primer-dimers. TM (107 bp): Forward 5′-ACCTTCCT CAATGCCAGTCAG-3′; Reverse 5′-GCCGTCGCCGTTCAGTAG-3′; S18 (250 bp): Forward 5′-CAGCCACCCGAGATTGAGCA-3′; Reverse 5′-TAGT AGCGACGGGCGGTGTG-3′.

Enzyme-linked immunosorbent assay (ELISA)

A thrombomodulin/BDCA-3 DuoSet® ELISA Kit (R&D Systems, MN, USA) was employed as per manufacturer instructions (with minor modifications) to measure absolute TM levels in HBMvEC conditioned media samples. Briefly, 96-well plates were coated with 50 μl/well of the capture antibody and incubated overnight. The plate was then blocked for 1 h by adding 150 μl of Reagent Diluent to each well. Media samples and human recombinant TM standards (31.25–2000 pg/ml) were added in duplicate at 50 μl/well, with incubations proceeding for 2 h. 50 μl of detection antibody was then added to each well for a further 2 h, followed by 50 μl of streptavidin–HRP to each well for 20 min (in the dark). 50 μl of substrate solution was then added to each well for a further 20 min (in the dark). Finally, reactions were terminated with the addition of 25 μl of Stop Solution to each well and the plate luminescence subsequently read at both 570 nm and 450 nm (wave correction was used to subtract the readings at 570 nm from 450 nm to control for optical imperfections in the plate).

Transendothelial permeability assay

For analysis of HBMvEC monolayer permeability, the transwell method of Rochfort et al. (2014) was employed. Briefly, HBMvECs were plated at high density (5×10^5 cells/well) into Millicell hanging cell culture inserts placed within 6-well dishes (Millipore; 0.4 μm pore size, 24 mm filter diameter). Complete media was added to the upper (luminal, 2 ml) and lower (abluminal, 4 ml) chambers of the Millicell insert within the 6-well dish and the cells were allowed to grow to confluency. Confluent HBMvECs within the upper chamber were next incubated for 30 min with either unconditioned media (0 ng of TM) or shear-conditioned media (3 ng or 6 ng of TM — i.e. 1.5–3.0 ng/ml) in the absence and presence of 2 units of thrombin (i.e. 1 U/ml) (note: conditioned media was pre-concentrated by centrifugal filtration to contain either 3 ng or 6 ng of released thrombomodulin for co-incubations with thrombin). Post-treatment, media in the upper and lower chambers was replenished, fluorescein isothiocyanate (FITC)-labelled 40 kDa dextran was added to the upper chamber (giving a final concentration of 250 μg/ml), and transwell diffusion allowed to proceed. Media samples (28 μl) were collected from the lower chamber after 3 h, diluted to a final volume of 400 μl with complete media, and monitored in 96-well format for FITC–dextran fluorescence. A TECAN Safire 2 fluorospectrometer was used with excitation and emission wavelengths set at 490 and 520 nm, respectively. Permeability is presented as % transendothelial exchange of FITC–dextran 40 kDa (%TEE FD40).

Statistical analysis

Results are expressed as mean ± s.d. Experimental points were typically performed in triplicate with a minimum of three independent experiments ($n = 3$). Statistical comparisons between control and experimental groups were by ANOVA in conjunction with a Dunnett's post-hoc test for multiple comparisons. A Student's t-test was also routinely employed for pairwise comparisons. A value of $P \leq 0.05$ was considered significant.

Results

Effect of proinflammatory cytokines on TM protein/mRNA levels and release in HBMvECs

Treatment of confluent HBMvECs with either TNF-α or IL-6 reduced TM protein levels by up to 70% at 100 ng/ml cytokine for 18 h (Fig. 1A). Reductions in TM mRNA levels of 65% and 53% were also observed

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