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The impact of the endothelial protein C receptor on thrombin generation and clot lysis

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ABSTRACT

Introduction: When thrombin is bound to thrombomodulin (TM), it becomes a potent activator of protein C (PC) and thrombin-activable fibrinolysis inhibitor (TAFI). Activation of PC is enhanced when PC is bound to the endothelial protein C receptor (EPCR). Activated protein C (APC) inhibits thrombin generation while activated TAFI (TAFIa) attenuates fibrinolysis. To determine the impact of diminished EPCR function on thrombin generation and fibrinolysis we generated cells that expressed TM and a variant of EPCR (R96C) that does not bind PC.
Methods: To determine the impact of EPCR on the generation of APC and TAFIa and how this affects thrombin generation and fibrinolysis we performed thrombin generation and clot lysis assays in the presence of cells expressing wild-type TM and EPCR (WT cells) or wild-type TM and the R96C variant of EPCR (R96C cells).
Results: In the presence of R96C cells, thrombin generation in normal plasma is increased, as a result of impaired PC activation when compared to WT cells. In addition, clot lysis is delayed in normal plasma in the presence of R96C cells, despite no increase in TAFI activation. In PC deficient plasma, clot lysis is delayed in the presence of WT and R96C cells as a result of increased TAFI activation.
Conclusions: We demonstrate that impaired EPCR function can be detected by thrombin generation and clot lysis assays on cells expressing TM and EPCR. We also demonstrated that deficiency in EPCR has procoagulant effects that lead to a delay in clot lysis.

1. Introduction

The protein C (PC) pathway is an important natural anticoagulant pathway that inhibits thrombin generation. When thrombin binds thrombomodulin (TM) on the endothelial cell surface, the substrate specificity of thrombin switches from fibrinogen to PC [1]. The thrombin-TM complex converts zymogen PC to its activated form, activated PC (APC) [2]. The endothelial protein C receptor (EPCR), which binds PC and presents it to the thrombin-TM complex, enhances PC activation by 8-fold in vitro and 20-fold in vivo [3,4]. Activated protein C (APC), along with its cofactor protein S inactivates coagulation factors (F) Va and VIIIa, thereby attenuating thrombin generation.

In addition to activation of PC, the thrombin-TM complex also activates thrombin-activable fibrinolysis inhibitor (TAFI) to activated TAFI (TAFIa) [5]. TAFIa inhibits fibrinolysis by removing the exposed C-terminal lysine residues from fibrin, thereby attenuating plasmin generation and clot lysis [6]. Although the kinetics of PC and TAFI activation by the thrombin-TM complex are similar [2,5], it is not well understood if PC-bound EPCR can affect TAFI activation by the thrombin-TM complex. Simultaneous activation of PC and TAFI on human endothelial cells (both umbilical vein and microvascular) that express both TM and EPCR, demonstrate that activation of PC and TAFI by the thrombin-TM complex are likely independent processes, and that the presence of EPCR does not affect TAFI activation [7]. How EPCR can affect fibrinolysis, however, remains unanswered.

Clinically, impairment of the anticoagulant PC pathway has been associated with an increased risk of venous thromboembolism [8–10]. PC deficiency, in which individuals have a congenital or acquired reduction in PC antigen and/or activity, is an independent risk factor for thrombosis and can be identified by ex vivo activation of PC by protac, an activator found in snake venom [11,12]. In vivo, however, activation of PC is accelerated by the binding of PC to EPCR on the vascular endothelium [4]. Thus, mutations that impair EPCR/PC binding interactions are predicted to increase the risk of thrombosis [13–15]. In support of this, targeted gene sequencing studies have shown that the frequency of

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mutations that impair EPCR-interactions are higher in patients with unprovoked VTE compared with controls [16–21].

In vitro, thrombin generation and clot lysis assays are commonly performed in the absence of cell surfaces, and therefore not sensitive to deficiencies in EPCR function. We hypothesize that impaired EPCR/PC binding will result in increased thrombin generation due to decreased levels of APC generated. We also hypothesize that increased amount of thrombin generated will lead to inhibition of fibrinolysis due to increased TAFI activation. To test these hypotheses we performed thrombin generation and clot lysis assays in plasma in the presence of cells that express TM and a variant of EPCR that does not bind PC.

2. Materials and methods

2.1. Generation of stable cell lines expressing TM and the EPCR variant R96C

Human embryonic kidney cells (HEK293) expressing both human wild-type TM and EPCR were established as previously described (known as WT cells) [22]. HEK293 cells expressing both wild-type TM and R96C EPCR (known as R96C cells) were established as follows. Generation of the EPCR variant R96C was performed as previously described [18,22]. HEK293 cells were stably transfected using 2 different pcDNA3.1(−) vectors containing TM and R96C EPCR cDNAs using Effectene transfection reagent (Qiagen, Valencia, CA) as described by the manufacturer. Transfection of HEK293 cells was performed in 6-well dishes in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Burlington, ON) containing 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO). 48 h post-transfection, the media was changed to DMEM with 10% FBS, 400 mg/mL of G418 and 200 mg/mL of hygromycin (Life Technologies, Burlington, ON). After 2 weeks of drug selection, drug-resistant colonies were isolated and expanded, and expression of cell surface TM and EPCR was assessed using a FASCCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

2.2. Flow cytometry

HEK293, WT and R96C cells were grown to confluency in 6-well dishes, detached with gentle pipetting, and suspended in 1 mL of phosphate buffered saline (PBS), pH 7.4 containing 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO). For comparison of TM and EPCR expression levels, human umbilical vein endothelial cells (HUVEC) (Lonza, Allendale, NJ) were grown to confluence in endothelial growth medium (EGM-2MV) (Lonza, Allendale, NJ), detached with 0.5% trypsin-EDTA (Life Technologies, Burlington, ON) and suspended in 1 mL of PBS. The cells were washed twice and incubated with 5 μg/mL of fluorescein isothiocynate (FITC)-labeled CTM1009, an anti-TM mouse monoclonal antibody or Alexa488-labeled JRK1535, an anti-EPCR mouse monoclonal antibody [3,22] for 30 min at 4 °C. Bound antibody was detected on the fluorescence-1 channel on a FASCCalibur flow cytometer. The fluorescence intensity of each sample was analyzed twice.

2.3. Thrombin generation assay on cells

WT cells, R96C cells, and HEK293 cells (no TM or EPCR) were cultured to 80% confluence in 100 mm dishes in DMEM containing 10% FBS and the appropriate drug selection reagent. At 80% confluency, the cells were washed and harvested in HEPES buffered saline (HBS) and total cell counts were performed using a hemocytometer. Cells were added (8×10^4) into the wells of a 96-well black microtiter plate and mixed with HBS (to a total volume of 20 μL), 5 μL recombinant human tissue factor (TF) (RecombiPlasTin 2G, final dilution in plasma 1/1000) (Instrumentation Laboratory, Bedford, MA) and 50 μL citrated normal human pooled plasma or plasma deficient in PC or TAFI (Affinity Biologicals, Ancaster, ON). To collect human plasma, informed consent was obtained from 10 healthy adult volunteers and approved by the McMaster Research Ethics Board. For normal human plasma, blood was obtained via venipuncture into 3.2% sodium citrate. Whole blood was centrifuged at 1500 ×*g* for 10 min at room temperature. Plasma was collected, pooled and stored at −80 °C. To determine the effect of TM and EPCR on thrombin generation, cells were pre-incubated with 500 nM of anti-TM antibody (CTM1009) or anti-EPCR antibody (JRK1535) [3,22] for 30 min at RT. Thrombin generation was initiated by 25 μL of a solution containing 30 mM $CaCl_2$ and 2 mM fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC) (Bachem, Bubendorf, Switzerland). Thrombin generation was monitored in a SpectraMax M5e plate reader (Molecular devices, Sunnyvale, CA) at 37 °C using the Technothrombin thrombin generation assay protocol (Technoclone, Vienna, Austria). Thrombin generation profiles were analyzed using Technothrombin thrombin generation assay software (Technoclone).

2.4. Plasma clot lysis in the presence of cells

PC-deficient, TAFI-deficient or normal plasma (50 μL) was added to flat-bottomed 96-well plates containing 8×10^4 cells (HEK293, WT, or R96C) and mixed with HBS (up to 25 μL). To initiate clot formation and clot lysis, 25 μL of a solution containing 2 nM tissue plasminogen activator (tPA, Aviva Systems Bio, San Diego, CA) 30 mM $CaCl_2$ and RecombiPlasTin 2G (final dilution in plasma 1/1000) in HBS was added. To determine the effect of TAFIa on clot lysis, an inhibitor of TAFIa, potato tuber carboxypeptidase inhibitor (PTCI) (Sigma, St. Louis MO) was added to the assay reaction mixture (final concentration 10 μM) prior to initiation of clot formation. Absorbance was monitored at 450 nm for 2 h at 37 °C in a SpectraMax plate reader. Clot lysis times were determined as the time from half maximal increase to half maximal decrease in absorbance as determined using SoftMax Pro software.

2.5. Protein C activation on HEK293 cells expressing TM and EPCR

To measure PC activation by cells expressing TM and EPCR, a modified thrombin generation assay was used. Briefly, HEK293, WT and R96C cells were added to 96-well plates containing plasma that was defibrinated prior to use by incubation with 50 U/mL of batroxabin (Sigma, St. Louis, MO) at 37 °C for 10 min. Thrombin generation was initiated as described previously in the methods. At 0, 5, 10, 20 and 30 min intervals, plasma was removed from the well and added to a solution containing citrate and 20 mM benzamidine for measurement of APC. To quantify APC, an APC enzyme capture assay was performed as previously described [23].

2.6. TAFI activation on HEK293 cells expressing TM and EPCR

As a result of the short half-life and thermal instability of TAFIa, it is difficult to measure TAFI activation in plasma at 37 °C. To enhance TAFIa stability in our experiment we used the TAFI variant with Ile at position 325 instead of Thr, which has a longer half-life without changes to its activation kinetics or activity [24]. We then prepared plasma that is deficient in both PC and TAFI (PCTDP). TAFI was immunodepleted from the PC-deficient plasma using anti-TAFI monoclonal antibody (mAb16) linked to Sepharose beads as described previously [25]. PC (Heamatologic Technologies, Essex Junction, VT) and/or TAFI were added back at physiological concentrations (70 nM) into PCTDP to produce PCTDP + PC + TAFI, PCTDP + TAFI or PCTDP + PC. To measure TAFI activation by cells expressing TM and EPCR in plasma, a modified thrombin generation assay was used. Briefly, HEK293, WT and R96C cells were added to 96-well plates containing defibrinated PCTDP + PC + TAFI, PCTDP + TAFI or PCTDP + PC. Thrombin generation was initiated as described previously in the methods. At 0, 5, 10, 20 and 30 min intervals, plasma was removed from the well and added to a solution containing 100 μM Phe-Pro-Arg-chloromethylketone (FPRck) and 150 μM Val-Phe-Lys-chloromethylketone (VFKck) (plasmin inhibitor) for measurement of TAFIa. To measure TAFIa, a fluorescent-based assay was used [26]. Briefly, recombinant plasminogen possessing S741C