Persistence of endothelial thrombomodulin in a patient with infectious purpura fulminans treated with protein C concentrate

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Introduction

Purpura fulminans (PF) is a rare life-threatening complication of bacterial sepsis that is characterized by a highly thrombotic subtype of disseminated intravascular coagulation (DIC) and mortality of up to 80%.¹ Patients with PF develop a severe deficiency in protein C (PC), a serine protease that is a key endogenous anticoagulant.² PC is activated at the endothelial surface by thrombin in the presence of the cofactor thrombomodulin (TM). Activated PC (APC) is cytoprotective³ and inhibits thrombin generation by cleaving coagulation factors Va and VIIIa.⁴ Therefore, TM is a crucial regulatory “switch” governing negative feedback of coagulation. The initiating event in PF is hypothesized to be loss of TM from the endothelial surface in response to infection.⁵ As a result, conversion of PC to APC is impaired, and coagulation proceeds unchecked.³ Although therapy with PC concentrate has been proposed as a strategy to target the underlying pathophysiologic lesion in PF,⁶ the belief that endothelial TM loss is an early event in PF that renders infused PC ineffective has limited its widespread adoption.^{2,5} We report herein data on the kinetics of TM loss in a patient with PF that support the use of supplemental PC in upfront treatment of severe cases.

Case description

A previously healthy 39-year-old male presented after experiencing 12 hours of sore throat, malaise, and an expanding purpuric rash on his face. Physical examination revealed mask-like purpura with periorbital sparing, scattered purpuric papules on the extremities, and livedo of the hands and feet (Figure 1A; supplemental Figure 1). Initial laboratory studies were consistent with severe DIC and multiorgan system failure (supplemental Table 1). Review of the peripheral blood smear showed neutrophils containing toxic granulations, Döhle bodies, and prominent vacuoles, as well as frequent rod-like Gram-negative basophilic inclusions (Figure 1B). The patient reported owning a new dog and was later noted to have a small puncture wound on his left fourth finger from a dog bite sustained 3 days prior to admission. Based on his history and the findings on peripheral blood smear, a presumptive diagnosis of PF due to *Capnocytophaga canimorsus* bacteremia was made. *C canimorsus* is a rare infection but one that carries a very high rate of PF, which occurs in ~30% to 40% of cases.^{7,8} Indeed, PF appears to complicate *C canimorsus* sepsis more frequently on a per-case basis than meningococcal disease, an infection that is broadly understood to be a common cause of PF.

Shortly after transfer to the medical intensive care unit, the patient experienced cardiac arrest with pulseless electrical activity requiring 10 minutes of cardiopulmonary resuscitation. After reestablishment of spontaneous circulation, he was treated with 100 IU/kg PC concentrate, 3100 IU antithrombin (AT)

Figure 1. Initial presentation and clinical course. (A) The patient as he appeared at presentation, following rapid development of a mask-like purpuric rash involving the face. (B) Polymorphonuclear leukocyte containing rod-like basophilic inclusions on Wright's stain (arrowhead; left panel), and Gram's stain (arrowhead; right panel). Original magnification $\times 1000$. (C) Plasma levels of PC, antithrombin (AT), and lactate during the first 10 days of hospitalization. (D) Activated partial thromboplastin time (aPTT), prothrombin time (PT), and fibrinogen during the same period. PC and antithrombin concentrates were administered twice daily starting 6 hours after presentation through day 10 of hospitalization.

concentrate, and a 5000-U IV bolus of heparin, followed by a continuous heparin infusion. In addition, he received 40 U of cryoprecipitate and 10 U of fresh-frozen plasma overnight.

The following day, the patient's admission PC activity level returned at 12% (normal range, 66%-140%), protein S was 30% (normal range, 70%-134%), and AT antigen level was 37% (normal range, 69%-127%). A skin biopsy specimen confirmed the presence of microvascular thrombi (supplemental Figure 2). Although blood cultures remained negative, *C canimorsus* was detected in the patient's peripheral blood using a polymerase chain reaction (PCR)-based assay performed at the Centers for Disease Control (see "Methods"). In addition to continued antibiotic therapy, the patient received therapeutic anticoagulation with IV heparin throughout his hospitalization and was administered PC and AT concentrates until hospital day 10, at which point he was able to maintain normal levels without supplementation. Limb necrosis was managed conservatively with meticulous examinations, wound care, and bedside debridement. He steadily improved and was discharged on hospital day 57. He ultimately retained use of all 4 limbs and only required amputation of the 4th and 5th distal phalanges of the left hand (supplemental Figure 3) and 9 of 10 phalanges of both feet. He has since returned to work as a graphic designer.

Methods

Molecular diagnosis of *C canimorsus* infection

Under research use for diagnostic confirmation, DNA was extracted from a 200- μ L blood sample using a QIAamp DNA Mini Kit, according to the manufacturer's instructions (QIAGEN, Valencia, CA). Two fragments of the 16S rRNA gene were targeted for amplification (supplemental Methods) from the purified DNA using an Expand High-Fidelity PCR System (Roche Diagnostics, Indianapolis, IN). Briefly, each 50- μ L reaction contained 10 ng of DNA, 2.5 U of polymerase, 1.5 mM $MgCl_2$, 200 mM deoxynucleoside triphosphates, and 100 nM primers fD1 and R530 (amplicon 1) and F530 and rP2 (amplicon 2). Amplification was performed on an ABI 9700 thermocycler (Applied Biosystems, Foster City, CA) using the following program: 94°C for 5 minutes, followed by 35 cycles of 94°C for 15 seconds, 50°C for 15 seconds, and 72°C for 90 seconds, with a final single extension of 72°C for 5 minutes and then held at 4°C. Amplicons were generated using primers previously described^{9,10} and were sequenced according to Morey et al¹¹ with a modified set of primers (supplemental Methods).^{10,12} Sequence data were assembled using Geneious v. 7.0.4 (Biomatters, Auckland, New Zealand), resulting in a trimmed

sequence length of 909 base pairs. The resulting sequence was compared with the publicly available database GenBank to identify sequences of related taxa.¹³ BLAST results indicated matches to clinically relevant strains of *C. canimorsus* sequences with pairwise identities between 99.8% and 98.2%.

Immunohistochemistry and real-time quantitative PCR for TM and E-selectin

This study was approved by the Partners Healthcare Institutional Review Board with a waiver of informed consent because of the use of deidentified discarded specimens obtained in the course of clinical care. The following antibodies were used for immunohistochemistry: E-selectin (Neuromics; M020039), intercellular adhesion molecule 1 (ICAM-1; Sigma; HPA004877), and TM (Abcam; ab6980). Grading of protein expression was performed by an author (O.P.) who was blinded to the time point and source of the specimens. All vessels in skin biopsies were analyzed for the presence of protein staining. Stain intensity and degree of vascular involvement were assessed using an arbitrary scale from 0 (no staining) to 3 (strong staining involving all vessels).

Real-time quantitative PCR for TM and E-selectin

RNA was extracted from formalin-fixed paraffin embedded skin biopsies using a RecoverAll Total Nucleic Acid Isolation Kit, per the manufacturer's instructions. RNA was quantitated using a NanoDrop 1000 Spectrophotometer. First-strand cDNA synthesis was performed using a SuperScript III kit (Invitrogen), and second-strand synthesis was performed using a NEBNext second strand synthesis kit (New England Biolabs). TaqMan probe and primer sets were obtained for the following genes: E-selectin, TM, GAPDH, and actin (Life Technologies, catalog numbers Hs00950401_m1, Hs00264920_s1, Hs02758991_g1, and Hs01060665_g1, respectively). TaqMan PCR was performed with 1 μ L of input DNA using a Prism 7900HT machine and Universal TaqMan Master Mix II (both from Life Technologies), per the manufacturer's directions. The relative fold difference in E-selectin and TM were calculated compared with the housekeeping gene GAPDH, as previously described, using the $\Delta\Delta$ Ct method.

Results and discussion

Response to PC concentrate and anticoagulation

After an initial bolus infusion of PC (100 IU/kg) and AT concentrate (3100 U), these agents were readministered every 12 to 24 hours through day 10 to maintain values >70%, and the patient also received 2 to 4 U of fresh-frozen plasma daily during the first week of hospitalization (see supplemental Table 2 for the relative PC content of different products). Markers of coagulopathy rapidly corrected, followed by improvement in the patient's lactic acidosis over 7 days (Figure 1C-D). Therapeutic anticoagulation with heparin proved difficult, likely due to the severity of inflammation present.¹⁴ Convalescent levels of circulating anticoagulant proteins, checked 86 days after initial presentation, were all within the normal range (PC, 151%; protein S, 115%; AT, 118%).

Expression of endothelial TM

Loss of TM at the endothelial surface is thought to be an early event in PF.⁵ We performed immunohistochemistry on serial skin biopsy

specimens taken on days 0, 3, 28, and 231 (day 0 denotes the first day of presentation) to characterize the kinetics of TM loss (Figure 2A-E). Coagulation-related laboratory results corresponding to each time point are shown in supplemental Table 3. Specimens were also examined for E-selectin (Figure 2F-J) and ICAM-1 (Figure 2K-O), markers of endothelial cell activation and viability, respectively. At presentation, our patient's endothelial TM protein expression was similar to that of normal control specimens (Figure 2P). Surface TM protein expression remained detectable through the third day of hospitalization before declining to an undetectable level. In contrast, E-selectin protein expression rose threefold between days 1 and 3 and remained elevated throughout the first 28 days of hospitalization, consistent with an ongoing endothelial inflammatory response. Protein expression of TM and E-selectin returned to baseline levels during convalescence (day 231).

TM loss is posttranscriptional

Previous in vitro studies have suggested 2 mechanisms for TM loss during inflammation: cleavage of the protein from the endothelial surface^{5,15} and downregulation of TM gene expression in response to inflammatory cytokines.¹⁶ We quantified TM and E-selectin messenger RNA transcript levels in our patient by real-time quantitative PCR of serial skin biopsy specimens (Figure 2Q). We found a 4.2-fold increase in TM transcript levels relative to GAPDH expression at the time of presentation, followed by a gradual decline to baseline levels through day 28. In contrast, E-selectin transcript levels were normal at presentation, increased 2.7-fold by day 3, and decreased thereafter to baseline levels. These data indicate that TM expression is significantly upregulated in response to inflammation and that endothelial TM loss during the early stages of PF is a posttranscriptional event. At convalescence, expression levels of TM and E-selectin in our patient were modestly lower than those of a healthy subject (Figure 2R).

In patients with sepsis complicated by PF, failure of the TM-PC system is hypothesized to be central to the disease process.^{3,5} This model posits that loss of TM early in the disease course impairs activation of endogenous and therapeutically administered PC. However, small trials and retrospective case series¹⁷⁻²⁰ have consistently suggested that PF patients benefit from supplemental PC, particularly in the prevention of amputations.⁶ Although results from this single case report should be interpreted with caution, our data support the idea that, during the hyperacute phase of PF, endothelial TM remains intact, and PC is consumed extremely rapidly (a process that requires the presence of functional TM). Endothelial TM loss occurs later and is a secondary event. Therefore, it is overwhelming thrombin generation and the resultant depletion of PC, not the absence of endothelial TM, that is the primary driver of microvascular thrombosis in PF. In our patient, PC concentrate was undoubtedly functioning in concert with other treatments that we administered, and the specific contribution of PC to his recovery is difficult to delineate with certainty. Additionally, due to the lack of sufficient biopsy tissue, we were unable to assay for other endothelial proteins (eg, endothelial PC receptor) that may have played a role in the disease process. We hypothesize that PC supplementation, together with other anticoagulant therapies, served to mitigate the effects of

Figure 2. Time course of expression of TM and E-selectin. (A-E) Immunohistochemical staining for TM on blood vessels (arrowheads) in skin biopsy specimens taken at various time points during the patient's hospitalization (days 1-28) and at convalescence (day 231) compared with a normal control specimen (scale bars, 20 μ m). Staining for E-selectin, a marker of endothelial cell activation (F-J), and for ICAM-1, a marker of cell viability (K-O), in the same biopsy specimens. (P) Quantification of the immunohistochemical staining of TM, E-selectin, and ICAM-1 in (A-O), represented as fold change from day 0. Expression of TM and E-selectin returned to baseline at day 231. (Q) TM and E-selectin transcript levels normalized to GAPDH expression at each time point, as determined by real-time quantitative PCR. (R) Quantification of the patient's convalescent TM and E-selectin transcript levels as a ratio of the transcript levels in a healthy subject.

prothrombotic stimuli from the microorganism as antibiotics gradually cleared the infection.

It should be noted that a large randomized trial of recombinant human APC (drotrecogin alfa) in unselected patients with septic shock failed to show benefit.²¹ The discrepancy between these results and those from smaller experiences that support the use of supplemental PC in PF may be due to the clinical heterogeneity of "severe sepsis," which includes patients without thrombotic DIC. Clinical trials of PC repletion combined with aggressive anticoagulation in patients with bona fide PF will be required to assess the efficacy of this approach.

In this article, we provide a mechanistic explanation for the therapeutic role of PC concentrate in PF by showing that TM transcription is upregulated in response to infection, and TM protein can persist at

the endothelial surface for up to 72 hours after initial presentation in some patients. This observation is consistent with previous work demonstrating that infused PC is rapidly converted to APC in PF patients²² and suggests that supplemental PC can be effective, even after patients present with apparent failure of the TM-PC pathway.

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Authorship

Contribution: P.K.B., N.L., R.S., K.E.S., A.S., and J.-A.L. conceived and designed the study and cared for the patient; P.K.B., A.R., O.P., A.B., F.D., J.M., B.H., B.R., and M.C. performed experiments and

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