Hemostatic and protein C pathway dysfunction in the pathogenesis of experimental cerebral malaria

by Niamh O'Regan, Kristina Gegenbauer, Eimear M. Gleeson, Kenji Fukudome, Jamie M. O'Sullivan, Clive Drakeford, Niall Dalton, Alain Chion, Teresa M. Brophy, Owen P. Smith, Roger J.S. Preston, and James S. O’Donnell

Received: December 2, 2021.
Accepted: April 8, 2022.


Publisher’s Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
Hemostatic and protein C pathway dysfunction in the pathogenesis of experimental cerebral malaria.

Niamh O’Regan¹, Kristina Gegenbauer¹, Eimear M. Gleeson¹,², Kenji Fukudome³, Jamie M. O’Sullivan¹,⁴, Clive Drakeford¹, Niall Dalton¹, Alain Chion¹,⁴, Teresa M. Brophy¹, Owen P. Smith²,⁵, Roger J.S. Preston²,⁶ and James S. O’Donnell²,⁴,⁶

¹ Haemostasis Research Group, Institute of Molecular Medicine, Trinity Centre for Health Sciences, St James’s Hospital, Trinity College Dublin, Ireland.
² National Children’s Research Centre, Our Lady’s Children’s Hospital, Crumlin, Dublin.
³ Department of Life Science, Saga University Organization for General Education, Saga, Japan.
⁴ Irish Centre for Vascular Biology, School of Pharmacy & Biomolecular Sciences, Royal College of Surgeons in Ireland, Dublin 2, Ireland.
⁵ Haematology Dept, Our Lady’s Children’s Hospital, Dublin, Ireland.
⁶ National Coagulation Centre, St James’s Hospital, Dublin, Ireland.

Running Title: Protein C pathway modulates ECM pathogenesis
Text word count: 1394
Figure count: 2
Reference count: 15
Scientific Section: LETTER TO THE EDITOR

ACKNOWLEDGEMENTS
This work was supported by funds from the NIH for the Zimmerman Program (HL081588); a Science Foundation Ireland Principal Frontiers for the Future (FFP) award (20/FFP-A/8952), a Health Research Board Investigator Lead Project Award (ILP-POR-2017-008) and a National Children’s Research Centre Project Award (C/18/1).

Author Contributions
N.O’R., K.G. E.M.G, J.M.O.S., C.D., N.D., A.C., and T.M.B., performed experiments; N.O’R., K.G. E.M.G, J.M.O.S., K.J., T.M.B., O.P.S., R.J.S.P. and J. S. O’ D. designed the research and analyzed the data. All authors were involved in writing and reviewing the paper.

Data sharing statement
All original data and protocols can be made available to other investigators upon request.
Disclosures
Roger Preston has received research grant funding awards from Novo Nordisk Daiichi Sankyo and Bayer.

James O'Donnell has served on the speaker’s bureau for Baxter, Bayer, Novo Nordisk, Boehringer Ingelheim, Leo Pharma and Octapharma. He has also served on the advisory boards of Baxter, Bayer, Octapharma CSL Behring, Daiichi Sankyo, Boehringer Ingelheim and Pfizer. James O'Donnell has also received research grant funding awards from Baxter, Bayer, Pfizer and Novo Nordisk.

Editorial correspondence should be addressed to:
Prof. James O'Donnell, Irish Centre for Vascular Biology,
Royal College of Surgeons in Ireland,
123 St. Stephen's Green, Dublin 2, Ireland.
Tel +353 (1) 416 2141; Fax +353 (1) 410 3570;
e-mail jamesodonnell@rcsi.ie
Accumulating data suggest that hemostatic dysfunction contributes to *Plasmodium falciparum* malaria pathogenesis.(1) In addition, specific mechanisms through which the protein C pathway modulates *P. falciparum* pathogenesis have been described.(1) We hypothesized that the anticoagulant and anti-inflammatory activities of recombinant activated protein C (APC) may possess therapeutic value in the setting of cerebral malaria CM. To address this hypothesis, we assessed hemostatic parameters in an established murine model of experimental cerebral malaria (ECM), and using the same model, investigated the ability of recombinant APC to ameliorate ECM. In keeping with findings in patients with severe *P. falciparum* malaria, we observed that dysregulated thrombin generation and protein C pathway dysfunction were both late features of ECM. Furthermore, pretreatment with a monoclonal anti-EPCR antibody that blocks protein C/APC binding prior to *P. berghei* inoculation significantly reduced overall survival. Conversely, mice treated with recombinant APC exhibited a marked attenuation in clinical ECM progression and parasitemia, in parallel with a significant increase in overall survival. All together, these findings confirm that hemostatic and protein C pathway dysfunction are both consistent features in human and ECM, and demonstrate for the first time a role for recombinant APC in reducing clinical progression and mortality in ECM.

Cerebral malaria (CM) is a life-threatening complication of *Plasmodium falciparum* infection characterized by ataxia, seizures, altered consciousness and coma. Although CM is associated with significant mortality, the biological mechanisms underlying its pathogenesis remain poorly defined. Significant coagulation cascade activation including elevations in levels of fibrin degradation products (FDPs) and thrombin-antithrombin (TAT) complexes are common in patients with *P. falciparum* infection.(2, 3) Furthermore, Moxon et al recently described overt DIC in 19% of children with retinopathy-positive CM.(3) Together, these findings suggest that hemostatic dysfunction may contribute to malaria pathogenesis. This hypothesis is supported by the observation that coagulation activation correlates with peripheral blood parasitemia levels, and is inversely-related to overall survival.(4, 5) Moreover, microvascular fibrin deposition has also been reported in postmortem studies of patients with fatal CM.(6)

A number of molecular mechanisms through which *P. falciparum* infection triggers coagulation activation have been described.(1) Recent studies have also highlighted specific mechanisms through which the protein C pathway influences *P. falciparum* pathogenesis.(6, 7) Importantly, both thrombomodulin (TM) and the endothelial protein C receptor (EPCR) can bind to PfEMP1 expressed on *P. falciparum* IE surfaces,(7, 8) thereby facilitating infected
erythrocyte (IE) cytoadhesion to endothelial cells (EC). PfEMP1 binding to EPCR also limits generation of anticoagulant activated protein C (APC), and inhibits EPCR-dependent PAR1-mediated protection of EC barrier integrity.\(^9\), \(^10\) The typical late clinical presentation of patients with CM makes it difficult to determine whether hemostatic dysfunction directly contributes to the pathogenesis of CM, or whether coagulation activation merely constitutes a secondary epiphenomenon. Therefore, in this study we sought to further investigate the role of coagulation activation and the protein C pathway in malaria pathogenesis using an established murine model of ECM, in which C57BL/6J mice were infected with \(P.\) \(berghei\) ANKA.\(^{11}\)

We have previously described in detail the murine experimental cerebral malaria (ECM) model. All mouse experiments were performed in compliance with the Irish Medicines Board and approved by the Trinity College Dublin BioResource Ethics Committee. Mice aged 8 to 10 weeks were infected by intraperitoneal injection of \(2 \times 10^6\) \(P.\) \(berghei\) ANKA. Following inoculation, malaria progression was monitored using a previously validated ECM clinical scoring system.\(^{11}\) \(P.\) \(berghei\) parasitemia levels were monitored by Giemsa-stained thin blood smears. Platelet counts were measured using a Sysmex haematology analyser (KX-21N). To prepare platelet-poor plasma, blood samples were centrifuged at 1500 g for 15 min at 20°C, aliquoted and stored at -80°C until use. Murine APTT was determined using a commercial kit (C.K. Prest, Stago) and time for clot formation determined using Amelung KC4 Micro Clinical Coagulation Analyzer (Amelung, Trinity Biotech, Ireland). Similarly, PT was determined using Hemosil recombiplastin 2G according to the manufacturer’s guidelines. Plasma levels of thrombin-antithrombin (TAT) complexes, soluble thrombomodulin (sTm) and soluble endothelial cell protein C receptor (EPCR) were quantified using specific commercial ELISAs (Abcam, Cambridge, UK and R&D Systems, UK) according to manufacturer’s instructions. To study the role of EPCR, ECM progression was assessed in mice pretreated with 50\(\mu\)g of an EPCR blocking antibody RCR-16,\(^{12}\) or isotype control antibody, immediately prior to \(P.\) \(berghei\) infection. Recombinant murine APC was generated, expressed, and purified as previously described.\(^{13}\) Consistent with previous studies assessing the anti-inflammatory properties of recombinant APC in murine endotoxemia models,\(^{14}\) mice were treated with \(10\mu\)g mAPC administered intravenously immediately prior to \(P.\) \(berghei\) infection. Subsequently another \(10\mu\)g of mAPC was administered 4 hours post-infection. Alternatively, mice were treated with two \(10\mu\)g mAPC boluses administered four hours apart on Day+3 following \(P.\) \(berghei\) inoculation.

Progressive clinical features similar to those observed in human CM were evident in mice from Day +3 following infection (Fig. 1A). Peripheral \(P.\) \(berghei\) parasitemia levels increased progressively (Fig. 1B), and mice typically died within 7-10 days. In keeping with previous findings in patients with severe \(P.\) \(falciparum\) malaria, a significant increase in APTT was
observed by Day +5 following *P. berghei* inoculation (Fig. 1C). (2) Again in keeping with human studies, (3) no significant changes in either PT or murine fibrinogen levels were seen through the course of *P. berghei* infection (data not shown). Nevertheless, by Day +5 after inoculation, median plasma TAT levels were increased approximately 2.5 fold (2.0ng/ml at Day 0 versus 5.5ng/ml at Day +5; \( P<0.01 \)) (Fig. 1D), which was similar in magnitude to the increase in plasma TAT levels observed in patients with CM. (2, 4, 6) In contrast to the early increase in plasma VWF levels that occurs within 24 hours following *P. berghei* infection, (11) plasma TAT levels remained normal for 72 hours post-infection. Murine plasma protein C and antithrombin levels were not significantly reduced during *P. berghei* infection (data not shown). However, again as reported in previous findings in children infected with *P. falciparum*, (3, 6) plasma levels of both sTM and sEPCR were significantly elevated in C57BL/6J mice following *P. berghei* infection (sTM 6.5ng/ml at Day 0 versus 21.6ng/ml at Day +5; \( P<0.001 \) and sEPCR 0.99ng/ml at Day 0 versus 3.50ng/ml at Day +5; \( P<0.001 \)) (Figs. 1E and 1F). In keeping with the hypothesis that hemostastic dysfunction is a relatively late feature in ECM, the increases in plasma sTM and sEPCR were not observed until Day +5 following inoculation. Collectively, these findings demonstrate that dysregulated thrombin generation represents a consistent feature of both human and experimental murine CM. Critically however, unlike the acute EC activation that represents an early hallmark in both murine and human malaria, hemostatic and protein C pathway dysfunction both develop at a much later stage.

To investigate whether protein C pathway dysfunction contributes to ECM pathogenesis, mice were pretreated with a monoclonal anti-EPCR antibody (RCR-16) previously shown to block protein C/APC binding (12), or an isotype control antibody, immediately prior to *P. berghei* inoculation. We observed significantly reduced overall survival (\( P<0.05 \)) in the cohort of mice treated with RCR-16 (Fig. 2A), suggesting that EPCR-dependent APC generation and/or signaling is important for controlling ECM development. Case studies involving a small number of patients with severe *P. falciparum* malaria treated with APC have reported variable effects. (15) Consequently, we further investigated whether administration of recombinant mAPC influenced ECM pathogenesis. Mice were treated with 10μg mAPC immediately prior to *P. berghei* infection, and a subsequent second 10μg mAPC dose was administered 4 hour later. Mice treated with recombinant mAPC exhibited a mild but significant reduction in parasitemia at Day+4 (Fig. 2B; \( P<0.001 \)). In contrast however, the APC-treated mice demonstrated significantly attenuated clinical ECM progression (Fig. 2C; \( P<0.001 \)) and weight loss (Fig. 2D; \( P<0.001 \)). Furthermore, mAPC administration also caused a significant increase in overall survival (Fig. 2E; \( P<0.001 \)). To investigate whether mAPC administered later in the course of ECM can still influence clinical progression, the effect of administering mAPC on Day+3 was investigated. Once again, clinical progression
(Fig. 3A) and overall survival (Fig. 3B) were both marginally improved in mice treated with mAPC compared to controls (P<0.05). Interestingly, however, the magnitude of this effect was markedly less than that observed in mice treated with mAPC on Day 0. This attenuated efficacy of APC administered later in the disease course is in keeping with the concept that CM is associated with progressive shedding of EPCR and TM from EC surfaces.(10)

In conclusion, our findings demonstrate that hemostatic and protein C pathway dysfunction are both consistent features in human and experimental murine CM. We also show for the first time that recombinant mAPC markedly reduces clinical progression and overall mortality in ECM. Further studies will be required to elucidate the molecular mechanisms through which APC modulates ECM pathogenesis, together with the optimal APC dosing and treatment regimen. Nevertheless, given the significant morbidity and mortality that are still associated with CM, novel adjunctive therapies to limit vascular dysfunction and slow disease progression are urgently required.
REFERENCES


FIGURE LEGENDS

Figure 1. Hemostatic and protein C pathway dysfunction constitute late features of ECM.

(A) Following I.P. inoculation with $2 \times 10^6$ *P. berghei* ANKA parasites, ECM progression was followed in WT C57BL/6J mice (n=12) using a validated clinical scoring algorithm. Results presented represent the mean values ± SEM unless otherwise stated. (B) Following *P. berghei* infection, peripheral blood parasitemia levels were determined each day using Giemsa-stained smears (n=12). By Day +5 following *P. berghei* infection, murine APTT levels (C) and plasma TAT complex levels (D) were both significantly increased (*P<0.05, **P<0.01, ***P<0.0001 respectively). Similarly, plasma levels of sEPCR (E) and sTM (F) were also both significantly elevated by Day +5. Data are expressed as mean values ± standard error of the mean (SEM). To assess statistical differences, data were analyzed using Student's unpaired 2-tailed t test. ECM clinical scoring data were assessed by two-way ANOVA analysis.

Figure 2. Recombinant APC significantly attenuates clinical progression and markedly improves overall survival in ECM.

(A) To determine if EPCR plays a role in ECM pathogenesis, mice were pretreated with the EPCR blocking antibody RCR-16 or an isotype control antibody prior to infection with $2 \times 10^6$ *P. berghei* ANKA parasites. Twelve mice were treated in the RCR-16 and 11 in the isotype control groups. Overall survival was significantly reduced in mice treated with RCR-16. To investigate whether recombinant mAPC administration influences ECM progression, mice were pretreated with 10μg mAPC immediately prior to *P. berghei* infection and a second 10μg mAPC dose was administered 4 hour later. Twelve mice were treated in the mAPC and control groups. Mice treated with recombinant mAPC exhibited (B) mildly reduced parasitemia at Day +4, (C) attenuated clinical ECM progression (D) reduced weight loss and (E) significantly increased overall survival (*P<0.05, **P<0.01, ***P<0.001).

Figure 3. Recombinant APC later in the course of ECM has only mild effects on clinical progression and survival.

To investigate whether mAPC later in the course of ECM can still influence clinical progression, two doses of mAPC were administered on Day+3 following *P. berghei* inoculation. Once again, (A) clinical progression and (B) overall survival were both mildly improved in mice treated with mAPC compared to controls. Mouse survival data were compared using a Log-rank (Mantel-Cox) Test.