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Rapid activation of endothelial cells enables *P. falciparum* adhesion to platelet decorated von Willebrand factor strings

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Abstract

During *Plasmodium falciparum* malaria infections, von Willebrand factor (VWF) levels are elevated, post-mortem studies show platelets co-localised with sequestered infected erythrocytes (IE) at brain microvascular sites, while *in vitro* studies have demonstrated platelet-mediated IE adhesion to TNF-activated brain endothelium via a bridging mechanism. This current study demonstrates how all these observations could be linked through a completely novel mechanism whereby IE adhere via platelet decorated ultra-large VWF strings on activated endothelium. Using an *in vitro* laminar flow model, we have demonstrated tethering and firm adhesion of IE to the endothelium specifically at sites of platelet accumulation. We also show that an IE pro-adhesive state, capable of supporting high levels of binding within minutes of induction can be removed through the action of the VWF protease ADAMTS-13. We propose that this new mechanism contributes to sequestration both independently of and in concert with current adhesion mechanisms.

Keywords

VWF; malaria; adhesion; *P. falciparum*; platelets

Introduction

P. falciparum malaria remains a scourge in many developing tropical regions of the world where it claims the lives of hundreds of thousands of children every year. A defining feature of one of the most severe forms of the disease, cerebral malaria (CM), is the sequestration of infected erythrocytes to endothelium in brain post-capillary venules. Adhesion is mediated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) to various host receptors such as CD36 1, to which almost all paediatric isolates bind 2. With little or no CD36 being expressed on brain endothelium, it had been assumed that CD36 adhesion was not involved in CM. A

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number of observations have challenged this assumption. Firstly, analysis of human post mortem brain sections from fatal CM, severe malarial anaemia (SMA) and non-malarial cases showed a strong correlation between platelet accumulation, which generally co-localised with malaria pigment, and disease severity 3. The possible mechanism underlying this association was further developed *in vitro*, where it was shown that platelets could act as a bridge between activated brain endothelium not expressing CD36 and IE that only bind to CD36 [4].

Endothelial activation is classically achieved using cytokines such as TNF, which induce the relatively slow process (hours) of *de novo* protein synthesis. We proposed a mechanism for rapid adhesion via the multimeric protein VWF 5, that mediates platelet adhesion to sites of vascular injury. The mature ultra-large, and physiologically most active, form of VWF is stored in specialised secretory vesicles in endothelial cells (EC) called Weibel-Palade (WP) bodies [6]. When released, these large VWF multimers unravel under flow to form platelet decorated strings that are cleaved and regulated by the endogenous plasma protease ADAMTS-13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13)7. Measurements of clinical plasma samples show that levels of VWF and its propeptide are elevated in malaria and are correlated to disease severity 5. Furthermore, a significant reduction in plasma ADAMTS-13 activity has been recently reported 8. However given the established limitations of current *in vitro* ADAMTS-13 assays [8-9], the translational significance of this reduction has not been defined. In addition, it remains unclear whether the high levels of circulating ultra-large VWF multimers present in children with severe *P. falciparum* malaria play a direct role in mediating the underlying pathophysiology.

Here we provide the first evidence that EC activation, and specifically the regulated release of VWF, is capable of mediating the rapid adhesion of IE's via CD36 dependent platelet bridging.

Materials and methods

P. falciparum strain ITO4-C24 culturing [10], and isolation of HUVEC[11] and fresh platelets[12] were performed as previously described. Platelet and IE binding flow adhesion assays were performed on confluent activated HUVEC monolayers.

Complete materials and methods are available in supplemental data (available on the Blood website; see the Supplemental Materials link at the top of the online article).

Results and Discussion

Mature IE bind to platelets on ultra-large VWF strings via CD36

Platelet decorated strings formed from ultra-large VWF multimers are large macroscopic structures visible under low-power microscopy that only form on activated endothelium [7; 13]. Using fluorescently labelled trophozoites, the mature adhesive form of the parasite, binding of IE's to platelet decorated strings was clearly visible (Figure 1A, Video S1 and S2). To confirm that these interactions were parasite mediated, the same experiment was performed replacing the trophozoite culture with uninfected erythrocytes or immature (non-adhesive) ring-stage parasites with only minimal levels of adhesion observed in both cases (Figure 1B). Cytoadherence by the strain ITO-C24 is known to be CD36-dependent, and as expected binding was completely inhibited in the presence of an anti-CD36 monoclonal antibody (Figure 1B). From these experiments binding was confirmed to be CD36 dependent and only possible in the mature trophozoite parasite form, which unlike ring stages express PfEMP-1, the protein mainly responsible for parasite adhesion and known to contain binding sites for CD36 [14].

To further characterise binding we used a monoclonal antibody to selectively block the VWF A1 domain, which is responsible for platelet binding via GP-Ib-IX-V. As predicted by our

original model [5], a dose-dependent reduction in the number of IE-bound strings was observed when blocking the A1 domain, while no effect was seen with the control (Figure 2A). In both cases the binding ratio of IE to platelets was unaffected, confirming that IE binding is proportional to the number of bound platelets. We concluded that IE are binding to platelets via CD36 and are not interacting directly with the VWF strings.

ADAMTS-13 has previously been shown to remove strings from activated endothelium *in vitro* [15]. In keeping with previous reports [7], the addition of 5 nM ADAMTS-13 both cleared tethered IE from the endothelium (Figure 2B), and ablated string formation if used to pre-treat the endothelium. Interestingly we observed a dose-dependent increase in the number of adherent platelet strings with a progressive reduction in recombinant ADAMTS-13 concentration (5 nM to 0.1 nM; data not shown). At this stage it is unclear how much string associated sequestration is necessary in our model to contribute to *in vivo* malaria pathology.

A dynamic model

Interestingly, while VWF strings are clearly tethered to the endothelium, the majority of the strings appeared to be distanced from, and not in direct contact with, the endothelial monolayer along much of their length. Free rotation of string sections, including bound IE was often observed. The dynamic detachment and reattachment of strings under flow, led to the concentration of strings into a foci or mesh (Video S2) as have previously been described *in vivo* in ADAMTS-13 −/− mice [16].

Implications in disease pathogenesis

This study establishes a new mechanism by which IE can sequester in vascular beds which do not express appropriate IE receptors. In contrast to classical activation pathways involving *de novo* protein synthesis, we show that a pro-adhesive surface is generated almost instantly through the regulated release of VWF and platelet tethering. Other clinical and experimental correlates suggest this novel model of adhesion could be of critical importance in malaria pathogenesis. Firstly, EC activation and specifically the release of WP body contents occurs very early on in experimental human infections before an individual becomes smear positive [17]. Although the basis of EC activation remains unknown, inhibition of histamine mediated signalling protects mice from CM [18-19], while *P. falciparum* is known to secrete a functional histamine release factor [20]. Finally, ADAMTS-13 levels are reduced, whilst there is an increase in circulating ultra large VWF multimers in severe malaria [8].

The potential role that this new model could play in malaria disease progression, either early on, throughout infection, or late on, is striking, and suggests that VWF is not just a marker of malaria disease severity, but rather is likely to be a primary player in the pathogenesis of malaria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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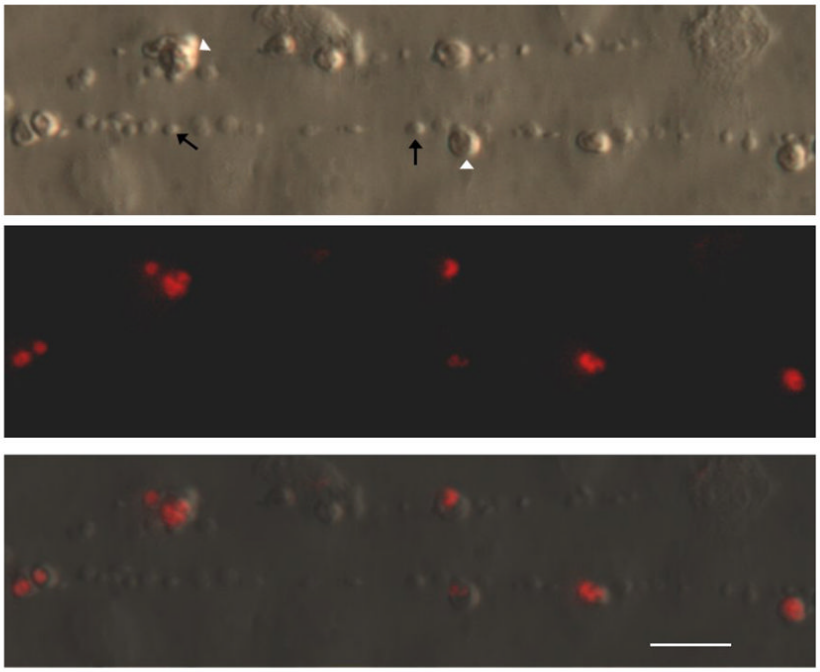

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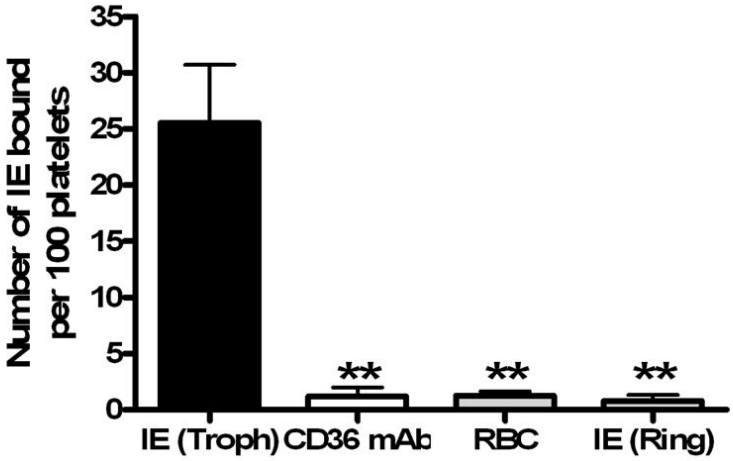

Figure 1. Only mature *P. falciparum* infected erthryocytes are able to bind to platelet decorated VWF strings on endothelial cells stimulated with histamine

(A) EtBr labelled parasites (Δ) are the only RBC species binding to the platelet (→) decorated VWF strings. Flow direction is from left to right. (B) Trophozoite (Troph) IE binding to strings (n=11) was significantly blocked by a CD-36 monoclonal antibody (n=4). Similarly, binding was not observed in the presence of uninfected RBC (n=4) or with ring stage IE (n=3). The bar shown is 20 μm, ** $p < 0.005$.

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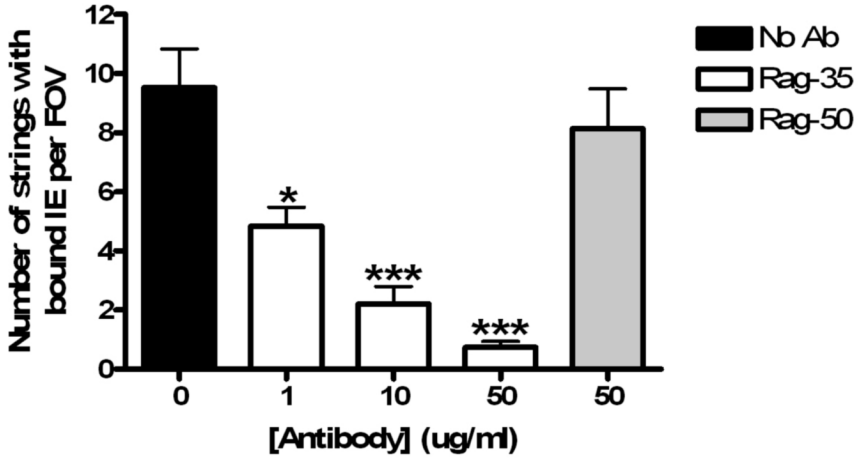

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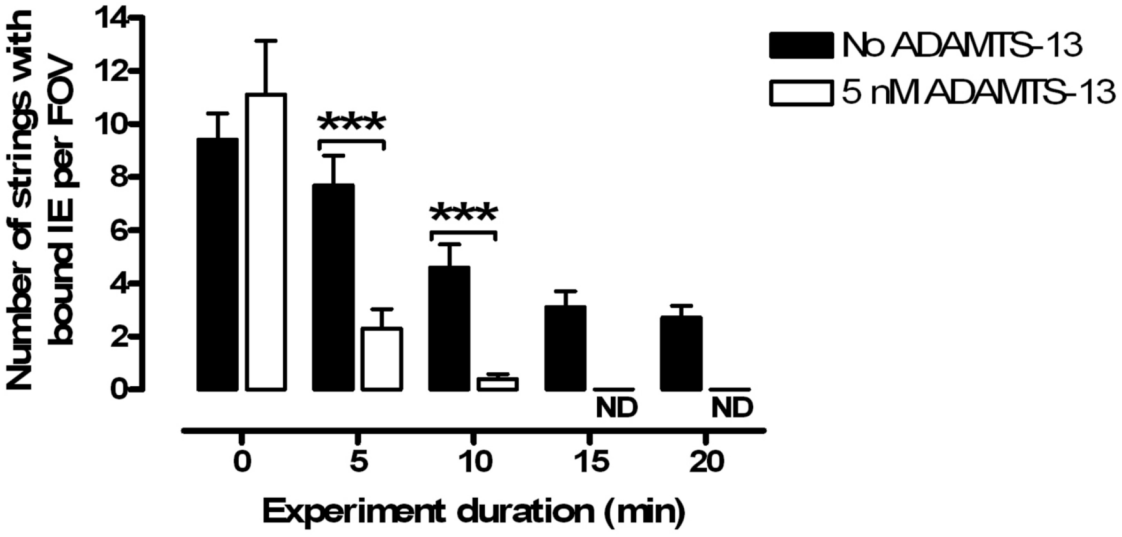

Figure 2. IE adhesion to endothelium is dependent on binding of platelets to VWF strings, which can be cleaved by recombinant ADAMTS-13

(A) Rag-35 blocks platelets binding to VWF strings in a dose-dependent manner (n=3). The control antibody (Rag-50) does not inhibit binding (n=2). (B) Strings can be rapidly cleaved from the endothelium by ADAMTS-13 (added at t = 5 min) in the wash media (n=3). All strings were removed after 15 min. ND = no detectable strings, * $p < 0.05$, *** $p < 0.0001$.