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Plasmodium falciparum-infected erythrocytes induce Tissue Factor expression in endothelial cells and support the assembly of multimolecular coagulation complexes

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Summary

Background—*Plasmodium falciparum* malaria infects 300–500 million people every year causing 1–2 million deaths annually. Evidence of a coagulation disorder, activation of endothelial cells (EC) and increase in inflammatory cytokines are often present in malaria.

Objectives—We have asked whether parasitized red blood cells (pRBC) interaction with EC induces Tissue Factor expression *in vitro* and *in vivo*. The potential of phosphatidylserine-containing pRBC to support the assembly of blood coagulation complexes was also investigated.

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Authors` Contributions

I. Francischetti, K. Seydel, R. Monteiro, J. Ward, study design, writing the paper, performing research, data analysis, vital reagents; C. Erexson, performing research; A. Noronha, G. Ostera; vital reagents; S. Kamiza, data analysis; R. Whitten, M. Molyneux, T. Taylor, data analysis, writing the paper, vital reagents.

Results—We demonstrate that mature forms of pRBC induce functional expression of tissue factor (TF) by endothelial cells (EC) *in vitro* with productive assembly of the extrinsic Xnase complex and initiation of the coagulation cascade. Late stage pRBC also support the prothrombinase and intrinsic Xnase complex formation *in vitro*, and may function as activated platelets in the amplification phase of the blood coagulation. Notably, *postmortem* brain sections obtained from *P. falciparum*-infected children who died from Cerebral Malaria and other causes display a consistent staining for TF in the EC.

Conclusions—These findings place TF expression by endothelium and the amplification of the coagulation cascade by pRBC and/or activated platelets as potentially critical steps in the pathogenesis of malaria. Furthermore, it may allow investigators to test other therapeutic alternatives targeting TF or modulators of EC function in the treatment of malaria and/or its complications.

Keywords

endothelial cell; malaria; prothrombinase; platelets; *Plasmodium falciparum*; Tissue Factor

P. falciparum malaria manifests as a spectrum ranging from asymptomatic infection through mild, severe, and fatal disease. Uncomplicated malaria is an acute febrile illness characterized by fever, chills, and headache [1,2]. In some patients, particularly non-immune individuals, malaria infection can become complicated by severe anemia and cerebral malaria (CM)[1,2]. Clinically, the term "cerebral malaria" is used to describe a syndrome consisting of decreased consciousness not attributable to other causes in a patient with *P. falciparum* parasitemia [2, 3]. These comatose patients often present with metabolic acidosis, hypoglycemia, and anemia. Recovery can be rapid and is usually complete, with a low incidence of long-term neurologic sequelae [2,3]. Although various hypotheses have been proposed, the mechanisms of CM pathogenesis remain incompletely understood [1,3]. *Post mortem* studies in children [4] have shown that this disease is a heterogeneous syndrome in which the sequestration of parasitized red blood cells (pRBC) in cerebral microvessels is a consistent finding [4–6]. Sequestration is mediated by various parasite-derived molecules located on the erythrocyte membrane. These molecules, collectively referred to as PfEMP-1 [7], interact in a synergistic manner with various endothelial cell surface receptors such as CD36 [8] and ICAM-1 [9]. Expression of these host receptors appears to be modulated, at least in part, by tumor necrosis factor alpha (TNF-α) a cytokine which concentration is increased in the plasma of malaria patients and that may play a role in the pathogenesis of the disease [10].

Sequestration has also been associated with localized and widespread activation of endothelial cells in mild and fatal cases of malaria [4–6]. A procoagulant state has been identified in these same patient populations, characterized by thrombocytopenia [11], hemostatic alterations [12], and microparticle production [13]. Hemostatic derangements may be contributing to the disease progression and organ failure observed in severe disease [12]. A systemic response characterized by increased levels of circulating cytokines and soluble adhesion molecules has also been observed in malaria in general and in cerebral malarial in particular [10].

No link has yet been described between sequestration, a coagulation disorder, endothelial cell activation, and the production of inflammatory cytokines, all of which have been separately described as features of severe malaria pathology. We hypothesized that parasitized red blood cells (pRBC) activate endothelial cells (EC) to express tissue factor (TF), a 47-kD protein that initiates the clotting cascade [14,15] and is increasingly recognized at the interface of blood coagulation and inflammation [16,17]. TF binds to coagulation FVIIa leading to FXa and FIXa formation with subsequent thrombin generation [18,19]. In this paper we describe three novel aspects related to malaria pathogenesis: *i*) interaction of pRBC with cultured microvascular endothelial cells (MVEC) *in vitro* is accompanied by TF expression by EC, *ii*) parasitized RBC supports the assembly of the multimolecular coagulation complex *in vitro*, leading to

amplification of the coagulation cascade, and *iii*) we demonstrated the expression of TF in cerebral vascular endothelial cells of patients dying of cerebral malaria and patients with parasitemia who died of non-malarial causes. TF expression was not found in cerebral vascular tissue of subjects dying without malaria, suggesting that TF and subsequent hemostatic derangements may be contributing to malaria pathogenesis.

Materials and Methods

Culture of human dermal MVEC

MVEC were purchased from Clonetics (San Diego, CA) and grown in the presence of EBM-2 Plus as described [20] in detail in the Supplemental data.

Culture of *P. falciparum* parasites

Mycoplasma-free parasites (3D7) were thawed and initially grown in a 5% suspension of purified human O^+ RBC in RPMI 1640 medium as described in detail in the Supplemental data.

TF expression by pRBC

Assembly of extrinsic Xnase in MVEC was carried out using a discontinuous assay as described in detail in the Supplemental data.

ELISA for TF

Human TF antigen was quantitated in triplicates with an enzyme-linked immunosorbent assay (ELISA) using Imubind Tissue Factor ELISA kit (American Diagnostica) as described in detail in the Supplemental data.

Reverse Transcriptase-PCR

Total RNA was isolated from MVEC cultures incubated with pRBC using TRIzol. RT-PCR was performed as described in detail in the Supplemental data.

Multimolecular coagulation assembly

Prothrombinase and intrinsic Xnase assemblies by pRBC using a discontinuous assay [21] as described in detail in the Supplemental data.

Pathology and IHC for TF

All patients included in this analysis met the standard clinical case definition of cerebral malaria during life [4]. The brain sections were fixed in buffered 10% neutral buffered formalin and embedded in paraffin casts before histologic sections were prepared. IHC was performed using the labeled polymer method as described in detail the Supplemental data.

Statistical analysis

Experiments were performed three or four times in triplicates or quadruplicates, and results are expressed as means ± SEM. Statistical significance was determined using Student`s *t* test or ANOVA (Bonferroni posttest comparison) using GraphPad Prism 3.0 software (GraphPad Software., Inc., San Diego, CA). Significance was set at $p \leq 0.05$.

Results

pRBC induce TF expression and extrinsic Xnase complex assembly by MVEC *in vitro*

To identify whether the interaction between mature pRBC (late-trophozoites and schizonts) and MVEC could induce TF expression, MVEC monolayers were incubated with 2% hematocrit of uninfected RBC or late-stage pRBC (0–50% parasitemia). FXa generation was measured as an estimate of TF expression. Figure 1A shows that FXa generation increased in a time- and parasitemia-dependent manner, reaching levels of ~40 pM with 50% parasitemia after 8 hrs. Unparasitized RBC and/or Hepes-AlbuMAX media alone induce negligible increase of FXa production. Control incubation of MVEC with TNF-α (10 ng/ml in AlbuMAX medium, 8 hrs) generated 206.5 ± 26 pM (not shown).

A number of additional controls were performed to confirm whether FXa generation was specifically mediated by TF expression. Figure 1B shows that FXa generation was completely blunted by sheep anti-human polyclonal antibody against TF and by recombinant Ixolaris, a specific recombinant Tissue Factor Pathway Inhibitor (TFPI) from tick salivary gland [20]. No inhibition was attained with the specific thrombin inhibitor, hirudin. We then determined whether TF expression was associated with functional assembly of the extrinsic Xase complex. Figure 1B shows that FXa was not generated when FVIIa was replaced by dansyl-Glu-Gly-Arg-chloromethyl ketone-treated FVIIa (DEGR-FVIIa), a catalytic site-occupied FVIIa which interacts with TF but does not activate FX. Likewise, incubation of the supernatant with the specific FXa inhibitor — antithrombin/heparin — followed by addition of chromogenic substrate S-2222, was accompanied by 100% inhibition of chromogenic substrate hydrolysis. These experiments indicate that TF is specifically expressed on the surface of EC after incubation with pRBC, with productive assembly of the extrinsic Xnase complex leading to FXa production. Other controls were performed to rule out that TF expression was mediated by proinflammatory cytokines potentially released by activated MVEC or contaminating lipopolysaccharide (LPS). Figure 1B shows that a combination of anti-TNF-α and anti-interleukin-1β monoclonal antibodies (mAb) with polymyxin B did not affect pRBC-induced TF expression by MVEC. LPS contamination was below 0.05–0.025 EU/ml using the Limulus Ameboid Assay (data not shown). Figure 1B also shows that TF expression was not detected in experiments using Transwell plates, which do not allow physical contact between pRBC and MVEC but permit free exchange of macromolecules less than 1 μm between both compartments.

We confirmed the expression of TF using an enzyme-linked immunosorbent assay (ELISA) specific for human TF. Figure 1C shows that incubation of MVEC with 2% hematocrit (5–50% parasitemia) is accompanied by TF expression reaching concentrations as high as 271.8 ±12 pg/ml. Maximum TF expression was attained at 1–2% hematocrit, at 50% parasitemia (inset). In contrast, incubation of MVEC with Hepes-AlbuMAX medium or unparasitized RBC produced similar TF levels of ~35 pg/ml. When Transwell plates were used, TF detection was ~10% of control (6.3%, 8%, and 12% for each assay) under identical experimental conditions. In addition, Figure 1D shows that an increase of TF expression (~20% of control) was detected by ELISA when supernatants collected from pRBC (kept 6 h at 37°C, and centrifuged for 10 min at 0.1g) were added to fresh MVEC. TNF-α (10 ng/ml, diluted in AlbuMAX medium) induced 1,135 ± 155 pg/ml TF (not shown).

To determine whether increases in cell expression of TF were accompanied by changes in TF mRNA expression, MVEC were incubated with 2% hematocrit (0% or 50% parasitemia) or TNF-α (10 ng/ml) for 3 hours and mRNA was isolated for reverse transcriptase polymerase chain reactions (RT-PCR). Figure 1E shows that steady-state TF mRNA levels were undetectable when RBC or Hepes-AlbuMAX medium were used, but present when incubation

was performed with pRBC or TNF-α. As a control, the identity of the band was checked by cloning and sequencing and it was found to be identical to human TF (gi 135666).

Temporal expression of specific genes has been reported during parasite development [22], and sequestration is associated with mature forms of pRBC [4–6]. Therefore, we examined whether TF expression was associated with a specific parasite developmental stage. Figure 1F shows that TF expression was detected after incubation of EC with late trophozoites/schizonts but was only marginally detectable with ring and early-mid trophozoite stages. Our results also demonstrate that pRBC did not inherently support the extrinsic Xnase assembly in the presence of FVIIa and FX, in contrast to lipidated TF (not shown).

pRBC support the assembly of the prothrombinase *in vitro*

Recently, it has been shown that pRBC expose phosphatidylserine on their surface as intracellular parasites mature to late trophozoite and schizont stages [23]. Because phosphatidylserine (PS) is critical for the assembly of multimolecular blood coagulation complexes [18,19], we attempted to determine whether late-stages pRBC support prothrombinase complex formation. In a first set of experiments, pRBC were incubated with FVa, FXa, and Ca^{2+} (prothrombinase) followed by addition of prothrombin to start reactions. Figure 2A shows that RBC did not induce thrombin generation. In contrast, 0.2% hematocrit at 5% parasitemia slightly increased thrombin formation, whereas at 50% parasitemia, the thrombin concentration reached 60 nM after 6 min. Figure 2B shows that 50% parasitemia consistently supported thrombin formation at hematocrits as low as 0.025%, and Figure 2C shows that thrombin production is exquisitely sensitive to FVa, a critical cofactor for prothrombinase assembly [18,19]. Figure 2D confirms that thrombin formation is mostly detected when mature, but not younger forms of pRBC are used in the experiments.

pRBC support the assembly of the intrinsic Xnase *in vitro*

In a second set of experiments, pRBC (0.2% hematocrit, 0–50% parasitemia) was incubated with FVIIIa, FIXa, and Ca^{2+} (intrinsic Xnase) followed by addition of FX to initiate reactions. Figure 2E shows that pRBC induces FXa formation in a parasitemia-dependent manner, and Figure 2F shows that hematocrits as low as 0.025% are effective. The specificity of the intrinsic Xnase complex formation was estimated by increasing concentrations of FVIIIa, an indispensable cofactor in the intrinsic Xnase assembly [18,19]. We detected FXa production at FVIIIa concentrations as low as 1.25 U/ml (Figure 2G). Next, the specificity of prothrombinase and Xnase complexes were confirmed using annexin V, a specific PS binding protein [18,19]. Figure 2H depicts a dose-response inhibitory curve showing inhibition of thrombin and FXa formation with an IC_{50} around 2.5 nM; complete inhibition of enzyme formation was attained with 25 nM annexin V. Figures 2 thus show that PS is functionally expressed by pRBC.

TF expression in the EC from the brain of *P. falciparum* malaria patients

Brain slides from the frontal cortex of thirteen parasitemic children and 10 aparasitemic cases were used for immunohistochemistry (IHC) purposes using a specific mAb for TF and counterstaining with hematoxylin (see Methods). All parasitemic patients who met both clinical (in life) and pathological (at autopsy) case definition of CM were designated as “CM cases”. When another cause of death was identified in a parasitemic patient, they were designated as “parasitemic controls”. The patients who died without *P falciparum* infection in a malaria-free area were named “aparasitemic controls”. For the IHC assays, NovaRed dye was used to exclude any confounding effects from the brown malaria erythrocytic pigment (hemozoin) that is released by ruptured schizonts [1,2]. In some cases, polarized light that identifies hemozoin was employed to confirm that staining was associated with sequestration.

Figure 3 (A, C and E) shows three different CM cases with positive staining for TF in EC and associated with sequestration — hemozoin and/or pRBC were detectable using polarized light (Figure 3,B, D and F). Staining in EC was patchy and focal and found mostly in post capillary venules, or pre-capillary arterioles but not in capillaries. Figure 3G shows one CM case with intense TF expression in EC but no sequestration was observed using polarized light (Figure 3H). Figure 3I is a CM case showing TF staining of EC (arrow) and aggregates of extracellular hemozoin (open arrow), the latter indicating that schizonts have ruptured following sequestration (Figure 3J). Positive staining was present in the "parasitemic controls" with one exception. All "aparasitemic controls" were negative, with the exception of one Burkitt`s lymphoma case. Positive staining was not detected in mononuclear cells or in the subendothelial space, and was consistently not found in the absence of the primary antibody (not shown). Table 1 summarizes our findings.

Figure 4 shows the potential sources for TF expression in malaria (see Figure Legends).

Discussion

In patients with malaria, pRBC containing young, unpigmented parasites circulate in the peripheral blood for the first 18–24 h of the 48-h life cycle of *P. falciparum* [1,2]. pRBC containing the more mature, pigmented trophozoites and schizonts are rarely seen in the peripheral blood, because they are sequestered in the vascular beds of various organs [4–6] including the brain [4]. Although multiple pathogenic mechanisms have been suggested to explain the disease [10,24], including the "sequestration" [25] , and "cytokine" [26] hypotheses no unifying model has met with a consensus among investigators. However, four pathologic features have been described in severe malaria in general and in CM in particular: *i*) sequestration of mature forms of pRBC in different vascular beds [4–6]; *ii*) EC activation as detected by staining for adhesion molecules ICAM-1, VCAM-1, and E-selectin [6,10]; *iii*) an increase in the concentration of inflammatory cytokines such as TNF-α, IL-1β, IL-6, and others in plasma [10]; and *iv*) activation of the blood coagulation cascade [12,27–33]. Collectively, these features suggest that pRBC and EC are involved with the pathogenesis of malaria, and may play an important role in the pro-coagulant state observed in the disease [12,27–33].

Because blood coagulation *in vivo* is initiated by TF [14–19] we attempted to identify the mechanism by which pRBC might induce TF expression. Using three independent *in vitro* assays, we show that late-stage pRBC interact with cultured EC *in vitro* and induce TF expression with productive assembly of extrinsic Xnase complex, and initiation of the coagulation cascade. TF antigen and transcript were also detected by ELISA and RT-PCR, respectively. Functional expression of FXa was found in the pM range, was 3–4 times higher that the basal conditions (albumax or RBC), but ~ 5 times lower than the values obtained with TNF-α (10 ng/ml). These values were compatible with the levels of antigen detected by ELISA (~ 10 pg/well or ~ 0.5–1 pM TF, in 200 μl) and may also be due to the non-optimum binding of FX to apoptotic/activated EC [34,35].

Our results with the Transwell suggest that physical contact or close proximity between pRBC and EC is required for most TF expression — only ~ 10% of the response was observed with non-contact incubation. This is in agreement with previous reports showing that Transwell prevents pRBC-induced apoptosis [34] and EC activation [35]. On the other hand, we detected a small increase of ~ 20% in TF expression by ELISA using the supernatant obtained from pRBC that were kept at 37°C for 6 hrs. These findings are also in agreement with a recent report showing partial induction of ICAM-1 expression in EC by the supernatant of pRBC [36]. Therefore, molecules produced/released by pRBC [37–40] may contribute in part, although modestly, to TF expression by EC. At present, however, it is not known how pRBC induce TF expression but signaling triggered by its interaction with EC [41], or the molecules

released by infected erythrocytes [38–40], and free radicals [42] may potentially contribute to this response. Also, the molecular mechanisms of TF gene expression triggered by pRBC remains unknown, but possibly involve NFkB, which is known to regulate TF expression in different cell types [14] and to be nuclear translocated in brain EC incubated *in vitro* with pRBC [36].

In a second set of experiments, the potential procoagulant activity of pRBC was investigated. Mature forms (late trophozoites and schizonts) of pRBC display PS [23], a negatively charged phospholipid expressed in activated platelets [18,19]. In the experiments described here, it was revealed that late-stage pRBC support productive assembly of both intrinsic Xnase and prothrombinase complexes with generation of FXa and thrombin, respectively. Both complexes were exquisitely sensitive to their respective cofactors FVa and FVIIIa and inhibited by annexin V, a PS-binding protein [18,19]. Assemblies were attained at a remarkable low hematocrit (e.g. 0.025 %) and parasitemia (e.g. 5%), indicating that minute amounts of pRBC presumably propagate the blood coagulation cascade *in vivo*. In fact, pRBC obtained directly from *P. falciparum*-infected patients shorten the clotting [43], and the recalcification time [44]. This indicates that pRBC are indeed procoagulant. Therefore, pRBC behaves functionally as activated platelets that, among other functions, operate as the limiting step in the amplification phase of the blood coagulation cascade [19]. These data are particularly important, because late-stage pRBC are associated with sequestration in the brain of CM patients [4–6]. It is plausible that initiation of the blood coagulation by endothelium TF may be amplified by pRBC and/or activated platelets at sites of sequestration. Therefore, a balance between pro- and anti-coagulant mechanisms may determine the pro-coagulant tonus in different vascular beds.

We have attempted to demonstrate whether sequestration in parasitemic children was associated with TF expression by brain EC. Accordingly, sections of the frontal cortex from children who died from CM were used for IHC assays using a specific mAB against TF. All except one of the malaria-infected cases have evidence of TF expression to a certain degree. In contrast, none of the aparasitemic patients displayed TF staining, with the exception of one of the three Burkitt`s Lymphoma cases, a condition where TF expression in the EC has been reported previously [45]. The presence of TF in the cerebral endothelium of children with severe malaria could serve to support activation of the coagulation cascade, a possibility corroborated by histopathologic findings of platelet accumulation [46] and fibrin deposition in the brain of some CM patients [2–6].

We cannot determine, however, whether TF was locally synthesized or was acquired from circulating particles via P-selectin/PSGL-1 interactions [47]. In some ECs, TF stained in patchy or granular structures that appear to be located on the cell surface, but it was not possible to determine whether TF was produced by ECs or was transferred to the EC surface from blood-borne microvesicles [47,48]. We did not detect positive staining for TF in mononuclear cells, but they cannot be excluded as a potential source of TF as described in other diseases [14–16,47,48]. In other slides, TF expression was found in the EC of vessels containing malaria pigment (hemozoin) but no sequestered parasites. Extra-erythrocytic hemoglobin is a marker of the schizont rupture that occurs once asexual replication is complete [22]. EC expression of TF was also found in the absence of hemozoin and pRBCs, suggesting that pRBC had been present, but had cleared, either as a result of the dynamic nature of sequestration or an effective anti-parasitic treatment. Our IHC studies also show variable degrees of TF staining in the five of six parasitemic children who died from non-malarial causes. The one ‘parasitemic control’ with no evidence of TF expression died from direct head trauma.

Therefore, TF expression appears to be a general feature of malaria, and not necessarily specific for CM. Consistent with this notion, widespread EC activation has been reported in

uncomplicated malaria [6,49], a condition where sequestration [4–6,50], thrombocytopenia [11], and activation of blood coagulation [12] are generally observed. According to several reports [27–33], these non-comatose patients present none or few clinical manifestations compatible with a coagulation disorder such as bleeding but the plasma levels of coagulation factors are decreased to a moderate extend although not exhausted. These patients often present *i*) PT, PTT and fibrinogen often near the normal range, *ii*) low plasma levels of PC and PCI-1 and *iii*) increased levels of TAT, PAI-1 and D-dimers [12,27–33]. This laboratory and clinical profile therefore resembles "compensated" disseminated intravascular coagulation (DIC), a term that has been applied to atypical cases of DIC in which a continuous or intermittent slow rate of initiation of intravascular coagulation occur *in vivo* [51]. In theses cases, concomitant increase in coagulation factor synthesis may occur (e.g. fibrinogen), and therefore changes in coagulation tests may not be always remarkable [51]. However, it remains unclear why 1% of malaria patients develop CM, and why in 5–10% patients of severe malaria patients develop typical DIC [1,2] . It may be that blood coagulation, while normally homeostatic [16,52] may contribute to disease when it undergoes decompensation [16,53]. Of note, thrombocytopenia in malaria is associated with disease severity and is predictive of fatal outcome in humans [11]. Moreover, accumulation of platelets and monocytes in the vessels of the brain [46], apoptosis of EC [54] and formation of EC microparticles [13]—known to bear TF [55] and PS and to participate in inflammation [47]—have been specifically reported in CM but not in uncomplicated cases [13,46,54]. What catalyzes the transition from uncomplicated to complicated malaria, and the relative contribution of TF, activated platelets, activated/apoptotic EC to the pathogenesis of both conditions remains to be elucidated.

Our IHC studies show that some vessels contain sequestration and no TF staining. Perhaps, the tightly regulated mechanism of blood coagulation [14–19] may play a role in preventing an uncontrolled EC activation with widespread TF expression. Actually, *in vivo* TF staining in non-human primates or in human EC has been reported in only few studies [14,48,56,57], and it is remarkable that our cases consistently show positive staining and notably in some cases, at sites of sequestration. It is important to recognize that other events reported in malaria such as hypoxia [2], fibrin formation [5,6], and apoptosis [54]—and known to induce TF expression by EC *in vitro* [58–60]—may also play a proinflammatory role as may molecules released from ruptured pRBC [38–40] and monocyte-derived cytokines [10]. One of these, TNF-α, has been found in the *P. falciparum* patients plasma [12,61] which has been reported to induce TF expression and NF-κB activation in EC *in vitro* [62]. In addition, monocytes express TF *in vitro* after incubation with pRBC [37] suggesting that it could contribute to the pro-coagulant tonus observed in the disease [12,27–33]. It has also been reported that *P. falciparum*-infected placentas present macrophages that stain positive for TF [63], and display monocytes infiltrates which appear to be associated with complication in pregnancy in malaria [64]. Finally, the plasma level of the pro-inflammatory cytokine HMGB1 [65] is increased in severe cases of malaria [66]. Therefore, it is plausible to speculate that in malaria the positive feedback loop between clotting and inflammation [16,17,52,67–68], may ultimately contribute to neutrophil, leukocyte, and platelet interactions with the endothelium, resulting eventually in endothelial injury, increased vascular permeability, vesiculation and cellular apoptosis in different vascular beds [69].

The finding that TF is expressed *in vivo* may have implications for our understanding of the pathogenesis of malaria. Tissue factor is a structural member of the cytokine receptor family [16,52] which signify the expansion of the adaptive immune system in vertebrates, indicating a close connection of the coagulation pathways with the host response to infection [16,52]. TF is increasingly recognized as the interface of blood coagulation and inflammation [16,17,52, 67,68], and reportedly plays a pivotal role in disease pathogenesis [70–74]. Our histological data to date suggest that expression of TF in cerebral vascular endothelium occurs in malaria, and our *in vitro* data indicate that this has the potential to activate the coagulation cascade and

thus contribute to the severe disease. Therefore, malaria may provide a natural disease model for studying the interface between EC activation, inflammation and blood coagulation. Identification of TF as a potently critical mediator of malaria pathogenesis suggests that therapeutic agents targeted at TF and/or EC [75] might be useful as adjunct treatment in patients with severe disease [76].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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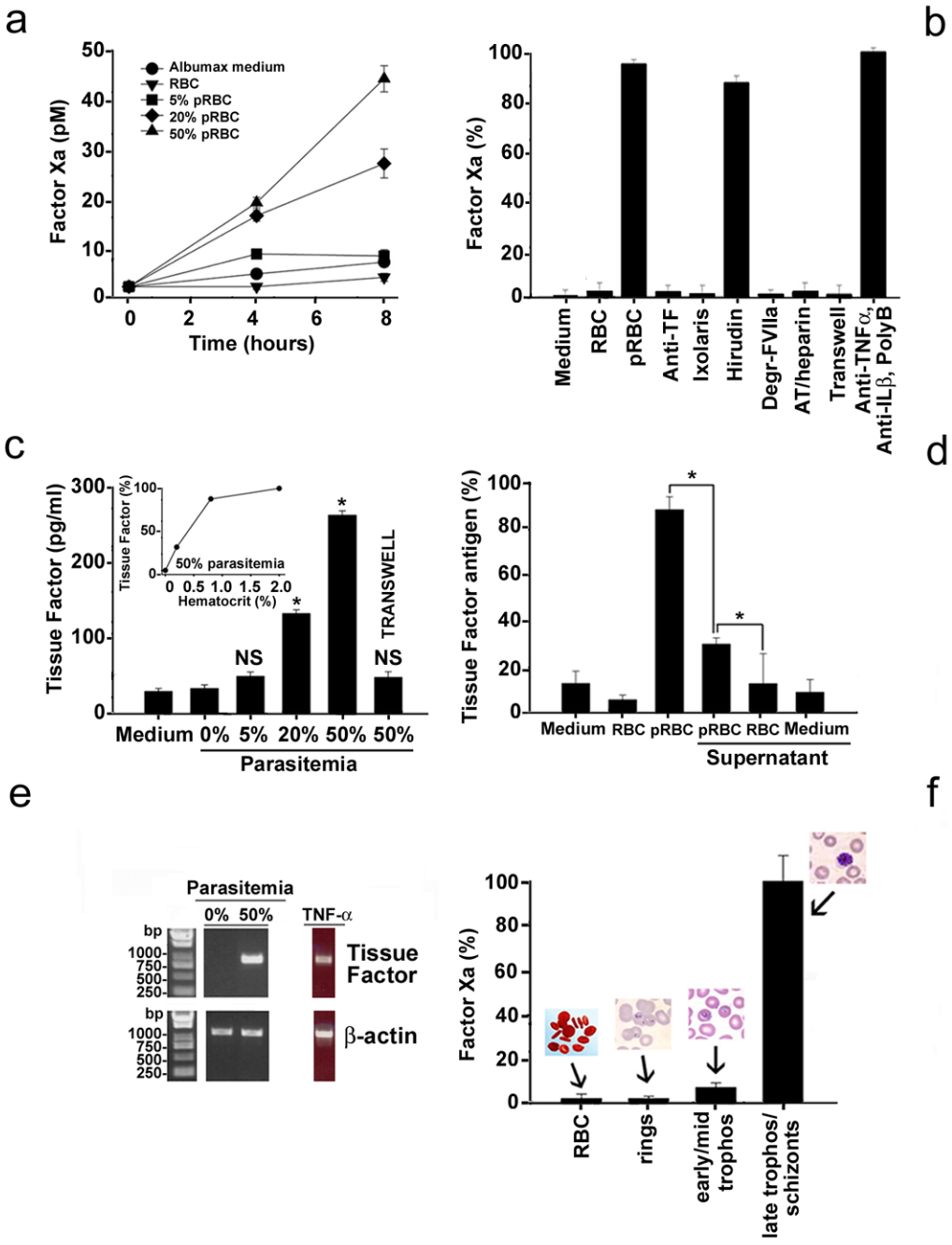

Figure 1. Parasitezed Red Blood Cells (pRBC) induce TF expression and productive assembly of the extrinsic Xnase complex in MVEC

(A) Functional assays for TF. (B) Controls. Degr-FVIIa, catalytic site blocked FVIIa; AT/heparin, antithrombin/heparin; PolyB, polymyxin B. (C) Antigen detection for TF (ELISA). Inset: hematocrit dependence. (D) Effects of pRBC supernatant in TF expression (antigen detection). (E) Transcript detection for TF (RT-PCR). (F) Stage-specificity of TF expression (functional assay). For each data point, results are the mean ± SE from three or four independent experiments, or a typical experiment is shown (*, $p<0.05$; NS, non significant). Legends are described in detail in the Supplemental data.

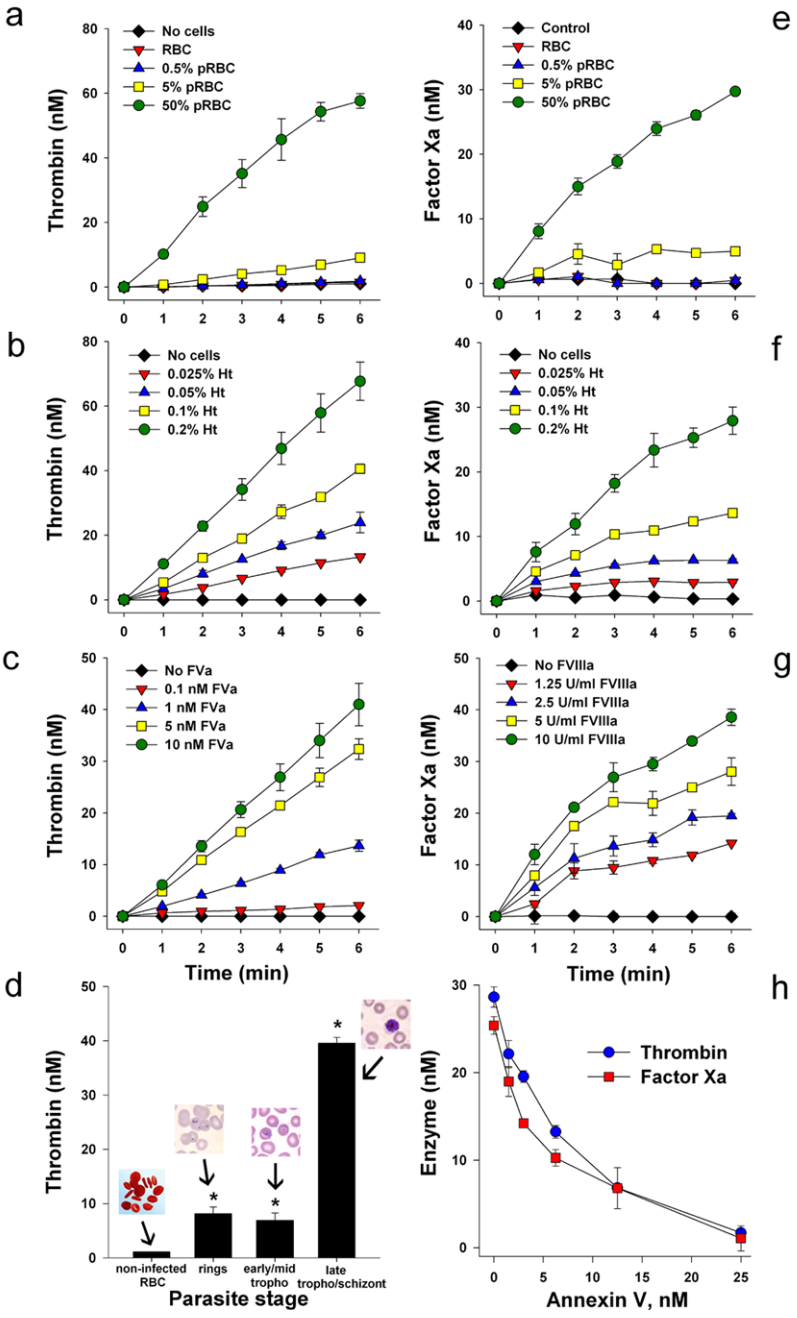

Figure 2. pRBC support assembly of prothrombinase and intrinsic Xnase complexes

(A) pRBC support prothrombinase assembly. (B) Hematocrit-dependent prothrombinase assembly. (C) FVa-dependent prothrombinase assembly. (D) Stage specificity of pRBC-dependent prothrombinase assembly. Reactions were initiated by addition of human prothrombin, and thrombin formation was estimated using chromogenic substrate (S2238). (E) pRBC support intrinsic Xnase assembly. (F) Hematocrit-dependent intrinsic Xnase assembly. (G) FVIIIa-dependent intrinsic Xnase assembly. Reactions were initiated by addition of human FX, and FXa formation was estimated using chromogenic substrate (S2222) (H) Effects of annexin V in the prothrombinase and intrinsic Xnase assemblies by pRBC. For each data point,

results are the mean ± SE from three independent experiments (*, $p<0.05$). Legends are described in detail in the Supplemental data.

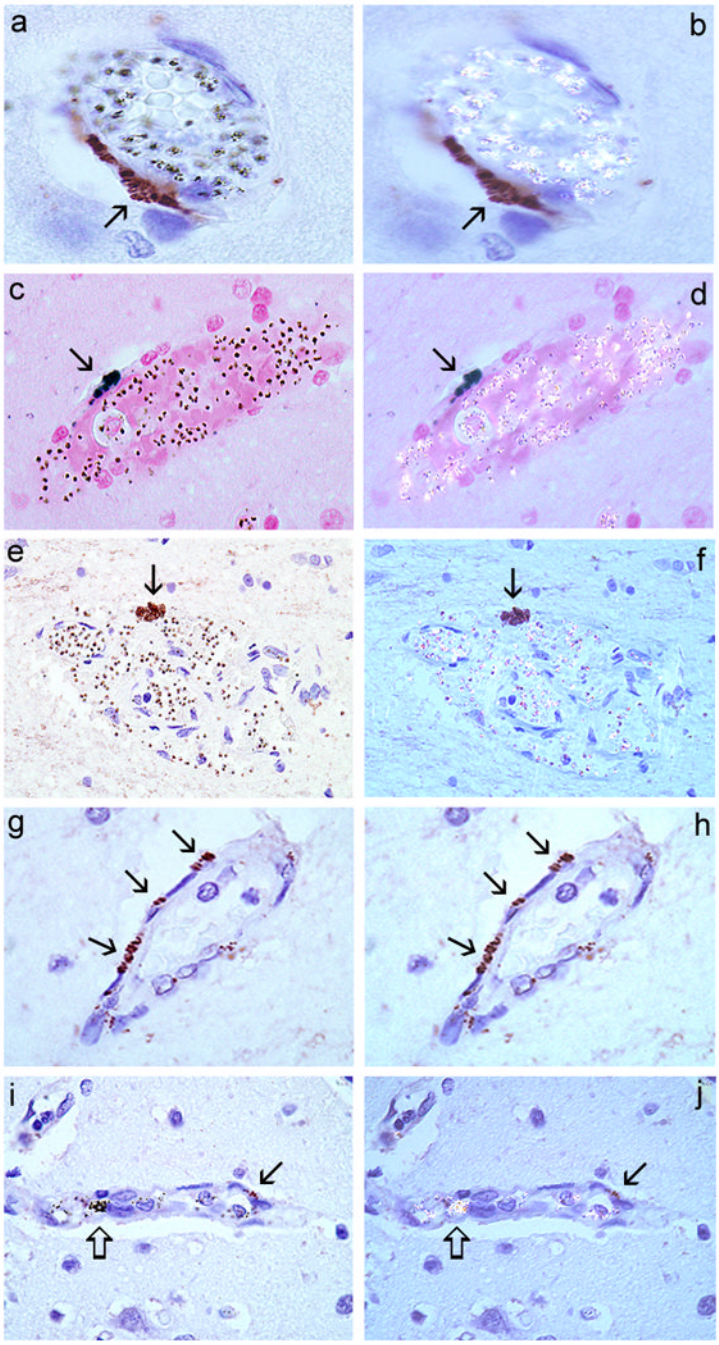

Figure 3. TF staining in EC of the frontal cortex of CM cases

Case 75 (A and B), case 68 (C and D), case 74 (E and F), case 38 (G and H), and case 69 (I and J). (A), (C) and (E) show TF staining of EC (arrow) associated with sequestration only as confirmed by polarized light (B, D, and F) that detects hemozoin (malaria pigment). (G) Shows TF staining of EC but no sequestration (H). In (I) TF staining of EC (arrows) is shown in the presence of aggregated hemozoin (open arrows) (J). Staining was never detected for any of the slides in the absence of primary anti-TF antibodies. Counterstaining: Hematoxilin. Immunohistochemistry for TF is described in detail in the Supplemental data.

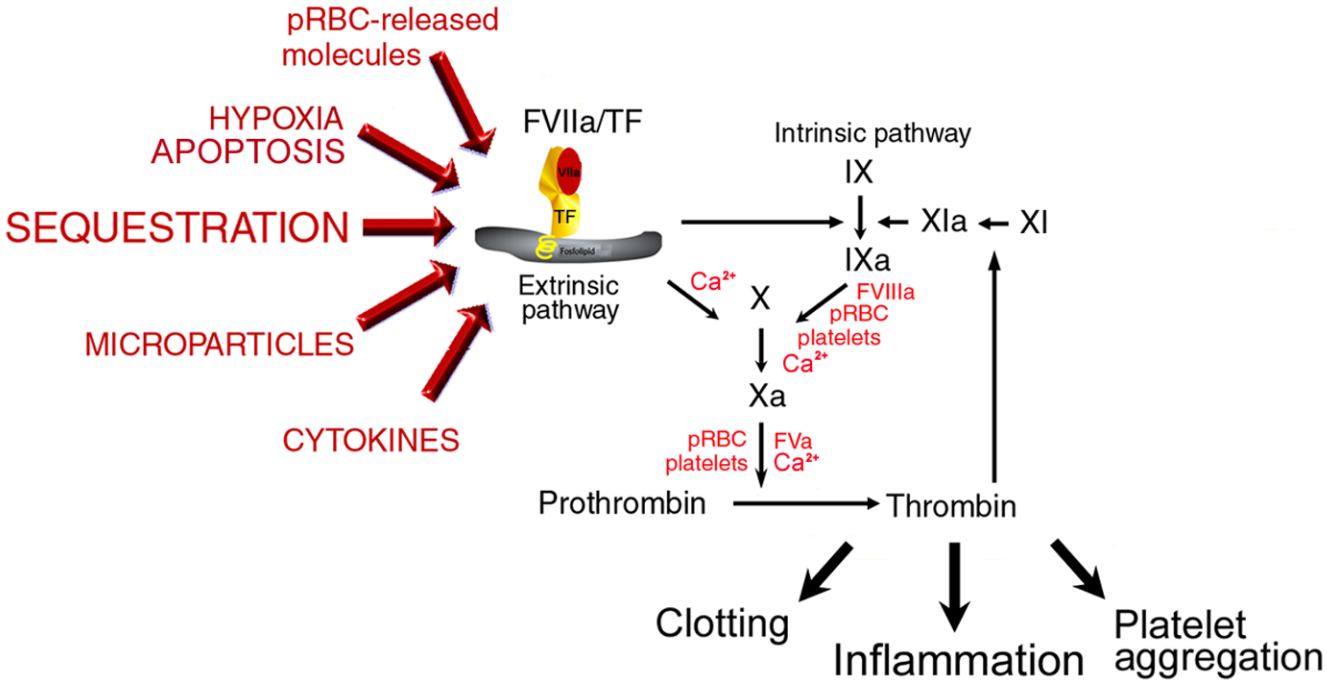

Figure 4. Tissue Factor expression in Malaria

Normal, quiescent endothelium does not express TF in the absence of biologic stimulation [14–19]. Sequestration of pRBC, in addition to cytokines [10], microparticles [13], hypoxia [2,3], fibrin [5,6], apoptosis [54], and proinflammatory molecules released by pRBC [38–40] potentially contribute to TF expression in microvessels of the brain and in other vascular beds [69]. Monocytes may also express TF and/or produce TNF-α and thus contribute to the procoagulant tonus observed in the disease [12,27–33]. Mechanistically, TF/FVIIa complex activates FIX or FX (extrinsic Xase complex) generating respectively FIXa and FXa, in the presence of Ca^{2+}. FXa, FVa, and prothrombin (prothrombinase complex) or Factor IXa, FVIIIa, and FX (intrinsic Xnase complex) assemble in the membrane of activated platelets and/or pRBC leading to amplification of blood coagulation, platelet aggregation [18,19], and inflammation [16,17]. The result is a convergence of signals leading to exacerbated TF expression that could sustain the coagulation-inflammatory cycle [16,17,47,52,67–68] in different vascular beds. Legends are described in detail in the Supplemental data.

Patient	Age*	Sex†/Race‡	Cause of death§	Parasitemia‖	Platelets¶	TF staining in EC#
Malawian "Cerebral Malaria cases" (parasitemic patients, malarial coma)						
37	6 mo	M/B	Cerebral Malaria	616,400	74,000	1 (9/286)
38	7 yrs	M/B	Cerebral Malaria	782,320	26,000	3 (121/267)
68	13 yrs	M/B	Cerebral Malaria	20,080	30,000	1 (21/267)
69	39 mo	F/B	Cerebral Malaria	81,840	NA**	1 (9/390)
74	8 yrs	F/B	Cerebral Malaria	280,000	NA	1 (3/259)
75	12 yrs	M/B	Cerebral Malaria	215,300	351,000	1 (33/299)
78	15 mo	M/B	Cerebral Malaria	637,000	73,000	1 (4/200)
79	15 mo	M/B	Cerebral Malaria	34,400	144,000	1 (1/196)
Malawian "parasitemic controls" (parasitemic patients, non-malarial coma)						
49	17 mo	M/B	Ruptured AVM	1,943	276,000	3 (50/169)
52	24 mo	M/B	Severe anemia	319,287	87,000	1 (4/203)
54	17 mo	M/B	Skull fractures	96,520	195,000	0 (0/167)
58	8 mo	M/B	Severe anemia	788	469,000	1 (1/110)
71	5 yrs	M/B	Subdural hematomas	314	346,000	1 (1/183)
NIH "aparasitemic controls"						
A90	9 yrs	F/W	Burkitt`s Lymphoma	NA	NA	1 (4/266)
A83	9 yrs	M/B	Osteosarcoma	NA	NA	0 (0/216)
A4	3 yrs	M/W	Burkitt`s Lymphoma	NA	NA	0 (0/226)
A88	8 yrs	M/I	Burkitt`s Lymphoma	NA	NA	0 (0/259)
A87	11 yrs	M/W	Osteogenic sarcoma	NA	NA	0 (0/259)
Brazilian "aparasitemic controls"						
A-08-6	6 yrs	F/B	Congenital cardiopathy	NA	NA	0 (0/269)
A-08-14	6 yrs	F/B	Congenital cardiopathy	NA	NA	0 (0/318)
A-02-12	7 yrs	F/B	Congenital cardiopathy	NA	NA	0 (0/163)
A-02-8	7 yrs	F/B	Congenital cardiopathy	NA	NA	0 (0/277)
Neural Tissue Array "aparasitemic controls"††						
TMA3001	54 yrs	NA	NA	NA	NA	0

*Age; mo (months); yrs (years).

[†] Sex; M (male), F (Female).

[‡] Race; B (Black), W (White), I (Indian).

[§] Cause of death; CM, cerebral malaria. AVM; arteriovenous malformation.

[//] Parasitemia; parasites counts/μl at admission.

[¶] Platelets; platelet counts/μl at admission.

[#] TF (Tissue Factor) staining in EC; 0, none; 1, mild; 2, moderate; and 3, severe immunoreactivity. [positive vessels/number of total vessels observed]. Vessel counting and scoring for TF performed by R.O.W.

[**] NA; not available.

[††] Frontal cortex obtained from neural tissue arrays TMA3001 (Chemicon Co.) was negative for all vessels examined.