Blood-brain barrier breakdown during cerebral malaria: Suicide or murder?

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Summary
Cerebral malaria, one of the most serious complications of Plasmodium falciparum infection, is characterized by the sequestration of parasitized red blood cells (PRBCs) in cerebral microvascular beds. The precise mechanisms involved in the onset of neuropathology remain unknown, but parasite sequestration in the brain, metabolic disturbances, and host immune responses all play a role. Sequestration of PRBCs is mediated by different endothelial cell surface receptors, mainly ICAM-1 and CD36. In vitro studies demonstrated that PRBC adhesion to endothelial cells induces over-expression of various adhesion molecules including ICAM-1, expression of iNOS, oxidative stress and finally apoptosis in endothelial cells. In vivo studies, in humans and in mice models of cerebral malaria brought striking evidence of the implication of brain infiltrating cytotoxic effector CDBT lymphocytes in the development of murine cerebral malaria pathogenesis. These cells probably act by direct cytotoxicity against endothelial cells. Cytotoxicity and apoptosis potentially lead blood-brain-barrier disruption and could contribute to the development of cerebral malaria. We propose a key role for endothelial cells in the pathogenesis of cerebral malaria, both by suicide / apoptosis, and / or by murder / cytotoxicity.

Keywords
Cerebral malaria, adhesion, apoptosis, cytotoxicity, endothelial cell

Introduction
Malaria kills an estimated 1.5 to 2.7 million people every year throughout the world (1). Cerebral malaria (CM) resulting from Plasmodium falciparum infection remains only partially understood. Although the physiopathology has been extensively investigated, cellular and molecular bases of the neurological pathology are still unclear, particularly the intricacy of the different factors described as being involved in the pathogenesis: sequestration of parasitized red blood cells (PRBCs) within microvessels; secretion of cytokines; modifications of the T cell repertoire; the immune status and the genetic background of the host; parasite factors (2).

The pathological feature common to all patients who die in the early stages of CM is microvascular sequestration of PRBCs: mature trophozoite and schizont forms of developing parasite disappear from the peripheral circulation and preferentially localize within the vascular beds of vital organs, such as the brain, lung, and kidney. Four species of malaria infect human, of which only P. falciparum causes sequestration, and only P. falciparum causes cerebral malaria. Autopsy specimens from patients, who have died from CM, almost invariably display sequestered parasites in the cerebral microcirculation. This is also found in some patients with non-cerebral disease at the time of death, but to a lesser extent (3). Whatever the clinical status of the patient is, parasite sequestration appears to be a common feature of all P. falciparum infections. These results indicate that although sequestration is necessary to cause the coma of CM, it is not sufficient.

According to studies performed in our laboratory and others, using in vitro co-cultures of P. falciparum and human endothelial cells, and various in vivo models of CM, we propose a key role for endothelial cells, both by suicide / apoptosis, and / or by murder / cytotoxicity.
BBB disruption during cerebral malaria, endothelial suicide?

Sequestration of PRBCs to the surface of the micro-vasculature of various organs including the brain and the lungs is mediated by different endothelial cell surface receptors including thrombospordin (TSP), CD36, intercellular adhesion molecule-1 (ICAM-1), E-selectin, vascular cellular adhesion molecule-1 (VCAM-1), CD31, αβ integrin, hyaluronic acid (4) and the membrane-bound form of fractalkine/CX3CL1 (5). The expression of part of these adhesion molecules is reportedly modulated by cytokines (6), such as Tumor Necrosis Factor-α (TNF-α). In humans, TNF-α up-regulates endothelial adhesion molecules ICAM-1 and VCAM-1 (6) and therefore increases sequestration of PRBCs within the microvasculature of the brain and other organs like lungs, kidneys, etc. In human and in murine models, blockage of cerebral vessels is also associated with the accumulation of activated leucocytes as well as with infected red blood cells (7, 8).

In his study comparing CM in ICAM(-/-) mice and wild-type ICAM-1(+/-) mice, Favre et al. (9) observed a post-mortem breakdown of the BBB in CM-positive ICAM-1(+/-) only, evidenced by a blue staining of the brain after IV injection of Evans blue. In an in vitro co-culture BBB model with human endothelial cell (HEC) and rat glioma cells, Dobbie et al (10) observed a decrease in barrier integrity evidenced by a 47% reduction in transendothelial electrical resistance after treatment of the co-culture with TNFα for 18 hours in comparison with non treated cells.

In Viet-Nam, Brown et al (11) compared human brains collected post mortem after cerebral malaria with brains collected post mortem after non malarial causes of death (control brains). By immunohistochemistry, while cell junction proteins such as ZO-1, occludin, and vinculin were constitutively high in control brains, they observed a generalized reduction in the expression of the corresponding antigens with even, in some cases, a focal loss of staining in the brains of patients dead from CM. Brown et al hypothesized that the sequestration of parasitized red blood cells in the brain via adhesion to receptors such as ICAM-1 and others, could result in the activation of cerebral endothelial cells and that a potential consequence of this activation could be a disruption of the BBB, resulting in the exposure of the brain parenchyma to plasma proteins. Perivascular macrophages would then encounter proteins leaking across the disrupted BBB and, as a consequence of activation or phagocytosis, could secrete proinflammatory and neuroactive mediators, such as TNFα, which could influence local neuronal function (11). BBB perturbation was further evidenced by an increase in immunoreactivity for fibrinogen in cases of cerebral malaria; indicating a widespread leakage of this plasma protein across the BBB.

Although the various P. falciparum components that are potentially involved in the production of inflammatory responses by the innate immune system remain to be elucidated, the glycosylphosphatidylinositol (GPI) anchor glycolipids of the parasite have been proposed as the prominent parasite components responsible for malaria pathogenesis (12, 13).

Schofield et al. have shown that the GPIs of P. falciparum can induce the expression of iNOS, up-regulate the expression of intracellular adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin in endothelial cells, implicating these processes in malaria pathogenesis (14). Such an induction of adhesion molecules expression increases PRBCs adhesion and could then contribute to the pathological process (for an extensive review concerning GPI, see Clark et al. (15)).

We recently demonstrated that PRBC adhesion induces apoptosis in human endothelial cells (HEC). PRBC adhesion modulated HEC gene expression such as TNF-α superfamily genes (Fas, Fasl, DR-6) and apoptosis-related genes (Bad, Bax, Caspase-3, SARP 2, DFF45/ICAD, IFN-γ Receptor 2, Bel-w, Bik and iNOS). Apoptosis was confirmed on the basis of 1) morphological modifications by electron microscopy, 2) Annexin-V-binding, 3) DNA degradation, and 4) caspase activity (16).

It is well established that cross-linking of adhesion molecules on the endothelial cell membrane can induce intracellular signalizing leading to various cellular responses such as apoptosis (17, 18), and in that model, the apoptosis induction was dependent on PRBC adhesion. In the same way, Jimenez et al. (19) demonstrated that the binding of the CD36 ligand thrombospordin-1 on endothelial cells is sufficient to transduce signal through p59/fyn and caspases leading to endothelial cell apoptosis.

Apoptosis of endothelial cells as a PRBC adhesion consequence might contribute to blood-brain barrier dysfunctions and lesions. In addition, it was demonstrated that apoptotic endothelial cells up-regulate the expression of cellular adhesion molecules on normal endothelial cells resulting in hyperadhesiveness (20), which might contribute and amplify the pathological process.

A critical role for nitric oxide (NO) and oxidative stress has been proposed to explain malaria physiopathology in humans, but is still a matter of controversy. Molecular oxygen is an important environmental and developmental signal that regulates cellular energetics, growth and differentiation. While oxygen, indispensable for the cell to obtain ATP, is often transformed into highly reactive forms, radical oxygen species (ROS), which are frequently toxic for the cell. Peroxynitrite (ONOO-) is a potent oxidant formed from the non enzymatic reaction between superoxide anion (O2-) and NO to form ONOO-. Peroxynitrite can oxidize lipids, proteins and nucleic acids, resulting in cell death (21).

The effects of ROS in malaria can be both beneficial and pathological, depending on the amount and place of production. Enhanced ROS production after the administration of pro-oxidants, which is directed against the intra-erythrocytic parasite, inhibits the infection both in vitro and in vivo (22–24). However, ROS are also involved in pathological changes in host tissue like damage of the vascular endothelial lining during cerebral malaria (11). Pro-oxidants may support the host defense against the parasite when working in or near the infected cell but potentially cause vascular damage when working on or near the vascular lining (21, 25).

We previously described a protective effect of MnTBAP, a permeable chemical SOD1 mimic, on PRBC-induced endothelial cells apoptosis, suggesting that the apoptotic process is mediated by O2-. In addition, MnTBAP also exhibits a peroxy-
Figure 1: BBB breakdown during cerebral malaria: endothelial suicide or murder? Environment in brain capillaries during CM is remarkably 'hostile'. Sequestration of mature forms of P. falciparum infected erythrocytes as well as proinflammatory cytokines secreted by activated leucocytes are likely to trigger synergistic mechanisms affecting the endothelial cell compartment, which is a major component of the blood brain barrier. We demonstrated in our study that endothelial apoptosis resulting from mitochondrial production of free radicals 

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susceptible C57BL6 mice accumulated in their brains (8). Histological examinations in WT susceptible and PFP-KO resistant mice showed similar tendencies: adherence of sequestrated PRBC to the endothelium of cerebral vessels and activation of endothelial cells. The striking histological differences between the two strains of mice concerned the severe destructive lesions selectively affecting WT brains, with disrupted micro vessels and widespread endothelial cell destruction.

We thus suggest that endothelial cells lining the brain microvasculature could be a crucial target of activated CD8 T lymphocytes. Infected erythrocytes could be internalized/phagocytosed by endothelial cells and consequently parasites derived peptides could be loaded in complexes and transported to the cell surface. The binding and the killing of endothelial cells by cytotoxic T lymphocytes could therefore enhance the blood-brain barrier dysfunctions and leads to a complete and irreversible disruption.

This proposed mechanism is further supported by a previous study showing that Beta 2 microglobulin which is a specific domain of MHC class I, is indispensable for the development of CM in murine models (29). Moreover, ability of endothelial cells to present antigens via MHC class I molecules has already been demonstrated (Marelli-Berg et al., 2001). Although endothelial phagocytosis of red blood cells has been described before (30) further experiments are required in which co-cultures of *Plasmodium* parasites, lymphocytes and endothelial cells are performed, in order to evidence a direct cytotoxicity against endothelial cells. By using our co-culture model of HEC and PRBCs (16), we observed the internalization/phagocytosis of RBC by endothelial cells, but only when endothelial cells were co-cultivated with infected RBCs and not with control uninfected erythrocytes (Fig. 2).

We also found increased expression of CCR2, CCR5 and CXCR3 in cytolytic effector cells compared with naïve CD8 cells (8). In addition, mice deficient for CCR5 are much more less susceptible to cerebral malaria than wild type mice (31). As it was studied in other neurological diseases, such as multiple sclerosis in human or experimental autoimmune encephalomyelitis (32, 33), recruitment of defined populations of lymphocytes may involve chemokine/chemokine receptors. It is well known that leukocyte binding to endothelium is a multistep process implying interactions of various adhesion molecules, chemotactants and their receptors (34, 35). The Blood-brain-barrier, a dynamic system of which endothelial cells is a major component, could then be considered as a ‘checkpoint for inflammation and immunity’ (36). Recently, the membrane-bound form of Fractalkine/CX₃CCL1 (FKN), a chemokine with a unique structure, has been identified as a receptor for *P. falciparum*-infected erythrocyte cytoadherence (5). FKN is particularly expressed as a membrane-bound form on endothelial cells activated by proinflammatory cytokines such as TNF-alpha, IFN-gamma and IL-1 (37, 38). Identification and blockage of chemotactic molecules specifically involved in CTL/endothelial interactions and leukocyte recruitment during CM could help to thwart the course of the disease.

**Conclusion**

The interaction between *P. falciparum*-infected erythrocytes and endothelial cells is clearly crucial to the pathogenesis of cerebral malaria, but to date, the understanding of the pathological process is only arising. Only hypothesis can be proposed, such as apoptosis of endothelial cells (16, 26), or/and a direct cytotoxicity against endothelial cells (8, 28), or/and an overenthusiastic production of the cytokines and ROS. *In vivo* data from humans are desperately needed to confirm or invalidate these hypotheses. Most of the data available agree with the view that cerebral malaria in African children is a collection of overlapping syndromes acting in different organs like kidneys, lungs, etc., with several mechanisms potentially combining and contributing to coma and death. In such a case, the use of anti-oxidant molecules, to prevent endothelial cells apoptosis, or the inhibition of endothelial cells killing by cytokines and lymphocytes could constitute a powerful therapeutic complement to anti-malarial molecules for the treatment of cerebral malaria.
References

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