Review Article

Cytoadherence and Severe Malaria

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Abstract

Malaria is a disease that causes enormous human morbidity and mortality. One feature of mature *Plasmodium falciparum*-infected erythrocytes leading to the development of severe malaria is thought to be cytoadherence and blockage of the microvasculature. Therefore, an understanding of mechanisms that mediate parasite adhesion leading to malaria pathology is needed to yield new treatments for malaria. However, to date, cytoadherence-associated pathology is still under debate. Is cytoadherence needed to develop severe malaria? This review will discuss the available information on associations of cytoadherence with the development of severe malaria.

Keywords: *cerebral malaria, cytoadherence, endothelium, malaria*

Introduction

Malaria is a serious burden, particularly to low and middle-income countries, and a major contributor to morbidity and mortality. The aetiological agents of malaria to humans are recognised as 6 distinct protozoan species of *Plasmodium*: *Plasmodium falciparum* (1), *Plasmodium vivax* (2), *Plasmodium malariae* (3), 2 species of *Plasmodium ovale* (*P. ovale curtisi* and *P. ovale wallikeri*) (4), and *Plasmodium knowlesi* (5), which was recently recognised as the 6th human *Plasmodium* after cross infection from long-tailed Macaca monkey to humans was reported in Malaysia (6–9). *P. falciparum* has often been seen as the most clinically significant infection due to an association with mortality and the intensity of infection in some regions of sub-Saharan Africa, but *P. vivax* has a wider geographical distribution, and its categorisation as benign has been challenged (10). What is clear is that an episode of *P. falciparum* malaria in a non- or semi-immune host can lead to severe malaria (SM) if untreated, with a high risk of death. However, recently a study from Papua New Guinea and Malaysian Borneo extended the pattern of severe disease by showing a strong association of *P. vivax* (11) and *P. knowlesi* (8) infection, respectively, to SM and death. Studies of the pathology of SM were started in the late of 19th century by 2 Italian pathologists Marchiafava and Bignami (12), where they found, post-mortem, the presence of higher parasite load in a comatose malignant blood fever patient compared with in those with benign fever. They saw high parasite levels and parasite pigment predominantly retained in the tissue microvessels compared with in the peripheral circulation, as well as the existence of necrosis and alterations in the endothelium of the cerebral vessels. This discovery and subsequent observations have led to a suggestion that the preferential accumulation of parasitised red blood cell (pRBC) in tissues might be linked to the disease and its severity. The manifestations of SM are highly variable and are determined by factors from both the human host and the parasite. The most common clinical features of SM are high fever, respiratory distress, vascular obstructions, metabolic disturbances (e.g., acidosis), multi-organ dysfunction (e.g., renal failure), severe anaemia, and cerebral malaria (CM); these features differ among areas of varying transmission intensity and between adults and children. This creates problems in comparing studies as the clinical definitions can vary and the impact of cytoadherence on these variable clinical outcomes is difficult to define. Some aspects of SM occur because the parasite has developed mechanisms to escape the host immune system, which we will discuss later, so one of the features of CM (an important subset of severe cases) is that it is more common

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in semi-immune children in sub-Saharan Africa (13).

It is still unclear how infection with *P. vivax* and *P. knowlesi* lead to SM, and it is possible that research on understanding *P. falciparum*–derived SM may help us to understand and predict how *P. vivax* and *P. knowlesi* act. Once thought to be unique to *P. falciparum*, the ability of the mature pRBC to undergo a range of adhesive interactions (cytoadhesion), such as the binding of pRBC with endothelial cells (sequestration) and the interaction of pRBC with non-infected RBC (rosetting) and with other pRBC (auto-agglutination), is now thought to be shared with other species. One of the big questions in *P. falciparum* research is, "Is parasite adhesive behaviour linked to SM?" This question can now be extended to *P. vivax* and *P. knowlesi* with the discovery of adhesion to endothelial receptors by RBC infected with these species (8,14,15), although the timing and extent of cytoadherence in these 2 species differs from that of *P. falciparum*, with the latter exhibiting earlier (from 15 hours post-invasion) and more pronounced sequestration, whereas only the schizont stages of *P. vivax* and *P. knowlesi* show this phenotype.

One molecule identified on the surface of *P. falciparum* pRBC, known as *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) encoded by *var* genes, has been correlated with *P. falciparum* cytoadherence (16–18). It has been thought that the antigenic switching between different PfEMP-1s constitutes an important virulence factor by facilitating the parasite's escape from the host's immune response, thus establishing chronic infection (19,20). Considering the potential harmful effect of *P. falciparum* cytoadherence to the host, early treatment or even prophylaxis would be highly desirable in preventing cytoadhesion and progression of disease. Unfortunately, falciparum malaria has become increasingly refractory to chloroquine (21,22), the cheapest and most widely available antimalarial, and this emergence of drug resistance in Southeast Asia and Africa was closely associated with the increased incidence of SM (23) . The World Health Organization advises all countries experiencing antimalarial drug resistance (including monotherapies such as chloroquine, amodiaquine, or sulfadoxine-pyrimethamine) to use combination therapies, preferably those containing artemisinin derivatives (artemisinin-based combination therapies, ACTs).

Recent clinical trials in Asia and Africa using ACTs showed improved recovery of SM patients (24,25), but mortality reported shortly after hospital admission (within 48 hours) was still high despite the administration of highly effective anti-parasitic drugs. This finding is consistent with our recent data showing that after exposure to drugs, killed pRBC were still able to cytoadhere (26), which has led us to suggest that this persistent mortality may be due to the effects of adherent pRBC in the microvasculature. Is there any way of reducing this mortality? Perhaps adjunct therapies that can block and reverse the pathogenic effect of pRBC adhesion will lighten the disease burden. However, before embarking on this course, what evidence is there that cytoadherence is involved in SM?

pRBC Cytoadhesion

Why and how does parasite cytoadherence–related morbidity take place? Several hypotheses associated with the binding of pRBC in the microvasculature have been proposed and reviewed elsewhere, such as i) changes of the RBC and pRBC rigidity (27–29), ii) pro-inflammatory induction of the adhesion-receptor expression (30,31), iii) binding of pRBC to specific adhesion receptors on endothelial cells (32), iv) endothelial activation (33–35), and v) malaria toxins (36), with various levels of evidence to support them. However, there are also more recent discoveries such as the relevance of platelets and microparticles as well as the role for the coagulation cascade in mediating pRBC binding to endothelial cells (37,38).

A major question is how *P. falciparum* has adapted to bind in the microvasculature to such an extent that mature pRBC are rarely seen in the peripheral circulation, unlike other human-invading malaria parasite species. An important difference in *P. falciparum* is the modification to the surface of the host erythrocytes to become rigid and inflexible by exporting specific proteins to the RBC membrane during the intra-erythrocytic stages. This reduction of flexibility of RBC makes their circulation through the microvasculature difficult and favours pRBC adhesion to endothelial cells (39).

In 1985, MacPherson et al. (40) reported higher levels of pRBC in the cerebral vessels of adults dying from CM compared with in non-CM cases, demonstrating the preferential accumulation of pRBC in the brain being linked to CM; this is consistent with the findings of Marchiafava and Bignami (12). The MacPherson study identified the contact point for pRBC in vivo as a knob-like structure, which had previously

been demonstrated in in vitro studies. Knobs are distortions on the surface of *P. falciparum* pRBC caused by deposition of knob-associated His-rich protein (KAHRP) at the cytoplasmic side of the pRBC membrane (41); these knobs contain several other proteins including PfEMP-1 as well as ring-infected and mature parasite–infected erythrocyte surface antigens (42). It is generally accepted that PfEMP-1 is largely responsible for pRBC adhesion in *P. falciparum*, and various associations between *var* gene expression and complicated or uncomplicated disease have been reported. However, are knobs essential to establish an interaction in the microvasculature? Some other *Plasmodium* species such as *P. brasilianum*, *P. vivax*, and *P. malariae* also have knob-like structures but do not always exhibit cytoadherence properties, suggesting that these membrane modifications are not identical to those seen with *P. falciparum* (14,43). Biggs et al. (44) demonstrated that knobless *P. falciparum* could bind to host receptors, although later work (45) showed that a KAHRP knockout line could not bind under more physiological flow conditions.

What Factors Mediate Adhesion of the pRBC in Host Microvasculature?

Several receptors on endothelial cells have been shown to support interactions with pRBC, including thrombospondin; CD36; immunoglobulin superfamily cell adhesion molecules, e.g., intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule, (VCAM), platelet endothelial cell adhesion molecule, and neural cell adhesion molecule; selectins, e.g., P-selectin and E-selectin; integrin αvβ3; globular C1q receptor; and glycoaminoglycans, e.g., chondroitin sulphate A (CSA) and heparin sulphate (35). With such a diverse collection of host receptors, how might one investigate associations between disease and specific adhesion phenotypes? It has been reported that ICAM-1 and CD36 are the most commonly used adhesion receptors by patient isolates, except in placental malaria (46), and correlations with severe and uncomplicated disease have been suggested (47), which has often been a starting point for clinical studies. It has been proposed that synergism (or at least co-operation) between these two receptors makes the binding of pRBC stronger (48,49). Therefore, it is not unusual that clinical studies examining the association between receptor usage and disease have concentrated on these two proteins, as indicated in Table 1 (31,45,46,50–58).

In addition to ICAM-1 and CD36, CSA is also one of the most common and successfully studied adhesion receptors. CSA provides the clearest example of an interaction of pRBC with an adhesion receptor in causing disease; however, this example of adhesion-related pathology does not come from endothelial cytoadherence but rather adhesion of pRBC to CSA in the placenta of pregnant women through a set of semi-conserved PfEMP-1 proteins (59–61). The restricted variation in this important facet of malaria pathology provides one of the most hopeful cases for the development of a disease-specific vaccine for malaria (62). In the case of placental malaria, the association of a specific *var* gene (*var2csa*) with adhesion and disease has been possible, but this has been much harder to define in other syndromes of SM, such as CM.

Another factor that has been postulated to have an association with host-mediated cytoadherence is the role of host pro-inflammatory cytokines. These cytokines have long been implicated in the pathogenesis of SM (63), where changes in cytokine plasma levels have paralleled the rise of temperature during fever paroxysms in SM (64), and an increase of pro-inflammatory cytokines, especially tumour necrosis factor (TNF), in CM in children especially from Africa and its correlation with mortality (65–68) have been observed. Nevertheless, how are these pro-inflammatory cytokines regulated and how might they mediate parasite adhesion and SM, or is this just a general effect? Other studies (33,69) have challenged the correlation of cytokines, especially TNF, towards malaria disease severity and claimed it is quite poor at predicting SM. It is thought that pro-inflammatory cytokines are central to the pathophysiology of systemic disease caused by infectious and non-infectious agents, and cytokines such as TNF and interleukin (IL)-10 have been proposed to have a protective role to clear the infections and to avoid inappropriate host responses that might lead to cell destruction and be harmful to the host. In the case of malaria and SM, high levels of pro-inflammatory cytokines TNF, IL-1, IL-6, IL-12, and interferon (IFN)-γ have been observed in patients with malaria, and low levels of IL-10 and tumour growth factor (TGF)-β have been correlated with fatal outcome (70). It is thought that these cytokines are produced by activated macrophages, dendritic cells, and, potentially, endothelial cells during the host response to pRBC and schizont rupture (36,71).

Several studies (72,73) showed that pRBC stimulate antigen-presenting cells macrophages

Table 1: Clinical studies on cytoadherence-related pathology in malaria

Study	Pathological feature	Adhesion molecule tested	Observation technique	Correlation with severity of disease
Marsh et al., 1988 (50) Subjects: 51 children Site: Gambia	• Cerebral malaria • Uncomplicated malaria	• CD36	• Static cell binding assay on C32 melanoma cells	Suggested that pH 6.9 mediates optimal binding of pRBC to CD36, but no correlation between parasite adhesion and disease.
Ockenhouse et al., 1991 (51) Subjects: 27 adults Site: Thailand	• Cerebral malaria • Acute renal dysfunction • Acute hepatic dysfunction	• CD36 • ICAM-1	• Static cell binding assay on C32 melanoma and CHO cells expressing CD36 or ICAM-1 • Protein binding assay	All patient isolates bound to CD36 purified protein and cells, but no association with disease.
Ho et al., 1991 (52) Subjects: 59 adults Site: Thailand	• Cerebral malaria • Severe malaria (without cerebral malaria or anaemia) • Uncomplicated malaria	• CD36	• Static cell binding assay on C32 melanoma cells incubated with TNF, IL-1, and IFN-γ	Severe malaria patient isolates showed higher binding to C32 melanoma cells compared with those of uncomplicated and cerebral malaria isolates. Cytokines did not enhance pRBC cytoadherence on C32 cells.
Chaiyaroj et al., 1996 (53) Subjects: 56 adults Site: Thailand	• Severe malaria with organ dysfunction	• CSA • CD36 • ICAM-1 • E-selectin • VCAM-1	• Static cell assays on C32 melanoma and CHO cells expressing E-selectin or VCAM-1 • Protein binding assay	All isolates bound to C32 melanoma cells. A small number of isolates adhered to ICAM-1, CSA, and TSP purified protein but not to E-selectin and VCAM-1 on CHO transfected cells. No correlation between severity and level of adhesion.
Udomsangpetch et al., 1996 (54) Subjects: 60 adults Site: Thailand	• Severe malaria with acute organ dysfunction • Cerebral malaria	• CD36 • ICAM-1 • E-selectin • VCAM-1	• Static cell binding assays on mouse L cells expressing CD36, E-selectin, ICAM-1, or VCAM-1	Patient isolates bound to CD36 10-fold higher than to ICAM-1, and no binding to VCAM-1 was observed. Therefore, CD36 binding could be associated with disease severity by allowing adhesion of a larger proportion of the parasite population.

Study	Pathological feature	Adhesion molecule tested	Observation technique	Correlation with severity of disease
Newbold et al., 1997 (55) Subjects: 150 children Site: Kenya	• Cerebral malaria • Severe anaemia • Non-severe malaria	• CD36 • ICAM-1 • VCAM-1 • E-selectin	• Static protein binding assay	CD36 was quantitatively the major receptor for all isolates, but some isolates bound strongly to ICAM-1, less to VCAM-1, and none to E-selectin. Binding to ICAM-1 was associated with disease, but not cerebral malaria.
Rogerson et al., 1999 (56) Subjects: 158 children Site: Malawi	• Severe malaria • Cerebral malaria • Severe anaemia	• CD36 • ICAM-1 • CSA • TM	• Static protein binding assay	Varied cytoadherence profiles from patient isolates; all isolates bound to CD36, and severe anaemia isolates had low binding to ICAM-1. No correlation with severe disease.
Heddini et al., 2001 (57) Subjects: 111 children Site: Kenya	• Severe malaria without cerebral malaria or severe anaemia • Cerebral malaria • Severe anaemia • Uncomplicated malaria • Control	• PECAM • CD36 • TSP • ICAM-1	• Static cell binding assays on mouse L cells expressing PECAM-1 and CHO cells expressing CD36 or ICAM-1 • Protein binding assays using FACS technique	Rosetting associated with blood group A and heparin-type receptors (e.g., heparin sulphate) were prone to severe malaria. Binding to multiple receptors promoted pRBC sequestration in the severe malaria group.
Cojean et al., 2008 (47) Subjects: 22 adults Site: France	• Uncomplicated malaria • Cerebral malaria • Severe malaria (without cerebral malaria)	• ICAM-1 • CD36	• Static cell binding assays on CHO cells expressing CD36 or ICAM-1	Binding of isolates from severe malaria showed no significant difference compared with uncomplicated malaria pRBC.
Chilongola et al., 2009 (46) Subjects: 155 children Site: Tanzania	• Uncomplicated malaria	• CD36	• Static protein binding assay	CD36 deficiency was protective in malarial anaemia.

Study	Pathological feature	Adhesion molecule tested	Observation technique	Correlation with severity of disease
Ochola et al., 2011 (32) Subjects: 101 children Site: Kenya	• Cerebral malaria • Severe anaemia • Uncomplicated malaria	• CD36 • ICAM-1	• Static and flow protein binding assays	High pRBC binding to CD36 was associated with uncomplicated malaria. High ICAM-1 binding under flow correlated with cerebral malaria.
Mayor et al., 2011 (58) Subjects: 46 children Site: Mozambique	• Severe malaria (with cerebral malaria, severe anaemia, respiratory distress, prostration) • Uncomplicated malaria	• CD36 • ICAM-1 • gC1qR	• Static protein binding assays	Higher levels of adhesion to gC1qR in isolates from children with multiple seizures.

Abbreviations: CHO = Chinese hamster ovary, CSA = chondroitin sulphate A, FACS = fluorescence activated cell sorting, gC1qR = globular C1q receptor, ICAM = intercellular adhesion molecule, IFN = interferon, IL = interleukin, PECAM = platelet endothelial cell adhesion molecule, pRBC = parasitised red blood cells, TM = thrombomodulin, TNF = tumour necrosis factor, TSP = trombospondin, VCAM = vascular cell adhesion molecule.

and dendritic cells probably through direct or indirect interaction of PfEMP-1 via CD36, and it is known that CD36 is expressed on the surfaces of macrophages and dendritic cells. The biological role of these interactions is not clear. For example, there is conflicting data about the effect of pRBC adhesion on dendritic cells: some studies showed that decreased activation was associated with cytoadherence (74), while others showed that this adhesion process was not required for the modulation of dendritic cell activity (75). These findings in the literature are variable due to the use of different host species and parasite strains, at pre-erythrocyte or blood stages of infection (76). How might cytokines support pRBC binding to microvascular endothelium? One thought is that it is through endothelial activation. For example, TNF is known to act by increasing the expression of host adhesion molecules. TNF binding to TNF receptor type 2 induces recruitment of signal transduction that activates effector molecules and transcription factors, leading to a strong increase in the expression of ICAM-1, VCAM, and E-selectin. The involvement of TNF in the upregulation of adhesion molecules has been clearly reported in different in vitro and in vivo studies (49,77,78). However, does TNF induction alone do enough to exacerbate SM? If pathology of malaria was the result of a high level of TNF, patients with SM could be treated using TNF specific antibody. However, a trial using a monoclonal antibody against TNF did not show any protection and, in fact, worsened neurological sequelae in patients (68).

The pattern of pathology in malaria is variable, and the profound cytokine-mediated changes and tissue oedema seen in other infections are not characteristic of this disease, although some signs of these pathologies are available. Thus, it seems that malaria pathology can be linked to a pro-inflammatory response, but this is not enough to explain the disease. This is consistent with recent studies that have shown that in children, TNF level was a poor discriminator of severity of disease, whereas proteins associated with endothelial activation (e.g., angiopoietin-1, angiopoietin-2, von Willebrand factor [vWF], soluble ICAM-1) were relatively good markers (69).

Endothelial activation, in response to inflammatory mediators, collectively increases the expression of adhesion molecules, including E-selectin, ICAM-1, and VCAM-1, on the cell surface through the activation of nuclear factor κB signalling transduction. Increases of P-selectin on endothelial cells following activation have also been reported (79). P-selectin is different from other adhesion molecules as it is stored in endothelial cell specific storage vesicles called Weibel–Palade bodies, together with other molecules such as vWF. How Weibel–Palade

bodies are activated in malaria infection is still unknown; the parasite has a protein that can cause basophils to release histamine, which is known as *P. falciparum* translationally controlled tumour protein (80), but some reports have also suggested that activated platelets and fibrin might mediate the release of P-selectin and vWF (81). vWF recently has been found to be a good prognostic marker for SM in children, and it has been thought that vWF might mediates pRBC binding on endothelial cell via ultra-large vWF multimers by producing a bridge via platelets (81–85).

Scientists have speculated that the febrile temperature seen as a part of malaria infection might enhance cytoadherence. We know that a fever is due, in part, to the increase in TNF seen during infection, but does temperature elevation help pRBC to bind to endothelial cells? Udomsangpetch et al. (86) showed that PfEMP-1 expression was accelerated by febrile temperature and increased cytoadherence. However, this is contrary to other findings where febrile temperature affected intra-erythrocyte growth, and upregulation of PfEMP-1 was not seen (87). Recently, Pattanapanyasat et al. (88) showed that febrile temperature induced and enriched expression of phosphatidylserine on the pRBC membrane surface. Several studies (89–92) have reported that phosphatidylserine promoted pRBC binding to CD36 and thrombospondin. Febrile temperature can also lead to endothelial cell disruption (93). In a clinical trial (94), the use of antipyretic intravenous ibuprofen was able to control fever but delayed parasite clearance. This finding suggests that ibuprofen and fever reduction does not act to reduce cytoadherence as might be expected from previous work, and there is some evidence that fever temperatures might act in the opposite way to reduce endothelial cell binding (Craig, unpublished observations). There is also evidence showing that febrile temperatures may increase pRBC rigidity (95), and this finding might cause at least some of the reduced RBC deformity, leading to the vascular flow obstruction seen in SM; however, there is a need for further studies to confirm this. In the absence of a consistent association with inflammation and malaria pathology as well as the observation of preferential pRBC accumulation in microvessels in SM, researchers have turned to cytoadherence, and many clinical studies (31,45,46,50–58) have attempted to correlate adhesion with disease (Table 1), particularly with CM where cerebral pRBC sequestration is an invariant feature of the disease.

How Might Cytoadherence Cause SM?

As mentioned earlier, complex interactions, including the host inflammatory response and endothelial activation, may contribute to SM, but how does cytoadherence itself modulate the severity of the disease?

When *P. falciparum* infects an RBC, the parasite expresses proteins that are transported to the RBC membrane, causing changes in rigidity and shape of the infected RBC. This may lead to difficulties in RBC flow through the microvasculature, and studies in Thailand (39) and Bangladesh (96) have shown that increased rigidity and reduced flow through blood vessels were associated with severe disease. Other studies on the retinal vasculature (97–99) have shown that micro-haemorrhages and vessel changes, thought to reflect blockage, were highly predictive of CM.

The hypothesis is that adhesion of pRBC in the deep vasculature leads to organ dysfunction. What evidence do we have to support this? As stated earlier, MacPherson et al. (40) showed that there was preferential pRBC accumulation in the brains of people dying of CM compared with in non-CM. One way that this might be taking place is that in some malaria infections, there is higher recruitment of pRBC to cerebral vessels due to the increased levels of receptors such as ICAM-1 (77) and the presence of parasites that are able to bind efficiently to these receptors (32). This clearly oversimplifies the potential mechanisms contributing to the preferential recruitment of pRBC in the brain, and there are likely to be several pathways by which this can be achieved. The role of the infecting parasite variant should not be ignored in this equation, and data from the analysis of pRBC in post-mortem tissues have shown the enrichment of specific PfEMP-1 variant types in the brains of children dying of CM (100).

How might the accumulation of pRBC in tissues lead to pathology? A simple explanation might be that localised ischaemia damages the endothelium, leading to disease. However, the histological evidence only partially supports an impact of endothelial cell destruction, and the relative reversibility of SM on treatment would argue against profound tissue damage. pRBC cytoadherence is known to activate the oxidative cascade (stress-activated protein kinase/c-Jun NH2-terminal kinase pathway), which can regulate gene transcription (101), rho-kinase (102), and nuclear factor κB (103) signalling via radical oxygen species to induce local endothelial activation (104).

Trans-endothelial electrical resistance experiments showed that when pRBC adhered to human brain microvascular endothelial cells, the integrity of the human blood–brain barrier (BBB) reduced 3-fold, causing increased permeability (105). The leakage of BBB leads to serum protein penetrating into the central nervous system (106). This influx of foreign substances activates the microglial cells that release pro-inflammatory cytokines, damaging astrocytes and glial cells that are crucial for BBB maintenance (107). It has also been suggested that interaction of serum protein with TGF-β receptors TGFBR1 and TGFBR2 could result in astrocyte dysfunction, followed by seizures and neuronal death (108).

The binding of pRBC to brain endothelial cells has also been reported to induce endothelial cell apoptosis (109,110). Pino et al. (109) have demonstrated pRBC modulation of the expression of endothelial cell genes such as TNF superfamily genes (Fas, Fas L, and DR-g) and apoptosis-related genes (Bad, Bax, Caspase-3, SARP2, DFF45/ICAD, IFN-γ Receptor 2, Bcl-w, Bik, and iNOS). Toure et al. (110) subsequently showed for the first time that clinical isolates could sometimes induce endothelial cell apoptosis, and Herbert et al. (111) showed that the presence of apoptotic cells might upregulate the expression of cellular adhesion molecules, resulting in hyperadhesiveness, leading to a greater accumulation of pRBC and subsequent endothelial cell apoptosis.

How Might We Alleviate the Symptoms of SM by Targeting Cytoadherence?

As described above, cytoadherence, which we believe may lead to some aspects of disease severity, is a process where mature pRBC erythrocytic stages escape from splenic clearance by binding to endothelial cells and promoting parasite growth in a relatively hypoxic environment. Therefore, can we use this information to devise treatments to prevent death or neurological sequelae?

Antiparasite drugs will still be the main treatment of choice to reduce mortality in patients with malaria; preferentially, these should kill the malaria parasite in early stages (in terms of the erythrocytic cycle) as destruction of non-adhesive ring stages will prevent the next wave of pRBC from sequestering. Therefore, artemisinin is a good choice as it kills ring-stage parasites; this might explain the reduced mortality seen in the field studies from South East Asia (24) and Africa (25) that compared artemisinin and quinine (which only kills mature pRBC). However, even with this welcome progress, there is still over 50% of the mortality recorded during first 48 hours after hospital admission that is largely unaffected by the use of ACTs. This may be because the pRBC has already sequestered to the endothelium. Therefore, there is a need for adjunct therapies to support the critically ill patients, to be used in combination with antimalarials such as artemisinin to remove the sequestered pRBC mass or reduce its effects on the host, while the standard drugs kill the parasite effectively.

To date, several compounds have been explored and screened for their potential to improve SM. N-acetylcysteine (NAC) is an antioxidant drug that is widely used in humans for the treatment of paracetamol overdose and has been shown to be able to reverse almost 72% of pRBC binding to CD36 (112). In addition, it also reduced the rigidity of pRBC (113). A pilot clinical trial study in Thailand (114) showed that NAC was able to normalise serum lactate (an indicator of SM) significantly in SM patients. NAC is thought to inhibit TNF release, thereby reducing cytoadherence. It is also a potent scavenger of free oxygen radicals, which are produced in response to TNF, and can mediate some toxic effects. However, despite these encouraging features, NAC has recently been shown to antagonise the action of artesunate (115), and clinical trials have been disappointing (116), with no reduction in TNF release.

Levamisole is an alkaline phosphatase inhibitor that is used as an antihelminthic drug. Using levamisole for treatment of endothelial cells in vitro showed that it was able to reduce the binding of *P. falciparum* pRBC through dephosphorylated ectodomain of CD36 (117,118). A clinical trial of Levamisole in combination with artesunate is currently underway, and so far, treatment with Levamisole has been shown to be safe and to cause the release of mature pRBC into the peripheral circulation (117). Epigalloyl-catechin-gallate, a naturally occurring polyphenol compound from green tea, was identified as being able to inhibit pRBC binding to ICAM-1 by 50% at micromolar concentrations (119) and has been postulated to synergise the effect of artemisimin on malaria by lowering the IC50 from 14 nM to 8.4 nM (120), but unfortunately, this compound does not appear to be able to reverse adhesion. This highlights the need to test potential anti-cytoadherence agents for inhibition and reversal.

Interventions based on adhesion-related pathology are not limited to attempts to modulate direct interactions of pRBC to specific adhesion molecules but include inhibiting endothelial cell dysfunction during cytoadherence. L-arginine is a substrate for nitric oxide (NO) synthesis by NO synthase. The rationale for the use of L-arginine

follows on from a study (121) in SM patients showing low NO production and low plasma arginine. In normal conditions, NO mediates host resistance to a wide variety of infectious microorganisms, and some in vitro studies have shown that it possessed antiparasitic effects by killing pRBC, as well as an anti-adhesion effect. NO is also a potent inhibitor of TNF production and other pro-inflammatory cytokines implicated in malaria immunopathology (122). Therefore, L-arginine is a good candidate to be used as an adjunct therapy for SM by improving endothelial function. Clinical studies measuring reperfusion parameters have been encouraging (123), and further work is needed in this area to provide better understanding of adhesion-related pathology in malaria and to conduct more clinical trials.

Erythropoietin (EPO) is a hormone produced by the kidney that modulates the survival of developing erythroid precursors and the production of new erythrocytes in the bone marrow. In SM patients, low EPO has been detected and correlated with severe anaemia. Injection of high doses of EPO in mice infected with *P. berghei* showed a significant reduction of pro-inflammatory cytokines TNF and IFN-γ (124) and an increased survival rate when used in combination with artesunate (125). Preliminary clinical trials of EPO in combination with quinine in CM children in Mali showed that it was safe and did not show any side effects (126).

As mentioned before, apoptosis is postulated to be one way in which cytoadherence can cause disease. Therefore, the use of anti-apoptotic agents should be advantageous. Fasudil is a Rho kinase inhibitor and widely used in humans for cardio- and neurovascular diseases. An in vitro study using clinical parasite isolates showed that fasudil has the potential to inhibit apoptosis mediated by *P. falciparum* pRBC adhesion to endothelial cells but showed no effect on reversing or inhibiting pRBC cytoadherence (127). It appears to be a promising adjunctive therapeutic approach for reducing neurological sequelae by reversing endothelial permeability through reducing NFκB activation and endothelial apoptosis (102). The use of statins to control blood cholesterol level has also been shown to be able to restore endothelial damage caused by pRBC cytoadherence (128). Atorvastatin appears to improve endothelial function by increasing NO production, protecting endothelial barrier integrity, reducing oxidative stress, and inhibiting inflammatory responses (129) through activated anti-apoptotic Akt cascade. There is also evidence that statins decrease ICAM-1 expression in stimulated endothelial cell and monocytes (130), but as yet, there is no evidence to show that they are able to reverse established pRBC adhesion.

Further work is needed in this area, such as a better understanding of adhesion-related pathology in malaria and more clinical trials. The latter are complicated by the need to record mortality as an outcome, making the number of patients that need to be recruited relatively large. This means that we need to have better measures of clinical success if this development is to be viable.

What Can We Do for the Future?

The use of adjunct treatments to reverse adhesion of sequestered pRBC is a rational approach to reduce disease severity, but the release of large amount of pRBC into the circulation could be damaging. Can the spleen deal with removing the released pRBC or might it lead to side effects such as splenic dysfunction? Better animal models would help to address this and identify and test lead compounds. Efforts are underway to develop humanised animal models and transgenic parasites (containing PfEMP–1 adhesion domains) that could provide a resource to study the pathophysiology of SM in humans. If we are to preserve some of the advantages gained using ACTs, then the design of new antiparasite drugs should incorporate the ability to kill ring stages as well as mature pRBC and gametocytes.

The need for rapid acting adjunct treatments is critical in order to reduce mortality in SM cases. Anti-adhesion therapies form a part of this portfolio, but we need to understand the biology of this interaction and have better tools to test potential therapies prior to clinical trials.

Authors' Contributions

Conception and design: MFMK, PRP
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