Big Endothelin in Patients with Complicated *Plasmodium falciparum* Malaria

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Plasma concentrations of big endothelin-1 were determined by ELISA in 18 patients with complicated *Plasmodium falciparum* malaria in Bangkok. Before therapy, elevated levels were recorded (21 ± 12 vs. 2.9 ± 1.1 pmol/L in age- and sex-matched healthy subjects; \( P < .001 \)). Even 7 days after therapy, elevated concentrations were seen (25 ± 14 pmol/L). Plasma endothelin levels were correlated with levels of tumor necrosis factor-\( \alpha \) (\( r = .632, P < .01 \)), and a negative correlation with platelet counts was seen (\( r = .783, P < .005 \)). No relation between plasma endothelin concentrations and parasitemia, fever, or other indices of severe infection (hypotension, renal, hepatic or pulmonary impairment, cerebral malaria) existed. During and after complicated malaria, increased levels of plasma endothelin could contribute to malarial pathology or reflect endothelial damage or both.

Endothelial inflammation as a result of the attachment of parasitized erythrocytes and tumor necrosis factor (TNF)-\( \alpha \) overactivity are hallmarks of severe malaria. TNF is known to mediate endothelin (ET) release and increase ET mRNA content in endothelial and epithelial cells [1]. TNF causes renal damage by recruiting leukocytes, accelerating fibrin accumulation, promoting cell lysis, and stimulating the release of vasoconstrictors, and ET causes renal dysfunction by evoking severe reductions in renal blood flow and glomerular filtration rate [2]. Endothelial inflammation and renal failure are common, but often lethal manifestations of severe falciparum malaria, but the relation between TNF and ET in this condition is unknown [3].

ET is produced by injured or ischemic endothelia and can stimulate immune cells to produce proinflammatory cytokines such as TNF and interleukin (IL)-6 [1]. Since TNF and IL-6 have been implicated in the development of endothelial cell damage during complicated falciparum malaria [4], we determined plasma concentrations of big ET, TNF, and IL-6 in 18 patients with severe *Plasmodium falciparum* infection to elucidate this vicious circuit.

**Methods**

**Patients and controls.** Patients with *P. falciparum* malaria conforming to the World Health Organization (WHO) criteria of severe malaria were included in the study [5]. A general physical examination was done on admission. All patients received standard antimalarial treatment with artesunate (120 mg statim intravenously, 60 mg every 12 h to a total of 600 mg). The response to antimalarial therapy was measured according to the WHO standardized classification system [5]. The rates of parasite clearance from peripheral blood and disappearance of fever were considered corroborative evidence of efficacy. Parasite clearance times were calculated from initiation of treatment until the first time that peripheral blood films were negative for asexual parasites. Fever clearance times were calculated from initiation of treatment until the first time that the temperature decreased to 37°C and remained below 37°C for at least 24 h. In addition, clinical efficacy was assessed by recording the duration of coma (cerebral malaria), duration of respirator therapy (respiratory failure), duration of dialysis (renal impairment), and incidence of other complications associated with severe malaria. The healthy control subjects were an age- and sex-matched group of local laboratory personnel. The ill controls were a group of patients with intestinal parasite infections (amebiasis, \( n = 8 \); taeniasis, \( n = 4 \); giardiasis, \( n = 6 \)).

**Laboratory assessments.** For routine patients, care assessments were done as described [6, 7]. Blood samples were obtained before and after initiation of treatment, at 24 h, and 7 days after therapy.

Blood specimens were centrifuged, EDTA-plasma separated, and immediately frozen at -70°C. Plasma levels of big ET were determined by ELISA (Biomedica, Vienna). For this assay, the detection limit was determined to be 0.2 pmol/L. Interassay and intrassay coefficients of variation were <10% and <6%, respectively. The cross-reactivity in this system is <1% with big ET 2 and 3, ET 1-3, and big ET 1-3. Plasma concentrations of TNF and IL-6 were determined by ELISA (RD Systems, Oxford, UK). In normal subjects and in patients with intestinal parasite infection, the mean ± SD levels of TNF were 4.5 ± 2.1 and 5.3 ± 2.6 pg/mL and the mean ± SD levels of IL-6 were 3.2 ± 1.2 and 4.1 ± 2.2 pg/mL (\( P > .01 \) for all).

**Statistical analysis.** Nonparametric tests were used. For comparison between patients and controls, Kruskall-Wallis and
Mann-Whitney-Wilcoxon U test were used. Serum levels on different days were compared with the Wilcoxon rank sum test. For correlation analysis, Spearman’s test was used. All analyses were two-sided, and differences with \( P < .01 \) were considered significant.

Results

Patients. All patients had acute complicated \( P. falciparum \) malaria, with a mean temperature on admission of 38.9 ± 0.6°C. The most frequent symptoms were fever, headache, nausea, and backache. The mean parasite count before treatment was 226.740/\( \mu \)L. The mean parasite clearance time was 75 ± 17 h. Fever persisted an average of 120 h after the start of treatment. Of the 18 patients, 15 required respirator therapy on admission. Fourteen patients had cerebral malaria: 6 had cerebral malaria only, 2 had renal failure only, and 8 had cerebral malaria plus renal failure.

Plasma levels of big ET-1. Plasma concentrations of big ET were significantly elevated in patients with complicated \( P. falciparum \) malaria (mean ± SD: 27 ± 12 vs. 2.9 ± 1 pmol/L in healthy subjects and 3.1 ± 1.2 pmol/L in patients with intestinal parasite infections). Big ET-1 levels were elevated from 5- to 50-fold in all patients on admission, and no change was observed during the first 7 days after initiation of therapy (figure 1). Before therapy, levels of TNF and IL-6 were significantly elevated (117 ± 62 and 281 ± 161 pg/L, respectively). An inverse relation between plasma ET levels and platelet counts (\( r = .783, P < .001 \)) and correlation between big ET and plasma levels of TNF-\( \alpha \) (\( r = .632, P < .005 \)) were detected (figure 2). In contrast, no relation between IL-6 and big ET was seen (\( r = .103 \)).

No differences in big ET levels between cerebral malaria (\( n = 14 \)) and noncerebral malaria (\( n = 4 \)) (26 ± 14 vs. 25 ± 13 pmol/L) and no difference between patients with renal failure (\( n = 10 \)) and with no renal failure (\( n = 8 \)) were seen (28 ± 12 vs. 26 ± 14 pmol/L; \( P > .01 \)). In addition, there was no correlation between big ET concentrations and markers of renal function (serum creatinine, blood urea nitrogen) and arterial blood pressure (diastolic, mean, or systolic) on admission (\( P > .01 \) for all). Serum levels of big ET did not differ between patients receiving hemodialysis for treatment of malaria-induced acute renal failure (\( n = 4 \)) and patients who did not require hemodialysis (\( n = 6 \)). Finally, there was no relation between plasma levels of big ET and parasite clearance time and fever clearance time.

Discussion

In patients with complicated \( P. falciparum \) malaria, plasma big ET concentrations are elevated throughout the first 7 days after initiation of therapy. Big ET, a small 38- --amino acid (aa) peptide, is the biologic precursor of ET (aa 1–21), the most potent vasoconstrictor known today. Big ET is cleaved in the intracellular compartment and on the cell surface of vascular, mesangial, and epithelial cells leading to the active ET (aa 1–21) and to the C-terminal fragment (aa 22–38) [1]. Plasma big ET levels on admission were closely related to thrombocytopenia and high TNF concentrations (\( r = .783 \) and .632, \( P < .01 \) for both). Both conditions are thought to be detrimental aspects in the antiparasite host immune response leading to the clinical complications of cerebral malaria, renal failure, or both. Up to 50% of adult patients with cerebral malaria have evidence of renal impairment, with elevated levels of serum creatinine and blood urea nitrogen [3]. Renal failure is mediated by various inflammatory cytokines, arachidonate metabolites, vasoactive substances, thrombogenic agents, and other factors. TNF and ET-1 have emerged as central mediators in this condition [2].

No relation between big ET and TNF levels and the incidence of renal failure, requirement of hemodialysis, or indices of renal function were seen. Plasma TNF concentrations are said to correlate with severity in falciparum malaria, and yet, in this study, there was a correlation between big ET levels but not with severity. This could be due to the different kinetics of both parameters found in patients’ plasma (no decrease of elevated big ET levels after therapy in contrast to a decrease in TNF concentrations). In addition, most of the renal effects of ET-1 and TNF occur at or adjacent to the site where the peptides are produced and thus plasma levels do not have to reflect their biologic role. Actually, both mediators were elevated in patients with malaria-associated renal dysfunction.

The sustained elevation of big ET could result from sustained endothelial damage [8]. Elevated plasma levels in malaria corre-
Figure 2. Relation between levels of big endothelin and plasma levels of tumor necrosis factor (TNF)-α (left) or platelet count (right) in patients with severe *P. falciparum* malaria.

spond well to this finding, since the consequence of high TNF concentration is endothelial cell activation, expression of endothelial adhesion molecules, and subsequent attachment of parasitized erythrocytes, leading to the recruitment of platelets and neutrophils and ultimately to vessel damage and the clinical complications of severe malaria [4, 9]. Severe vessel wall damage during severe malaria is also suggested by an elevation of soluble adhesion molecules during malaria. In addition, markers of subendothelial matrix (laminin, collagen type I degradation products) have been found in acute infection [7] (unpublished data).

The negative correlation between plasma ET and platelet count merits special attention since ET-1 has been demonstrated to be devoid of a direct effect on platelets. However, it was recently shown that ET-1 may indirectly modulate platelet activation by interfering with the antiaggregating effect of neutrophils. Actually, in the presence of ET-1, the neutrophils appear to provide platelet activation rather than inactivation. Since this effect was not inhibited by an anti-CD18 antibody, it can be considered to be independent of the neutrophil-aggregating effect of ET-1. ET-1 is thought to act as an in vivo negative modulator of the platelet antiaggregating effects of neutrophils through a platelet-activating factor–dependent mechanism [10, 11]. Since both leukocytes and platelets surround sequestered parasitized erythrocytes, high plasma ET-1 concentrations could be implicated in the pathogenesis of platelet activation, aggregation, and sequestration with subsequent thrombocytopenia [12].

ET-1 also causes monocyte production of prostaglandin E₂, TNF, IL-6, and IL-8 and substances that cause neutrophil production of superoxides [1]. Since these mediators are implicated in severe falciparum malaria [4], ET-1 could act as a proximal mediator and thus be accessible for a therapeutic intervention [13, 14]. However, since ET-1 levels are increased in a broad array of conditions, such as shock, sepsis, adult respiratory distress syndrome, burns, pulmonary hypertension, myocardial infarction, thrombolysis and reperfusion, and hepatic failure, the significance of high ET in malaria remains to be determined [1, 7, 15].

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References

Field Evaluation of a Polymerase Chain Reaction–Based Nonisotopic Liquid Hybridization Assay for Malaria Diagnosis

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In a blind field evaluation of a nonisotopic liquid hybridization assay for detection of malaria parasites, 100 blood samples were tested from an area in which malaria is endemic; light microscopy was used as the reference test. Sensitivity, specificity, and positive and negative predictive values of the hybridization assay were 100%. One sample that was microscopy-negative and hybridization-positive was positive when reexamined. Another sample that was microscopy-positive and hybridization-negative was negative at reexamination. The detection limit of the test was ≤0.0005% parasitemia. Four samples with mixed infections were misdiagnosed by microscopy as single-species infections. Four samples diagnosed as mixed infections by microscopy and single infection by the hybridization test had no evidence of a second Plasmodium species upon reexamination. The polymerase chain reaction–based nonisotopic liquid hybridization assay was better than conventional light microscopy in detecting low-grade parasite infection and offers an exceptional advantage for detecting mixed infections.

A definite diagnosis of malaria on the basis of clinical symptoms is very difficult [1]. For the past century, laboratory diagnosis of malaria has depended on visualization of the parasite by light microscopy. This procedure is simple and inexpensive but time and labor intensive and requires expert knowledge of the morphologic differentiation of Plasmodium species [2]. In addition, detection of mixed infections is a problem even to well-trained microscopists because 1 species can dominate the course of an infection [3]. Tests of alternatives to microscopy for malaria diagnosis have had varying specificities and sensitivities, and most do not match microscopy results [4–6].

Because of the limitations of the various procedures for malaria diagnosis, new approaches are warranted and badly needed. We sought to develop a fast, reliable, specific, sensitive, and field-usable malaria diagnosis assay. Our efforts resulted in a polymerase chain reaction (PCR)–based nonisotopic liquid hybridization assay [7]. Here we report the results of a blinded evaluation of this assay under field conditions that assessed the sensitivity, specificity, and predicted values of positive and negative tests compared with those of light microscopy.

Materials and Methods

Subjects. We evaluated 100 3- to 16-year-old students (55% were girls) who attended three schools in the Assembo Bay area of Kisumu, western Kenya, where malaria is endemic.

Microscopic diagnosis. Thick and thin blood films were prepared by fingerprick during the collection process. Slides were stained with Giemsa and analyzed for the presence of parasites and for parasite species. Analyses were done by expert microscopists from the Kenya Medical Research Institute (KEMRI) and the