Endoglin in African Children with *Plasmodium falciparum* Malaria: A Novel Player in Severe Malaria Pathogenesis?

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**Background.** Molecular mechanisms involved in the pathogenesis of severe *Plasmodium falciparum* malaria—specifically, cerebral malaria—are still unclear. Transforming growth factor β (TGF-β) family members are important regulators of inflammation that influence malaria pathogenesis. The soluble form of the auxiliary receptor endoglin (sEng) may play a role in malaria pathogenesis.

**Methods.** Serum levels of sEng were measured using enzyme-linked immunosorbent-assay in Gabonese children with cerebral malaria (*n* = 7), severe malaria (*n* = 43), or uncomplicated malaria (*n* = 43) and were compared with levels in healthy control subjects (*n* = 25) and in another infectious disease group (*n* = 8).

**Results.** Serum sEng levels were higher in patients with cerebral malaria and all patients with severe malaria when compared with levels in patients in the other infection group and the healthy control group. Furthermore, sEng correlated significantly with disease severity. Only 7% of patients with uncomplicated malaria and none of the control patients (patients in the other infection group or the healthy control group) had serum levels higher than 12 ng/mL, whereas this was found in 85.7% of patients with cerebral malaria and 46.5% of patients with severe malaria.

**Conclusions.** High sEng levels may attenuate anti-inflammatory response resulting in clinical deterioration of patients with *P. falciparum* malaria. Our results further corroborate the role of the vascular compartment, especially the endothelium, in severe malaria pathogenesis.

Malaria is one of the main global causes of death from infectious disease, with an estimated 250 million cases leading to nearly 1 million deaths in the year 2006 [1]. Cerebral malaria (CM) is an important, potentially fatal complication occurring in the course of a *Plasmodium falciparum* infection. CM is a neurovascular pathology [2] with pathogenic mechanisms that are far from being completely understood. It has now been widely recognized that microvascular dysfunction and endothe-

The Journal of Infectious Diseases 2009; 200:1842–8

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DOI: 10.1086/648476
<table>
<thead>
<tr>
<th>Variable</th>
<th>Malaria group, by disease severity</th>
<th>Control group, by infection status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cerebral (n = 7)</td>
<td>Severe (noncerebral) (n = 43)</td>
</tr>
<tr>
<td>Female sex, no. of patients</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>Age, median months (IQR)</td>
<td>30 (15–48)</td>
<td>30 (9–86)</td>
</tr>
<tr>
<td>Onset of signs and symptoms, median days</td>
<td>4 (1–7)</td>
<td>3 (0–30)</td>
</tr>
<tr>
<td>Body temperature, mean °C at admission</td>
<td>38.7 ± 0.97</td>
<td>38.8 ± 1.2</td>
</tr>
<tr>
<td>sMODS, median score (IQR)</td>
<td>21 (19–26)</td>
<td>17 (13–23)</td>
</tr>
<tr>
<td>WBC count, mean ×10^3 cells/µL ± SD</td>
<td>12.1 ± 8.1</td>
<td>10.3 ± 5.7</td>
</tr>
<tr>
<td>RBC count, mean ×10^6 cells/µL ± SD</td>
<td>3.2 ± 1.1</td>
<td>2.9 ± 1.1</td>
</tr>
<tr>
<td>Hb level, mean g/dL ± SD</td>
<td>7.1 ± 2.2</td>
<td>6.7 ± 2.5</td>
</tr>
<tr>
<td>Platelet count, mean ×10^4 platelets/µL ± SD</td>
<td>169 ± 152</td>
<td>216 ± 129</td>
</tr>
<tr>
<td>Parasite count, median ×10^7 parasites/µL (IQR)</td>
<td>120 (9.5–703)</td>
<td>56 (0.2–900)</td>
</tr>
</tbody>
</table>

**NOTE.** Hb, hemoglobin; IQR, interquartile range; NS, not significant; RBC, red blood cell; sMODS, simplified multi-organ dysfunction score; WBC, white blood cell.

* By means of analysis of variance, unless otherwise indicated.
* By χ² test.
* By Kruskal-Wallis test.

Binding of TGF-β1 dimers to TGF-β –receptor II (TGF-βRII) initiates recruitment of TGF-β–receptor I (TGF-βRI) with subsequent phosphorylation and conformational changes in their intracellular domain and downstream activation of smad transcription factors [13, 14]. One TGF-βRI, the activin receptor-like kinase 5 (ALK5), is broadly expressed in most cell types [15]. In endothelial cells, binding and activation of ALK5 has growth inhibitory effects and is thought to increase vessel stability [15]. Endothelial cells also express the other TGF-βRI ALK1 and the TGF-β co-receptor endoglin (Eng or CD105, also referred to as TGF-βRIII), both acting in a similar manner: TGF-β1 signaling via Eng or ALK-1 on endothelial cells is associated with vessel destabilization, endothelial cell proliferation and migration, by an indirect inhibition of TGF-β1-ALK5 signaling [16, 17]. Eng is highly expressed in endothelial cells, especially in inflamed tissue [18–21]. Being a marker of angiogenesis [22], expression of Eng is induced by hypoxia, and Eng has antiapoptotic properties under hypoxic stress in endothelial cells [23]. The soluble form of Eng (sEng), released from the cellular membrane into circulation most likely by cleavage through matrix metalloproteinases (MMP) [24], has been shown to cause endothelial dysfunction [25]. sEng has been found antagonizing the membrane-bound form by inhibition of TGF-β1 binding and signaling in endothelial cells, inhibition of nitric oxide synthase (NOS)-dependent vasodilation, and abrogation of anti-inflammatory effects of TGF-β1 [26, 27].

In patients with malaria, low levels of circulating TGF-β1 are described in the literature [8, 9], inversely correlating with disease severity. To our knowledge, sEng has not been investigated in the context of experimental or human malaria. The purpose of this study was to determine levels of circulating sEng in patients with severe malaria (SM) and uncomplicated malaria (UM) and to compare them with levels of sEng in healthy control subjects (the HC group) or patients with infectious diseases other than malaria (the OI group).

**PATIENTS AND METHODS**

**Study site, participants and ethics committee.** Ninety-three children who were hospitalized with *P. falciparum* malaria in the pediatric ward of the Albert Schweitzer Hospital and the Regional Hospital of Lambarencé, Gabon, from October 2005 through May 2006 were prospectively enrolled. Twenty-five healthy sex- and age-matched volunteers served as healthy control subjects (the HC group) or patients with infectious diseases other than malaria (the OI group).

**Clinical definition of malaria and study groups.** Malaria was defined as a blood smear result positive for *P. falciparum* and the presence of fever (axillary temperature ≥37.5°C or rectal temperature ≥38°C) or previous episodes of fever before hospital admission (assessed by parents or guardian) in a child presenting with signs and symptoms of SM or UM [30]. SM was defined according to the World Health Organization (WHO) criteria from 2000 [30], including hyperparasitemia (>10% parasitized red blood cells), severe anemia (hemoglobin, <5 g/dL), prostration (inability to sit or, in younger children,
to drink unaided), jaundice (clinically assessed by examination of conjunctiva and the palm of the hand or, if assessable, a bilirubin level of conjunctiva and the palm of the hand or, if assessable, a bilirubin level >3 g/dL) or respiratory distress (the presence of abnormal deep breathing with nasal flaring and chest recession). Children with ≥1 convulsion within 24 h before admission to the hospital and a Blantyre Coma Score (BCS) of ≤2 at admission to the hospital and no other apparent cause of coma were classified as having CM. Age-matched children without signs and symptoms of infection at the time of investigation and a negative peripheral blood smear result for malarial parasites served as healthy control subjects.

Blood collection and physical examination. Venous blood samples were collected before treatment was given and 24 h after hospital admission with use of sterile Sarstaedt S-Monovette serum tubes containing coagulation activator or sterile Sarstaedt S-Monovette citrate plasma tubes containing 0.106 molar citrate. Serum was obtained by centrifugation at 3000 g for 15 min after 30 min of blood coagulation time after collection. Plasma was obtained by centrifugation at 1500 g for 10 min within 30 min of blood collection. Both serum and plasma samples were stored at −80°C until use. Physical examination at hospital admission was conducted by the same investigator, and the clinical course of patients was reported until hospital discharge. Disease severity was assessed at hospital admission with use of the simplified Multi-Organ-Dysfunction-Score (sMODS) [31, 32].

Management of patients. According to local guidelines at this time, patients with SM were treated with intravenous quinine or sulfadoxine-pyrimethamine in case of UM. According to local guidelines at this time, patients with SM were treated with intravenous quinine or sulfadoxine-pyrimethamine in case of UM. Children with OI were treated with intravenous quinine or sulfadoxine-pyrimethamine in case of UM. Children with OI were treated with intravenous quinine or sulfadoxine-pyrimethamine in case of UM.

Laboratory analysis. White and red blood cell counts, hemoglobin levels, and platelet counts were determined with use of an automated hematology analyzer. Parasitological analysis and assessment of parasitemia was done according to the Lambaréné method [33]. Thick blood films were Giemsa stained and examined by 2 different experienced microscopists. Slides were considered to have negative findings if parasites were not detected after examination of 100 oil-immersion fields of the thick smear.

sEng assays. Serum concentrations of sEng were measured using commercial enzyme-linked immunosorbent assays (ELISA) (RnD Quantikine DNDG00) in accordance with the manufacturer’s instructions. In addition, in 10% of samples serum and citrate plasma values were compared to luate influence of sampling procedures on sEng differences. Serum and plasma samples were used in a 5-fold dilution. Furthermore, 4 selected plasma samples were reanalyzed after ultracentrifugation at 20,800 g for 1 h, as recently described for pelleting of circulating microparticles [34].

Statistical analysis. To reach equal variance and normal distribution, data of sEng serum levels were logarithmically transformed. Levels of sEng were compared between the groups of interest using 1-way analysis of variance. P values for post hoc analysis were Bonferroni corrected. For nonnormally distributed data, the Kruskal-Wallis test and Wilcoxon rank-sum test (when only 2 groups were compared) were used, respectively. A cutoff level was calculated as the mean value for healthy control subjects plus 2.5-fold standard deviation. Values above the cutoff level of 12 ng/mL were considered to be elevated. Repeated measures of sEng levels were analyzed by paired Student’s t test or for nonnormally distributed data by Wilcoxon signed rank test. Proportions were analyzed by χ² test. Correlations between different variables were analyzed by Spearman correlation. Calculations were done using SPSS 15 (Insightful), and graphs were drawn by GraphPad Prism, version 5.00 (GraphPad Software).

RESULTS

Characteristics of study population. Ninety-three children with P. falciparum malaria were enrolled in the study. On the basis of WHO criteria, 50 children were classified as having SM, and 43 were classified as having UM. Twenty-five children served as healthy control subjects, and 8 children with other infections, including acute infectious gastrointestinal disease (3 children), respiratory tract infection (3), and urinary tract infection (2). All 8 children with other infections than malaria experienced convulsions in their disease history as reported by parents. Demographic data, clinical characteristics, and laboratory findings are shown in Table 1. The sMODS was statistically significantly different between study groups (P<.001). They were higher for patients with CM than for patients with SM (P<.001), patients with UM (P<.001), or patients in the OI group (P<.001).

Serum sEng levels. sEng serum levels at hospital admission were higher in patients with SM and patients with CM when compared with serum levels in patients with UM (SM vs UM, P<.001; CM vs UM, P = .001), children with other infections (SM vs OI, P = .001; CM vs OI, P = .016), and healthy control subjects (SM vs HC, P<.001; CM vs HC, P<.001) (Figure 1). There was no difference between the CM and SM groups, between the HC and UM groups, or between the HC and OI groups. Significantly higher proportions of patients with SM and especially patients with CM demonstrated elevated sEng levels >12 ng/mL, as shown in Table 2. After 24 h of treatment, serum sEng levels decreased in patients in the CM group (P = .006), patients in the SM group (P = .006), and patients in the UM group (P = .032). The group of patients in the OI group showed no statistically significant decrease in serum sEng levels after 24 h of treatment. Serum sEng levels after 24 h of treatment were still significantly higher in patients with SM than they were in patients in the UM group (P = .015), patients in the OI group (P = .008), or patients in the HC group (P<.001). Patients in the
Figure 1. Serum soluble endoglin (sEng) levels in children with cerebral malaria (CM; n = 7), other forms of severe malaria (SM; n = 43), uncomplicated malaria (UM; n = 43), children with infections other than malaria and convulsions (OI; n = 8), and healthy control subjects (HC; n = 25) at admission to the hospital. Mean values are indicated by the line through the scatterplots, dotted line indicates sEng cutoff value of 12 ng/mL. Statistical comparison between groups was done using 1-way analysis of variance.

CM group did not show significantly higher sEng serum levels, compared with patients in the SM or UM groups, but did have significantly higher levels compared with those in the OI group (P = .023) and the HC group (P = .035). Patients in the UM group did not significantly differ from patients in the OI group or HC group. Patients in the OI group did not have statistically significantly higher levels of sEng after 24 h of treatment, compared with levels in the HC group.

Comparison of serum and citrate plasma sEng levels yielded a significant correlation (Spearman correlation coefficient R = .843; P < .001). After ultracentrifugation, sEng plasma levels were significantly reduced, compared with precentrifugation analysis (Wilcoxon signed-rank test P < .05).

**sEng and clinical parameters in children with malaria.** A significantly higher proportion of patients with elevated sEng levels had signs and symptoms of neurological impairment (Table 3). There was a statistically significant inverse correlation between BCS and sEng serum levels at hospital admission (Spearman correlation coefficient R = -.0344; P < .001). Other signs and symptoms of SM, such as jaundice and respiratory distress, but not severe anemia or hyperparasitemia, were associated with higher proportions of patients with elevated sEng levels (Table 3). The sMODS correlated significantly with sEng levels at hospital admission (Spearman correlation coefficient R = .511; P < .001) and after 24 h of treatment (Spearman correlation coefficient R = .362; P = .001).

**DISCUSSION**

In this study, we showed that circulating serum sEng is elevated in children with severe *P. falciparum* malaria but not in children with UM. The percentage of children showing elevated sEng levels above the cutoff level of 12 ng/mL was higher among patients with SM (46.5%) and was particularly high among children with CM (85.7%) whereas this was found in only 7% of patients with UM and in none of the control groups (including both the OI group and the HC group). sEng correlated significantly with clinical signs and symptoms of disease severity. We therefore hypothesize that sEng may play an important and, up to now, unknown role in the pathophysiology of severe and complicated *P. falciparum* malaria. Moreover, our results emphasize the role of endothelial cells in malaria pathogenesis. Furthermore, because elevated sEng levels accurately discriminated patients with SM, especially those with CM, from patients with UM, it may function as a novel marker for CM.

sEng competes for TGF-β1 binding to its receptors on endothelial cells and thereby abrogates the anti-inflammatory effects of TGF-β1 [27]. Absence of required anti-inflammatory response during SM may be partially explained by findings of low levels of circulating TGF-β1 and high levels of circulating sEng, which directly inhibits TGF-β, as already demonstrated for other disease entities [27, 35]. Systemic inhibition of TGF-β by high levels of circulating sEng have been shown to induce preeclampsia in pregnant rats [27], which is a disease pattern of pregnancy characterized by systemic endothelial dysfunction, multiple microvascular ischemia, hypertension and proteinuria. In another very recent murine study, TGF-β signaling was inhibited by systemic expression of sEng, leading to numerous abnormalities in the retinal microcirculation, with impaired perfusion of the superficial vascular plexus and vascular leakage [35]. It is tempting to speculate that TGF-β inhibition via high sEng levels could play a role in malarial retinopathy, influencing development of retinal whitening, vessel changes, retinal hemorrhages, and papilledema (reviewed in [36]). Malarial retinopathy is a newly established clinical feature that distinguishes comatose patients with typical histopathological features of CM from those with alternative pathologies [36, 37]. Because malarial retinopathy uniquely reflects the cerebrovascular state during malaria infection, further elucidation of sEng and other members of the TGF-β superfamily in this context may contribute importantly to a better understanding of the pathophysiology of SM, especially CM.

Several clinical and experimental studies on perfusion abnormalities during CM indicate that cerebral hypoxia is likely to be one of the major contributors to CM pathogenesis [36, 38–40]. Hypoxia induces Eng expression in endothelial cells in culture, with concomitantly significantly elevated levels of sEng in the cell culture supernatant [23]. The presented results could therefore be interpreted as a cerebral microvascular hypoxia with subsequent elevation of circulating sEng levels, acting as one of the pathogenic factors contributing to the development of SM, especially CM.
Table 2. Study Groups and Soluble Endoglin (sEng) Cutoff Value

<table>
<thead>
<tr>
<th>Patient group</th>
<th>No. of patients</th>
<th>No. (%) of patients, by sEng level</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral malaria</td>
<td>7</td>
<td>1 (14.3)</td>
<td>6</td>
</tr>
<tr>
<td>Severe malaria</td>
<td>43</td>
<td>23 (53.5)</td>
<td>20</td>
</tr>
<tr>
<td>Uncomplicated malaria</td>
<td>43</td>
<td>40 (93)</td>
<td>3</td>
</tr>
<tr>
<td>Other infection</td>
<td>8</td>
<td>8 (100)</td>
<td>0</td>
</tr>
<tr>
<td>Healthy control</td>
<td>25</td>
<td>25 (100)</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE. The cutoff value was summarized mean healthy control group plus 2.5 standard deviations. Fisher’s exact test was used for comparison to the healthy control group, with Bonferroni correction for multiple comparisons.

Table 3. Clinical Characteristics of Patients with Malaria with Elevated (>12 ng/mL) and Nonelevated (<12 ng/mL) Serum Levels of Soluble Endoglin (sEng)

<table>
<thead>
<tr>
<th>Variable</th>
<th>sEng level</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;12 ng/mL (n = 64)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥12 ng/mL (n = 29)</td>
<td></td>
</tr>
<tr>
<td>Prostration</td>
<td>17 (26.6)</td>
<td>18 (62.1)</td>
</tr>
<tr>
<td>Impaired consciousness</td>
<td>8 (12.5)</td>
<td>12 (41.4)</td>
</tr>
<tr>
<td>Convulsions</td>
<td>11 (17.2)</td>
<td>15 (51.7)</td>
</tr>
<tr>
<td>Respiratory distress</td>
<td>12 (18.8)</td>
<td>18 (62.1)</td>
</tr>
<tr>
<td>Jaundice</td>
<td>14 (21.9)</td>
<td>16 (55.2)</td>
</tr>
<tr>
<td>Severe anemia</td>
<td>10 (15.6)</td>
<td>10 (34.5)</td>
</tr>
<tr>
<td>Hyperparasitemia (≥20% packed red blood cells)</td>
<td>3 (4.7)</td>
<td>3 (10.3)</td>
</tr>
<tr>
<td>sMODS, median score (range)</td>
<td>15 (10–23)</td>
<td>19 (13–26)</td>
</tr>
</tbody>
</table>

NOTE. Data are no. (%) of patients, unless otherwise indicated. Proportions between cutoff sEng levels are analyzed by χ² test. sMODS, simplified multiorgan dysfunction score.

Furthermore, imbalance between vasoconstrictive and vasorelaxant endothelium-derived substances circulating in the plasma of children with malaria has recently been demonstrated [41]. Reduction of circulating TGF-β has a decreasing effect on the endothelial formation of nitric oxide (NO) [27], which is a potent vasoactive molecule with anti-thrombogenic effects. The host protective and anti-inflammatory roles of NO have been intensively studied in malaria pathology (reviewed in [2]). NO is generated via enzymatic conversion of L-arginine to L-citrulline by 3 isoforms of the nitric oxide synthases (NOS) [42], iNOS, eNOS, and nNOS. NO derived from ECs, constitutively formed by the enzyme endothelial NOS (eNOS), mediates vasodilatation, modulates expression of cell adhesion molecules, and inhibits endothelial cell activation and platelet aggregation [43]. Most recently, it was demonstrated that a mutation in the eNOS gene and a specific eNOS haplotype has a protective effect against CM, probably enhancing eNOS expression and NO production [44]. Interestingly, in ECs from patients with hereditary hemorrhagic teleangiectasia—a pathology due to heterozygous mutations in either the Eng or Alk1 gene and characterized by multiple vascular malformations—eNOS has been shown to be downregulated [45].

sEng is most likely released into circulation by cleavage of the membrane-bound Eng through MMPs [24]. Recently, we demonstrated that MMPs are significantly elevated during SM [46], which may partially explain high circulating sEng levels attributable to increased shedding by MMPs.

During CM and SM with coma, increased numbers of endothelial microparticles (EMPs) circulating in the plasma have already been demonstrated by Combes et al [47], which indicates endothelial activation. EMPs are membrane elements that express surface antigens from their cell of origin and might be detected by ELISA coincidentally because of their small size (0.05–1 µm) [48]. Because we found that serum and plasma contain comparable levels of sEng and that ultracentrifugation of plasma significantly reduces levels of sEng, it is worth mentioning that our results might reflect elevated numbers of endoglin-positive EMPs circulating in the plasma of malaria patients. Because EMPs are suspected to be critically involved in SM and especially CM pathogenesis [49], our results underline...
the necessity of further investigation in the field of endothelial activation, shedding of plasma EMPs, and especially the role of endoglin-positive EMPs in the development of SM and CM.

In the present study, we found an association of malaria severity and levels of serum sEng, summarized by a correlation of sEng with sMODS, which is a quantitative score of clinical severity in 10 organ systems [31, 32, 50]. Significantly higher proportions of patients with jaundice, respiratory distress, and signs and symptoms of neurological dysfunction—such as convulsions, prostration or impaired consciousness with a low BCS—yielded elevated sEng levels. Furthermore, we found that sEng levels higher than a cutoff value of 12 ng/mL of sEng are highly specific and sensitive for CM and correlate significantly with signs and symptoms of clinical disease severity.

It should be noted that the small number of patients with CM in our cohort is a limiting factor of our study. We suspect that, for this reason, it was not possible to demonstrate a statistically significant difference in sEng serum levels between patients with CM and patients with other forms of SM.

In summary, sEng serum levels are significantly elevated in patients with SM, especially those with CM, and sEng serum levels accurately discriminate patients with SM and/or CM from patients with UM. Elevated sEng levels in patients with SM could explain previous findings of low levels of circulating TGF-β1 in patients with malaria [8, 9], indicating an inhibition of anti-inflammatory effects of TGF-β by high levels of circulating sEng with a consequential deterioration of clinical characteristics of malaria. Our results further corroborate the hypothesis that members of the TGF-β family, in particular Eng and sEng, play critical roles in malaria pathogenesis and warrant further investigation.

References


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