Expression of Tissue Factor, the Clotting Initiator, on Macrophages in Plasmodium falciparum–Infected Placentas

Takahisa Imamura, Tatsuo Sugiyama, Luis E. Cuevas, Rachel Makunde, and Shin Nakamura

1Division of Molecular Pathology, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, Kumamoto, 2Department of Pathology, Akita University School of Medicine, Akita, and 3Department of Cellular and Molecular Biology, Primate Research Institute, Kyoto University, Inuyama, Japan; 4Liverpool School of Tropical Medicine, Liverpool, United Kingdom; 5Bombo Hospital, Tanga, Tanzania

Plasmodium falciparum malaria during pregnancy is an important cause of maternal and infant mortality. Maternal malaria is associated with insufficient placental growth and function and with fetal growth retardation. Histologically, infected placentas are characterized by the presence of parasitized erythrocytes and mononuclear cells in intervillous spaces, malarial pigment, fibrin deposits, and thickening of the trophoblast basement membrane [1]. The massive intervillous accumulation of mononuclear cells and perivillous fibrin deposition, together with parasitized erythrocytes, are associated with an increased risk of low birth weight and premature delivery, respectively [2]. These findings suggest that there is a link between the accumulation of intervillous mononuclear cells and fibrin deposition with insufficient placental and fetal growth in maternal malaria infection.

Patients with P. falciparum malaria have low concentrations of antithrombin III, high concentrations of the thrombin–antithrombin III complex [3, 4], and high procoagulant activity in peripheral blood monocytes [5]. The hemostatic state of malaria-infected patients could cause organ failure because of insufficient blood supply; however, the mechanism of the coagulation activation is not well understood. P. falciparum–infected erythrocytes stimulate monocytes to express tissue factor (TF)–like activity in vitro [5]. TF triggers the cascade reaction of coagulation factors by interacting with clotting factor VII/VIIa and ultimately yielding thrombin that converts fibrinogen to fibrin [6]. Monocytes or macrophages and endothelial cells express TF on the cell membrane by various inflammatory stimuli, including cytokines such as interferon (IFN)–γ and tumor necrosis factor (TNF)–α [7]. These cytokines are elevated in cultures of placental blood cells from malaria-infected women and are associated with low birth weight [7], suggesting that monocyte TF expression in the placenta may induce coagulation activation in situ that leads to placental insufficiency. However, there is no in vivo evidence of TF expression of monocytes of malaria-infected placentas. In this study, we investigated TF expression and the consequent fibrin deposition on P. falciparum–infected placentas to describe the induction mechanisms of blood coagulation in malaria infection.

Subjects, Materials, and Methods

This study was conducted at Bombo Hospital in Tanga, Tanzania, in April and May 1999. Women who had recently given birth, with normal delivery and no history of hereditary erythrocyte
disorders, were eligible for enrollment. The human immunodeficiency virus (HIV) infection status of participants was not established, but the seroprevalence of HIV in the region is 6%–8%.

Newborns and their placentas were weighed at birth. About 5 mL of placental blood and tissue samples were taken from the maternal surface of the placenta within 1 h after delivery. The blood was kept in EDTA for malaria diagnosis and leukocyte count. Thick and thin films, stained with Field’s and Leishman’s stains, respectively, were prepared and examined microscopically with a ×100 objective lens to identify malaria parasites. Samples were classified as malaria positive or negative. Infected placentas were chosen randomly from parasite-positive placentas. A sample was considered to be negative if no parasites were found after examination of 100 fields on the thick film. Negative control samples were selected from mothers who delivered a single full-term baby (i.e., gestation of 36–40 weeks) after an uneventful pregnancy and whose placental specimens did not contain parasites or malarial pigment.

The mean (±SD) weights of the newborns and placentas of infected women (parity 1, n = 3; parity 2, n = 1; and parity 6, n = 1) were 2.7 ± 0.7 kg and 530 ± 103 g, respectively, whereas those of noninfected women (parity 1, n = 2; parity 2, n = 1; parity 4, n = 1; and parity 6, n = 1) were 3.4 ± 0.6 kg and 620 ± 57 g, respectively. A 1-cm² specimen was cut from the placenta for histology and immunohistochemistry. The tissue, including all layers from the fetal to the maternal-side villi, was fixed and kept in a tissue container with 10% buffered formalin. The sample was obtained from an area between the center and the edge of the placenta that did not show complications, such as infarctions or hematomas.

Parasite density was measured in thick blood films by leukocyte count obtained with a hemocytometer. The count was taken in a section of the thick film where the white blood cells (WBCs) were evenly distributed and the parasites were well stained. Asexual parasites and 100 WBCs were systematically counted. The parasite count was repeated 3 times in different areas, and an average of the 3 counts was taken. Asexual parasite density per microliter of placental blood was calculated as follows: WBC count × parasites counted against 100 WBCs/100. The mean parasite density was 21,488 parasites/μL (range, 2346–80,640 parasites/μL), which indicates that the severity of malaria infection varied, as there is a direct correlation between density of parasites in blood and placentas.

Anti-TF murine monoclonal antibody (MAb) (K108) recognizes the human TF N-terminal region, TF1–83 [8]. A murine MAb reacting only with fibrin but not with fibrinogen was obtained from Immunotech. A murine MAb against human macrophage (KP-1) and APAAP (alkaline phosphatase anti-alkaline phosphatase) and LSAB/HRP (labeled streptavidin biotin/horseradish peroxidase) kits were purchased from Dako. These antibodies were used at a concentration of 2 μg/mL for immunohistochemical studies. Non-specific IgG was isolated from mouse serum by use of a HiTrap protein G column (Pharmacia Biotech), according to the manufacturer’s instructions.

Immunohistochemical staining was performed on paraffin-embedded tissue sections (2-μm thick) of formalin-fixed placentas. After deparaffinizing and hydration through xylene and graded alcohol series, sections were autoclaved in 50 mM citrate buffer (pH 6.0) for 10 min. The sections then were washed in 10 mM Tris-HCl buffer (pH 7.3) containing 150 mM NaCl (TBS) for 5 min (TBS wash) and incubated in 0.3% H₂O₂ in methanol to quench endogenous peroxidase activity. After the TBS wash, sections were incubated in 3% rabbit serum for 30 min to eliminate nonspecific binding of antibodies. After an incubation with a MAb (2 μg/mL TBS) at 4°C overnight, sections were stained by use of the APAAP kit and fast red TR salt (for TF and fibrin) or the LSAB/HRP kit and dianinobenzidine tetrahydrochloride (for macrophages), according to the manufacturers’ instructions. These sections were washed in distilled water, lightly counterstained with Mayer’s hematoxylin, and mounted in Aquatex (Merck). As a control, we used normal mouse IgG instead of the MAbs.

TF-positive cells and macrophages in villi and the intervillous space were counted at random in 5 microscopic high-power fields (×400) in an immunohistochemically stained section, and the total count was shown. Fibrin extent in a microscopic field of fibrin-immunostained sections was measured by digital camera (model DP50; Olympus). Fast red spots were scanned, and the percentage occupancy in a microscopic field (×40) was calculated by computer-assisted image analysis with Mac Scope software (Mitani). The percentage of the area occupied by red spots for a whole field was measured in 3 microscopic fields at random, and the average was used as the percentage of fibrin occupancy in each section. Statistical analysis was done by Student’s t test.

Results

Numerous leukocytes were present in the intervillous spaces of P. falciparum–infected placentas. The majority of these leukocytes, including malarial pigment (hemozoin)–containing cells, were stained by anti-TF MAb. The fibroblast-like cells and macrophages in the villi were also stained, but endothelial cells and syncytiotrophoblasts were TF negative (figure 1A). TF-positive leukocytes in the intervillous space were mononuclear cells, and some adhered to the syncytiotrophoblast layer of villi (figure 1B). Since nonspecific mouse IgG did not stain the cells (figure 1C), it is clear that these mononuclear cells express TF.

TF-positive mononuclear cells resembled macrophages morphologically, and most were confirmed to be macrophages by positive staining for antimacrophage MAb (figure 1D). The quantitative histopathologic analysis showed that most TF-positive cells were macrophages, accounting for >70% of the total leukocytes in infected placentas (table 1). These results indicate that macrophages accumulated in the intervillous space express TF in P. falciparum–infected placentas. In comparison, noninfected placentas had a marginal amount of leukocytes in the intervillous space, and few of the macrophages were TF positive. The majority of TF-positive cells in noninfected placentas were fibroblast-like cells and macrophages in the villi (figure 1G; table 1), which is consistent with previous observations [9].

Fibrin depositions with a meshlike structure were mostly seen in the intervillous spaces of P. falciparum–infected placentas and narrowed and/or plugged intervillous spaces (figure 1E). Fibrin fibril-surrounded macrophages (figure 1F) appear to show that macrophages are associated with fibrin deposition.

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Figure 1. Immunohistochemical staining of placentas with (A–F) or without (G and H) malaria infection. A, B, and G, Anti–tissue factor monoclonal antibody (MAb); D, antimacrophage MAb; E, F, and H, antifibrin MAb; C, nonspecific mouse IgG. Original magnifications: A, C, G, and H, ×60; B and D, ×240; E, ×120; F, ×360.
Some macrophage-aggregated masses packed in fibrin were seen. There was a negligible amount of fibrin deposited in the intervillous space and around TF-positive fibroblist-like cells in noninfected placentas (figure 1H). The tissue occupancy ratio of fibrin was 5-fold higher in infected than in noninfected placentas (table 1). The mean fibrin occupancy correlated significantly with the mean number of TF-positive cells in placentas (correlation coefficient, 0.8976). These results indicate that, presumably initiated by macrophage-expressed TF, coagulation occurs in the intervillous spaces and suggests that the resulting fibrin disturbs blood flow in *P. falciparum*-infected placentas.

### Discussion

This is the first report of TF expression of macrophages accumulated in the intervillous space of *P. falciparum*-infected placenta. Since monocytes isolated from peripheral blood do not express TF without stimulation, the macrophages must have been activated in the infected placentas. *P. falciparum*-infected erythrocytes induce monocytes to express TF in vitro [5] and can be a direct stimulant of TF expression. Glycerol-phosphatidylinositol of *P. falciparum*, a macrophage activator [10], may result in macrophage TF expression, whereas its TF-inducing capacity has not been proved. The high serum TNF-α levels correlated positively with the number of circulating parasitized erythrocytes, suggesting that parasitized erythrocytes induce the production of TNF-α in plasma and, together with IFN-γ, stimulate monocyte TF expression [6]. The high levels of a Th1-type cytokine, IFN-γ, and a proinflammatory cytokine, TNF-α, in cultures of placental blood cells from malaria-infected women [11] and the high procoagulant activity in peripheral blood monocytes [4] may support this indirect pathway of monocyte TF expression by parasitized erythrocytes. Taken together, it seems likely that macrophages express TF in *P. falciparum*-infected placentas.

TF-expressing macrophages present in the intervillous space were surrounded by coagulation factors in plasma. The fact that *P. falciparum*-infected erythrocytes expose phosphatidylserine on the cell surface [12] indicates a supply of this important coagulation cofactor from parasitized erythrocytes. The cascade reaction of coagulation factors is accelerated >10,000-fold [6] when they assemble on phospholipids, especially phosphatidylserine. Decreased plasma levels of antithrombin III and protein C [4, 5], which are negative regulators of the coagulation reaction, would allow for an efficient reaction of coagulation in plasma. It is thus likely that the coagulation reaction initiated by macrophage TF is promoted, resulting in significant fibrin deposition in the infected placentas (figure 1E). In contrast, TF in fibroblast-like cells and macrophages in the villi (figure 1G) appears to be incapable of inducing the coagulation reaction (figure 1H). Because the TF of these intravillous cells is remote from circulating factor VII, they cannot form a complex with this initiating factor. This, in addition to the lack of phospholipids, could explain why little fibrin deposition was seen in noninfected placentas (figure 1H), despite the presence of TF (figure 1G).

The adhesion of *P. falciparum*-infected erythrocytes to the syncytiotrophoblasts via chondroitin sulfate A [13] and/or hyaluronic acid [14] has been proposed as the method for sequestering the erythrocytes in the intervillous space, leading to placental insufficiency and low birth weight. Macrophages phagocytose the cell after specific recognition of the phosphatidylserine on the cell [15]. Macrophages presumably accumulate in response to the phosphatidylserine on the surface of the infected erythrocyte [12]. Interacting with these erythrocytes, macrophages would be stimulated to express TF [5] and ultimately produce fibrin.

Fibrin nets generated around macrophages and the infected erythrocytes facilitate the formation of large and firm masses of cells (figure 1E and 1F). The masses of cells and fibrin probably result in intervillous space narrowing and plugging more effectively than erythrocyte aggregates initiated by adhesion of *P. falciparum*-infected erythrocytes to the syncytiotrophoblasts, disturbing blood supply to the placenta and the fetus. This possibility is supported by the correlation between fibrin deposition and trophoblast necrosis [2] and the association of massive intervillous accumulation of mononuclear cells, perivillous fibrin deposition, and parasitized erythrocytes with increased risk of low birth weight and premature delivery [3]. Conversely, coagulation activation is a host response linked to inflammation, and fibrin deposition may actively assist in walling off *P. falciparum* infection and accelerating its resolution.

Our results may indicate an association of macrophage TF expression and consequent fibrin formation. This should be investigated to establish its role in maternal and fetal complications of *P. falciparum* malaria infection.

### Acknowledgment

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**Table 1.** Histologic analysis data of placentas with or without malaria infection.

<table>
<thead>
<tr>
<th>Placental findings</th>
<th>Infected (n = 5)</th>
<th>Noninfected (n = 5)</th>
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</thead>
<tbody>
<tr>
<td>K108</td>
<td>258 ± 33</td>
<td>34 ± 5</td>
</tr>
<tr>
<td>KP-1</td>
<td>246 ± 57</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>Total leukocytes</td>
<td>336 ± 51</td>
<td>43 ± 15</td>
</tr>
<tr>
<td>Fibrin occupancy, %</td>
<td>4.9 ± 0.7</td>
<td>0.9 ± 0.5</td>
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NOTE. Data are mean ± SD positive cells/5 high-power fields (magnification, ×400), except for fibrin occupancy. The average percentage area occupancy of fibrin was measured in 3 microscopic fields. K108, anti-tissue factor murine monoclonal antibody (MAb); KP-1, murine MAb against human macrophage. P < 0.001, infected vs. noninfected for all variables.
References