

Short Report: Elevated Levels of Vascular Endothelial Growth Factor (VEGF) and Soluble Vascular Endothelial Growth Factor Receptor (VEGFR)-2 in Human Malaria

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Abstract. In cerebral malaria, the binding of parasitized erythrocytes to the cerebral endothelium and the consequent angiogenic dysregulation play a key role in pathogenesis. Because vascular endothelial growth factor (VEGF) is widely regarded as a potent stimulator of angiogenesis, edema, inflammation, and vascular remodeling, the plasma levels of VEGF and the soluble form of the VEGF receptor (sVEGFR)-1 and -2 in uncomplicated malaria patients and healthy adults were measured by enzyme-linked immunosorbent assay (ELISA) to examine their roles in malaria. The results showed that VEGF and sVEGFR-2 levels were significantly elevated in malaria patients compared with healthy adults. Moreover, it was confirmed that malarial parasite antigens induced VEGF secretion from the human mast cell lines HMC-1 or KU812 cell. This is the first report to suggest that the interaction of VEGF and sVEGFR-2 is involved in the host immune response to malarial infection and that malarial parasites induce VEGF secretion from human mast cells.

Malaria is a complex disease that results in the death of more than one million people every year.¹ The most lethal form of malaria is caused by infection by *Plasmodium falciparum* that induces severe anemia and/or cerebral malaria (CM), which are considered to be the most serious complications leading to mortality. Although the detailed pathophysiology of CM remains far from resolved, it is thought that the binding of parasitized erythrocytes to the cerebral endothelium and the consequent angiogenic dysregulation play a key role in the disease pathogenesis. Thus, the pathogenesis of severe malaria is closely related to pathophysiological changes of blood vessels such as endothelial cell activation, increased vascular permeability, and blood-brain barrier dysfunction. A recent study showed that vascular endothelial growth factor (VEGF) and its receptor-related molecules are overexpressed in the brain tissues of CM patients.² Increased levels of VEGF are often detected in tissues and biologic samples from malaria patients.

The VEGF was first identified as a potent stimulator of vascular endothelial permeability and was subsequently reported to promote the proliferation, migration, and survival of endothelial cells.^{3,4} The VEGF is characterized by its highly specific mitogenic activity for endothelial cells and its angiogenic effect observed both *in vitro* and *in vivo*. In addition to its role in promoting endothelial permeability and proliferation, VEGF may also contribute to inflammation and coagulation.⁵ *In vitro*, VEGF induces the expression of cell adhesion molecules, including E-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1, in endothelial cells and promotes the adhesion of leukocytes.^{6,7} Moreover, VEGF signaling up-regulates tissue factor mitochondrial RNA (mRNA), protein, and procoagulant activity.⁸ The proinflammatory/procoagulant effects of VEGF are mediated, at least in part, by the activation of NF- κ B, Egr-1, and nuclear factor of activated T cells (NFAT) transcription factors.^{9–11} The VEGF has been implicated as a pathophysiological mediator in several human diseases, including rheumatoid arthritis, cancer, and inflammatory bowel disease.

Recently, it was reported that mast cells are the main source of VEGF and can rapidly release VEGF from a preformed pool, which is then sustained by the secretion of newly synthesized VEGF.^{12,13} Mast cells play an important role in inflammation¹⁴ and in the host defense against foreign pathogens.^{15–17} Our recent reports have indicated the importance of mast cell activation through Toll-like receptor (TLR) 4 or the binding of Fc ϵ R₁ to IgE antibody for the protection from malarial infection.^{18,19}

In this study, the level of VEGF and the soluble form of its receptors, vascular endothelial growth factor receptor (sVEGFR)-1 and -2, in the plasma from uncomplicated malaria patients and healthy adults was compared to examine the potential role of these molecules in the host immune response to malarial infection. The specific activity of malarial antigens on the secretion of VEGF by human mast cell lines was also studied.

A total of 73 malaria patients with uncomplicated malaria including 55 *P. falciparum* and 18 *Plasmodium vivax* patients were enrolled in this study. The 55 *P. falciparum* patients (parasite density: 5.2×10^3 – $9.8 \times 10^5/\mu\text{L}$) consisted of 46 Asian (39 males and 7 females), 6 Africans (5 males and 1 female), and 3 Caucasians (2 males and 1 female), whereas the 18 *P. vivax* patients (parasite density: 7.2×10^3 – $3.5 \times 10^4/\mu\text{L}$) consisted of 16 Asians (14 males and 2 females) and 2 Africans (2 males). The participants ranged from 18 to 67 years of age and were either tourists or business travelers visiting malaria-endemic countries in South-East Asia, South Asia, or Africa. The clinical manifestations of uncomplicated malaria were defined according to World Health Organization (WHO) criteria.²⁰ The plasma from 15 Asian patients with febrile illness without an obvious source of infection and from 26 healthy adults was also collected to use as a control. The study design was approved by a committee (headed by Dr. Mariko Honda) at Jikei University School of Medicine in Tokyo, where the experiments were performed. The informed consent was obtained from all of the participants. All plasma samples were collected before the treatment of the patient, and in addition, four plasma samples were also obtained from the Asian *P. falciparum* patients at convalescence after the treatment. The plasma was stored at -80°C until used. The VEGF, sVEGFR-1, and sVEGFR-2 levels in the plasma and culture supernatants were measured using a sandwich enzyme-linked immunosor-

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bent assay (ELISA) kit (R&D Systems, Minneapolis, MN). The human mast cell/basophil line KU812 cells²¹ and the human mast cell line HMC-1 cells²² were maintained in RPMI M 1640 medium or IMDM (Invitrogen, Grand Island, NY). The KU812 or HMC-1 cells (10^6 /mL) were treated with 20 μ g/mL of soluble malarial parasite or normal erythrocyte crude antigen for 24 hr. The culture supernatants were then collected and analyzed by ELISA to determine the VEGF concentration. Soluble antigens of malarial parasites were prepared from *P. falciparum* (FCR-3 strain)-infected human erythrocytes cultured *in vitro* or from the blood of *P. vivax*-infected patients. Briefly, malarial parasite-infected erythrocytes were lysed with 0.5% saponin and then washed with phosphate buffered saline (PBS). After two rounds of freeze-thawing, the sample was sonicated at 5A for 30 sec, and then used as crude malarial parasite antigens in the experiments.¹⁹ Normal erythrocyte crude antigen was also prepared from normal erythrocytes using the same method.

The total amount of VEGF was measured in patients infected with *P. falciparum* or *P. vivax* and compared with the levels in the febrile illness and healthy adult groups. The relationship between malarial infection and the VEGF response before drug administration was also examined. The total VEGF concentration in patients with *P. falciparum* or *P. vivax* infections was significantly higher than those in the febrile illness and healthy adult groups (Table 1). However, no significant difference in the VEGF, VEGFR-1, and -2 levels between the *P. falciparum* and *P. vivax* patients (Table 1) or among the different races was observed (data not shown). In addition, a significant association between the age or sex of the participants and the incidence of malaria was not observed. Moreover, there was no significant correlation between parasitemia and the levels of VEGF level (data not shown).

One potential ligand of VEGF is the soluble form of VEGFR-1 (sVEGFR-1 or sFlt-1), which is generated by the differential splicing of VEGFR-1 mRNA.²³ We measured the sVEGFR-1 and -2 levels in the plasma from the malaria patients, febrile illness patients, and healthy adult groups. The sVEGFR-1 plasma level in each of these groups was not significantly different and was very low compared with sVEGFR-2, suggesting that sVEGFR-1 might not participate in malarial infections. Interestingly, the sVEGFR-2 plasma level in malarial patients was dramatically increased compared with the febrile illness patients and healthy adults, whereas the amount of sVEGFR-2 in *P. falciparum* and *P. vivax* patients was not significantly different (Table 1). Finally, four post-treatment plasma samples were obtained from *P. falciparum*-infected

patients, and the VEGF and VEGFR-2 levels in the plasma were determined. The results showed that their levels had returned to comparable levels with the febrile illness patients and healthy adult group after the treatment (data not shown).

To investigate the possibility that malarial antigens can induce VEGF production from mast cells, *in vitro* VEGF production in HMC-1 cells and KU812 cells was examined after their stimulation with malarial antigens. The HMC-1 and KU812 (1×10^6 cells/mL) were stimulated with 20 μ g/mL of *P. falciparum*, *P. vivax*, or normal erythrocyte crude antigens overnight, and then the amount of VEGF in culture supernatants was measured by ELISA. A large amount of VEGF was observed in the supernatants of HMC-1 and KU812 cells after stimulation with both *P. falciparum* and *P. vivax* crude antigens (Table 2), which suggests that malarial parasites induce VEGF secretion from human mast cells during infection.

Recently, Muehlenbachset and others²⁴ reported that the plasma level of sVEGFR-1 was elevated in first-time mothers with either placental malaria, hypertension, or both. However, it has been reported that sVEGFR-1 is abnormally overexpressed in the placenta of preeclamptic patients and is reasonable for the major pathological symptoms on the maternal side such as hypertension and renal dysfunction.²⁵ Clark and others²⁶ reported that the serum from pregnant women contains sFlt-1 (sVEGFR-1), which was not present in the serum from men or nonpregnant women. The serum levels of placenta-derived sVEGFR-1 are also elevated before and during preeclampsia.^{25,27} It is still unclear whether the elevation of sVEGFR-1 observed in the placental malaria is caused by malarial infection or another serious disease affecting pregnancy. In this study, the observed plasma sVEGFR-1 levels in the malarial patients and control groups were low, which is consistent with findings previously reported for healthy volunteers under non-pregnancy-related conditions.²⁵ Generally, sVEGFR-2 has much higher plasma levels than sVEGFR-1, and the kinase activity of VEGFR-2 is approximately 10-fold higher than that of VEGFR-1, suggesting that VEGFR-2 plays a more important role in VEGF-mediated effects *in vivo*.²⁸

Recently, Jain and others²⁹ examined the plasma levels of 30 biomarkers in human malaria using a multiplex bead-based cytokine immunoassay and found that VEGF was protective against CM-associated mortality. The VEGF is known to bind to two membrane-anchored receptors, VEGFR-1(Flt-1) and VEGFR-2 (KDR/Flk-1), on endothelial cells that result in the MAPK signaling cascade. To examine the role of these receptors in murine malaria, *Plasmodium berghei* ANAK was used to infect VEGFR-1 knockout (KO) mice and mice treated with an antagonistic peptide specific to VEGFR-2. Although increased parasitemia was observed in mice treated with the

TABLE 1
Plasma VEGF, sVEGFR-1, and sVEGFR-2 levels in malaria patients and healthy adults*

Subject	VEGF (pg/mL)	sVEGFR-1 (pg/mL)	sVEGFR-2 (pg/mL)
<i>Plasmodium falciparum</i> malaria	62.5 \pm 19.9 [†]	38.9 \pm 15.9	4414 \pm 428 [‡]
<i>Plasmodium vivax</i> malaria	60.9 \pm 20.4 [†]	30.1 \pm 9.3	3839 \pm 587 [‡]
Febrile illness§	13.1 \pm 9.1	25.9 \pm 8.7	2595 \pm 324
Healthy adults	11.5 \pm 8.6	20.9 \pm 11.3	2576 \pm 522

*VEGF = vascular endothelial growth factor; sVEGFR = soluble vascular endothelial growth factor receptor.

[†] $P < 0.01$ (vs. febrile illness and healthy adults).

[‡] $P < 0.01$ (vs. febrile illness and healthy adults).

[§] Patients with febrile illness without an obvious source of infection.

TABLE 2
Vascular endothelial growth factor (VEGF) production in human mast cell lines

Human mast cell	<i>Plasmodium falciparum</i> antigen (pg/mL)	<i>Plasmodium vivax</i> antigen (pg/mL)	Normal erythrocyte antigen (pg/mL)
HMC-1	612.1 \pm 75.3*	375.4 \pm 38.9*	56.2 \pm 8.4
KU812	22494 \pm 97.8*	1692.6 \pm 193.5*	203.5 \pm 79.2

* $P < 0.01$ (vs. normal erythrocyte antigen).

Plasmodium falciparum, *P. vivax*, or normal erythrocyte antigen: *P. falciparum*, *P. vivax*, and normal erythrocyte antigen were prepared according to a previously reported method.⁷ Briefly, *P. falciparum*-infected, *P. vivax*-infected, or normal erythrocytes were lysed with 0.05% saponin in phosphate buffered saline (PBS) at 37°C for 20 min, washed with PBS and then centrifuged at 2,280 g. The pellet was sonicated and used for *in vitro* stimulation of the cells as crude malarial or normal erythrocyte antigens.

antagonistic peptide to VEGFR-2, parasitemia was much lower in the VEGFR-1 KO mice. These findings suggest the importance of VEGF and VEGFR-2 interaction in the host response to malarial infection (Furuta, unpublished data).

We have recently attempted to examine the possible roles of mast cells and VEGF in malaria using mast cell-deficient (W/W^v) and control littermate (+/+) mice. When W/W^v and +/+ mice were infected with *P. berghei* ANKA (PbA), +/+ mice showed lower parasitemia and higher VEGF levels when compared with W/W^v mice. The diminished resistance to infection in the W/W^v mice was considered to be caused by the lack of mast cells and the low amount of VEGF as W/W^v mice reconstituted with the bone marrow-derived mast cells (BMMCs) of +/+ mice recovered resistance to PbA infection and had high VEGF serum levels. Moreover, increased parasitemia was observed in antiVEGF antibody-treated mice compared with nontreated mice (Furuta, unpublished data). Although their precise role is currently unknown, these results clearly suggest the involvement of mast cells and mast cells-derived VEGF in malarial infection. With regard to the mechanism by which mast cells interact with malarial parasites, we have proposed the following explanation. Mast cells are derivatives of hematopoietic progenitor cells that migrate into virtually all vascularized tissues where they complete their maturation. Mature mast cells are normally located in perivascular tissues and close to blood vessels. It is probable that soluble malarial parasite antigens (or the debris of destroyed malarial parasites) leak out of blood capillaries into the perivascular tissues and activate mast cells. We also propose that the spleen is important for the interaction of mast cells and malarial parasites, as parasitized erythrocytes or damaged parasites are filtered from the blood stream in the red pulp of the spleen where mast cells are known to be located.

On the basis of similarities in lineage development and the activation as well as release of mediators, basophils and mast cells have long been considered to be closely related to one another. However, the role of basophils has not yet been explored in human malarial infections. Although mast cells are typically located in tissues and in close association with blood vessels, basophils normally circulate in the blood. In our previous study described above,¹⁹ when mast cell-deficient W/W^v and control +/+ mice were infected with PbA, parasitemia in W/W^v mice was higher than +/+ mice. The resistance to PbA infection in W/W^v mice was recovered by the transfer of BMMCs of +/+ mice. This finding suggests a protective role for mast cells in murine malaria. As to the role of basophils in murine malaria, because basophils were present equally in W/W^v and +/+ mice, it suggests that basophils are not directly involved in the protection against malaria.

In this study, both HMC-1 and KU812 cells secreted VEGF after stimulation with malarial parasite antigens, suggesting the direct activation of mast cells by malarial parasites. As mast cells express TLR1, 2, 3, 4, 6, 7, and 9, it is possible that they are activated directly by molecules that interact with TLRs.²⁹ It has been reported that glycosylphosphatidylinositol and hemozoin derived from malarial parasites function as a ligand for TLR2 or TLR9 on dendritic cells,^{30–32} and recently, we have reported that malarial peroxiredoxin recognizes TLR4 on mast cells and induces cytokine production.¹⁸ Taken together, these findings suggest that malarial parasite components activate mast cells through TLRs to secrete various kinds of inflammatory mediators.

In conclusion, we have confirmed that plasma VEGF levels are elevated in malarial patients compared with febrile illness patients or healthy adults, and is accompanied with an increased level of sVEGFR-2, but not sVEGFR-1. *In vitro* studies showed that malarial parasite antigens induce VEGF secretion from the human mast cell lines KU812 and HMC-1. This is the first report to indicate that the interaction between VEGF and VEGFR-2 is involved in the host immune response to uncomplicated malaria. These findings may be useful for the development of diagnosis and prognostic markers for malarial infection, and will hopefully provide the basis for a new strategy in antimalarial therapy.

Received April 21, 2009. Accepted for publication October 12, 2009.

Acknowledgment: The KU812 cell line was provided by Riken Bioresource Center (Tsukuba, Japan).

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