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Host Vascular Endothelial Growth Factor Is Trophic for *Plasmodium falciparum*-Infected Red Blood Cells

Navakanit Sachanonta[1], Isabelle M. Medana[2], Rachel Roberts[2], Margaret Jones[2], Nicholas P.J. Day[3], Nicholas J. White[3], David J.P. Ferguson[2], Gareth D.H. Turner[2,3] and Emsri Pongponratn[1]

SUMMARY *Plasmodium falciparum*, the protozoan parasite responsible for severe malaria infection, undergoes a complex life cycle. Infected red blood cells (iRBC) sequester in host cerebral microvessels, which underlies the pathology of cerebral malaria. Using immunohistochemistry on post mortem brain samples, we demonstrated positive staining for vascular endothelial growth factor (VEGF) on iRBC. Confocal microscopy of cultured iRBC revealed accumulation of VEGF within the parasitophorous vacuole, expression of host VEGF-receptor 1 and activated VEGF-receptor 2 on the surface of iRBC, but no accumulation of VEGF receptors within the iRBC. Addition of VEGF to parasite cultures had a trophic effect on parasite growth and also partially rescued growth of drug treated parasites. Both these effects were abrogated when parasites were grown in serum-free medium, suggesting a requirement for soluble VEGF receptor. We conclude that *P. falciparum* iRBC can bind host VEGF-R on the erythrocyte membrane and accumulate host VEGF within the parasitophorous vacuole, which may have a trophic effect on parasite growth.

Infection by the protozoan parasite *Plasmodium falciparum* remains a major health problem in developing countries, with a high level of morbidity and mortality.[1] Malaria presents with a range of clinical manifestations, from non-symptomatic infection in immune individuals through acute disease and the serious complications of severe malaria.[2] Neurological involvement during severe malaria, termed cerebral malaria (CM), is associated with coma and seizures.[3] The pathophysiology of cerebral malaria is a controversial area. Neuropathological studies of the brain in patients dying of CM show sequestration of infected red blood cells (iRBCs), which bind to vascular endothelial cells and accumulate in cerebral microvessels.[4] This process is due to specific molecular adhesion between parasite ligands such as *P. falciparum*-erythrocyte membrane protein-1 (PfEMP1) expressed on the iRBC surface, and host receptors on endothelial cells including intracellular adhesion molecules (ICAM-1) and CD36.[5] The degree of parasite sequestration is quantitatively linked to the occurrence of coma.[6, 7]

Other mechanisms have been proposed to account for the effects of iRBC sequestration on brain function, including sequestration of host leukocytes and platelets, metabolic competition, or inhibition of neuronal function due to release of local neu-

From the [1]Department of Tropical Pathology and [3]Mahidol Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, [2]Nuffield Department of Clinical Laboratory Sciences, John Radcliffe Hospital, Oxford University, Oxford, UK.
Correspondence: Emsri Pongponratn
E-mail: tmepp@mahidol.ac.th

roactive mediators.[8] Local mechanical obstruction in the cerebral microvasculature may decrease the supply of oxygen and nutrients to the brain parenchyma. Induction of local hypoxia could be exacerbated by systemic effects of malaria infection such as anemia, hemodynamic shock and renal failure.[9] There is evidence that iRBCs alter host endothelial cell function by direct contact due to cytoadherence and indirectly by the release of soluble factors.[10] Direct adhesion dependent mechanisms play a part in the pathogenesis of CM by causing endothelial cell dysfunction and changes to blood-brain barrier (BBB) permeability, by signaling *via* receptors such as ICAM-1.[11,12] Other studies have shown that some alterations to BBB permeability induced by iRBC *in vitro* are non-adhesion dependant and work 'at a distance'. It has long been speculated that the parasite releases factors which interfere with host cell function, or induce host pathways causing such changes. A recent study demonstrated that a parasite homologue of macrophage inhibitory factor interferes with host monocyte migration and activation.[13]

In order to detect patterns of endothelial and neuronal injury in the brain in CM, we have examined post mortem brain samples and delineated novel patterns of secondary neuropathological injury in CM, such as axonal injury and neuronal stress responses.[14,15] It is unclear whether hypoxic responses occur in the brain in malaria, as have been characterized predominantly in oxygen sensing tissues such as the kidney[16] or hypoxic areas in solid tumors.[17] There is a widespread oxygen sensing system in all tissues, which allows regulation of gene expression in response to decreased oxygen levels. Such responses are predominantly controlled by the expression of hypoxia inducible transcription factors-1 alpha (HIF-1α) and -2 alpha (HIF-2α).[18,19] These coordinate expression of numerous genes involved in energy metabolism, cellular transport and angiogenesis. The latter includes vascular endothelial growth factor (VEGF)[20] and its receptors VEGF-R1 (Flt-1) and -R2 (Flk-1/KDR).[21] VEGF has a key role in angiogenesis, but also plays an autocrine role in survival and function of mature endothelial cells.[22]

We made the unexpected observation that immunostaining for VEGF and activated (phosphorylated) VEGF receptor in the brain of CM patients, not only demonstrated host endothelial cell staining, but also showed positive staining on a proportion of *P. falciparum*-iRBCs. A previous report had investigated VEGF as an angiogenic protein in brain tissue from adult patients with CM[23], but did not report VEGF production or localization within iRBCs. To investigate this phenomenon further, we examined expression of VEGF and VEGF-R immunoreactivity in cultured iRBCs, and investigated its effects on iRBC growth and response to drug treatment *in vitro*.

MATERIALS AND METHODS

Immunohistochemistry for VEGF on post mortem brain samples

Specimens were taken at autopsy from adult Vietnamese patients who had died of severe falciparum malaria on the Malaria Research Ward, Centre for Tropical Diseases, Ho Chi Minh City, Viet Nam; part of a large double blinded trial of artemether *versus* quinine in Viet Nam reported by our research group in 1996.[24] On admission, malaria infection was confirmed by quantification of parasites on peripheral blood smears and subsequently by polymerase chain reaction (PCR) on peripheral blood samples.[25] Cases for immunostaining included cerebral malaria (CM, n = 10) and deaths from severe, non-cerebral malaria (NCM, n = 10), as defined clinically by WHO guidelines.[1] Autopsy was performed with consent from the family, and autopsy and specimen collection protocols were approved by the Ethical and Scientific Committee of the Centre for Tropical Diseases. Subsequent use in immunohistochemical studies was approved by the Central Oxford Research Ethics Committee (ref C01.002).

Brains were fixed in 10% buffered formalin for a minimum of 4 weeks, a formal brain cut was performed and samples taken from various areas of the brain. These were embedded in paraffin and processed using standard histological methods, then stored as cassettes at 26°C. Slides of three selected areas of the brain (cerebral cortex, thalamus, and brainstem) were stained using standard hematoxylin and eosin staining, and examined to judge the pathological features of the disease. For immunohistochemistry, unstained sections of 4 µm thickness were freshly cut on Snowcoat Xtra slides (Surgipath, UK). We examined immunostained sections from each case from the cerebral cortex, thalamus, and brainstem. Controls for VEGF and VEGF-R immunostaining included breast, lung, and renal tumors previously used to optimize staining for these individual antibodies.[26, 27]

Immunohistochemistry

Immunohistochemistry was performed using the following antibodies or antisera; VEGF (clone VG-1, mouse monoclonal),[27] VEGF-R2 (phosphorylated KDR, mouse monoclonal), VEGF-R1 (Flt-1, rabbit polyclonal) (both Santa Cruz, California, USA). The phosphorylated KDR (pKDR) protein[26] was assessed using the monoclonal antibody 34a (made at the Nuffield Department of Clinical Laboratory Sciences, Oxford) against the Y1214 tyrosine residue of the KDR protein. Sections were deparaffinized using histoclear and rehydrated using graded alcohol to water. The sections for VEGF and Flt-1 immunostaining underwent standard antigen retrieval methods by pressure cooking[28] for 2 minutes in 0.05 M Tris/0.2 mM disodium EDTA (Sigma, Poole, UK). Sections for pKDR staining were microwaved in 0.01 M sodium citrate buffer (pH 6.0) for 3 minutes, as detailed previously.[26,27] Subsequent secondary detection of antibody binding was performed using the Envision (HRP) kit (DAKO, St Neots, UK) following manufacturer's instruction. The dilutions of antibodies were determined using optimization on appropriate control tissues. Negative controls included omission of primary antibody. Positive controls were included on each run of staining and consisted of sections of ovary for VEGF and Flt-1, breast carcinoma sections for pKDR. Immunostaining was visualized by addition of di-amino benzidine chromogen (DAB) method and counterstained with Gills Hematoxylin and mounting in Aquamount (BDH) for examination by light microscopy.

Confocal microscopy

Immunofluorescence staining of iRBCs was performed to examine the site of localization of VEGF, Flt-1, pKDR and immunostaining in *P. falciparum*-iRBCs, infected with the A4 and 3D7 strains, cultured using standard methods as detailed below.[29] These strains are laboratory clones originally derived from patient isolates.[30] *P. falciparum*-iRBCs and uninfected RBC controls were synchronized, cultured in 10% human serum or serum-free medium overnight, harvested at late trophozoite stage at 5% parasitemia. Parasites were concentrated to a volume of 0.5 ml then spread on a glass slide and fixed with 100% ethanol at 26°C for 15 minutes, air dried and frozen at -20°C until use. Slides were incubated for 30 minutes with the primary antibodies including the red cell membrane protein markers spectrin (clone RG-26) and Glycophorin A/CD235a (clone JC-159, both gifts from Jackie Cordell, LRF Immunodiagnositics Group, NDCLS, Oxford), neat VG-1 recognizing VEGF, 1:50 dilution of anti-Flt-1 or 1:20 dilution of anti-pKDR. After washing with Tris-buffered saline (TBS), the slides were incubated in darkness for 45 minutes with appropriate isotype specific secondary conjugated antibodies including goat anti-mouse IgG2a (Alexafluor 568 nm) for RG26, goat anti-mouse IgG1 (488 nm) for VG-1, goat anti-mouse IgM for pKDR (488 nm), goat anti-rabbit IgG for Flt-1 (568 nm) and goat anti-mouse IgG for Glycophorin A (647 nm) (Molecular Probes, Cambridge BioScience, Cambridge, UK). After washing with TBS, the sections were mounted with DAKO fluorescence mounting medium containing TOTO-3 iodide (1:500) (Molecular Probes Europe BV) to stain nuclei. Staining was visualized using a Zeiss LSM 510 confocal microscope using Bitplane Imaris Software.

In vitro* culture of *P. falciparum

P. falciparum strains A4 and 3D7 were cultured according to standard methods.[29] Parasites cultured in serum-free medium, according to the method of Rogerson *et al.*,[31] were maintained for a minimum of 24 hours before immunofluorescence staining and washed three times in serum-free medium before staining, to prevent cross reactivity with soluble VEGF in human serum used in the standard growth medium. For experiments on the trophic effect of VEGF, parasite cultures were synchronized using sorbitol lysis and gradient centrifugation to obtain a synchronized culture of ring form iRBCs. Trophozoite-iRBCs were harvested approximately 6-10 hours after synchronization and checked by microscopy on smear preparations.

Investigating the effects of VEGF on parasite growth *in vitro*

To investigate whether exogenous VEGF was trophic for *P. falciparum* growth *in vitro*, or abrogated the effects of drug treatment on inhibition of growth, iRBC (strain 3D7, ring stage) suspensions were adjusted to 5% hematocrit and 2% parasitemia. We examined the effects of serum or serum-free cultured medium, addition of exogenous VEGF (Sigma, UK) at concentrations of 100 or 1,000 pg/ml, and antimalarial drug exposure, using artesunate at IC90 (5 nM) for 1 hour (see Table 1). These concentrations of VEGF were selected to reflect the range of VEGF in serum in reports of control and disease states.[32] IRBCs exposed to artesunate were washed prior to reculture in serum or serum-free medium +/- VEGF for 54 hours (experiments performed in triplicate). Specimens were collected at 0, 6, 24, 30, 48 and 54 hours and % parasitemia, parasite stage, and the proportion of dead (pyknotic) parasites assessed using Giemsa stained thin smears. Differences in parasitemia, stage, and amount of parasite death were compared between groups using analysis of variance (ANOVA), performed using SPSS 11.5 statistical software.

RESULTS

VEGF staining of *P. falciparum*-iRBCs in post mortem tissues and *in vitro*

We found vascular endothelial staining for both VEGF and, to a lesser extent, Flt-1 and pKDR on endothelial cells and neurons, the details of which will be published elsewhere (Medana *et al.*, in preparation). However, of note we demonstrated positive staining of sequestered *P. falciparum*-iRBCs by VEGF was seen in 10/20 (50%) of malaria cases (Fig. 1a). These included cases of cerebral (6/10 = 60%) and non-cerebral (4/10 = 40%) malaria cases. Although other controls showed staining of plasma proteins in the vascular space, the pattern of parasite staining appeared specific for several reasons. Careful examination at high magnification (under oil) showed staining within the parasites themselves, rather than just on the surface of the iRBC. Not all cases showed VEGF staining of parasites, and within positive cases not all iRBCs were stained.

We confirmed staining of *Plasmodium* parasites by anti-human VEGF antibody *in vitro*, using immunofluorescence confocal imaging of *in vitro* cultures of *P. falciparum* (strain A4). This showed intracytoplasmic staining of a proportion of mature trophozoites and schizonts with anti-human VEGF monoclonal antibody (Fig. 1b). Using confocal microscopy, the staining was predominantly localized to the parasitophorous vacuole and the cytoplasm of the developing parasite (Fig. 1c), confirmed by a 3D image rendering in the z-axis (Fig. 1d).

Immunofluorescence for VEGF-R on the surface of cultured *P. falciparum*-iRBC

Confocal immunofluorescence confirmed glycophorin A on the surface of both uninfected and iRBC cultured in both control and serum-free medium, which was unaffected by drug treatment. Both

Table 1 Effects of VEGF and artesunate treatment on parasite growth *in vitro*

Group	Culture conditions	Added VEGF (pg/ml)	Artesunate (nM)	24 hours	48 hours	54 hours
				% parasitemia/ pyknosis	% parasitemia/ pyknosis	% parasitemia/ pyknosis
1	Serum (control)	Serum	0	3/1	6.2/12	6.8/20
2	Serum + VEGF	100	0	2.8/5	7.8*/5	9.2*/12
3	Serum + VEGF	1,000	0	2.4*/2	7.2*/3	10.5*/12
4	Serum-free (control)	0	0	1.4/9	3.7/2	2.7/5
5	Serum-free + VEGF	100	0	2.2*/0	3.2*/11	2.1*/12
6	Serum-free + VEGF	1,000	0	3.3*/2	2.6*/6	2.5*/3
7	Serum + drug (control)	Serum	5	2/34	0.5/66	0.7/66
8	Serum + drug + VEGF	100	5	2.7*/6	1.4*/14	1.4*/23
9	Serum + drug + VEGF	1,000	5	1.8/15	1.2*/38	1.2*/30
10	Serum-free + drug (control)	0	5	1.8/38	1.1/60	0.3/69
11	Serum-free + drug + VEGF	100	5	2.2*/15	1.3*/21	1.4*/19
12	Serum-free + drug + VEGF	1,000	5	2.1/15	1.7*/17	1.1*/21

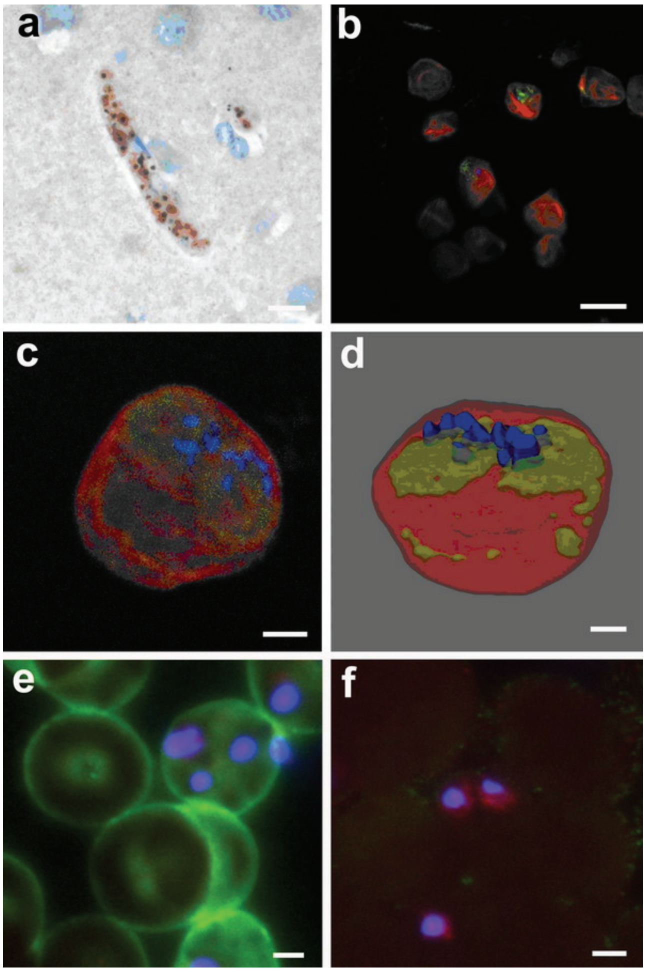

Fig. 1 VEGF and VEGF-R staining of *P. falciparum*-iRBCs: 1a) Immunoperoxidase staining of a sequestered vessel in a case of cerebral malaria showing strong staining for VEGF in iRBCs (haematoxylin counter-stain, bar = 20 μm); 1b) Double immunofluorescence labeling of A4 strain cultured *P. falciparum*-iRBCs showing red cell membrane labelled red (spectrin) and parasites labelled with VEGF (green) (bar = 10 μm); 1c) Confocal microscopic image showing staining for spectrin (red), VEGF (green), and TOTO-3 nuclear staining of the *Plasmodium* parasite (blue) with positive staining for VEGF in the parasitophorous vacuole, but not the red cell cytoplasm (bar = 1 μm); 1d) Rendered 3D image of the same parasite showing the distribution of VEGF staining predominantly within the parasite cytoplasm but also some spreading around a rim under the red cell membrane, corresponding to the parasitophorous vacuole (bar = 1 μm); 1e) Immunofluorescence staining of iRBC A4 strain cultured in normal serum medium, stained with pKDR (green), Flt-1 (red), and TOTO-3 (blue, parasite nuclei) showing surface staining for pKDR on infected and uninfected RBCs (bar = 1 μm); 1f) Immunofluorescence staining of iRBC A4 strain cultured in serum-free medium, stained with pKDR (green), Flt-1 (red), and TOTO-3 (blue, parasite nuclei) showing loss of surface staining for pKDR on infected and uninfected RBCs, with focal intracellular Flt-1 staining (bar = 1 μm).

uninfected and iRBCs showed staining of pKDR and, to a lesser extent, Flt-1 on their surface (Fig. 1e). This staining was almost abolished on both uninfected and iRBCs grown in serum-free medium compared to culture in normal serum medium (Fig. 1f). pKDR staining was seen predominantly on the iRBC surface and there was minimal staining within the iRBC, although occasional iRBC showed faint Flt-1 staining within iRBC. Therefore no convincing evidence of strong staining for VEGF-R was seen within iRBC cultured in either normal or serum-free medium, in contrast to the intracellular staining seen for VEGF in iRBC in post mortem tissues and on confocal immunofluorescence staining.

Effect of VEGF on parasite growth *in vitro*

The effects of exogenous VEGF on parasite growth *in vitro*, and rescue following drug treatment, were examined *in vitro* (Table 1, Fig. 2). The control groups, from a synchronized ring stage culture of 2.6% parasitemia at 0 hour showed normal stage development to trophozoite (33%) and schizont (4%) stages at 24 hours with a small number of pyknotic, dead parasites (1%). Subsequent reinvasion (with no dilution by addition of new uninfected RBCs to the culture) increased the parasitemia to an average 6.2% at 48 hours and 6.8% at 54 hours (n = 3).

Addition of 100 and 1,000 pg/ml VEGF, respectively, to the parasites cultured in 10% normal serum medium showed a mild acceleration in parasite stage development. The rate of conversion to trophozoites and schizonts was not significantly increased at 30 and 48 hours, but the total number of schizonts at 54 hours was significantly lower in both VEGF treated groups, compared to the control, due to a more rapid stage conversion into the next cycle of ring forms. There was no significant difference in stage conversion rates between the two VEGF concentrations. However, there was a significant increase in % parasitemia during later development, at 48 hours (7.2, 7.8) and 54 hours (9.2, 10.5), respectively, compared to the control group ($p < 0.03$ and $p < 0.02$). There was also a significant decrease in the amount of pyknotic, dead parasites at both 48 and 54 hours (5 *versus* 12%, and 12 versus 20%, $p < 0.05$) in VEGF treated parasites compared to controls.

Of note this effect was not seen when parasites were cultured in serum-free medium. Unsurprisingly, growth rate of iRBCs in serum-free medium were significantly lower than those cultured in 10% serum medium. This was seen both in delayed stage development (through trophozoite to schizont stages) and reinvasion, with an increase in the number of pyknotic iRBC, and significantly lower levels of parasitemia compared to the control serum group at all time points ($p < 0.05$). Addition of VEGF to serum-free medium at concentrations of 100 and 1,000 pg/ml showed significant rescue of the rate of stage conversion at 6-30 hours of culture ($p < 0.05$). However, there was no significant difference be-

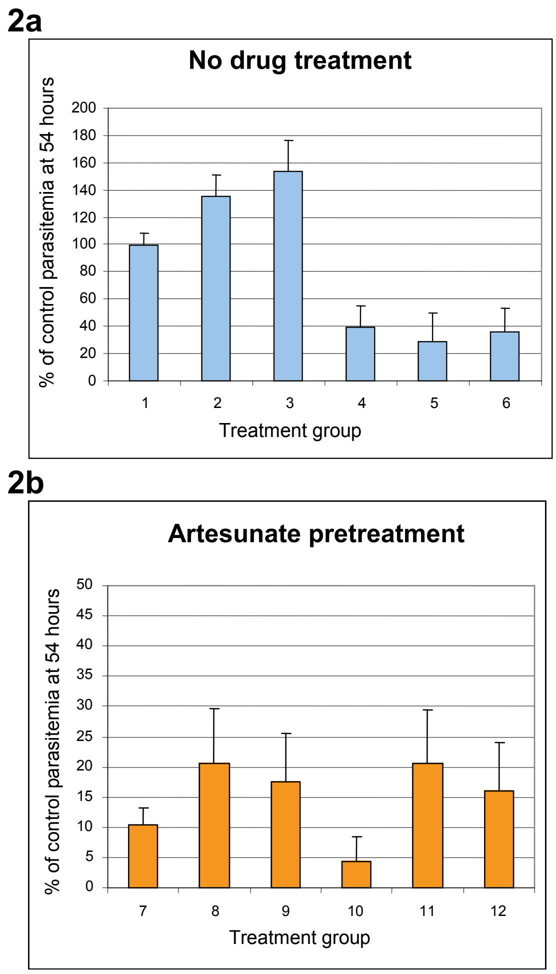

Fig. 2 2a) Effect of exogenous VEGF on *P. falciparum*-iRBCs growth in normal (10% serum) medium (groups 1-3), and serum-free medium (groups 4-6). VEGF was added to culture media at 100 (groups 2 and 5) or 1,000 (groups 3 and 6) pg/ml and their growth rate compared to controls (groups 1 and 3) over the course of 54 hours as described in the methods and Table 1. Addition of VEGF at both concentrations caused a significant increase in final parasitemia at 54 hours compared to control ($p < 0.02$) which was not seen in the serum-free group; 2b) Effect of exogenous VEGF on *P. falciparum*-iRBCs growth following pretreatment with artesunate in normal (groups 7-9) and serum-free medium (groups 10-12) as described in the methods and Table 1. Addition of VEGF at both concentrations caused a significant increase in final parasitemia compared to control ($p < 0.05$) which was not seen in the serum-free group. No significant differences were seen between the two VEGF concentrations.

tween final parasitemia at 54 hours between the controls and VEGF treated iRBC (Fig. 2a), and the proportion of dead, pyknotic parasites was equivalent to control, serum-free culture.

Effect of VEGF on parasite growth after drug exposure

The effect of VEGF on parasite growth was examined after an initial period of pretreatment with artesunate, followed by washing and removal of the drug, and subsequent culture. This was calculated as an IC90 dose, and effectively killed the control group, leading to > 66% pyknosis and stable ring stage parasitemia after 48 hours of subsequent culture, with no stage progression at 54 hours.

However, addition of VEGF to serum medium rescued the parasites from this drug effect, with significantly increased parasite stage development (from 24 hours on) and significantly increased total % parasitemia ($p < 0.05$) from 6 hours post drug treatment. There was also a highly significant decrease in the proportion of pyknotic parasites (decreased from 66% to 23% at 100 and 30% at 1,000 pg/ml, respectively, at 54 hours post exposure, $p < 0.01$). This effect was duplicated in the serum-free medium group (Fig. 2b).

DISCUSSION

We performed a study of the interactions between host (human) VEGF and the intraerythrocytic stage of *P. falciparum*. This arose from our novel finding of VEGF accumulation in iRBC sequestered in post mortem brain tissues from patients dying of malaria. The true incidence of parasite staining is probably higher than the 50% of cases in which we observed immunoreactivity for VEGF, as not all of these cases had observable sequestered parasites, due to treatment effects and decreasing levels of sequestration with increasing time to death.[6,7] Parasite staining for VEGF appeared specific because it was not present in every case, and not present in all sequestered iRBCs within a positive case. Culture of parasites in serum-free medium and measurement of the media over the course of several cycles of schizogony showed no evidence for release of immunoreactive VEGF into the supernatant on ELISA (data not shown). No parasite homologues to human VEGF or VEGF-R were found on a GenBank search of the 3D7 strain malaria genome. These findings implied that either the parasites seen in tissue samples had concentrated circulating host VEGF or were releasing a *Plasmodium* derived product that cross-reacts with the VG-1 antibody to human VEGF. Confocal microscopy showed accumulation of immunoreactive VEGF within the parasitophorous vacuole around the parasite in later intraerythrocytic stages.

Given that sequestered parasites in post mortem samples had been subjected to drug treatment before death, it seemed possible that exogenous VEGF, produced by host cells such as leukocytes or endothelial cells, or circulating VEGF bound to soluble forms of VEGF-R within serum, might be being concentrated within iRBC. We hypothesized that this may represent a stress response to drug treatment, or a trophic factor for parasite growth. The concentration of VEGF in normal human serum varies according to disease state, and can also be affected by release of stored VEGF from platelets[32], but most studies show a range of 100-1,000 pg/ml in serum in control and disease states. Addition of these concentrations of exogenous VEGF to *in vitro* parasite cultures showed a non-significant increase in stage transition and a significant increase in parasitemia at 48 and 54 hours post culture. Whilst these effects were abrogated by culture in serum-free medium, there was still an increase in stage transition.

Following drug pretreatment before culture, exposure of parasites to an IC90 dose of artesunate caused biological death of the culture, with ~ 66% of parasites dying, and a stable ring stage culture at 48 hours onwards with no stage transition. Parasites pretreated with drug and then cultured in serum-free medium was worse. However, addition of exogenous VEGF to these cultures partially rescued parasite growth, both in terms of stage transition and increased final parasitemia, with a much decreased rate of parasite death. These levels were still, however, much lower than in the equivalent non-drug treated groups. These results imply that host VEGF is trophic to parasite growth *in vitro*, and can rescue parasite growth in the face of stress from drug treatment. The abrogation of these effects when parasites were grown in serum-free medium suggests a requirement for soluble VEGF-R from serum, as well as possible roles for other soluble factors in human serum.

Since the parasite did not express or produce VEGF-R, we reasoned that host VEGF must be admitted to the parasitophorous vacuole either *via* passive transport or active uptake bound to soluble VEGF-R. Some staining for Flt-1 and pKDR was seen on the surface of uninfected and iRBC. The finding that this surface staining was almost abolished following culture in serum-free medium suggests that the staining seen on the iRBC membrane reflects passive adsorption of soluble VEGF-R from serum onto the RBC surface. Whether this mediates the passage of VEGF into the parasitophorous vacuole, as seen in parasites in brain sections and in culture, is unclear. Our experiments did not demonstrate appreciable quantities of VEGF-R within the parasitophorous vacuole, with only rare occasion of iRBC showing localization of Flt-1 within the vacuole. It is thus more likely that VEGF-R becomes adsorbed onto the iRBC surface passively, possibly due to changes in the RBC surface membrane following parasitization, and transport of VEGF into the parasitophorous vacuole is mediated separately. The composition and passage of proteins and metabolites between the vacuole and the iRBC membrane is tightly controlled by a number of mechanisms, including parasite induced changes to RBC membrane proteins, lipid rafts and specific transport proteins.[33,34] Several biological examples exist of pathogens which produce VEGF homologues, for instance the ORF viruses, where VEGF release causes characteristic neovascularization and is critical for successful infection.[35,36] However, our data indicate that the VEGF localized around the *P. falciparum* parasite within the iRBC is derived from the host, not the parasite.

Whether this accumulation of VEGF or adsorption of soluble VEGF-R onto the surface of iRBC has a pathogenic effect in the human host is unknown. VEGF has been shown to increase cell adhesion molecule expression such as ICAM-1 on endothelial cells, which would aid sequestration.[37] Increased local concentrations of receptor-immobilized VEGF could also increase blood-brain barrier (BBB) permeability through its action on junctional proteins.[38,39] We and others have observed a decrease in the expression of the junctional proteins and mRNA levels in association with malaria parasite sequestration.[12,40] VEGF signaling, through VEGF-R1, and the neuropilin receptors, is known to have neuroprotective effects.[41] The possibility that *Plasmodium*-iRBCs may be altering host endothelial or neuronal function by interfering with BBB permeability or neuroprotective VEGF signaling pathways is intriguing and requires further research.

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