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Brain endothelial cells increase the proliferation of *Plasmodium falciparum* through production of soluble factors

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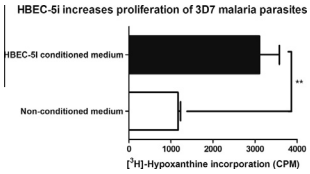
HIGHLIGHTS

- Human brain endothelial cells increased growth of malarial parasites.
- The manner of increase is through production of soluble factors.
- This production of soluble factors was disrupted with the lysis of endothelial cells.
- We also observed this in different brain endothelial cell lines and parasite strains.
- These factors were heat resistant and of low molecular weight.

GRAPHICAL ABSTRACT



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ABSTRACT

We here describe the novel finding that brain endothelial cells *in vitro* can stimulate the growth of *Plasmodium falciparum* through the production of low molecular weight growth factors. By using a conditioned medium approach, we show that the brain endothelial cells continued to release these factors over time. If this mirrors the *in vivo* situation, these growth factors potentially would provide an advantage, in terms of enhanced growth, for sequestered parasitised red blood cells in the brain microvasculature. We observed this phenomenon with brain endothelial cells from several sources as well as a second *P. falciparum* strain. The characteristics of the growth factors included: <3 kDa molecular weight, heat stable, and in part chloroform soluble. Future efforts should be directed at identifying these growth factors, since blocking their production or actions might be of benefit for reducing parasite load and, hence, malaria pathology.

1. Introduction

Malaria continues to be one of the most important infectious diseases in the world. Its medical and social impacts on developing countries have been devastating. One of the most dangerous manifestations of *Plasmodium falciparum* infection is cerebral malaria (CM), which even with anti-malarial therapy has a mortality rate

Abbreviations: CM, cerebral malaria; HBEC, human brain endothelial cells; PfHRP-2, *Plasmodium falciparum* histidine rich protein-2; MCM, malaria complete medium.

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of about 19% (John, 2007). One approach to investigating CM has been *in vitro* cultures (Hunt and Grau, 2003), which have helped in studying the interactions between different cell types and their potential roles in the pathogenesis of CM by modelling the lesion observed in post-mortem studies (Silamut et al., 1999). In CM, important components of the lesion such as brain endothelial cells, peripheral blood mononuclear cells, platelets and infected red blood cells (iRBC) have been studied extensively (Schofield and Grau, 2005). iRBC have been shown to interact with peripheral blood mononuclear cells (Baratin et al., 2005; Binh et al., 1997; Corrigan and Rowe, 2010; D'Ombrain et al., 2007; Hensmann and Kwiatkowski, 2001; Horowitz and Riley, 2009; Khaw et al., 2013; Rhee et al., 2001; Struik et al., 2004; Waterfall et al., 1998), endothelial cells (Adams et al., 2000; Chakravorty et al., 2008; Esslinger et al., 1994; Hughes et al., 2010; Johnson et al., 1993; Tripathi et al., 2007; Tripathi et al., 2009; Viebig et al., 2005), or both concurrently (Khaw et al., 2013). Examples of the interactions between endothelial cells and iRBC *in vitro* include an increase in adhesion molecule expression (Viebig et al., 2005; Wu et al., 2011), induction of apoptosis in endothelial cells (Gillrie et al., 2012; Pino et al., 2003; Wilson et al., 2008), and potentiation of interferon-γ production by blood mononuclear cells (Khaw et al., 2013).

Endothelial cells are an essential part of the blood–brain barrier, along with pericytes, basal lamina, astrocytes and perivascular endfeet (Abbott et al., 2006; Medana and Turner, 2006). They are a key interface between iRBC and the host, particularly in CM, where they sequester *Plasmodium*-infected red blood cells in the brain microvasculature, as evident in African children (Dorovini-Zis et al., 2011; Schofield and Grau, 2005) and in animal models (de Souza and Riley, 2002; Hunt and Grau, 2003). It is believed that such sequestration may benefit the parasite by avoiding clearance in the spleen and by taking advantage of favourable conditions in the microvasculature (Fonager et al., 2012; Ho and White, 1999; Krucken et al., 2005; Sherman et al., 2003). This sequestration is widely believed to contribute to the pathogenesis of CM, which can be the result of low microcirculatory flow (Medana and Turner, 2006) in addition to expression of pro-inflammatory cytokines (Hunt et al., 2006). We recently have shown that brain endothelial cells promote the production of the pro-inflammatory cytokine interferon-γ in co-cultures of *P. falciparum* iRBC and mononuclear cells (Khaw et al., 2013). Most *in vitro* studies have focused on how the iRBC adhere to and activate endothelial cells (Chakravorty et al., 2007; Ockenhouse et al., 1991; Tripathi et al., 2009; Viebig et al., 2005), leaving open the issue of whether the association modifies the development of the parasite. We hypothesised that iRBC-endothelial cell interactions in the microvasculature might promote the growth, as well as the survival, of *P. falciparum*. In this study, we describe the novel finding that endothelial cells can enhance the growth of malaria parasites *in vitro* by producing one or more low molecular weight growth factor(s).

2. Materials and methods

2.1. Ethics statement

Human erythrocytes were purchased from the Red Cross Blood Service, Sydney, and were not tested for haemoglobinopathies or metabolic disorders. Donors gave written informed consent. The studies were approved by the University of Sydney Human Ethics Committee.

2.2. P. falciparum culture

P. falciparum cultures were maintained using methods modified from those described by Trager and Jensen (1976). In brief, parasites were grown in group O^+ human erythrocytes in Malaria Complete Medium (MCM) consisting of RPMI 1640 fortified with 2 mmol/L glutamine (Thermo Fisher Scientific, USA), glucose (10 mmol/L, Amresco, USA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (25 mmol/L, Research Organics, USA), sodium bicarbonate (32 mmol/L, Research Organics, USA) and albumax II (0.5%, w/v, Life Technologies, USA). Cultures were contained in sealed T25 or T75 flasks (Corning, USA) at 5% haematocrit and flushed with a gas mix of 5% O_2, 5% CO_2, and 90% N_2 (Coregas, Yennora, New South Wales). The *P. falciparum* lines used in this study were 3D7 (Walliker et al., 1987) and CS2 (Beeson and Brown, 2004).

2.3. Endothelial cell culture

The immortalised human brain endothelial cell line 5i (HBEC-5i) (Wassmer et al., 2006) was cultured in 25 cm^2 (T25) or 75 cm^2 (T75) flasks (NUNC, Denmark, pre-coated overnight with 0.1% gelatine (w/v in distilled water, Sigma–Aldrich, USA) using Dulbecco's Modified Eagle Media: Nutrient Mixture F-12 (Thermo Fisher Scientific, USA) enriched with 10% (v/v) FBS and 30 μg/mL Gentamycin (Life Technologies, USA) at 37 °C with 5% CO_2 in a humidified atmosphere. Immortalised hCMEC/D3 (Weksler et al., 2005), primary ACBRI 376 (Cell Systems, Kirkland, WA, USA) and primary cAP-0002 (Jusof et al., 2013) (Angio-Proteomie, USA) endothelial cells were grown in EBM-2 (endothelial basal medium-2, LONZA, Switzerland), fortified with 5% (v/v) FBS, 1.4 μmol/L hydrocortisone, 5 μg/mL ascorbic acid (Sigma–Aldrich, USA), 1 in 100 dilution of chemically defined lipid concentrate (Life Technologies, USA), 10 mmol/L HEPES and 1 ng/mL basic fibroblast growth factor (Sigma–Aldrich, USA).

2.4. Pf-Histidine rich protein-2 enzyme-linked immunosorbent assay (PfHRP2 ELISA)

Histidine-rich protein II (HRP2) was measured for estimation of parasite growth, using a modification of the ELISA method described in 2005 by Noedl et al. (2005). In short, 96-well ELISA plates (Nunc, Denmark) were coated first with 100 μL/well of anti-HRP2 IgM antibody solution (Immunology Consultants Laboratories, Inc., USA) before overnight incubation at 4 °C. Next, the wells were washed 3 times with 0.05% Tween 20 (v/v) prior to blocking for 1 h with 200 μL/well of 1% (w/v) skim milk in DPBS at room temperature. After washing, freeze-thawed samples were added at 100 μL per well for 1 h before being replaced with the IgG horse peroxidase-coupled detection antibody (Immunology Consultants Laboratories, Inc., USA) in Dulbecco's phosphate buffered saline with 1% (v/v) Tween 20 and 2% (w/v) bovine serum albumin (BSA). Following washing, 50 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) (Dako, Botany, New South Wales) was added to the sample at a 25% (v/v) final concentration and the reaction was stopped by addition of 50 μL 2 mol/L sulphuric acid. Spectrophotometric analysis was performed at 450 nm using a Spectramax 190 (Molecular Devices, USA).

2.5. [^{3}H]-Hypoxanthine incorporation assay

P. falciparum growth in the absence of endothelial cells was assessed using the [^{3}H]-hypoxanthine incorporation method (Golenser et al., 1981). iRBCs, at starting 2% parasitemia and 0.5% haematocrit, were incubated in a hypoxia chamber in a low oxygen gas mixture (90% N_2, 5% O_2 and 5% CO_2) at 37 °C for 72 h, with the addition of [^{3}H]-hypoxanthine (1 μCi/well) for the last 24 h. The incubation was terminated by placing the plate in a −80 °C freezer. Subsequently, cells were harvested using the Mach III Harvester 96® (Tomtec, USA), with radioactivity measured using a MicroBeta