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The role of heme-oxygenase-1 in pathogenesis of cerebral malaria in the co-culture model of human brain microvascular endothelial cell and ITG *Plasmodium falciparum*-infected red blood cells

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ABSTRACT

Objective: To investigate the role of human host heme-oxygenase-1 (HO-1) in pathogenesis of cerebral malaria in the *in vitro* model.

Methods: The effect of human host HO-1 [human brain microvascular endothelial cell (HBMEC)] on hemoglobin degradation in the co-culture model of HBMEC and ITG *Plasmodium falciparum*-infected red cells (iRBC) through measurement of the enzymatic products iron and bilirubin.

Results: Following exposure to the HO-1 inducer CoPPIX at all concentrations, the HBMEC cells apoptosis occurred, which could be prominently observed at 15 μM of 3 h exposure. In contrast, there was no significant change in the morphology in the non-exposed iRBC at all concentrations and exposure time. This observation was in agreement with the levels of the enzymatic degradation products iron and bilirubin, of which the highest levels (106.03 and 1753.54% of baseline level, respectively) were observed at 15 μM *vs*. 20 μM at 3 h *vs*. 24 h exposure. For the effect of the HO-1 inhibitor ZnPPIX, HBMEC cell morphology was mostly unchanged, but significant inhibitory effect on cell apoptosis was seen at 10 μM for the exposure period of 3 h (37.17% of baseline level). The degree of the inhibitory effect as reflected by the level of iron produced was not clearly observed (highest effect at 10 μM and 3 h exposure).

Conclusions: Results provide at least in part, insight into the contribution of HO-1 on CM pathogenesis and need to be confirmed in animal model.

1. Introduction

Malaria remains one of the most important infectious diseases in the world, raiding developing countries in terms of morbidity and mortality [1]. Cerebral malaria (CM) is one of the most severe pathological complications of *Plasmodium falciparum* (*P. falciparum*) infection manifesting as coma that may lead to death [2]. However, mechanism of the pathogenesis of CM is not completely understood. Several factors associated with pathogenesis and severity of *P. falciparum* infections have been reported, but major factors involve the production of cytokines (IL-4 and IL-12) and tumor necrosis factor (TNF-α) [3,4]. The hypothetical role of heme-oxygenase-1 (HO-1) enzyme in pathogenesis of severe malaria has been proposed as one of the important factors that may be linked with susceptibility and severity of malaria infections [5]. HO-1 is the human enzyme involved in heme degradation process to release the nontoxic products iron, carbon monoxide, and biliverdin/bilirubin. This process therefore significantly influences iron supply that support the growth of *P. falciparum* [6]. It is hypothesized that polymorphisms in the promoter region of the *HMOX1* gene encoding HO-1 might confer protection against severe malaria [5,7–11]. Two single nucleotide

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polymorphisms (SNPs), *i.e.*,T(-1135A) and G(-1135)A, and a (GT)n repeat length polymorphism in the *HMOX1* promoter have been reported to link with susceptibility and severity of malaria disease. The prevalence of patients with short $(GT)_n$ alleles was significantly higher in patients with CM than those with uncomplicated malaria [8]. This suggests that short length $(GT)_n$ repeat in patients with CM possibly results in higher expression level and activity of HO-1 enzyme. Short $(GT)_n$ alleles in the promoter region may therefore represent a genetic risk factor for CM as it may directly enhance the transcription of HO-1 in malaria patients, and increase the products of heme degradation, *i.e.*, carbon monoxide, iron, and bilirubin in the brain [12]. Results from previous studies on the possible role of HO-1 and the risk of malaria infection and disease severity to CM are however, controversial. While the association between the short $(GT)_n$ repeat alleles and risk of severe malaria was reported in Gambia, Myanmar, and Angola[[5,7,8]], lack of such association was reported from Thailand [9] and Ghana [13]. The aim of the study was to investigate the role of human host HO-1 on CM pathogenesis in the *in vitro* model. Specifically, the effect of human host HO-1 (human brain microvascular endothelial cell: HBMEC) on hemoglobin degradation in the ITG *P. falciparum* ITG-infected red blood cells (iRBC) was investigated through the measurement of the enzymatic products iron and bilirubin.

2. Material and methods

2.1. Study design

ITG *P. falciparum*-iRBC was co-cultured with HBMEC to investigate the role of human host HO-1 in the hemoglobin degradation process in iRBC. The model mimics at certain degree, the pathogenesis of CM. The co-culture experiment was divided to 6 groups (3 controls and 3 experimental groups) as follows: Group 1: HBMEC co-cultured with normal RBC (HBMEC/nRBC); Group 2: HBMEC co-cultured with normal RBC, with exposure to HO-1 inducer CoPPIX (HBMEC/nRBC + CoPPIX); Group 3: HBMEC co-cultured with normal RBC, with exposure to HO-1 inhibitor ZnPPIX (HBMEC/nRBC + ZnPPIX); Group 4: HBMEC co-cultured with iRBC (HBMEC/iRBC); Group 5: HBMEC co-culture with iRBC, with exposure to HO-1 inducer CoPPIX (HBMEC/iRBC + CoPPIX); Group 6: HBMEC co-cultured with iRBC, with exposure to HO-1 inhibitor ZnPPIX (HBMEC/iRBC + ZnPPIX).

2.2. Cultivation of ITG P. falciparum l iRBC

ITG strain *P. falciparum* used in the experiment was continuously maintained in culture at Liverpool School of Tropical Medicine, University of Liverpool, UK according to the method described by Trager and Jensen [14]. In brief, parasite was grown in group O^+ human RBCs in Malaria Complete Medium (MCM) consisting of RPMI 1640 fortified with 2 mmol/L glutamine (Thermo Fisher Scientific, Massachusetts, USA), glucose (10 mmol/L: Amresco, Ohio, USA), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) (25 mmol/L: Research Organics, Ohio, USA), sodium bicarbonate (32 mmol/L: Research Organics, Ohio, USA), and albumax II (0.5%, w/v: Life Technologies, California, USA). The parasite was cultured in a 25 cm^2 (T25) or 75 cm^2 (T75) flask (Corning, California, USA) with 5% hematocrit, and 5% O_2, 5% CO_2 and 90% N_2 (Core-gas, Michigan, USA). Three days before the experiment, the culture was synchronized with sorbitol. Schizonts and mature trophozoites were enriched via magnetic separation with an AutoMACSH (MiltenyiBiotec, BergischGladbach, Germany). The experiment was performed twice (triplicate each) using these two late stages of *P. falciparum* as they are the predominant forms that sequester in the brain microcirculation in CM.

2.3. Human brain microvascular endothelial cell culture

The line 5i (HBMEC) was cultured in a 25 cm^2 (T25) or 75 cm^2 (T75) flask (Corning, California, USA) pre-coated overnight with 1% gelatin (w/v in sterile PBS: Sigma-Aldrich, Missouri, USA) for 1 h in Dulbecco's Modified Eagle Media (Thermo Fisher Scientific, Massachusetts, USA). Nutrient Mixture F-12 (Thermo Fisher Scientific, Massachusetts, USA) was enriched with 10% (v/v) FBS and 30 μg/mL gentamycin (Life Technologies, California, USA) at 37 °C with 5% CO_2 in a humidified atmosphere.

2.4. Co-culture experiment

The nRBC and iRBC were co-cultured with HBMEC at the ratio of 100:2 cell density in a flat bottom microplate (Nunc, Roskilde, Denmark) under normoxic conditions. HBMEC was seeded at 2×10^4 cells per well into a 1% (w/v) gelatin (Sigma-Aldrish, Missouri, USA) pre-coated microplate and incubated at 37 °C for 1 h to achieve confluence. To investigate the time- and concentration-dependent inducing effect on HO-1, the co-cultures in group 2 (HBMEC/nRBC + CoPPIX) and 5 (HBMEC/iRBC + CoPPIX) were exposed to the HO-1 inducer Co-protoporphyrin IX (CoPPIX: Sigma-Aldrish, Missouri, USA) at the concentrations of 5, 10, 15 and 20 μM for 0, 3, 6, and 24 h. To investigate the time- and concentration-dependent inhibitory effect on HO-1, the co-cultures in group 3 (HBMEC/nRBC + ZnPPIX) and 6 (HBMEC/iRBC + ZnPPIX) were exposed to the HO-1 inhibitor Zn(II) protoporphyrin IX (ZnPPIX: Sigma-Aldrish, Missouri, USA) at the concentrations of 5, 10, 15 and 20 μM for 0, 3, 6, and 24 h. In groups 1 (HBMEC/nRBC) and 4 (HBMEC/iRBC), the co-cultures were incubated for 0, 3, 6, and 24 h. At the end of each incubation period, the co-culture was centrifuged (25 °C, 300 ×*g* for 3 min) to separate cell supernatant. Morphology of the HBMEC in the cell sediment was examined under light microscope (×10). Concentrations of iron and bilirubin in cell supernatant were determined using QuantiChrom Iron Assay Kit (Bioassay Systems, California, USA) and QuantiChrom Bilirubin Assay Kit (Bioassay Systems, California, USA), respectively, according to the manufacturers' instructions. Concentrations of TNF-α and IL-10 in cell supernatant were determined using Human ELISA Kit (Pierce Biotechnology, California, USA) according to the manufacturer's instructions. The ratio of IL-10 and TNF-α levels was used as a criterion for severity of malaria pathogenesis. The ratio of greater than 1 is associated with non-severe malaria, while that of less than 1 is associated with severe malaria [1]. Data are present as mean and/or range (minimum–maximum) values where appropriate.

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