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Natural hemozoin stimulates syncytiotrophoblast to secrete chemokines and recruit peripheral blood mononuclear cells

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ABSTRACT

Background: Placental malaria is associated with local accumulation of parasitized erythrocytes, deposition of the parasite hemoglobin metabolite, hemozoin, and accumulation of mononuclear cells in the intervillous space. Fetal syncytiotrophoblast cells in contact with maternal blood are known to respond immunologically to cytoadherent *Plasmodium falciparum*-infected erythrocytes, but their responsiveness to hemozoin, a potent pro-inflammatory stimulator of monocytes, macrophages and dendritic cells, is not known.

Methods: The biochemical and immunological changes induced in primary syncytiotrophoblast by natural hemozoin was assessed. Changes in syncytiotrophoblast mitogen-activated protein kinase activation was assessed by immunoblotting and secreted cytokine and chemokine proteins were assayed by ELISA. Chemotaxis of peripheral blood mononuclear cells was assessed using a two-chamber assay system and flow cytometry was used to assess the activation of primary monocytes by hemozoin-stimulated syncytiotrophoblast conditioned medium.

Results: Hemozoin stimulation induced ERK1/2 phosphorylation. Treated cells secreted CXCL8, CCL3, CCL4, and tumor necrosis factor and released soluble intercellular adhesion molecule-1. Furthermore, the dependence of the hemozoin responses on ERK1/2 stimulation was confirmed by inhibition of chemokine release in syncytiotrophoblast treated with an ERK pathway inhibitor. Hemozoin-stimulated cells elicited the specific migration of PBMCs, and conditioned medium from the cells induced the upregulation of intercellular adhesion molecule-1 on primary monocytes.

Conclusions: These findings confirm an immunostimulatory role for hemozoin and expand the cell types known to be responsive to hemozoin to include fetal syncytiotrophoblast. The results provide further evidence that syncytiotrophoblast cells can influence the local maternal immune response to placental malaria.

1. Introduction

Malarial infection is detrimental to pregnancy outcome, contributing to preterm labor, intrauterine growth retardation, and low birth weight (LBW). The World Malaria Report, 2008 reports that an estimated 243 million malaria cases occur each year [1]; in sub-Saharan Africa, at least 25% of pregnant women have evidence of malarial infection at delivery [2]. In infections with *Plasmodium falciparum*, infected red blood cells (iRBCs) sequester in the intervillous space (IVS) of the placenta by binding to receptors on the syncytiotrophoblast (ST) [3]. This is often accompanied by an intense accumulation of maternal inflammatory cells, leading to a condition referred to as placental malaria (PM). In malaria endemic areas, an estimated one of every five cases of LBW is attributable to PM, and maternal infection contributes to 75,000–200,000 infant deaths annually [2].

During the intraerythrocytic stage of malaria, parasites digest hemoglobin in the food vacuole, resulting in the production of potentially toxic heme metabolites [4]. To protect itself from

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oxidative damage, the parasite detoxifies the heme by converting it to an insoluble crystal called malarial pigment or hemozoin [5]. Synthetic hemozoin (sHz; also known as β-hematin) can be chemically synthesized from hemin chloride under acidic conditions and is structurally similar to natural Hz (Hz) (reviewed in [6]). Both Hz and sHz have been used extensively to probe the host response to this ubiquitous parasite byproduct, a response that has been unequivocably shown to be pro-inflammatory [7].

PM-associated placental histopathology is characterized by the presence of Hz extracellularly in the IVS, often trapped in fibrin, and in maternal macrophages [8]. Hz accumulates in tissue and remains for several months, even after parasite clearance [9]. Several epidemiological studies have demonstrated the presence of Hz in the placental tissue of women even in the absence of detectable placental or peripheral parasites [8,10]. Importantly, Hz has been shown to induce cytokine and chemokine secretion by hematopoietic cells [11,12]. During PM, Hz-laden macrophages secrete the chemokines CCL2, CCL3 [13], and CXCL8, and the pro-inflammatory cytokine tumor necrosis factor (TNF) [14]. However, high levels of placental Hz-laden leukocytes were also shown to suppress *ex vivo* cytokine and prostaglandin E_2 production by intervillous blood mononuclear cells [15,16], suggesting that acute and chronic exposure to Hz may have differential effects in the placenta, similar to what has been proposed for dendritic cells [17]. Considered together, these findings suggest that Hz is a critical determinant in the placental immune environment during PM.

While the presence of Hz in the IVS during PM is widely reported, it can also be found in the ST [8], in limited cases at high levels (Fig. 1). The extent to which this cell can respond to Hz, however, has not been studied. We have previously observed that cultured ST respond to cytoadherent *P. falciparum* by activating the mitogen-activated protein kinase (MAPK) JNK, secreting cytokines and chemokines, and eliciting migration of peripheral blood mononuclear cells (PBMCs) [18–20]. In the present study, exposure of ST cells to Hz resulted in the secretion of chemokines and other soluble products in an ERK1/2 MAPK-dependent manner. The secreted products elicited the migration of normal PBMCs and induced the upregulation of Intercellular Adhesion Molecule (ICAM)-1 on primary peripheral blood monocytes.

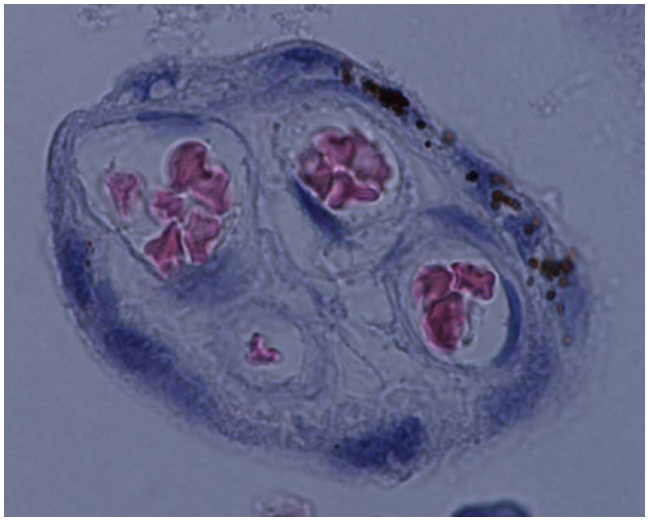

Fig. 1. Hz uptake into ST *in vivo*. Hematoxylin and eosin-stained placental tissue from a malaria-exposed Kenyan woman shows clear evidence of intracellular localization of Hz in ST, suggesting that ST immune responses to Hz, as reported herein, have the potential to influence the placental immunological environment *in vivo*.

2. Materials and methods

2.1. Collection of placentae and trophoblast cell culture

Term placentae were obtained from women delivering either by elective cesarean section at Athens Regional Medical Center, Athens, Georgia (for trophoblast isolation), or by vaginal delivery at Siaya District Hospital in Siaya, Kenya (for histopathology), after written informed consent was obtained. This study was reviewed and approved by the University of Georgia and Athens Regional Medical Center Institutional Review Boards; collection of placentae for histopathology of PM was further approved by the Kenya Medical Research Institute Ethical Review Committee. Primary cytotrophoblast cells were isolated and cultured as described previously [19]. The cells were exposed to varying concentrations of Hz (prepared as described below) within a physiologically relevant range (0.1–20 μg/mL) [21]; 10 μg/mL was deemed optimal for immunologic activation of the ST cells (Fig. 1S). ST cells were also stimulated with 10 μg/mL *Escherichia coli* lipopolysaccharide (LPS; Sigma Aldrich, St. MO) or 10 μg/mL synthetic Hz (prepared as described below) and cultured for up to 24 h. Kenyan placentae were preserved in Streck Tissue Fixative (Streck Laboratories, Omaha, NE), paraffin-embedded, and 5 μm sections stained with hematoxylin and eosin.

2.2. Preparation of natural Hz

Natural Hz was prepared as previously described [21] using *in vitro* cultures of *P. falciparum* iRBCs (strain FCR3). The preparation was ascertained to be endotoxin-free by the use of the Limulus Amoebocyte Lysate gel-clot test (Cambrex Corp. East Rutherford, NJ).

2.3. Preparation of sHz

sHz was prepared as previously described [22] with some modifications. HPLC-purified hemin (MP Biomedical, Solon OH) was dissolved in NaOH and the heme precipitated by addition of glacial acetic acid for 12 h at 80 °C. Crystalline sHz was washed five times with E-Toxate water (endotoxin free water; (Sigma–Aldrich, St. MO), five times with sodium bicarbonate to fully remove free heme, and finally five times with E-Toxate water. The dried sHz was suspended in endotoxin-free PBS at a final concentration of 1 mg/mL, treated with polymyxin B (Sigma–Aldrich, St. MO) to remove trace contamination with endotoxin, and then washed 10 times with endotoxin free PBS to remove the polymyxin B. The sHz was confirmed to be endotoxin free as described above and stored at −20 °C.

2.4. Immunoblotting

Protein lysates from primary ST were prepared as previously described [20]. Thirty-five μg/lane of the lysates (12 μg for LPS-stimulated cells) were separated on a 10% SDS-PAGE and transferred to nitrocellulose membrane. The immunoblotting was performed as previously described, using unconjugated mouse monoclonal antibodies against phosphorylated ERK1/2, JNK1/2 and p38 and rabbit anti-mouse HRP-conjugated antibodies (Cell Signaling Technology Inc., Danvers, MA) as recommended by the manufacturer [20]. Proteins were detected using an enhanced chemiluminescence reagent (Pierce, Rockford, IL) with blue autoradiography film (Genesee Scientific, San Diego, CA). Membranes were stripped with stripping buffer (2% SDS; 62.5 mM Tris–HCl, pH 6.7; 100 mM 2-mercaptoethanol) and reprobed with antibodies against non-phosphorylated forms of the relevant MAPK (Cell Signaling Technologies), which served as loading controls for densitometry analysis (using ImageJ software v.1.43) [23].

2.5. Cytokine, chemokine and cell surface receptor detection by ELISA

Supernatants from stimulated ST were collected over a 24 h time course and stored at −85 °C until used for measurement of CCL3, CCL4, TNF, soluble TNF receptor-1 (sTNFR1), sTNFR2, CXCL8, macrophage migration inhibitory factor (MIF) and soluble ICAM-1 (sICAM-1) by ELISA (R&D Systems, Inc. Minneapolis, MN). Limits of detection were, respectively, 1 pg/mL for CCL3, 2 pg/mL for CCL4, TNF, sTNFR1 and −R2, 4 pg/mL for CXCL8 and sICAM-1, and 8 pg/mL for MIF. IL-10, CCL5 and CCL17 were also assayed but were not detected (data not shown). Protein concentrations were determined using a curve obtained from known concentrations of standards included in each assay plate.

2.6. Inhibition assays

In some experiments, ST cells were treated with 10 μM of the MEK1/2 inhibitor (PD98059; Cell Signaling Technology, Inc. Beverly, MA), an ERK1/2 inhibitor, for 30 min before and during stimulation with Hz or LPS. Supernatants collected over a 24 h time course were assayed for the secretion of cytokines, chemokines and other immune factors as above.