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Artesunate and erythropoietin synergistically improve the outcome of experimental cerebral malaria

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ABSTRACT

Cerebral malaria (CM) is a severe neurological syndrome in humans and the main fatal cause of malaria. In malaria epidemic regions, despite appropriate anti-malarial treatment, 10–20% of deaths still occur during the acute phase. This is largely attributable to poor treatment access, therapeutic complexity and drug resistance; thus, developing additional clinical adjunctive therapies is an urgent necessity. In this study, we investigated the effect of artesunate (AST) and recombinant human erythropoietin (rhEPO) using an experimental cerebral malaria (ECM) model—C57BL/6 mice infected with *Plasmodium berghei* ANKA (PbA). Treatment with the combination of AST and rhEPO reduced endothelial activation and improved the integrity of blood brain barrier, which led to increased survival rate and reduced pathology in the ECM. In addition, this combination treatment down-regulated the Th1 response during PbA infection, which was correlated with the reduction of CCL2, TNF-α, IFN-γ, IL-12, IL-18, CXCL9 and CXCL10 levels, leading to reduced accumulation of pathogenic T cells in the brain. Meanwhile, AST and rhEPO combination led to decreased maturation and activation of splenic dendritic cells, expansion of regulatory T cells, and increased IL-10 and TGF-β production. In conclusion, these data provide a theoretical basis for clinical adjunct therapy with rhEPO and AST in human cerebral malaria patients.

1. Introduction

Malaria is elicited by five species of the protozoan parasites from the genus *Plasmodium*, and is transmitted to humans through the bites of female *Anopheles* mosquitoes. About 429,000 patients die annually as a result of severe malaria, and 90% of all malaria deaths occur in children under 5 years of age [1]. Cerebral malaria (CM) is a severe neurological syndrome in humans resulting from *Plasmodium falciparum* infection [2], and 5–30% of surviving children experience subsequent neurological disabilities [3]. Primary treatment for severe malaria is parenteral quinine or the artemisinin derivative AST; the latter has become an indispensable tool for malaria therapy [4,5]. Artemisinin derivatives have many advantages over quinine; most notably, they have better safety profiles and fewer serious side effects [6,7]. A large multicenter, multi-country, open-label randomized clinical trial on the treatment of malaria in Southeast Asia definitively showed decreased mortality with AST (15%) as compared to quinine (22%) treatment [8,9]. Artemisinin derivatives kill circulating ring-stage parasites, and prevent parasitic maturation and sequestration in deep organs, including the brain [7,10,11]. However, despite appropriate anti-malarial treatment, 10–20% of deaths still occur during the acute phase of CM [4,5]. To improve CM outcome, it is strategically important to explore new adjunct therapies.

A few studies have assessed the pathogenesis of neurologic and cognitive deficits in experimental cerebral malaria (ECM) in rodents. Considering the neuroprotective effect of erythropoietin (EPO), it was proposed as an adjunctive therapy during the acute phase of ECM to reduce the initial death rate. EPO is a 30.4 kDa growth factor and cytokine that controls cell proliferation, immune modulation, metabolic homeostasis, vascular function, and cytoprotection [12]. In our previous study, we confirmed the protective roles of recombinant human erythropoietin (rhEPO) in ECM and showed that EPO could down-regulate the inflammatory response by directly inhibiting the differentiation and function of splenic dendritic cells (DCs) [13]. More recently, it was reported that elevated levels of EPO are associated with decreased acute neurologic deficits in children with CM [14]. Thus, we hypothesize that anti-malarial therapy in combination with cell protective therapy may improve *Plasmodium* infection outcomes for high-risk patients at the early stages of this syndrome. Among the potential cell protective drugs, EPO is one of the most promising drugs

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with its capacity to prevent programmed cell death, reduce the development of pro-inflammatory cytokines, and enable tissue regeneration [15–17]. Recent studies have indicated beneficial effects of EPO or EPO analogs after acute lung injury [17,18] or during sepsis [19,20]. To determine the effect of EPO as a potential new combination therapy to improve ECM outcomes, we used the *Plasmodium berghei* ANKA (PbA) murine malaria model and identified synergistic effects of high-dose EPO in combination with AST treatment.

2. Materials and methods

2.1. Mice and parasitic infection

Female C57BL/6 of 6–8 weeks of age were purchased from the Benxi Animal Institute (Liaoning, China) and maintained at the Animal Care Facilities of the China Medical University. Infections were initiated in C57BL/6 mice via intraperitoneal (i.p.) injection of 1×10^6 PbA-parasitized erythrocytes. Parasitemia was determined by light microscopy of Giemsa-stained thin blood smears. Mortality was monitored daily. Clinical ECM scores were defined as the presence of the following signs: ruffled fur, hunching, wobbly gait, limb paralysis, convulsions, and coma. Each sign was given a score of 1. Animals with scores ≥ 4 were considered to have severe ECM. All procedures of animal manipulation were approved by the Institutional Animal Care and Use Committee of China Medical University, China.

2.2. EPO and/or AST treatment

All experimental mice were randomized into seven groups: normal control, AST group (AST); rhEPO group (EPO); PbA-infected group (PbA); PbA-infected AST treatment group (PbA + AST), PbA-infected EPO treatment group (PbA + EPO), and PbA-infected EPO and AST combination group (PbA + EPO + AST). Normal control, AST-treated and EPO-treated groups comprised the total control group and received no PbA infection, while the PbA group was parasite-infected group and received no treatment. In the PbA + AST group, mice were treated daily by oral gavage of 40 mg/kg AST (Sigma, St. Louis, MO) dissolved in 5% sodium bicarbonate in phosphate-buffered saline (PBS) at 2 to 4 days post-infection (p.i.). In the PbA + EPO group, mice were treated daily by intravenous injection of 50 U rhEPO (Roche, Basel, Switzerland) dissolved in PBS at 2 to 4 days p.i. In the PbA + EPO + AST group, mice were simultaneously administered intravenous injections of 50 U rhEPO in PBS and oral gavage of 40 mg/kg AST at 2 to 4 days p.i. [21–23].

2.3. Histopathology and immunohistochemistry

Three mice from each of the seven groups were euthanized at 5 days p.i. Brains were immediately harvested, fixed in 4% paraformaldehyde for 24 h, and embedded in paraffin. Serial 4-μm-thick brain sections were mounted and stained with hematoxylin and eosin, then examined for microvascular obstruction and leakage. To evaluate the presence of white blood cells (WBC), we counted the mononuclear cells. To detect ICAM-1 and VCAM-1 along the endothelial lining, immunohistochemical staining was performed with specific polyclonal antibodies against ICAM-1 (Abcam, Cambridge, UK) and VCAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using a previously described method [13,24], followed by incubation with biotin-conjugated goat anti-rabbit IgG secondary antibodies. Streptavidin-conjugated peroxidase was added, and color development was performed using 3-amino-9-ethylcarbazole as the substrate. In each experiment, ICAM-1-positive (ICAM-1^+) or VCAM-1-positive (VCAM-1^+) vessels were visualized by microscopy at 400× magnifications for each section. Vessels were enumerated in 20 randomly selected fields per mouse.

2.4. Blood-brain barrier integrity

Blood-brain barrier (BBB) integrity was evaluated by intravenous injection with 200 μL of 2% Evans Blue solution (Sigma, St. Louis, MO, USA) at 5 days p.i. as previously described [25]. Mice were euthanized by i.p. injection of 0.2 mL/20 g chloral hydrate followed by cervical dislocation and perfused with 20 mL of PBS. Brains were removed rapidly and incubated in 2 mL of formamide for 48 h at 37 °C to determine changes in 100 μL of Evans Blue extracted from brain tissue by measuring absorbance at 620 nm [26,27].

2.5. RNA extraction and quantitative real-time PCR

Brain samples were removed and approximately 100 mg was rapidly placed into 1 mL of TRizol (Invitrogen, Carlsbad, CA, USA). Samples were then homogenized via a Fastprep homogenizer (Qbiogene, Santa Ana, CA, USA). Chloroform (0.2 mL) was added and the lysate was mixed thoroughly. After centrifuging at 12,000 × *g* for 20 min at 4 °C, the aqueous layer was transferred to a new tube. RNA was precipitated with 500 mL of isopropanol and pelleted by microfuging at 12,000 × g for 20 min at 4 °C. The pellet was washed with 70% v/v alcohol and resuspended in RNAse-free water. Contaminating DNA was removed by subjecting 2 mg total RNA to DNase I digestion. DNase-treated RNA samples were subsequently stored at − 80 °C. Total RNA was used for reverse transcription with the oligo (dT) primer. The resulting cDNA samples were used for qRT-PCR assays performed using a SYBR Green quantitative PCR kit (Takara, Kusatsu, Japan) for CXCL9 and CXCL10. Primer sequences used here are described in Table 1. PCR was performed for 40 cycles on an ABI PRISM 7700 apparatus (Applied Biosystems, Foster City, CA, USA). Threshold values were calculated and mRNA was quantified using PE Biosystem software. β-actin was used as an internal control to determine the relative ratio of each target gene. A normal control sample value was taken as 100%, and treated values were calculated based on the control. The PCR specificity was confirmed by melting-curve analysis.

2.6. Measurement of cytokines by ELISA

For quantification cytokines, splenocytes were harvested from mice and the supernatants were collected after culturing for 48 h. Levels of CCL2, TNF-α, IFN-γ, IL-12, IL-18, TGF-β, and IL-10 were measured using commercial enzyme linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol (R & D Systems, Minneapolis, MN, USA). Optical density (OD) values were measured at 450 nm using a microplate reader. The concentration of cytokines in each sample was calculated via a standard curve generated using recombinant cytokines, and all cytokines in splenocytes had pg/mL sensitivity.

2.7. Flow cytometry

Splenocytes from a portion of each group were collected to differentiate T-helper 1 (Th1) type cells. Unless otherwise indicated, antibodies were purchased from BD Biosciences (San Jose, CA, USA). To measure intracellular cytokine production derived from Th1, 1×10^7 fresh splenocytes were stimulated in 12-well plates with phorbol myristate acetate (50 ng/mL) and ionomycin (0.5 μg/mL). After 2 h,

Table 1
Primer sequences.

Target gene or mRNA	Primer 5′-3′	
	Forward	Reverse
β-actin	GATTACTGCTCTGGCTCCTAGC	GACTCATCGTACTCCTGCTTGC
CXCL9	CCGAGGCACGATCCACTACA	AGTCCGGATCTAGGCAGGTTTG
CXCL10	GCCGTCATTTTCTGCCTCAT	GCTTCCCTATGGCCCTCATT