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Plasmodium falciparum exhibits markers of regulated cell death at high population density *in vitro*

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ABSTRACT

The asexual erythrocytic cycle of the protozoan parasite *Plasmodium falciparum* is responsible for the pathogenesis of malaria and causes the overwhelming majority of malaria deaths. Rapidly increasing parasitaemia during this 48 hour cycle threatens the survival of the human host and the parasite prior to transmission of the slow-maturing sexual stages to the mosquito host. The parasite may utilise regulated cell death (RCD) to control the burden of infection on the host and thus aid its own survival and transmission. The occurrence of RCD in *P. falciparum* remains a controversial topic. We provide strong evidence for the occurrence of an apoptosis-like phenotype of RCD in *P. falciparum* under conditions of high parasite density. *P. falciparum* was maintained *in vitro* and stressed by allowing growth to an unrestricted peak parasitaemia. Cell death markers, including morphological changes, DNA fragmentation, mitochondrial polarisation and phosphatidylserine externalisation were used to characterise parasite death at the time of peak parasitaemia and 24 h later. At peak parasitaemia, mitochondrial depolarisation was observed, together with phosphatidylserine externalisation in both parasitised- and neighbouring non-infected erythrocytes. DNA fragmentation coincided with a decline in parasitaemia. Fewer merozoites were observed in mature schizonts at peak parasitaemia. Growth recovery to near-peak parasitaemia was noted within two intraerythrocytic cycles. The combination and chronological order of the biochemical markers of cell death suggest the occurrence of an apoptosis-like phenotype. The identification of a RCD pathway in *P. falciparum* may provide novel drug targets, particularly if the pathway differs from the host machinery.

1. Introduction

The asexual erythrocytic cycle of the protozoan parasite *Plasmodium falciparum* is responsible for the pathogenesis of malaria. With every mature schizont-infected erythrocyte capable of releasing approximately 20 new merozoites every 48 h [1], a rapid increase in parasitaemia threatens to overwhelm the host prior to transmitting the slow-maturing gametocytes to the female *Anopheles* mosquito vector [1]. By controlling its own population density, the parasite may be capable of reducing the burden of infection on the host, thereby benefitting its own survival and transmission [2–4]. A number of mechanisms of self-limitation have been proposed, including: (i) increasing the rate of conversion to gametocytes, thereby relocating resources from reproduction to transmission; (ii) limiting the number of merozoites released per mature schizont, (iii) altering the invasion capabilities of merozoites; (iv) regulating population synchronicity and development time; or (v) altering the rate of cell death [2,4]. Regulated cell death (RCD), may offer the most effective mechanism of self-limitation through controlling parasite cell death independent of the host immune system [2].

The Nomenclature Committee on Cell Death (NCCD) strives towards uniform nomenclature in studies describing cell death processes. According to their recommendations, two distinct cell death categories are recognised: (i) accidental cell death (ACD), cellular demise brought about in an uncontrolled fashion as a result of immediate damage after extreme physical, chemical or mechanical stimuli; and (ii) regulated cell death (RCD), which is cell death initiated by genetically encoded machinery [5]. Although it should be noted that the term programmed cell death (PCD) has been previously used to refer to RCD, the NCCD describes PCD as referring only to instances of RCD "that occur as part of a developmental program or to preserve physiologic adult tissue homeostasis" [5]. Therefore, to avoid confusion, the present study uses RCD to refer to a controlled cell death pathway executed by parasite-derived machinery. Furthermore, in keeping with NCCD recommendations,

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apoptosis and autophagy are recognised as possible RCD phenotypes in *P. falciparum* [6]. Regulated necrosis may also present as a RCD phenotype [7] but should not be confused with necrosis due to ACD [5–7].

Apoptosis has been well-documented in vivo during parasite development in the *Anopheles* mosquito and in *in vitro* cultures in the ookinetes and zygotes of *P. berghei* [8–11], although others have reported very little evidence to support these findings [12]. It has been proposed that apoptosis in the sexual stages of *P. berghei* serves as a developmental bottleneck that limits the burden of infection on the anopheline vector [9]. Although an ever-proliferating body of evidence suggests that RCD does occur in *P. falciparum*, the phenotype remains controversial. Several studies have reported the occurrence of an apoptotic or apoptosis-like phenotype [2,13–20], an autophagy-like phenotype [21], necrosis [22], or simply a non-descript phenotype [16,17,21,23]. Some overlap between phenotypes has also been noted. Differences may be partly explained by the diverse combinations of strains, stimuli and biochemical markers used to characterise cell death [24]. Biochemical markers of metazoan RCD have been extrapolated to protozoa, however, RCD may involve features of multiple cell death pathways [6], or may manifest as a unique pathway in protozoans, which may offer novel drug targets.

Although studies have generally focused on drug pressure [2,16,17,21,23,25–27], several natural stimuli encountered by *P. falciparum* during malaria illness have been proposed to induce RCD in the parasite, including heat stress similar to febrile episodes [13,22,28] and starvation [2]. However, only one other study has specifically addressed the occurrence of RCD after high parasitaemia [19]. The present study confirms and expands those findings and shows the appearance of biochemical markers that support the occurrence of an apoptosis-like phenotype of RCD under the stress of high parasitaemia.

2. Materials and methods

2.1. Reagents

The APO-DIRECT TUNEL kit and FITC Annexin V Apoptosis Detection Kit II were obtained from Becton Dickinson (BD Pharmingen, San Diego, CA, USA). Thiazole orange (TO), hydroethidine (HE), 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$], carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Albumax II and RPMI (No. 13018-015) were obtained from Gibco (Life Technologies, NY, USA). Erythrocytes were obtained from healthy donors of any blood group.

2.2. P. falciparum culture

The 3D7 strain of *P. falciparum* was maintained asynchronously according to established methods [29] with some modifications [30]. Briefly, parasites were maintained in complete culture medium [RPMI 1640 with 20 mg/L L-glutamic acid and 2 g/L D-glucose (Gibco, Gran Island, NY, USA, formulation 13,018), supplemented with 0.5% Albumax, 0.21% sodium bicarbonate, 47 mg/L gentamycin and 47 mg/L hypoxanthine] at 5% haematocrit in washed donor erythrocytes. Cultures were seeded as 5 mL cultures in 25 cm^2 sealed culture flasks. Complete culture medium was changed daily and cultures were maintained continually at 37 °C in the dark. Optimal pH was maintained by daily gassing with a mixture of 2% O_2, 5% CO_2 and 93% N_2 for 1 min per flask, at 2.5 L/min flow rate. Parallel to parasitised cultures, non-infected erythrocytes taken from the same erythrocyte stock were maintained under identical conditions. Stock parasites used for seeding experimental and control cultures were asynchronously maintained at low mixed parasitaemia (0.5–5%), with trophozoite/schizont populations not exceeding 2%.

2.3. Achieving in vitro stress from high parasitaemia

Asynchronous cultures were seeded from the same stock at different starting parasitaemia levels, with experimental cultures designated to undergo high population stress seeded above 5%, while non-stressed control cultures were diluted with erythrocytes to a parasitaemia of ~1.5%. Samples of 1.5 mL were taken from each culture at 24 and 48 h. After samples were collected at 48 h, culture flasks were placed upright to minimise any possible effect caused by low culture volumes. Duplicate experimental and control cultures were maintained for the assessment of biochemical markers of cell death.

2.4. Microscopy of Giemsa-stained smears

Giemsa-stained smears were made daily of all cultures to monitor parasite morphology. Dry smears were prepared directly from parasite cultures and stained with a Rapi-Diff II stain kit (Clinical Sciences Diagnostics, South Africa), a modified Giemsa stain. Smears were fixed and stained sequentially with an acid- and basic stain, according to the manufacturer's recommendations. Stained smears were observed with an Olympus BX53 microscope, under an Olympus Plan C UIS2 100 X/1.25 oil immersion objective. Microscopic images were captured with an Olympus SC50 digital camera and processed with Olympus Stream Basic v.1.9.3 software. Slight adjustments to contrast, sharpness and brightness were made with Microsoft Powerpoint 2010, to enhance the quality of images. All microscopic images include a scale bar.

2.5. Comparing the number of merozoites produced per schizont

Schizonts were photographed from Giemsa stained smears of asynchronous cultures under stress from high parasitaemia and parallel control cultures. At least 50 schizonts per group were captured and quantified. Only mature schizonts, with clear individual merozoites, derived from single parasite infections, as judged by the presence of a single concentrated area of haemozoin, were considered. Individual merozoites in each schizont were counted by two scientists. Where possible, counts were performed blinded. The average numbers of individual merozoites per schizont were compared for stressed and control cultures at peak parasitaemia (24 h).

2.6. Flow cytometry

Flow cytometric analyses were performed on a Beckman Coulter Gallios flow cytometer (Beckman Coulter Inc., Miami, FL, USA). Excitation for all assays was by 488 nm argon laser at 22 mW. Emission was detected with the use of 545/40BP (525 ± 20 nm, FL1) and, where indicated, 575/30BP (575 ± 15 nm, FL2) filters. Optical alignment was monitored daily with Beckman Coulter Flow Check Pro fluorospheres (Beckman Coulter Inc., Brea, CA, USA). Post-acquisition analyses were performed with Beckman Coulter Kaluza (v1.1) software.

2.7. Thiazole orange flow cytometry for parasitaemia

Parasitaemia was determined daily with the use of the nucleic acid-binding dye TO, according to an established method [31]. Samples of 10 μL were collected from each culture and diluted to 1 mL in Sorenson's phosphate buffer (47 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7.2) with a final TO concentration of 1 μM (diluted from a 10 mM stock in methanol). Samples were incubated at room temperature in the dark for 20 min and analysed within 1 h. Erythrocytes were gated on a forward- (FS INT log, 269 V, Gain 1.0) versus side-scatter (SS INT log, 336 V, Gain 1.0) dot plot and analysed on a single-parameter thiazole orange (FL1 INT log, 443 V, Gain 1.0) histogram, with regions for uninfected, ring-infected and trophozoite- or schizont-infected erythrocytes delineated. Approximately 50,000 events in the erythrocyte gate were counted.

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