APOPTOTIC MACHINERY IN *PLASMODIUM FALCIPARUM* GROWING IN A CONTINUOUS CULTURE

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ABSTRACT

Apoptosis and indeed metacaspase have not been sufficiently evaluated to show their role in *Plasmodium falciparum.* To study apoptosis, synchronized *P. falciparum* cultures were initiated at a parasitemia of 0.5 % at 5 % hematocrit and allowed to grow in conditions that were not limiting for nutrients and red blood cells until the parasites crashed. Parasite growth was monitored by making Giemsa-stained thin smears that were examined microscopically and by flow cytometry following staining of cells with SYBR green. The following apoptotic processes were evaluated at every stage of the growth curve: DNA fragmentation by TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate assay), mitochondria membrane potential collapse by TMRE (tetra methyl rhodamine ester dye). Expression of metacaspse gene was evaluated by RT-PCR and blotted membranes were probed with polyclonal antibodies for human caspases. *P. falciparum* initiated at 0.5% ring-stage parasitemia doubled every 48 hours to 1.63 % parasitemia, but the rate of growth declined at 3.68 % and 4%. Consistently, the culture then crashed at about 6% parasitemia when the growth requirements were not limiting. At lower parasite density the malaria parasites were morphologically healthy, but thereafter, the proportion of healthy parasites declined. DNA fragmentation as indicated by TUNE,L positive staining increased from 0.2 % at early parasitemia to 5 % by the time the culture crashed. TMRE staining of the mitochondria showed collapse of mitochondrial membrane potential as the parasite density increased. By qRT-PCR, expression of metacaspase gene was not evident in the ring stages until the parasite density approached 4%. The metacaspase gene was present in trophozoites at 0.5% and 4% parasitemia while in the schizonts it was present at all parasitemia levels. Western blot analysis by anti-caspase 3 and 7 revealed presence of 45 and 28 KDa fragments as it has been reported in other studies. Unlike the metacaspase gene, the protein expression was observed in all the stages. In the ring stages, the protein expression was highest at parasite density of 1.36 % and lowest during the crash. The protein expression pattern was similar in the schizont stages, while at trophozoites stage protein abundance increased as the density increased. Taken together, these results indicate existence of apoptotic machinery in *P. falciparum* that seem to operate in tandem with parasite density. These findings offer important insights into *P. falciparum* survival strategies that could open new avenues for designing rational therapeutic interventions for malaria.

CHAPTER ONE

1.0. INTRODUCTION

The development and propagation of malaria parasites in their vertebrate host is complex and involves various host and parasite factors. Nevertheless, there are a number of observations that suggest occurrence of a parasite-mediated auto regulation of population density. For example the *in vitro* growth rate between strains differ and the evolution of parasitemia in fast growing strains seems to be quelled by parasite density even when the red blood cells (RBC) supply is not limiting (Ginsburg and Hoshen, 2002). Parasite density is an obvious parameter associated with the disease severity, and therefore chemoprophylaxis that solely limits parasitemia reduces morbidity and mortality (Menon *et al.,* 1990). Therefore, understanding the mechanisms involved in quorum sensing could lead to development of new ways of treating malaria.

One of the physiological mechanisms of reducing cell number is by apoptosis (Domen, 2001). The first description of the "death gene" *ced-3* was in *Caenorhabditis elegans* that was found to encode a protein homologous to mammalian protease interleukin 1β-converting enzyme (ICE or caspases-1) (Yuan *et al.,* 1993). Following this discovery, a family of related proteases have been described and later termed the caspase (Cystein Aspartate- Specific Proteases) (Alnemri *et al.,* 1996). These cysteine proteases are now known to play a crucial role in the execution of apoptosis. Apoptosis is a normal process by which the body disposes of genetically damaged or unwanted cells. Apoptosis is characterized by morphological and biochemical changes that result in cell death (Alnemri *et al.,* 1996; Samali *et al.,* 1999). Despite earlier thought that apoptosis is limited to

multicellular organisms, in the recent past growing evidence has ascertained the occurrence of apoptosis-like events in unicellular parasites including Apicomplexan parasite *Plasmodium* (Picot *et al.*, 1997; Al-Olayan *et al.*, 2002; Olivier *et al.*, 2003). Caspase like activity has been observed in *P. berghei* ookinettes and has been incriminated in regulation of parasite numbers (Al-Olayan *et al.*, 2002). A putative metacaspase protein has been identified in the genome of *P. falciparum* (Bozdech *et al.*, 2003; Llinas *et al.*, 2006) Gene ID PF14_0363 supporting the existence of caspase like gene in *P. falciparum*. This study attempted to identify and characterize apoptotic events involved in control of *P. falciparum* cell numbers. It is hoped that the phenomenon of apoptosis will in future be exploited for control of malaria parasitemia and thereon control severe disease

1.1. Rationale of the study

The identification of a *P. falciparum* metacaspase gene following the complete sequencing of *P. falciparum* genome indicates the possibility that the parasite use this gene to initiate apoptosis. This finding contradicts earlier data that suggests that protozoa do not contain caspases (Aravind and Koonin, 2002). There are a number of indications to suggest that malaria parasites auto-regulate their numbers: Firstly, the development of parasitemia in fast growing strains seems to be quelled by parasite density even when the RBC supply is not limiting and *in vitro*, the parasitemia of *P. falciparum* cultures increases by a factor of 3-8 within 48 hours whereas theoretically it should increase 16 fold. It is tempting to speculate that the disparity between expected and observed parasite density is a function of self-regulation by the parasites. The studies reported here sought to identify apoptotic machinery that accompany the rise and fall of *P. falciparum* cell density growing in an *in*

vitro culture system. This phenomenon is also observed *in vivo* where parasitemia in non-immune hosts appears self-limiting and rarely exceeds 5%.

1.2. Hypothesis

P. falciparum utilize apoptotic machinery to auto-regulate parasite density.

1.3. Main Objective

Determine whether *P. falciparum* utilizes apoptotic machinery to auto regulate its parasite population.

1.4. Specific objectives

1. Determine whether the decline in *P. falciparum* cell numbers in an *in vitro* Culture system is associated with up-regulation of apoptotic machinery.
2. Determine the presence of *P. falciparum* metacaspase gene and gene product during the asexual development and at different parasite density.

CHAPTER TWO

2.0. LITERATURE REVIEW

Malaria is one of the most important infectious diseases that afflict millions of people worldwide (Greenwood and Mutabingwa, 2002). *P. falciparum*, the causative agent of the most virulent human malaria, is responsible for hundreds of millions of illnesses and more than 1 million deaths each year. The control of malaria is increasingly being hampered by the increasing resistance of malaria parasites to available antimalarial drugs (Shunmay *et al.,* 2004). It is therefore imperative that new chemotherapeutic targets against malaria parasites be identified if the high mortality and morbidity is to be curtailed. Among potential new targets for antimalarial chemotherapy are cysteine proteases (Mithu *et al.,* 2007).

2.1. Life cycle of *Plasmodium falciparum*

As shown in Figure 1, the development and propagation of the malaria parasites is a complex process and involves the *Anopheles* mosquito, and the human host.

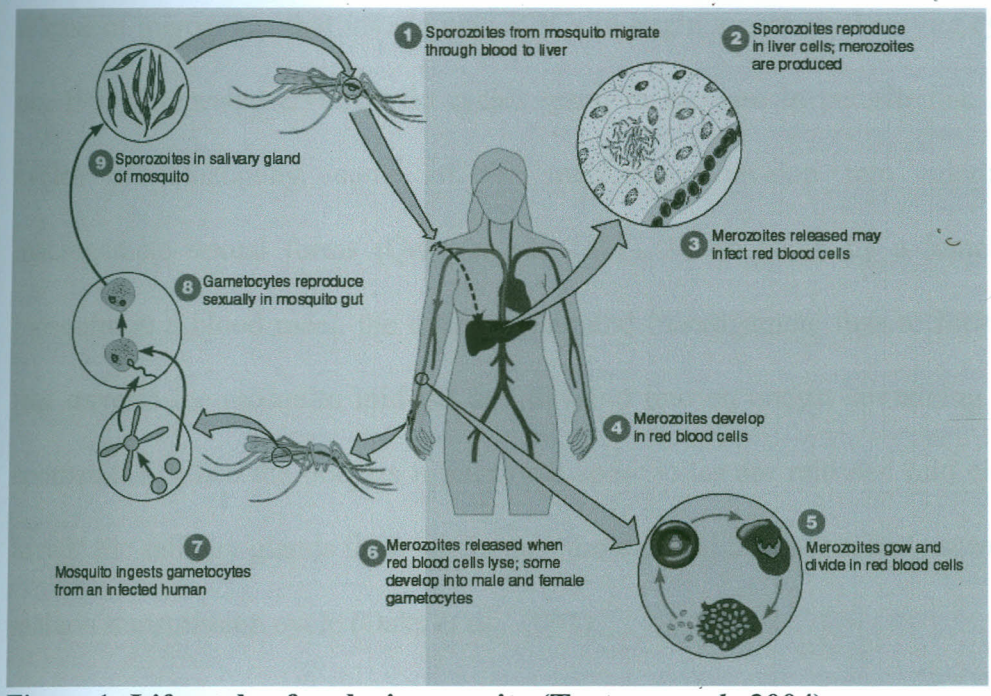


Figure 1: Life cycle of malaria parasite (Tortora *et al.*, 2004)

The life cycle has two distinct phases: asexual (schizogony) and sexual stage (sporogony). The asexual reproduction takes place in the liver (pre-erythrocytic stage) and in the RBCs (erythrocytic stage), while sexual reproduction takes place in the *Anopheles* female mosquito (Tortora *et al.,* 2004). In the process of taking a blood meal, the female *Anopheles* mosquito inoculates sporozoites into the vertebrate host. Within 30 minutes, the sporozoites are carried in blood to the liver where they invade the hepatocytes (Tortora *et al.,* 2004). In *P. falciparum,* the hepatic phase takes 5–8 days during which period the sporozoites proliferate to produce thousands of merozoites (Till *et al.,* 2002). The merozoites are then released into the blood stream and quickly invade the RBCs to commence the erythrocytic phase. Merozoites in the RBCs develop into young trophozoites called ring forms, which then develop into mature trophozoites. The mature trophozoites then develop into schizonts, each containing 12-16 merozoites (Till *et al.,* 2002). This development, called schizogony has a 48 hours cycle and results in

release of merozoites that invade new RBCs to begin a new erythrocytic cycle (Oaks *et al.,* 1991). Repeated erythrocytic cycles result in increase in parasitemia. After several cycles of schizogony, some of the merozoites develop into microgamete and macrogamete sexual forms (Oaks *et al.,* 1991). When taken by a female *Anopheles* mosquito in a blood meal, the microgamete and macrogamete fuse to form an ookinete that traverse the mosquito mid-gut and develop into an oocyst containing thousands of sporozoites. When the oocysts rupture, the sporozoites are released into circulation and invade the salivary glands from where they are injected into a human host to start another malaria transmission cycle (Oaks *et al.,* 1991).

2.2. *Plasmodium falciparum* and malaria severity

Parasite density is an obvious parameter associated with the disease severity (Menon *et al.*, 1990). In non-immune individuals, occurrence of low parasite burden, suggests a parasite-mediated auto-regulation of the population density. In absence of specific anti- parasitic immune responses in non-immune individuals, it is reasonable to assume that certain strains of *P. falciparum* cause severe disease because they have imbalance between cell proliferation and cell loss. This assumption is strengthened by the following observations: 1) chemoprophylaxis that solely limits parasitemia reduces morbidity and mortality (Menon *et al.*, 1990); 2) the *in vitro* growth rate between strains differ; 3) the evolution of parasitemia in fast growing strains seems to be quelled by parasite density even when the RBC supply is not limiting (Ginsburg and Hoshen, 2002) and 4) *in vitro*, the parasitemia of *P. falciparum* cultures increases by a factor of 3-8 within 48 hours whereas theoretically it should increase 16 fold (Marcel and Katja, 2004). It is tempting to speculate that the disparity between expected and observed parasite density is a function of self-regulation.

2.3. Apoptosis

One of the physiological mechanisms of controlling cell numbers is by apoptosis. Apoptosis, or programmed cell death, is a normal component of development and health of multi-cellular organisms (Samuilov *et al.,* 2000). Cells may die in response to a variety of stimuli, but during apoptosis they die in a controlled, regulated manner. This makes apoptosis distinct from another form of cell death called necrosis that result from acute cellular injury in which uncontrolled cell damage leads to rapid cell swelling and lysis. Apoptosis, by contrast, is a process in which cells play an active role in their own death, hence the name cell suicide (Samuilov *et al.*, 2000).

At the cellular level, apoptosis is characterized by controlled auto-digestion through the activation of endogenous proteases that result in cytoskeletal disruption, cell shrinkage, and membrane blebbing (Samuilov *et al.,* 2000). Due to activation of endonucleases, the nucleus undergoes condensation as DNA is fragmented into oligonucleosomes. This process can be detected using terminal deoxynucleuotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) test (Figarella *et al.,* 2005). Even before DNA damage is evident, mitochondria show collapse in the electrochemical gradient as measured by change in the membrane potential ($\Delta\Psi$). Changes in mitochondria $\Delta\Psi$ lead to the insertion of proapoptotic proteins into the membrane resulting in pore formation and subsequent release of cytochrome c into the cytoplasm (Samuilov *et al.,* 2000). Loss of $\Delta\Psi_m$ can be detected by a flourescence cationic dye 5, 5, 6, 6-tetrachloro-1, 1, 3, 3-tetraethylbenzainidazolocarbocyanin iodide, commonly known as JC-1 (Smiley *et al.*, 1991).

Although apoptotic cells maintain their plasma membrane integrity unlike in necrosis, there occurs alteration in the plasma membrane and this signals neighboring phagocytic cells to engulf them and complete the degradation process (Fadok *et al.,* 1998). Cells not immediately phagocytosed break down into apoptotic bodies. Another key feature that distinguishes apoptosis from necrosis is that in apoptosis dying cells are eliminated without induction of inflammatory responses.

The main functions of apoptosis as reviewed by Vaux *et al.,* (1994) are the elimination of cells during development of multicellular organisms in tissue modeling e.g. limb bud development in vertebrates and phylogenetic vestiges such as anurans tails and gills, the maintenance of homeostasis of cells of the immune system by eliminating auto reactive lymphocytes and regulating antigen receptor repertoire selection during T cell maturation and removal of tumorous or damaged cells (Heussler *et al.,* 2001). It has been shown that apoptosis can serve as a defense mechanism against virus and other infectious agents such as intracellular bacteria and parasites.

As demonstrated by Welburn *et al.,* (1997), unicellular parasites also use apoptosis to keep their cell population in check. The survival of the parasite species is at risk if the parasites kill its host before some of the population has been transferred to the next host or vector. An interesting concept has been proposed by Marcel and Katja, (2004) that *P. falciparum* does not rely exclusively on the varying immune systems of the host to control parasite density. They proposed three hypothetical mechanisms that *P. falciparum* can use to regulate parasitemia: - vary the rate of cell division, vary the rate of invasion into

uninfected erythrocytes and vary the rate of cell death. Programmed cell death (PCD) is the most effective of these hypothetical mechanisms because it slows down the increase of parasitemia and decreases the percentage of infected host cells independent of the host's immune system (Marcel and Katja, 2004). As suggested by Debrabant *et al.,* (2003), cell death plays an important role in the control of the malaria parasite population.

Existence of apoptosis in protozoa suggests that even these unicellular organisms do not live in isolation but are associated in communities where communication occurs between individuals. This interaction between protozoa is similar to the quorum sensing (QS) and biofilm formation in bacteria as suggested by Miller *et al.*, (2001); Donlan, (2002). QS as is known in bacteria, refers to regulation of gene expression in response to fluctuations in cell population density and is mediated by signaling molecules released into the environment. *In vitro* studies and epidemiological data provide evidence that malaria parasites exhibit QS (Dyer and Day, 2003). The process relies on production of low molecular mass-signaling molecules called auto-inducers, the extracellullar concentration of which is related to the population density (Dyer and Day, 2003).

Apoptosis has been shown to occur in two distinct phases; biochemical and morphological phases (Lazebnik *et al.,* 1993; Solary *et al.,* 1993). Following pro-apoptotic stimuli (e.g. lack of growth factors, DNA damage), a trigger mechanism activates central molecular machinery of apoptosis (initiation phase). In the second phase, the molecular executioner machinery becomes fully activated and the degradation phase takes place, following which the hallmarks of apoptosis become evident (Saraste and Pulkki, 2000).

Biochemical hallmarks of apoptosis involve the internucleosomal DNA fragmentation. The DNA is degraded by endogenous DNases that digest the internucleosomal regions into double stranded DNA fragments of 180 to 200 base pairs (Wyllie *et al.,* 1980). Electrophoretic separation of these fragments in agarose gel yields the characteristic DNA ladder (Vaux and Korsmeyer, 1991). The DNAse responsible for the fragmentation during apoptosis include DNA fragmentation factor (DFF) (Liu *et al.,* 1998) and caspase activated DNAse (CAD) (Enari *et al.,* 1998). These enzymes are selectively activated upon cleavage by caspase 3 or other members of caspase family (Tang and Kidd, 1998).

One of the most important caspase-mediated changes in the cell is the loss of plasma membrane lipid asymmetry. That is, the lipids of the planar membrane are normally non randomly distributed; some are predominantly localized to one or the other side. For example, sphingomyelin is mostly found on the outer leaflet, while phosphatidylserine and phosphatidylethanolamine are mostly on the inner leaflet of the plasma membrane. This is due to the action of phospholipid translocases that maintain the orientations of some of the lipids (Bevers *et al.,* 1999).

Changes in the mitochondrial $\Delta\Psi_m$ is one of the key features of apoptosis and loss of $\Delta\Psi_m$ is often associated with the early stages of apoptosis (Samuilov *et al.,* 2000). Mitochondrial dysfunction has been shown to participate in the induction of apoptosis and has even been suggested to be a central feature in the apoptotic pathway that leads to opening of the mitochondrial permeability transition pore which in turn induce depolarization of the $\Delta\Psi$ and release of apoptogenic factors (Ly *et al.,* 2003). These factors

include the cytochrome c (cyto-c) which is released into the cytosol where it interacts with apoptosis-activating factor (Apaf)-1 leading to activation of the caspases (James and Green, 2004).

Morphologically, the onset of apoptosis is characterized by shrinkage of the cell and nucleus and condensation of nuclear chromatin into sharply delineated masses that become marginated against the nuclear membrane. The nucleus breaks up and the cell detaches from the surrounding tissue, the plasma membrane seals to form apoptotic bodies packed with cellular organelles and fragments of nucleus (Saraste and Pulkki, 2000).

The morphological and the biochemical changes that occur during apoptosis are carried out by a family of cysteine proteases called caspase (Cysteine Aspartate Specific Proteases). Caspases were first implicated in apoptosis following the discovery that CED-3 (*Caenorhabditis elegans* death gene- 3), the product of a gene required for cell death in the nematode *Caenorhabditis elegans* is related to the mammalian interleukin-1β-converting enzyme (ICE or caspase-1) (Kohler *et al.,* 2002). Although caspase-1 has no obvious role in cell death, it became the first member of a large family of proteases which have distinct roles in inflammation and apoptosis. In apoptosis, caspases function in both cell disassembly (effectors) and in initiating the disassembly in response to proapoptotic signals (initiators) (Thornberry and Lazebnik, 1998).

Caspases occur as inactive proenzymes in normal cells and require cleavage at specific internal aspartate residues that separate large and small subunits for induction of full

enzymatic activity (Thornberry and Lazebnik, 1998). Studies on substrate specificity, prodomain structure and biological function have revealed that caspases are activated in a self- amplifying cascade (Martin and Green, 1995; Thornberry and Lazebnik, 1998). Activation of the upstream or initiator caspases, such as caspases 2, 8, 9 and 10, by pro-apoptotic signals leads to protealytic activation of the downstream or effector caspases 3, 6 and 7. The effector caspases actually cleave a set of vital proteins and initiate the apoptotic degradation phase including DNA degradation (Saraste and Pulkki, 2000).

There are two major pathways of caspase activation: the first one is initiated by ligation of the death receptors (Ashkenazi and Dixit, 1998), which include Fas and TNF (Tumor Necrosis Factor) receptors. Caspase 8 is the most upstream caspase in this pathway (Hirata *et al.,* 1998), which is activated by a signaling complex of the receptors. The second pathway is mitochondrial mediated (Kroemer *et al.,* 1997; Slee *et al.,* 1999), which integrates various apoptotic signals. The mitochondrial pathway is a target of some apoptosis regulating proteins of the B cell lymphoma 2 (Bcl-2) families (Kluck *et al.,* 1997; Yang *et al.*, 1997). The mitochondria pathway also amplifies receptor-mediated apoptosis. The key checkpoint of this pathway is the release of cytochrome-c and other caspase activating factors, such as the apoptosis inducing factor (AIF) into the cytosol from the mitochondrial transmembrane space (Kluck *et al.,* 1997). Caspase 9 is the most upstream caspase in the mitochondrial apoptotic pathway. Activation of pro-caspase 9 requires a cytosolic complex that includes cytochrome-c and *C. elegans* death gene CED-4 homologue called apoptosis protease-activating factor (Apaf-1) (Li *et al.,* 1997).

Upon activation, caspases cleave specific substrates: the caspases recognize a 4 amino acid sequence on the target substrate. For example, the preferred recognition sequences for caspase-1 and -3 are Tyrosine-Valine-Alanine-Aspartate and Aspartate-Glutamine-Valine-Aspartate, respectively that include an aspartic acid residue. The cleavage occurs at the carbonyl end of the aspartic acid residue (Thornberry *et al.,* 1997). The presence of apoptosis can be studied by demonstrating the activation of downstream caspases. Western blotting of target proteins that have been cleaved by caspases or demonstration of caspases activity by enzyme assay can also be used (Suurmeeijer *et al.,* 1999).

The importance of caspases in the apoptotic process has been documented by several findings: (a) Over expression of caspases efficiently kills cells, (b) synthetic or natural inhibitors of caspases effectively inhibit apoptosis induced by diverse stimuli, and (c) knockout animals lacking certain caspases show profound defects in apoptosis (Ekert *et al.,* 1999; Chang *et al.,* 2002).

For a long time, apoptosis was assumed to be confined to metazoan organism but recent findings indicate that unicellular organisms also undergo apoptosis. To date features typical of mammalian programmed cell death have been described in several species including the Kinetoplastids (*Trypanosoma brucei rhodesiensis, T. cruzi* and *Leishmania major*, (Ameisen *et al*., 1995; Moreira *et al.,* 1996; Welburn *et al.,* 1996; Ameisen, 1998; Arnoult *et al.,* 2002; Lee *et al.,* 2002; Figarella *et al.,* 2005). Features typical of apoptosis including condensation of chromatin, DNA fragmentation, the appearance of phosphatidylserine (PS) on the outer leaflet of the plasma membrane and eventual

formation of apoptotic bodies have been demonstrated in *P. berghei* (Al-olayan *et al.,* 2002). Picot *et al.,* (1997) previously reported oligonucleosomal DNA fragmentation in the erythrocytic stages of chloroquine treated *Plasmodium.* Recently DNA fragmentation and mitochondrial membrane potential disruption have been shown in erythrocytic stages of 3D7 and 7G8 isolates of *P. falciparum* treated with apoptosis- inducer etoposide or antimalarial chloroquine (Benoit *et al.,* 2007). Although clear homologues of metazoan caspases encoding genes in the *Plasmodium* have not been identified, two Cystein proteases have been annotated (PF13-0289 and PF- 0363) in the *P. falciparum* genome database (Bozdech *et al.,* 2003; Llinas *et al.,* 2006). The two proteases have 24 % sequence identity and have the conserved catalytic dyad histidine and cysteine required for catalysis activity and contains a putative caspase recruitment domain in the N-terminal amino acid sequence (Benoit *et al.,* 2007). Presence of caspase-like proteins in *Plasmodium* was also suggested by use of caspase inhibitors (Z-VAD.fmk (Benzyloxycarbonyl-Val-Ala-Asp flouromethylketone) and Z.DEVD.FMK (Benzyloxycarbonyl-Asp-Glu-Val-Asp-flouromethylketone)) that inhibited *Plasmodium* apoptosis leading to suggestion that caspase-like activity and aspartate specificity may be crucial for apoptosis in *Plasmodium* (Al-Olayan *et al.,* 2002). In addition to metacaspase gene, apoptosis related gene (PfARP) has been purified and characterized in *P. falciparum* although the role of this protein in growth, multiplication and stage progression has not been identified (Mithu *et al.,* 2007).

In this study, an *in vitro* model of studying the phenomenon of apoptosis in the erythrocytic stage of *P. falciparum* is described. This model may provide important insights as to how

apoptosis could be exploited to come up with new strategies of curtailing disease severity associated with parasite density.

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Study design

This was a descriptive study where a Dd2 clone which is derived from W2-MEF under mefloquine pressure (Guinet *et al.,* 1996; Oduola *et al.,* 1988) of *P. falciparum* was obtained from cryopreserved stocks at the US Army Medical Research unit- Kisumu, Kenya and used to initiate culture growth. Highly synchronized cultures (>98% young rings) (Lambros and Vanderberg, 1979) were allowed to grow in conditions that were not limiting in RBCs and nutrients until the parasites crashed (Trager and Jensen, 1976). At every developmental stage (time 0 for rings at 0.5%, 24 hours for trophozoites at 0.5% and 34 hours for schizonts at 0.5%) and until the crash of the parasite culture, aliquot were taken and used to assess parasitemia, parasite morphology and presence of selected apoptotic features, namely: DNA fragmentation, stability of mitochondrial membrane potential and expression of metacaspase gene and protein. Uninfected RBCs that were maintained in culture in a similar manner to the parasite cultures were used as negative control to ensure that all the apoptotic features detected were derived from the parasites and not human RBCs.

3.2. Maintenance of *P. falciparum* Cultures

P. falciparum cultures were maintained using continuous culture conditions following the method of Trager and Jensen, 1976 with minor modifications. A clone of *P. falciparum,* Dd2 isolate was used to initiate parasite growth in washed group O^+ human erythrocytes

diluted to 5 % hematocrit in complete RPMI 1640 media (Sigma-Aldrich Corporation, St Louis Missouri, USA) supplemented with 0.2% bicarbonate, 25 mM HEPES, 50 μg/ml gentamicin and 10 % heat inactivated human serum. Cultures were maintained in 25 cm^2 Corning flasks (Corning incorporated Corning NY, USA), with daily replacement of growth medium and gas mixture (O_2 – 2 %; CO_2 – 5.5 %; N_2 – 92.5 %) to meet the nutrient requirements and maintain optimal pH.

3.3. Synchronization of *the P. falciparum* cultures

Synchronized cultures were obtained by enriching for young ring stage trophozoites using 5% D- sorbital which lyses RBCs containing late rings stage and other mature parasites stages (Lambros and Vanderberg, 1979). Briefly, 6 mL of culture at 2% parasitemia of mainly young rings (10-12 h post invasion) were spun at 600 χ *g* and the pellet re-suspended in 6 mL 5% D-sorbitol. After 10 min incubation at room temperature (25 ° c), the cells were washed twice in RPMI 1640 medium (pH 7.2) containing 25 mM HEPES and 0.2% sodium bicarbonate (all from Sigma-Aldrich Corporation, St Louis Missouri, USA), diluted to 5% hematocrit and cultured as described in section 3.2. This treatment was repeated every 48 hours until >98% of the parasites were synchronized in the ring stage as confirmed by microscopy.

3.4. Experimental set-up

Synchronized cultures containing >98% young rings were grown to a density of 4% parasitemia and then sub-cultured to give 8 flasks at 0.5% parasitemia. Flask 1 was used for

assays at 0.5% parasitemia for ring, trophozoite and schizont stages while the rest of the flasks were grown as described section 3.2. At the next parasite density, flask 2 was used for assays for ring, trophozoites and schizonts stages. This was continued with the other culture flasks till the culture crashed. At every developmental stage (time 0 for rings at 0.5%, 24 hours for trophozoites at 0.5% and 34 hours for schizonts at 0.5%) and at similar hours until the crash of the parasite culture, aliquots were taken and used to assess parasitemia, parasite morphology and presence of selected apoptotic features, namely: DNA fragmentation, stability of mitochondrial membrane potential and expression of metacaspase gene and protein.

3.5. Monitoring parasite growth rate by light microscopy

Following initiation of the culture at parasite density of 0.5%, at every developmental stage and subsequent parasitemia levels, 10 µL aliquots was obtained from the culture and used to prepare Giemsa thin blood films. Light microscopy was then used to monitor the level of parasitemia stained by counting a minimum of 10,000 erythrocytes and check the morphological changes in the parasites.

3.6. Monitoring parasite growth rate by Flow-cytometry

Percentage parasitemia was also scored using SYBR Green (Molecular Probe, Eugene,OR, USA) staining, a cell permeable dye that stains nucleic acids. For this assay, a stock of 10,000χ SYBR Green was diluted to 1x in PBS, pH 7(8 g of NaCl, 0.2 g of KCl, 1.44 g Na_2HPO_4 and 0.24 g of KH_2PO_4 in 1000 ml of H_2O). Parasite culture suspension was

diluted to 2 % hematocrit and 100 µl was incubated with 200 µl of 1x SYBR Green for 30 minutes at room temperature. The cells were washed three times in 1x PBS and then resuspended in 400 µl PBS in FACs tube. Uninfected RBCs that were maintained in a similar manner as the parasites were used as negative control to set acquisition gate. The cells were analyzed by flow cytometry by acquiring 20,000 events for each sample on FL1 using BD Facscan, SAN JOS Califonia installed with BD cellquest Pro.version 5.2 acquasition software (BD Biosciences US).

3.7. Monitoring of DNA fragmentation by fluorescent microscopy and flow-cytometry

3.7.1 Fluorescent microscopy method

DNA fragmentation is an early event in apoptosis and although it can be observed on agarose gel following electrophoresis in high molecular weight DNA, in *P. falciparum* DNA fragments is only visible after labeling the nicked DNA. A common method for revealing DNA strand breaks is by incorporating labeled nucleotides to the exposed 3'- OH terminal of DNA ends in an enzymatic reaction utilizing terminal deoxynucleotidyl transferase (TdT). In this study, tetramethylrhodamine (TME red) labeled nucleotides obtained from Roche Diagnostics, (Germany) was used as a tracking dye for DNA fragmentation.

For this assay, a 500 µl aliquot of the cell suspension culture was removed and pelleted by centrifuging at 3000 rpm for 3 minutes. The cell pellet was washed twice with PBS and re-suspended in equal volume PBS, pH 7.2 before finally diluting them to a concentration of 20,000 cells/µl in PBS. 500 µl of the cell suspension was then concentrated on a slide by centrifuging in a cytospin (Shandon cytospin 4, Thermo Electron corporation USA) at 500 rpm for 3 minutes. The cells were then fixed with 4 % Para formaldehyde in PBS for 1 hour at room temperature. The slides were rinsed with PBS and then incubated in freshly prepared permeabilisation solution (0.1 % Triton X-100 in 0.1 % sodium citrate) for 2 minutes on ice. The slides were rinsed twice with PBS and allowed to dry. For each sample, a positive and a negative control sample was included. For a positive control, the sample slide was incubated with DNase 1 (Invitrogen, Carlsbad, CA) at a final concentration of 0.003 U /µl in DNase buffer (50 Mm Tris-HCL, pH 7.5,1 mg /ml BSA) for 10 minutes at 25 ° C to induce DNA strands breaks. The slides were rinsed in PBS to remove excess DNase 1. Terminal deoxynucleotidyl transferase nick end labeling (TUNEL) reaction mixture was prepared by diluting TUNEL enzyme solution by 1:10 in labeling solution. The positive control slide and the sample slide were incubated with 50 µl of TUNEL reaction mixture ensuring a homogeneous spread and allowed to sit in a humidified incubator for 1 hour at 37°C. For the negative control, 50 µl of labeling solution without terminal transferase enzyme was added and incubated in a humidified incubator for 1 hour at 37 ° C. Smears were then examined under oil immersion using x100 objective of an Olympus microscope (Olympus America, NY) at an excitation wavelength in the range of 520-560 nm and detection in the range of 570-620 nm. Photomicrographs were captured using

Olympus MagnaFire camera (Olympus America, NY) using MagnaFire acquisition software.

3.7.2 Flow-cytometry method

100 µl of the cell suspension at 20,000 cells/µl as prepared in section 3.6 was aliquoted in three eppendorf tubes and labeled "sample, positive control and negative control". The cells in the tubes were fixed by adding 100 µl of freshly prepared 4 % Para formaldehyde fixative solution for 1 hour at room temperature. The fixative was removed by centrifuging at 300 *g* for 10 minutes. Cells were washed once with 200 µl of PBS by centrifugation method and resuspended in 100 µl permeabilisation solutions (0.1 % Triton X- 100 in 0.1 % sodium citrate) solution for 2 minutes on ice. The cells were then washed in PBS. For the positive control tube, the cells were incubated with Dnase 1 in Dnase buffer at a final concentration of 0.003 U/µl for 10 minutes at 25 ° C and washed twice with 200 µl PBS by centrifugation. Thereafter, for the positive control and the test sample, the cells were incubated with 50 µl of TUNEL reaction mixture for 1 hour at 37 ° C. For the negative control, 50 µl of labeling solution without terminal transferase enzyme was added and cells incubated for 1 hour at 37 ° C in the dark. The cells were then washed and re-suspended to a final volume of 500 µl and then analyzed on FL3 by flow cytometry at an excitation wavelength in the range of 520-560 nm and detection in the range of 570-620 nm. The negative control sample was used to set the gates. 20,000 events were then acquired for each sample BD Facscan, SAN JOS Califonia installed with BD cellquest Pro.version 5.2 acquasition software (BD Biosciences US).

3.8. Monitoring of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$) collapse is a hallmark of apoptosis and permeability transition is an important step in induction of cellular apoptosis. The $\Delta\Psi$ collapse was monitored using Tetra methyl Rhodamine ethyl ester (TMRE) (Invitrogen Molecular Probes, Eugene, Oregon USA), a cell permeable cationic dye that is accumulated by healthy cells in the mitochondria in proportional to mitochondrial potential.

For this assay, an aliquot of 100 µl of cell culture was incubated with 25 nM TMRE for 15 minutes at 37 ° C in the dark. The parasites were washed in PBS twice by centrifuging at 3000 rpm for 3 minutes. The cells were then washed twice in PBS and re-suspended to a final volume of 400 µl and analyzed on FL3 channel by flow cytometry. Uninfected RBCs that were placed under the same culture conditions as malaria parasites were used as negative control and used to set the acquisition gate. 20,000 events were acquired for each sample BD Facscan, SAN JOS Califonia installed with BD cellquest Pro.version 5.2 acquasition software (BD Biosciences US).

3.9. Analysis of Metacaspase gene expression

3.9.1. RNA isolation

RNA was isolated using commercially available kit as recommended (Invitrogen, life technologies,California). To ensure that equal number of iRBCs were used for RNA

isolation at different parasite densities, 100 µL of well suspended culture cells was run on a Coulter Counter (Ac. T 5diff CP, Beckman coulter Inc. Miami Florida. USA) to obtain RBCs count per µL. Numbers of iRBC were calculated by multiplying % parasitemia with RBC count. A total of 5*10^5 iRBC was considered adequate for RNA isolation. Briefly, after working out the volume of the culture that will contain 5*10^5 iRBC, the required volume was removed and pelleted by centrifugation at 956 χg for 5 minutes at 4°C. The pellet was resuspended to 200 µL of lysis buffer provided in the kit and then vortexed to disrupt the cells. The suspension was centrifuged at 12000 χg for 2 minutes at 25 ° C. The supernatant was transferred to a clean tube and 200 µL of 100 % ethanol was added and then mixed by pipetting up and down to disperse the precipitate. The preparation was applied to RNA spin cartridge and centrifuged at 12 000 χg for 15 seconds at 25 ° C. The eluate was discarded and the spin cartridge placed on a clean wash tube. 700 µL of wash buffer I (Invitrogen, RNA Isolation kit) was added and centrifuged again at 12,000 χg for 15 seconds at 25 ° C. The elute was discarded and the RNA spin cartridge was washed twice using 500 µL of wash buffer II (Invitrogen, RNA Isolation kit) containing ethanol. The RNA was finally eluted by adding 30 µL of RNAse-free water provided in the kit and centrifuged at 12 000 χg for 15 seconds at 25 ° C. The eluted RNA isolated was stored at – 70 °C until required.

3.9.2. Quantitative reverse transcriptase real time PCR (QRT - PCR)

A one–step RT-PCR kit (Applied Biosystem, Roche, Branchburg New Jersey. USA) was used to reverse transcribe and amplify the RNA template as recommended by the manufacturer. Metacaspase and Seryl tRNA synthetase gene sequences were obtained from

PlasmoDB library and used to design primers using Primer Express 3.0 software (Applied Biosystems, California. USA). The *P. falciparum* Seryl tRNA synthetase gene was used for normalization of metacaspase gene transcripts. The following primer pairs were bought from Applied Biosystem and used for one–step RT – PCR. Metacaspase primers: forward 5′ -CAT CCT TGT CCC ATC AAT CTT TT-3′, reverse primer 5′-ATG GAA ACC CTC CTT AAA ATT AG-3′. Seryl tRNA synthetase primers: forward 5′-TAT CAT CTC AAC AGG TAT CTA CAT CTC CTA-3′, reverse 5′-TTT GAG AGT TAC ATG TGG TAT CAT CTT TT-3′. Reverse transcription and amplifications were done using ABI PRISM 7300 PCR system (Applied Biosystems). The PCR reactions were carried out in 96-well plate (Applied Biosystems Cat. No.4306737) each containing 25 µL Power SYBR Green PCR master mix (1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 1.25 U/µL AmphliTaq Gold DNA polymeras), 0.25 U/µL Multiscribe reverse transcriptase, 0.4 U U/µL RNAse inhibitor, 2.5 µL RNA, 0.9 µM metacaspase primers, 1 µM seryl tRNA synthetase primers and PCR water to a final volume of 50 µl. The PCR mix also contained ROX dye as an internal reference. Reverse transcription step was done at 48°C for 30 minutes followed by heat inactivation of reverse transcriptase enzyme at 95 °C for 10 minutes. The amplification was then done for 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. A dissociation analysis step was included to check the specificity of the amplification. The reporter dye (SYBR Green) signal was measured against the internal reference dye signal to normalize for non-PCR-related fluorescence. The signals were reported as threshold cycle (CT) which represents the refraction cycle number at which a positive amplification reaction is just measurable.

3.9.3. Analysis of metacaspase protein by Western blotting

To ensure that equal number of iRBCs was used for protein extraction at different parasite densities, 100 µL of well suspended culture cells was run on a Coulter Counter and numbers of iRBCs calculated as described in section 3.9.1. After working out the volume of the culture that would give the required number of iRBC, the specified volume was removed and pelleted by centrifugation at 3000 rpm for 3 minutes at 4 ºC. The pellet was re-suspended to 100 µL using culture media devoid of serum and stored at -80 °C until all the samples were ready for analysis. Cell lysates were then thawed from the -80 °C freezer and 10 µL used for protein quantification using BCA protein assay kit (Pierce, Rockford, IL).

After quantification of protein, a volume containing 1.5 µg of the protein was added to an equal volume of 2X SDS sample buffer (100 mM Tris-HCL, pH 6.8, 4 % SDS, 0.2 % bromophenol and 25 % gycerol) and then resolved on an 12 % SDS-PAGE gel (5% stacking) by electrophoresis at 120 volts for 1 hour. The protein was then electro-blotted onto the nitrocellulose membrane (Invitrogen, Carlsbad, CA). Thereafter, the membrane was blocked at 4 ºC overnight with gentle agitation using 1 X casein in PBS containing 10 % Tween-20. Thereafter, the membrane was incubated for 1 hour with 1:500 dilution of rabbit polyclonal anti-human caspase-3 or 7 (BIOMOL, USA). The membranes were then washed three times in PBS containing 10 % Tween-20 and then incubated for 1 hour at room temperature with 1:20,000 dilution of anti-rabbit IgG conjugated to horseradish peroxidase in 1X casein. The blot was then washed as indicated above. The blot was then developed by chromogenic peroxidase reaction with 3, 3′ – diamino benzidine (Pierce

Biotechnology, Inc) before being exposed to an x – ray film (Kodak Biomax film) for 30 seconds.

To confirm equal loading of *P. falciparum* protein, gels loaded with 1.5 µg of protein for each parasite stage and density were stained with Commassie blue stain kit (Invitrogen, Carsbad, CA) as recommended by the kit manufacturer.

CHAPTER FOUR

4.0. RESULTS

4.1. Growth characteristics of *P. falciparum* Dd2 isolate

The growth curve of synchronized Dd2 isolate is shown in Figure. 2. Parasites seeded at a starting ring stage parasitemia of ~ 0.5% doubled after every schizogonic cycle but only up to a parasitemia of 4-5%. Thereafter, the growth stagnated as determined by both microscopy and SYBR Green measurements. However, while parasitemia count by microscopy declined steadily after day 6 of culture, parasitemia by SYBR Green measurements remained steadily elevated. It is worth remembering that SYBR Green stains for nucleic acids of live and dying parasites while for light microscopy, only viable parasites inside the RBCs are scored.

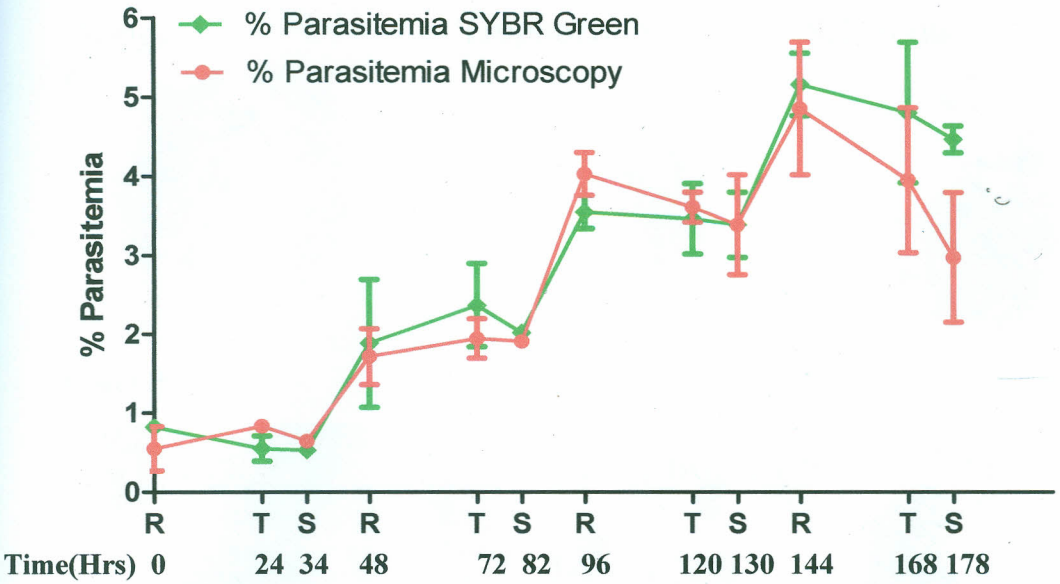


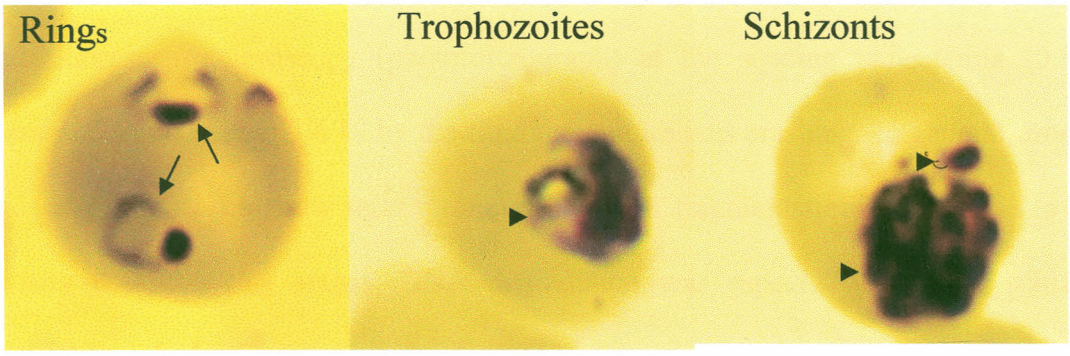
Figure 2: In vitro growth pattern of P. falciparum Dd2 isolate as measured by microscopy (red curve) and SYBR Green (green curve). R - Rings, T - Trophozoites, S – Schizonts.

By both methods, parasitemia initiated at ~ 0.5% doubled at every schizogony and did not increase beyond 4-5%. Parasitemia by microscopy declined after the 4% parasite density, but remained elevated by SYBR Green measurements. Each time point represents a mean and the bars are standard deviations of the mean

4.2. Parasite morphological abnormalities coincide with crash of parasite culture

Figure 3 shows photomicrograph of Dd2 parasites at 0.5% initiation parasitemia (panel A) and at 4 % (panel B). At initiation, the parasites had the classical signet ring-like morphology which changed to an irregular rounded trophozoites 12 hours later and eventually changed to a mature schizonts with numerous nuclei 9 hours thereafter (Figure 3, panel A). At 4% parasitemia that coincided with parasite culture crash, the ring stages were smaller with intensely stained nuclei (Figure 3, Panel B). Trophozoites and schizonts at this parasitemia appeared to have lost the smoothness of their plasma membrane with the parasites becoming moribund or morphologically abnormal that indicates crisis forms in the parasite population.

Panel A



Panel B

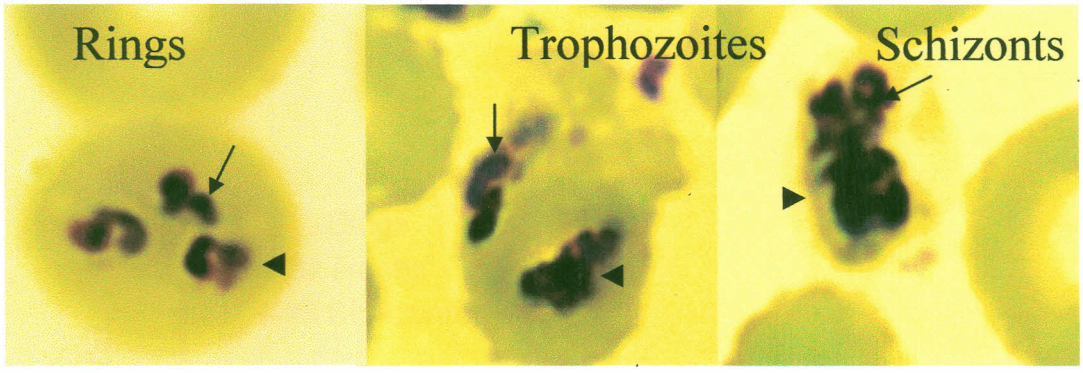


Figure 3: Photomicrographs of Geimsa stained malaria parasites at initiating parasitemia and at 4% parasitemia when the culture crashed.

At 0.5% parasitemia, rings have healthy morphology and have the "classical signet ring" (panel A, arrow) and trophozoites and schizonts have intact plasma membrane and numerous nuclei (panel A, arrow head). In contrast, at 4% parasitemia (panel B), the nuclei in all stages are shrunken (arrow), plasma membrane is not smooth (arrow head) and looks morphologically abnormal.

4.3. *P. falciparum* culture crash and concomitant morphological changes are heralded by classical biochemical hallmark of apoptosis

4.3.1 DNA fragmentation increases as parasitemia increases

DNA fragmentation, a characteristic feature of cells undergoing apoptosis can be revealed when labeled nucleotides are incorporated to the exposed 3-OH terminal of DNA ends. As

shown in Figure 4, DNA fragmentation was evident by both flow cytometry (bar graph) and fluorescent microscopy (photomicrograph) at the seeding parasitemia (0.5%) but increased steadily to ~ 5-6 % by the time the culture was crashing. In general, trophozoite and schizonts stages show more pronounced DNA fragmentation in response to parasite density than the ring stages. DNA degradation was especially high for schizonts as parasites crashed.

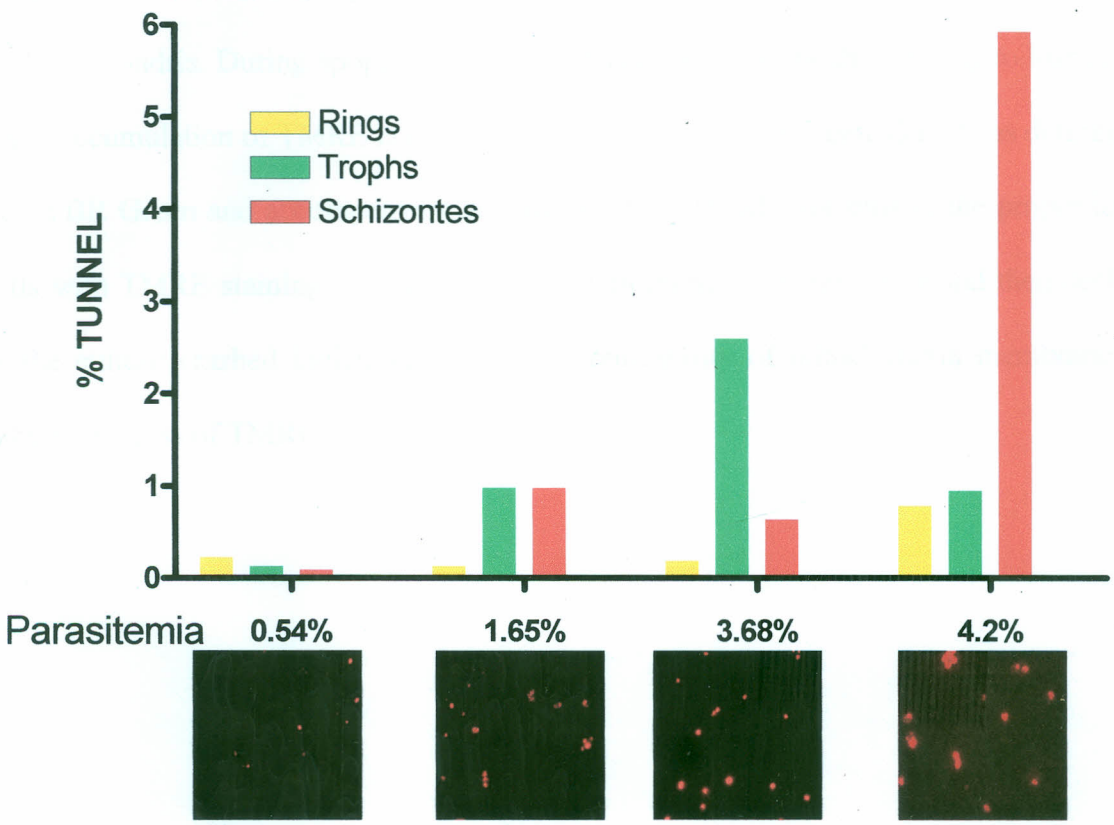


Figure 4: Level of fragmented DNA using terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL).

Evidence of DNA fragmentation as measured by TUNEL is discernible albeit at very low level by flow cytometry (bar graph) and by fluorescent microscopy (photomicrographs) at starting parasitemia but increases dramatically by the time the parasite culture crashes.

Photomicrographs show TUNEL positive cells for rings at 0.54% parasitemia, 1.65% and 3.68% for trophozoites and 4.2% for schizonts.

4.3.2 Mitochondrial membrane potential disruption increases as parasitemia increases

Changes in the mitochondrial $\Delta\Psi_m$ is a key features of apoptosis and can be revealed by staining with TMRE, a cell permeable cationic dye that is accumulated by healthy cells in the mitochondria. During apoptosis, mitochondria become permeable leading to loss of $\Delta\Psi_m$ and accumulation of TMRE is reduced. Figure 5 shows the parasite density as determined by SYBR Green and accompanying accumulation of TMRE. As shown, the proportion of cells with TMRE staining increased steadily with increased parasitemia and then declined as the culture crashed indicating increased permeability of mitochondria membrane and subsequent loss of TMRE.

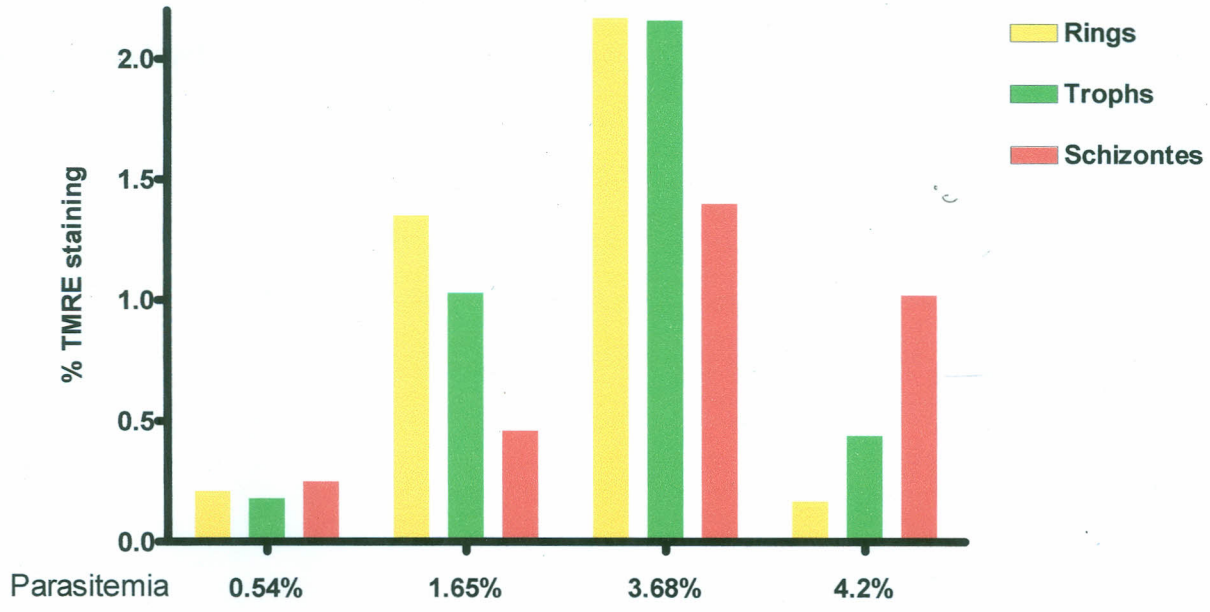


Figure 5: Bar graph showing accumulation of TMRE in the mitochondria in each of the developmental stages in relation to parasite density.

4.4. *Plasmodium falciparum* metacaspase gene expression analysis using Real – time RT–PCR.

QRT-PCR was used to assess the level of expression of metacaspase gene as *P. falciparum* parasitemia increased. A house keeping gene, seryl tRNA gene was used as the normalizing control for the target gene. To confirm qRTPCR results, PCR amplicons were then resolved on a 2% agarose gel (Figure 6). As shown in Figure 6, the Ct values for the seryl tRNA gene were about the same (mean 21.92±0.55, Table 1), indicating presence of equal amount of RNA in all the preparations. For metacaspase gene, amplification was stage dependent and in general, the transcript level as reflected by Ct values was very low (Table 1) and barely detectable on agarose gel (Figure 6). For ring stage, metacaspase was absent until the parasite density reached 4.2%. For the trophozoites, the metacaspase gene was

detectable at 0.54% and 3.68% parasitemia while in the schizonts; it was present at all parasitemia levels.

Table 1: Ct values of seryl transferase (House keeping gene) and metacaspase gene.

Key: R – Rings, T- Trophozoites, S – Schizonts.

% Parasitemia	0.54			1.63			3.68			4.2		
Growth stages	R	T	S	R	T	S	R	T	S	R	T	S
Ct values/ seryltransferase	21.42	21.40	21.85	22.67	21.54	20.63	21.47	21.9	22.34	22.24	23.54	22.15
Ct values /metacaspase	Undet.	40.97	38.04	Undet.	Undet.	42.21	39.59	36.01	38.47	39.33	Undet.	40.89

The seryl tRNA gene amplified at relatively equal level (mean Ct value of 21.92±0.55) in all the RNA preparations. For metacaspase gene, the transcript level was very low (Ct values ≥38) and barely detectable on agarose gel (Figure 6). For ring stage, metacaspase was absent until the parasite density reached 4%. For the trophozoites, the metacaspase gene was detectable at 0.54% and 3.68% parasitemia while in the schizonts; it was present at all parasitemia levels.

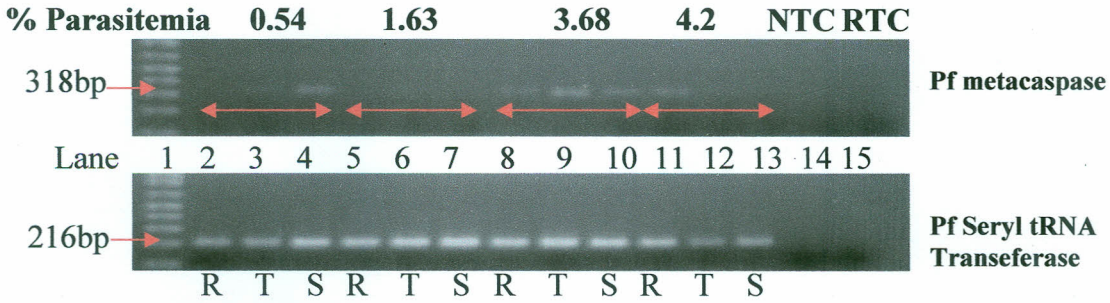


Figure 6: Agarose gel picture of metacaspase (318 bp) and Seryl transferase genes (216 bp) at different parasite densities. Key: Rings(R), Trophozoites (T), Schizonts (S), NTC = non template control, RTC = reverse transcriptase control.

The seryl tRNA gene was amplified at relatively equal level in all the RNA preparations. For metacaspase gene, amplification was stage dependent and in general, the transcript level was very low and barely detectable on agarose gel. For ring stage, metacaspase was absent until the parasite density reached 4%. For the trophozoites, the metacaspase gene was detectable at 0.54% and 3.68% parasitemia while in the schizonts; it was present at all parasitemia levels.

4.5. Metacaspase protein expression increases as parasitemia increases

Figure 7A shows a western blot of malaria parasite protein probed with human anti-caspase 7 polyclonal antibody. The expected fragments of 45 and 28 kDa were observed in all the developmental stages (Figure 7, lanes 2-13), but absent from uninfected erythrocyte preparation (lane 14). Similar results were obtained with rabbit anti-human caspase 3 antibodies, but the results were much better with caspase 7 antibody. Judging from protein band intensity, metacaspase protein was up-regulated at parasite density of 1.63% in all the developmental stages and remained up-regulated in subsequent parasitemia except in the ring (lanes 8 and 11). Figure 7B shows a coomassie stained gel and confirms equal loading of protein in all the wells.

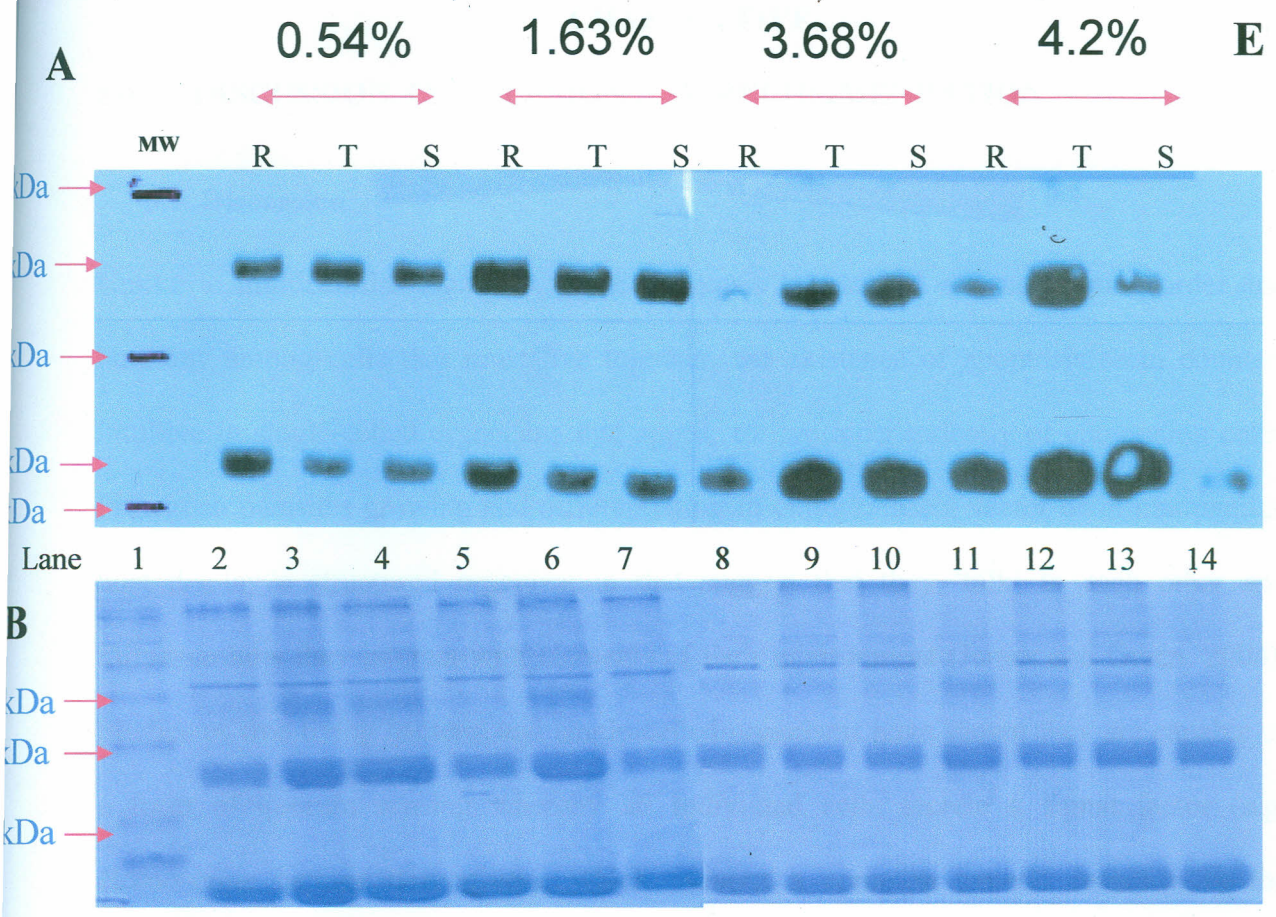


Figure 7: Western blot analysis of P. falciparum protein extract probed with rabbit anti-human caspase-7 polyclonal antibody (A) and Coomassie stained gel (B).Mw- Standard molecular weight marker, R – Rings, T – Trophozoites, S- Schizonts, E – Erythrocytes.

Protein fragments of 45 and 28 kDa were observed in all the developmental stages (lanes 2-13), but absent from uninfected erythrocyte preparation (lane 14). Metacaspase protein was up-regulated starting from parasite density of 1.63% in all the parasite stages and remained up-regulated in subsequent parasitemia levels except in the ring (lanes 8 and 11).

CHAPTER FIVE

5.0. DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1. Discussion

Unlike in multicellular organism where apoptosis is essential for maintaining order and harmony among cells that must live together, the existence of apoptosis seem counter-intuitive in single-celled organisms that appear to live independently of companion cells. Protozoan parasites growing and differentiating in culture, insect vector or in mammalian host display a structured organization that suggest that they 'talk' to each other and influence how they interact with elements of their environment (James and Green, 2004). Studies of free-living bacteria and single-celled organisms have shown they are capable of a form of quorum sensing whereby all individual cells secrete a signal factor (e.g. Homoserine lactone derivatives), which induces a common genetic response in the population once a threshold concentration is reached (Greenberg, 2003) Such factors have been identified in protozoan organisms such as *Dictyostelium* (Saito *et al.,* 2006), *Plasmodium* (Billker *et al.,* 1998) and *Trypanosoma brucie* (Vassella *et al.,* 1997). Therefore, the driving force for quorum sensing and apoptosis responses in parasitic protozoa is population-density control that ensures optimum parasite density for the available resources as well as guaranteeing the survival of the host, at least until the transmission of the progeny to the next host is assured.

In this study and as has been described previously (Choukri *et al.*, 2001; Marcel and Katja, 2004), Dd2 *P. falciparum* parasitemia increased by a factor of 1.5 to 2 within 48 hours instead of the theoretical 16-32 fold (Choukri *et al.,* 2001). Thereafter, the culture crashed,

even when the nutrients were not limiting (Figure 2). In order to determine whether apoptosis was involved in the Dd2 population density control, experiments were set that examined the use of apoptotic machinery at various stages of the parasite growth curve.

Early in the growth curve (Figure 3, panel 1); the morphological features were those of healthy malaria parasites. Parasites with compromised morphological features were seen later in the growth curve as parasitemia increased (Figure 3, panel 2). Morphological features that are consistent with apoptosis included cell shrinkage, (pyknotic parasites) and shrinkage of the nuclei due to chromatin condensation (Samuilov *et al.,* 2000). To explore whether occurrence of these abnormalities were related to existence of apoptotic machinery, molecular assays were used to probe for apoptotic signatures at various stages of the parasite growth curve.

An important molecular indicator of apoptosis is DNA fragmentation whereby the DNA is degraded at the internucleosomal regions by endogenous DNAses into double stranded DNA fragments of 180-200 base pairs (Wyllie., 1980). The degraded DNA can be revealed by electrophoretic separation of these fragments in agarose gel or by incorporating labeled nucleotides to the exposed 3'-OH terminal of nicked DNA ends by the terminal deoxynucleotidyl transferase (TdT) (Vaux and Korsmeyer, 1991). Using the TUNEL method, evidence of DNA fragmentation was discernible albeit at very low level by flow cytometry and by fluorescent microscopy at the seeding parasitemia (0.5%) but increased dramatically to 5% by time the parasite culture crashed (Figure 4). DNA fragmentation was reported in chloroquine treated *P. falciparum* in culture (Picot *et al.,* 1997) and in the

ookinates of *P. berghei* (Al-Olayan; *et al.*, 2002). In this study, DNA fragmentation in untreated asexual stages of *P. falciparum* parasites in a continuous culture has been shown. Most of the DNA degradation was evident at the trophozoites and schizont stages (Figure 4) a finding that probably reflects variations in cellular DNA content of the different life cycle stages. This variation can therefore be attributed to the fact that trophozoites and schizonts have the highest amount of DNA as earlier had been reported by Nyakeriga *et al.*, 2006.

During apoptosis death stimuli induces mitochondrial membrane permeabilization that results in loss of mitochondria membrane potential. This loss can be revealed as loss of TMRE staining inside the mitochodria. As shown in Figure 5, the proportion of cells with intact mitochondria as revealed by TMRE staining declined as parasite density increased. The uptake of TMRE in the mitochondria varied among the stages, with the ring showing the greatest mitochondrial response (Figure 5). This can be speculated to be as a result of differing level of metabolic activities in the three asexual blood stages as earlier reported by Learngaramkul *et al.*, 1999, although further biochemical evidence will be required to validate this observation. This is the first report in literature on the use of TMRE to study depolarization of mitochondria in *P. falciparum.*

Caspases in metazoan organisms or their equivalent, the metacaspases in the unicellular organisms, are pivotal for many of the biochemical and morphological changes that accompany apoptosis (Thornberry *et al.*, 1998). Studies conducted by Al-Olayan *et al.*, (2002) gave the first indication of existence of caspase like proteins in *Plasmodium.* In their

study use of classical caspase inhibitors Z-VAD.fmk and Z.DEVD.FMK that inhibited *Plasmodium* apoptosis leading to suggestion of occurrence of caspase-like activity with aspartate specificity in *Plasmodium*. Recently a metacaspase gene that possesses histidine and cysteine residues that typically form the catalytic dyad in this family of proteases has been annoted in the plsmoDB (Bozdech *et al.*, 2003; Llinas *et al.*, 2006). In this study evaluation of whether the expression level of *P. falciparum* metacaspase gene and/or protein show sensitivity to changes in parasite density. As has been shown by micro-array data the gene was expressed in all the asexual stages which was in agreement with the observation made in this study (Figure 6). However there was large variations in levels of gene expression in the different parasite stages and at different parasite densities that was observed (Figure 6). It was confirmed that the seeming differential expression of this gene may not be due to variation in quantity or quality of RNA by parallel analyses of expressing level of a house keeping gene, the seryl tRNA gene. For ring stages, metacaspase was not expressed until the parasite density of 4% was attained (Figure 6, lane 8). Expression of metacaspase gene was evident in the trophozoites at 0.5% and 4% parasitemia while in the schizonts, metacaspase was present at all parasitemia levels (Figure 6). It was not clear what the cause of this large variation in the transcripts, but it could reflect changes in mRNA stability as the parasites proceed through different development stages.

Gene transcriptional activity does not necessarily translate into protein expression (Blair *et al.*, 2002), it was therefore important to explored changes in metacaspase protein expression as parasite density changed. The proteolytic activation of caspases has been

reported to be highly conserved in higher eukaryotes and depends on prodomain removal and cleavage of protein fragments (Thornberry *et al.*, 1998). Using anti-human caspase 3 and 7 polyclonal antibodies to probe for *P. falciparum* metacaspase, two protein fragments were detected in all the asexual stages (Figure 7). Metacaspase protein cleavage products of 45 kDa and 28 kDa (Figure 7) were observed as earlier reported by Al-olayan *et al.*, 2001. The expression level of metacaspase protein increased with rise in parasite density and was highest as sudden parasite density decline occurred (Figure 7). For some reason, the increase in expression level of metacaspase protein was more pronounced in the trophozoites. This can be attributed to fact that the trophozoite stage is the metabolic maturation phase of the parasite and is characterized by extensive RNA and protein sysnthesis compared to ring and schizont stages (Bozdech *et al.*, 2003; Choukri *et al.*, 2001). Absence of similar protein fragments in uninfected human erythrocytes indicates that the protein detected by the antibodies came exclusively from the parasites.

5.2. Conclusion

It is hereby concluded that, the four key indicators of apoptosis, namely DNA fragmentation, collapse of mitochondrial membrane potential, up-regulation of metacaspase gene and protein transcripts accompanied collapse of malaria *in vitro* culture that was maintained under conditions that were not limiting for nutrients or RBCs. These are strong indicators that *P. falciparum* has functional apoptotic machinery whose activation appear to be in tandem with increase in parasite density. These findings offer important insights into the parasite survival strategies that could open new avenues for designing rational therapeutic interventions for malaria.

5.3. Recommendation

This findings and other earlier findings indicate presence of a density dependent process that limit expansion of the malaria parasite population that may one day be exploitable to inhibit further parasitemia growth. It is therefore recommended that:

a) In depth studies of the control mechanisms of the apoptotic processes, including identification of parasite molecules involved and their mode of action is undertaken. This may in future present new opportunities for depiction of therapeutic targets.

b) Studies should be conducted to determine whether apoptosis can be used to screen for sensitivity of malaria parasites to antimalarials.

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