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Plasmodium falciparum proteases hydrolyze plasminogen, generating angiostatin-like fragments

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ABSTRACT

Malaria is a disease caused by *Plasmodium* parasites and remains one of the most prevalent and persistent maladies, affecting hundreds of millions of people. In the present work, we evaluated the capability of *Plasmodium falciparum* proteases to hydrolyze the multifunctional protein plasminogen, which is implicated in angiogenesis and coagulation processes by the generation of angiostatin and plasmin, respectively. Using fluorescence microscopy, we visualized the internalization of FITC-labeled plasminogen in erythrocytes infected by *P. falciparum* and showed that the parasites are able to hydrolyze the protein. The cleavage of plasminogen by the *P. falciparum* proteases was also observed by SDS-PAGE, followed by immunoblotting with anti-angiostatin antibody. N-terminal sequencing of the main generated fragments indicated that they are comprised in the five plasminogen kringle domains, suggesting as being angiostatin-like peptides. This assumption was reinforced by the demonstration that the products of plasminogen processing mimic angiostatin functions, including the capability to inhibit angiogenesis and to stimulate calcium response in endothelial cells *in vitro*. However, no plasmin activity was detected after plasminogen hydrolysis by *P. falciparum*. Nonetheless, exogenous tissue plasminogen activator (tPA) activated plasmin in infected erythrocytes, suggesting that the uptake of plasminogen by *P. falciparum* may be modulated by the vertebrate host. Taken together, the data presented here provide evidence for the processing of host plasminogen by malaria parasites to generate active fragments that may modulate host physiology events during malaria infection.

1. Introduction

Malaria is responsible for more than a million death each year and represents the most significant human parasite infection [1]. The resistance to drugs is increasing among the *Plasmodium* population, and much effort is being devoted in the development of new drugs and treatment strategies to control the disease [1].

Plasmodium development occurs with the acquisition of a large number of macromolecules from the host, providing the building blocks for parasite biomolecules and establishing an interrelationship between the host and parasite. The disease causes a multi-organ dysfunction syndrome that is often associated with morbidity. Severe malaria generally results in cerebral cytoadherence and sepsis, activation of the blood coagulation cascade and increased pro-inflammatory cytokine levels. The disease consists of intravascular fibrin formation [2] and modification of host hemodynamics [3].

Plasminogen is a multifunctional protein with a molecular mass of 92 kDa and consists of an N-terminal domain (PAN-domain with 77 amino acid residues), five tandem structures called kringle domains (approximately 90 residues each) and a catalytic domain corresponding to plasmin [4,5]. Plasminogen participates in coagulation, fibrinolysis, inflammation pathways and hemodynamics through plasmin activation and release of angiostatin [6–8]. Angiostatin includes the first four plasminogen kringle domains [9] and presents anti-proliferative activity in endothelial cells [10–12]. In addition, angiostatin can trigger calcium signaling in endothelial cells [13] and modulate the inflammatory process through the inhibition of macrophage migration and leukocyte recruitment [14,15]. Patients with cerebral malaria were found to accumulate angiostatin in the cerebral tissue [16], which was capable of modulating endothelial cell growth [17].

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Proteolysis is tightly involved in the *Plasmodium* intraerythrocytic cycle events, including invasion, egress of erythrocytes and degradation of host proteins [18]. More than 90 proteases have been identified in the *Plasmodium* genome, and these proteases are involved in many aspects of *Plasmodium* cell metabolism and regulation [19]. Among the best-studied enzymes of *Plasmodium falciparum* are the falcipains, which are papain-like cysteine proteases that are present in the parasite's food vacuole [20–22]. Falcipain-2 and falcipain-3 are essential for the hydrolysis of hemoglobin, and the absence of falcipain-3 halts parasite development [23]. Acting as the aspartyl proteases plasmepsins, the falcipains quickly degrade hemoglobin, generating the amino acids needed for parasite development [24] and control of the osmotic stability of the cell [25].

Besides the well-studied hemoglobin degradation by *P. falciparum*, it has been suggested that other molecules from the plasma can be imported by the parasite [26–28]. *Plasmodium* induces the formation of New Permeability Pathways (NPP) in the erythrocytes that provide essential nutrients to the intracellular parasite [29]. These modifications are important to the host cell, as it lacks any transport machinery for macromolecules. The uptake of nucleotides and amino acids by the parasite through the NPP has been described, but no reports of protein transport using this process have been presented. Plasma proteins can modulate *P. falciparum* growth and are essential for proper parasite development *in vitro* [30,31]. However, the use of host proteins for metabolic and signaling events by the pathogen is poorly described.

Recently, Bagnaresi et al. [32] reported that *P. falciparum* can generate bradykinin, and other kinins, by the intracellular proteolysis of imported kininogen. These peptides elicited a calcium response in human umbilical vein endothelial cells (HUVEC) *in vitro*, revealing new aspects of malaria physiology. In the present work, we show the ability of malaria parasites to hydrolyze plasminogen and generate active angiostatin-like fragments that may play an important role in the modulation of host physiology during malarial infections.

2. Materials and methods

2.1. Proteins and reagents

Plasminogen purified from human plasma was purchased from R&D Systems (Minneapolis, MN, USA) and tissue plasminogen activator (tPA) was from Sigma–Aldrich (St. Louis, MO, USA). Monoclonal anti-human angiostatin kringle 1–3 and anti-plasmin antibodies were also obtained from R&D Systems (Minneapolis, MN, USA). Other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA), unless otherwise indicated.

2.2. Parasites

P. falciparum was cultured in flasks using RPMI 1640 medium (Atená Biotecnologia, Campinas, SP, Brazil), which was supplemented with inactivated human plasma (10%) as previously described [33]. Parasitemia was verified by Giemsa-stained smears. The parasites were isolated from the infected erythrocytes by selective lysis using 10 mg/mL saponin in phosphate-buffered saline (PBS), followed by centrifugation at 2000 × *g* for 10 min at 4 °C. Then, the isolated parasites were washed twice in PBS to remove the red cell membranes.

2.3. FITC labeling of plasminogen

Fluorescein isothiocyanate isomer I (FITC; 10 μg/μL) was conjugated to human plasminogen (1 μg/μL) according to the manufacturer's specification. Protein and FITC in a 1:10 (v/v) ratio were incubated in 1 M Na_2HCO_3, pH 9.0, for 3 h at 37 °C. The labeled protein (FITC-plasminogen) was separated from the unbound FITC in an ÄKTA purifier system using a HiTrap desalting column (GE Healthcare, Uppsala, Sweden). FITC absorbance was monitored at 239 nm and 490 nm, and the protein content was monitored at 280 nm. The fractions that showed the highest absorbance at these three wavelengths, ensuring the coupling of the fluorescent probe to the protein, were collected. The fraction containing the free FITC was discarded.

2.4. Internalization of FITC-labeled plasminogen

Erythrocytes infected with *P. falciparum* were resuspended in PBS buffer (10^3 cells/mL), and 1 mL of this suspension was incubated with 0.6 μM FITC-plasminogen for 30 min at 37 °C. The cells were placed on microscopy glass bottom dishes (MatTek Corp, Ashland, MA, USA) that were pre-incubated with poly-L-lysine for 1 h at room temperature to enhance cell adhesion. The images were acquired on an Axio Observer Z1 inverted microscope system equipped with an MRc camera (Carl Zeiss, Göttingen, Germany), with the excitation and emission wavelengths set at 488 nm and 505–550 nm, respectively. As control, uncoupled FITC was incubated using the same conditions.

2.5. Detection of plasminogen by immunofluorescence assay

Infected erythrocytes (10^3 cells/mL) were incubated with 0.3 μM human plasminogen for 30 min, washed twice in PBS (2 min, 300 × *g*) and fixed with a solution of 4% paraformaldehyde and 0.075% glutaraldehyde in PBS for 30 min. Subsequently, the fixed cells were permeabilized in 0.1% Triton-X100 in PBS for 30 min, followed by incubation for 20 min with 0.15% glycine and 0.24% NH_4Cl in PBS. The cells were then washed once more with PBS, and a 3% BSA/PBS solution was added. After washing again with PBS, the cells were incubated for 2 h with anti-angiostatin antibody produced in mouse and diluted 1:250 in 3% BSA/PBS solution. After three more washes with PBS, the cells were incubated for 1 h with 1:500 Alexa 594-conjugated anti-mouse IgG (Life Technologies, Carlsbad, CA, USA), and the nuclei were stained for 5 min with 1 μg/mL DAPI (Life Technologies, Carlsbad, CA, USA). The cells were washed again with PBS, and the coverslips were mounted using polyvinyl alcohol medium with DABCO to reduce bleaching. All the steps described above were performed at room temperature. The images were acquired on an Axio Observer Z1 inverted microscope (Carl Zeiss, Göttingen, Germany).

2.6. Western blotting

Human plasminogen (0.3 μM) was incubated with isolated *P. falciparum* parasites (10^6 cells/mL) in 50 mM sodium phosphate buffer, pH 6.5, at 37 °C for 30 min in the presence or absence of the inhibitors E-64 (5 μM), PMSF (0.5 mM), pepstatin (1 μM) and *ortho*-phenathrolin (5 mM). These inhibitors, which are specific for cysteine, serine, aspartyl and metalloproteases, respectively, were pre-incubated for 10 min at 37 °C prior to the addition of plasminogen. Then, the samples were centrifuged for 2 min at 400 × *g*, and the supernatants were subjected to SDS-PAGE (12.5%) before transferring to a nitrocellulose membrane (Millipore, Bedford, MA, USA). The membranes were incubated overnight at 4 °C with the primary anti-angiostatin (1:1000) and anti-plasmin (1:250) antibodies in PBS containing 5% non-fat milk. The membranes were then incubated for 1 h at 4 °C with the secondary peroxidase-conjugated anti-mouse antibody (Millipore, Bedford, MA, USA) in PBS containing 5% non-fat milk and revealed with 4-cloro-1-naftol (0.6 mg/mL) and a peroxide solution (30%).