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Research paper

Interaction of *Trypanosoma evansi* with the plasminogen-plasmin system

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ABSTRACT

Trypanosoma evansi is a widely-distributed haemoflagellated parasite of veterinary importance that infects a variety of mammals including horses, mules, camels, buffalos, cattle and deer. It is the causal agent of a trypanosomiasis known as Surra which produces epidemics of great economic importance in Africa, Asia and South America. The main pathology includes an enlarged spleen with hypertrophy of lymphoid follicles, congested lungs, neuronal degeneration and meningoencephalitis, where migration of the parasites from the blood to the tissues is essential. Most cells, including pathogenic cells, use diverse strategies for tissue invasion, such as the expression of surface receptors to bind plasminogen or plasmin. In this work, we show that *T. evansi* is able to bind plasminogen and plasmin on its surface. The analysis of this binding revealed a high affinity dissociation constant (K_d of 0.080 ± 0.009 μM) and 1×10^5 plasminogen binding sites per cell. Also a second population of receptors with a K_d of 0.255 ± 0.070 μM and 3.2×10^4 plasminogen binding sites per cell was determined. Several proteins with molecular masses between ~18 and ~70 kDa are responsible for this binding. This parasite-plasminogen interaction may be important in the establishment of the infection in the vertebrate host, where the physiological concentration of available plasminogen is around 2 μM.

1. Introduction

Trypanosoma evansi is a flagellated hemoparasite that infects horses, camels, cows, sheep, pigs and other economically important mammals. Particularly affected are large farms where the parasite is responsible for substantial economic losses in the productivity of horses used for cattle management (Kubes, 1939; Roberts and Janovy, 2000). The disease known as "mal de cadeiras" or "derrenguera" caused by this parasite is considered as a widely distributed, endemic disorder in South America, Africa, Asia and Australia (Lun and Desser, 1995). Although it is known that this trypanosomiasis is not manifested in humans, recent reports described this parasite's ability to infect a minority population of human carriers of a defective form of the protein APOL1 (Joshi et al., 2005). In horses, the disease is characterized by a progressive weakness, limb edema, fever, loss of appetite, anemia, enlarged spleen with hypertrophy of lymphoid follicles, congested lungs and neuromotor disorders such as lack of coordination of the limbs and paraplegia, which appear to be the results of neuronal degeneration and meningoencephalitis (Brun et al., 1998; García et al., 2000).

T. evansi is a monomorphic parasite considered by some authors as a subspecies of *T. brucei* that has evolved by losing the kDNA (Carnes et al., 2015). This parasite exhibits itself only as a bloodstream trypomastigote form; however, it has also been found in the cerebrospinal fluid and invading as well other tissues such as kidney, brain, bone marrow, liver and spleen (Chizoba and Bedu, 2005; Berlin et al., 2009; Tejero et al., 2009; Rodrigues et al., 2009). In addition, this parasite has a known preference for connective tissue in the host, where it disrupts the collagen bundles and destroys the fibroblasts that produce and maintain the collagen (Boid et al., 1980). The disruption of the connective tissue, along with vascular damage, may release large quantities of cytosolic and mitochondrial enzymes into the serum, thereby causing further tissue damage (Boid et al., 1980).

Several studies have demonstrated that *T. evansi* is also able to invade the central nervous system causing necrotizing encephalitis (Tuntasuvan et al., 1997; Tuntasuvan et al., 2000; Rodrigues et al., 2009). Since diminazene aceturate, a trypanocidal drug, does not cross the blood-brain barrier (Jennings and Gray, 1983; Kennedy et al., 1997), parasites in the central nervous system survive antitrypanosomal therapy. This, together with a change in their surface

Abbreviations: PBS, phosphate buffer saline; PA, plasminogen activator.

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glycoproteins may lead to recurrence of the parasitemia (Jennings and Gray, 1983). The mechanism by which trypanosomes penetrate the blood-brain barrier is not clear, but it is assumed that invasion of the central nervous system occurs where the blood-brain barrier has been disrupted, either directly by the parasites or by release of chemical mediators, including cytokines and proteases (Girard et al., 2003; Masocha et al., 2004).

Many pathogenic organisms produce their own proteolytic enzymes for tissue invasion. However, an interesting known strategy is to take advantage of the mammalian host fibrinolytic system, which is responsible for the breakdown of fibrin and extracellular matrix during tissue remodeling (Ploplis and Castellino, 2000; Bergmann and Hammerschmidt, 2007). The most important fibrinolytic enzyme is plasmin, a serine-protease product of plasminogen activation by two mammalian activators, the tissue plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA). Proteolytic activity is an important factor in the pathogenesis of many organisms; it is associated with tissue damage due to invasiveness of the pathogen within the host. The pathogen-associated proteolysis may have several functions, including: (i) Acquiring nutrients by releasing tissue amino acids or peptides. (ii) Degrading proteins of the immune system (antibodies, complement proteins), (iii) Enabling the activation of proenzymes, such as those of collagenases and metalloproteases.

Receptors for plasminogen are expressed in a wide range of prokaryotic and eukaryotic cells (Lähteenmäki et al., 2001; Bhattacharya et al., 2012; Sanderson-Smith et al., 2012; Figuera et al., 2013). The pathogen-plasminogen interaction was first reported by Tillett and Garner (1933), and ever since, the number of pathogenic organisms described to interact with the plasminogen-plasmin system has become quite large (Lähteenmäki et al., 2005; Sanderson-Smith et al., 2007; Figuera et al., 2013). It is proposed that plasmin activity on the pathogen's surface, directly or indirectly facilitates degradation of the extracellular matrix, as well as the activation of metalloproteases, enabling dissemination and invasion of the organism (Lähteenmäki et al., 2000; Lähteenmäki et al., 2005). Pathogens exploiting the plasminogen-plasmin system are known to immobilize plasminogen, plasmin and/or t-PA on their cell surface, activating the plasminogen by t-PA and avoiding plasmin inhibition by α2-antiplasmin (Plow et al., 1995). Thus, a pathogen with the surface bound plasmin activity can dissolve fibrin clots and/or degrade extracellular matrix and basement membranes upon adhesion, allowing it to migrate through tissue barriers (Lähteenmäki et al., 2005; Bhattacharya et al., 2012; González-Miguel et al., 2015).

Currently two kinetoplastids, *Leishmania mexicana* and *Trypanosoma cruzi,* have been described to bind plasminogen-plasmin on their surface, and this phenomenon has been invoked as a means to facilitate cell invasion and migration in the host (Avilán et al., 2000; Almeida et al., 2004; Maldonado et al., 2006). In this paper we report the capability of *T. evansi* of binding plasminogen on its surface, which could be related to the pathogenic process of this parasite.

2. Materials and methods

2.1. Parasites

Parasites of the Venezuelan *Trypanosoma evansi* strain TEVA 1 isolated from a horse (kindly donated by Dr. Alfredo Mijares, Instituto Venezolano de Investigaciones Científicas, IVIC), were used to infect Wistar rats of about 250 g. Parasites collected from whole blood were cryopreserved. The rats were infected by intraperitoneal injection of 1×10^6 parasites. Parasites were harvested from whole blood obtained by cardiac puncture from anaesthetized rats in the presence of PBS-glucose-sodium citrate buffer (57 mM Na_2HPO_4, 3 mM NaH_2PO_4, 44 mM NaCl, 1% (w/v) glucose, 3.5% (w/v) sodium citrate pH 8.1), when the parasitemia exceeded 1×10^8 parasites ml^{-1}. Finally, parasites were purified from blood by ionic exchange chromatography as described by Lanham and Godfrey (1970).

2.2. Plasminogen purification, activation to plasmin and plasminogen-antibodies production

Plasminogen was purified to homogeneity from freshly taken rat plasma according to the protocol described by Deutsch and Mertz (1970) as modified by Avilán et al. (2000). Activation of the plasminogen to plasmin was carried out using 5 U of urokinase (Obtained from Choay Laboratory, Paris-France) in PBS for 1 h at 25 °C. For the preparation of rabbit antibodies against rat plasminogen the protocol described previously was followed (Avilán et al., 2000).

2.3. Binding assay using plasmin activity

To evaluate plasminogen binding to *T. evansi* parasites, two systems were performed: (*i*) 100 μl of purified parasites (1×10^8 cells) were incubated for 60 min in the presence or absence of 5 U urokinase on microtiter plates at 5 °C. This method allows to detect bound plasminogen already present on parasites purified from rat blood; (*ii*) 100 μl of purified parasites (1×10^8 cells) were incubated with 100 mM ε-aminocaproic acid in PBS-glucose for 60 min to eliminate any bound plasmin(ogen), and subsequently washed three times with PBS-glucose by centrifugation. Thereafter, the parasites were incubated with 2 μM rat plasminogen for 90 min at room temperature in the presence or absence of 100 mM ε-aminocrapoic acid to determine if the plasminogen binding is to lysine residues. After treatment parasites were washed with PBS-glucose. Parasites were then incubated with 5 U urokinase. In both cases, plasmin activity was detected using the chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (Sigma-Aldrich) at 0.6 mM, and the absorbance at 405 nm was determined using a microtiter reader.

2.4. Binding assay using anti-plasmin(ogen) antibodies

Motile *T. evansi* parasites (1×10^8 cells) were incubated in PBS-glucose with different concentrations of either plasminogen or plasmin in the presence or absence of different concentrations of ε-aminocaproic acid. Then, the cells were washed three times with PBS-glucose by centrifugation, resuspended in 200 μl PBS-glucose containing 0.5% (w/v) gelatin and placed ($\sim 5 \times 10^6$ cells) in microtiter plates (Maxi Sorp surface, Nalgene Nunc International, USA). Non-specific binding sites on the plates were blocked with 0.5% (w/v) gelatin in PBS. The wells were washed with PBS-glucose and 0.5% (w/v) gelatin and then incubated for 1 h at room temperature with anti-rat plasminogen antiserum diluted in PBS-glucose plus 0.5% (w/v) gelatin. The washing procedure was repeated and the peroxidase-conjugated secondary antibody added for 1 h incubation at room temperature. Following washing, 0.012% (v/v) H_2O_2 and 0.04% (w/v) *o*-phenylene-diamine in citrate buffer, pH 5 were added. The absorbance at 450 nm was recorded after 15 min incubation.

2.5. Quantification of plasminogen and plasmin bound to T. evansi parasites

To quantify the specific amount of either plasminogen or plasmin bound to *T. evansi* parasites, cells were extensively washed