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Leishmania amazonensis: early proteinase activities during promastigote–amastigote differentiation in vitro

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Abstract

Leishmania proteinase activity is known as parasite differentiation marker, and has been considered relevant for leishmanial survival and virulence. These properties suggest that *Leishmania* proteinases can be promising targets for development of anti-leishmania drugs. Here, we analyze the activities of four proteinases during the early phase of the *Leishmania amazonensis* promastigotes differentiation into amastigotes induced by heat shock. We have examined activities of cysteine-, metallo-, serine-, and aspartic-proteinase by hydrolysis of specific chromogenic substrates at pH 5.0 and at the optimal pH for each enzyme. Our results show that metallo-, serine-, and aspartic-proteinases activities were down-regulated during the shock-induced transformation of promastigotes into amastigotes. In contrast, cysteine-proteinase activity increased concomitantly with the promastigote differentiation. Immunocytochemical localization using two anti-cysteine-proteinase monospecific rabbit antibodies detected the enzyme in several cell compartments of both parasite stages. Our results show different proteinase activity modulation and expression during the early phases of the shock-induced parasite transformation.

Keywords: *Leishmania amazonensis*; Proteinases; Cathepsin; Cysteine proteinases; Amastigote-like

1. Introduction

Protozoan parasites of the genus *Leishmania* cause a broad spectrum of disease in humans, ranging from self-healing skin lesions to fatal visceralizing leishmaniasis, depending on the species of the parasite and host factors (Herwaldt, 1999). *L. amazonensis* is a causative agent of cutaneous leishmaniasis and also often causes disseminated cutaneous leishmaniasis. It may be a rare cause of visceral leishmaniasis and post-kala azar dermal leishmaniasis (Barral et al., 1991), although this finding has not been substantiated by other authors.

Leishmania presents two major developmental forms: extracellular, flagellated promastigotes which multiply in the midgut of the sand fly vector and intracellular, non-motile amastigotes that live within macrophages of the vertebrate host (Sacks and Kamhawi, 2001). Promastigotes can be easily cultured in cell-free media, while amastigotes need to be extracted from animal lesions or infected cell cultures (Chang, 1980; Saraiva et al., 1983). Promastigotes of several *Leishmania* species grown in vitro either at acidic pH and/or elevated temperatures, transform into amastigote-like forms (Gupta et al., 2001). The ability to axenically cultivate amastigotes provides not only a limitless source of these forms that can be used for different studies, but also allows the characterization of different aspects of developmentally

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regulated change during the promastigote–amastigote transformation process.

Leishmania proteinase activity has been recognized for long time and is considered as marker of parasite differentiation (Coombs et al., 1982; Leon et al., 1994; Pral et al., 1993, 2003; Rosenthal, 1999; Sajid and McKerrow, 2002; Ueda-Nakamura et al., 2002). The majority of the proteinases detected in *Leishmania* are cysteine-proteinases (North et al., 1990) and metallo-proteinases (Bouvier et al., 1985). In fact, there is now evidence that metalloproteinases are mainly expressed in promastigotes (Schneider et al., 1992), whereas cysteine-proteinases are predominant in amastigote forms (Mottram et al., 1997; Traub-Cseko et al., 1993). Because of their relevance for parasite survival and virulence (Chang et al., 1990; Coombs and Baxter, 1984; Mottram et al., 1996) *Leishmania* proteinases have been suggested as promising targets for development of anti-leishmania drugs (Mottram et al., 1997; Souza et al., 1992; Werbovetz, 2002).

The present study was undertaken to analyze the activity of four different proteinases (cysteine-, metallo-, serine-, and aspartic-proteinases) during the early phase of *L. amazonensis* promastigote–amastigote in vitro transformation. Our results show that cysteine-proteinase activity increased but metallo-, serine-, and aspartic-proteinase activities were down-regulated during the promastigote transformation into amastigotes. Immunocytochemical localization using two anti-cysteine-proteinase monospecific rabbit antibodies detected the enzyme in several cell compartments of both parasite stages. Our results show different proteinase activity modulation and expression during the early phases of the heat shock-induced parasite transformation.

2. Materials and methods

2.1. Leishmania culture and differentiation

Leishmania amazonensis (MHOM/BR/77/LTB0016) was obtained from *Leishmania* Type Culture Collection-WFCC World Data Center on Microorganisms Directory (Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil). Promastigotes were cultured in BHI medium supplemented with 10% heat-inactivated fetal calf serum (FCS) at 26 °C. In vitro promastigote–amastigote differentiation was carried out as described (Bates et al., 1992; Leon et al., 1994). Briefly, promastigotes from late log phase of growth (10^7 cells/mL) were washed three times in phosphate buffer saline, pH 7.2, PBS (3000*g*, 15 min, 5 °C) and incubated in Schneider's *Drosophila* medium with 20% FCS, pH 5.5, at 34 °C. Lesion amastigotes were obtained from BALB/c mouse footpad lesions with 4–6 weeks of infection.

2.2. Proteinase activity and inhibition assay

The enzymatic activity of different proteinases was determined in *L. amazonensis* extracts. Parasites washed in PBS, were lysed in 10 mM Tris–HCl, pH 6.8, containing 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate-Chaps (40 min, 4 °C). The enzymatic activity was evaluated in 10 μg of soluble extracts obtained after centrifugation (30,000*g*, 30 min, 4 °C) in 50 mM sodium acetate, pH 5.0 (final volume of 500 μL, 25 °C, 60 min). The chromogenic substrates included: N-Cbz-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser β-naphthylamide (for aspartic-proteinase; Roth and Reinharz, 1966), optical density determined at 300 nm, pGlu-Phe-Leu *p*-nitroanilide (for cysteine-proteinase; Filippova et al., 1984) and N-Cbz-Gly-Gly-Ler *p*-nitroanilide (for metallo-proteinase; Lyublinskayc et al., 1974) at 405 and 247 nm for Na-*p*-Tosil-L-Arg methyl ester (for serine-proteinase; Aguiar et al., 1996). Inhibition assays were performed with, at least, one specific inhibitor for each proteinases class: cysteine-proteinase [(E-64; L-*trans*-epoxysuccinyl L-leucylamide-(4-guanidino) butane)], metallo-proteinase (O-phe; 1,10-phenanthroline), serine-proteinase (PMSF; phenylmethylsulfonyl fluoride), aspartic-proteinase (PepA; Pepstatin A).

The variation of the absorbance at each wavelength was followed for 60 min and the velocity of the reaction defined using the formula $v = [s - so]/(t - to)$, where v is the velocity, $[s - so]$ is the final substrate concentration minus initial substrate concentration, and $(t - to)$ is the final time minus initial time. The assays were controlled for self-liberation of the conjugated chromogenic over same time interval. The enzymatic activity was expressed in nanomoles min^{-1} mg of $protein^{-1}$. All the chromogenic substrates and inhibitors were obtained from Sigma Chemical, St. Louis, Missouri, USA. Protein concentration was determined as described (Lowry et al., 1951).

2.3. Antisera

Polyclonal antibody anti-heat-inactivated commercial papain was raised in rabbits as described (Alves et al., 2000). Antibody against a synthetic peptide from COOH-terminal region of *Leishmania* cysteine proteinase (peptide II [pII] VGGGLCFE; Alves et al., 2001) was obtained after rabbit immunization with the peptide II conjugated with slug hemocyanin (Sigma). Conjugate was prepared using 2 mg of the peptide and 2 mg slug hemocyanin dissolved in 2 mL of 0.5% glutaraldehyde in PBS, during 16 h at 4 °C with gentle agitation, followed by dialysis in PBS at 4 °C. An *L. amazonensis* promastigote stage-specific monoclonal antibody (MAb), 3A1-La, was also used (Chaves et al., 2003).