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Research paper

Functional attribution of LdISP, an endogenous serine protease inhibitor from *Leishmania donovani* in promoting infection

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ABSTRACT

Leishmaniasis, a parasitic disease caused by unicellular eukaryotic protozoa of the genus *Leishmania*, affects more than 12 million people worldwide. Events of leishmaniasis are based on the infection of the mammalian host, precisely macrophages, where both host and parasite derived proteases and endogenous inhibitors are significant. Pathogen derived protease inhibitors have generated considerable interest as they often act as an agent promoting infection and parasitic survivability. An endogenous serine protease inhibitor from Indian strain of *Leishmania donovani* was previously identified by our group and named as LdISP. It has been found to inhibit neutrophil elastase (NE), responsible for natural inflammation process. However, LdISP's role in progression of infection or the proteomics based structural exposition has not been explored. The present study is aimed to localize and validate the potential role of LdISP in infectivity. We found that LdISP localized endogenously and treatment of infected host cells with LdISP curbs ROS and NO production. Additionally, i*n silico* studies are carried out to predict the putative amino acid residues of LdISP involved in the inhibition process. Taken together, our results demonstrate that LdISP eventually exerts a pronounced role in *L. donovani* infection.

1. Introduction

Leishmaniasis, a parasitic disease caused by unicellular eukaryotic protozoa of the genus *Leishmania*, affects more than 12 million people worldwide [1]. The recent number may be more than that due to the refugee crises in the Middle East [2]. The etiological characteristics of Leishmaniasis in different zones are varied, so are the available clinical interventions [cdc.gov.com]. To overcome rampant problems like drug toxicity and resistance more specific approaches exploiting modern studies should be adapted.

The advent of novel treatment(s) has become increasingly aimed at molecular targets, which needs apt understanding derived from advanced studies of the genes, proteins and/or other biomolecule(s), known to be involved in dissemination of a particular disease. Recently, the role of serine protease inhibitors have been studied in several prokaryotic and eukaryotic parasites [3–5], including *Leishmania* [6,7] in the events of pathogenesis. The protein data banks reposit several of these encoded proteins including ecotin like proteins from different *Leishmania* spp for example *L. major, L. braziliensis* and *L. infantum* (http://www.uniprot.org).

Specific inhibitory character of such protein(s) most often corroborate with infection progression and establishment of parasitism. Moreover, the unique features of this ecotin like protein make them ideal subject of study to understand evolution of parasitic survivability and horizontal gene transfer [8].

Mostly, if not all chemotherapeutic interventions used to combat leishmaniasis is based on pentavalent antimonials that are toxic and prone to drug resistance [9]. On the other hand we are witnessing both usage and targeting of proteases and inhibitors to curb many parasitic diseases [10–12]. Therefore, it is essential to apprehend each fundamental factors responsible for disease progression, which could help to come up with effective novel therapeutic trends. LdISP, an inhibitor of serine proteases has first been identified, purified and characterized in our laboratory from Indian strain of *Leishmania donovani* (MHOM/IN/1983/AG83) [13] although, the precise regulation or mechanism of action of LdISP has not yet been demonstrated.

The aim of the present investigation has been focused on the localization of LdISP in Indian strain of *Leishmania donovani* (MHOM/IN/1983/AG83) and assertion of its role in infection progression using cell line model (RAW 264.7). Additionally to understand putative structural characteristics of LdISP, *in silico*

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approaches such as modeling and docking have also been employed.

2. Materials and methods

2.1. Reagents & software

M199 and RPMI (Invitrogen), FITC(Sigma), PE Rat Anti-Mouse Cd11b (BD Pharmingen™/BD Biosciences), FITC tagged Anti-*Leishmania* Major Surface Protease (GP-63) Monoclonal Antibody (Cedarlane, Burlington, ON). The molecular modeling studies were performed in Discovery Studio 2.5 software suite and Autodock.

2.2. Culture of L. donovani

Leishmania donovani strain MHOM/IN/1983/AG83 promastigotes were cultured at 22 °C in medium 199 with Hanks' salt containing HEPES, L-glutamine, 10% heat-inactivated fetal calf serum (FCS), penicillin at 50 U ml^{-1}, and streptomycin at 50 μgml^{-1} (Gibco BRL/Life Technologies, Middlesex, UK) [13].

2.3. Culture of macrophages and L. donovani infection

The murine macrophage cell line RAW 264.7 was cultured at 37 °C under 5% CO_2 in RPMI 1640 supplemented with 10% heat-inactivated FCS, 10 mM HEPES (pH 7.3), 100 Uml^{-1} penicillin, and 100 μg/ml streptomycin [14].

Macrophages were infected with *L. donovani* promastigotes in suspension culture. 1×10^7 MΦ/ml and 8×10^7 *L. donovani* promastigotes/ml were combined giving an approximate ratio of 1/8 and incubated for 24 h at 37 °C in ~5% CO_2 to allow internalized parasites to transform into amastigotes. To assess the parasite load, treated and untreated infected macrophages were fixed on slide, Giemsa stained and examined under light microscope (Zeiss Axio Observer. D1)[Fig. 5].

2.4. Raising antibody and FITC conjugation

A polyclonal antibody against LdISP was raised in New Zealand white rabbit [Animal ethics accreditation number 147/1999/CPCSEA] by subcutaneous injection of 80 μg of purified protein LdISP emulsified in Freund's adjuvant as mentioned by Choudhury et al. [15]. Polyclonal antibody was purified from the antiserum by protein A agarose (Sigma Chemical Co., St. Louis, MO) affinity chromatography [15]. The peak fractions were concentrated and kept frozen at −80 °C. Further the anti LdISP-Ab conjugated with fluorescein isothiocyanate (FITC) by using the Pierce EZ-Label FITC protein labeling kit [www.drmr.com/abcon/FITC].

2.5. Localization of LdISP by confocal immunofluorescence microscopy

L. donovani promastigotes of 5th passage and metacyclic promastigotes were centrifuged at 3000 g for 10 min, washed in PBS, and resuspended at a concentration of 10^7 cells/ml. Cells were layered to poly-L-lysine-coated glass coverslips (BD) and were fixed in PBS containing 4% paraformaldehyde for 30 min. Infected macrophages were fixed following same procedure. Coverslips were rinsed once with PBS to remove the fixative and were incubated in a blocking solution consisting of PBS supplemented with 5% FCS, 0.1% Tween 20, and 0.1% Triton X-100 for 1 h at room temperature. FITC tagged anti LdISP-Ab were diluted 1:500 in blocking solution and were incubated with the promastigotes, metacyclic promastigotes and infected macrophages. After incubation of prefixed coverslips were rinsed six times for 5 min with PBS and were observed. The incubation and all subsequent steps were performed in the dark. Images of fixed and transfected promastigotes, metacyclic promastigotes and infected macrophaged with FITC tagged Anti LdISP Ab (1:500 dilution) were collected using respective filters using confocal/fluorescent microscope (OLYMPUS IX81 and Zeiss Axio Observer. D1).

2.6. Assessment of NO and ROS

In each cell culture supernatant of the infected, LdISP (60 ng/ml) treated and untreated macrophages, the concentration of nitrite (NO_2^-) and ROS were determined. For NO measurement,100 μl of Griess reagent was mixed with equal volume of cell culture supernatant and incubated at room temperature for 10 min [14]. The absorbance of the chromophore formed was measured at 540 nm in a microplate reader (Smart Spec 3000; Bio-Rad). Nitrite was quantitated with $NaNO_2$ as a standard and the data were expressed in μM. Post treatment with LdISP (60 ng/ml) the level of ROS was monitored by the cell permeant probe H2DCFDA and analyzed by Flow cytometer (BD FACS Calibur) [16] and data have been extrapolated as comparative bar chart [Fig. 4] LPS (10 ng/ml) is used as control.

2.7. Infectivity assessment

LdISP (60 ng/ml) is treated 6 h post parasite charge. Macrophages (non infected, infected and treated) were washed (200 × g, 10 min, 4 °C) and resuspended in 2 ml of cold PBS. The cells were centrifuged again, resuspended in 300 μl of PBS, and stored on ice to facilitate detachment of remaining adherent cells. To differentiate LdISP treated infective macrophages against untreated for the assessment of infection, shifting of FSC and SSC trends were studied in a flow cytometer. Information on the rate of infection was obtained by comparing both FSC and SSC signals with that of non treated but infected cells. A total of 50,000 events were counted using a FACS Calibur cytometer and analyzed using CellQuest Pro software (Becton Dickinson). The experiment was carried out parallel by staining with fluorophores PE (Phycoerythrin) and FITC (Fluorescein isothiocyanate), tagged to AntiCD11b (CD11b is a macrophage surface marker) and Anti gp63 (gp63 is a parasite surface glycoprotein) respectively. The aforementioned fluorophore tagged antibodies are used to assess the relative increment of parasite burden by counting the mean fluorescence.

2.8. In silico studies

The amino acid sequence of ECOT2_LEIIN protein from *Leishmania infantum* was obtained from Uniprot database [17] bearing the accession no. A4HWE9. The amino acid sequence was used to search for suitable templates in the Protein Data Bank (PDB) [18] using the tool BLAST [19]. The best BLAST search result in terms of e-value and query coverage was the crystal structure of a monomeric form of general protease inhibitor, Ecotin in absence of a protease, chain A (PDB ID: 1IFG) with 57% sequence similarity, 87% query coverage and an e-value of 4×10^{-25}. The amino acid sequence of 1IFG, A Chain was used to build the three dimensional structure of ECOT2_LEIIN protein using the tool MODELLER.

MODELLER implements comparative protein structure modeling by satisfaction of spatial restraints [20]. The three dimensional model so generated was checked for the stereochemical qualities using the PROCHECK tool in SAVES server. The PROCHECK tool checks if there is any amino acid which is present in the disallowed regions of the Ramachandran plot. It was observed that the built model of ECOT2_LEIIN does not have any amino acid in the disallowed region of the Ramachandran plot [Fig. 8B].