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Evaluation of *Leishmania donovani* disulfide isomerase as a potential target of cellular immunity against visceral leishmaniasis

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ABSTRACT

In *Leishmania* species, protein disulfide isomerase (PDI) – a redox chaperone is primarily associated with virulence and survival. The precise mechanism, especially in relation to redox changes and its effects on immunological responses in visceral leishmaniasis (VL) is not completely understood as yet. Therefore, we purified a recombinant *PDI* from *Leishmania donovani* (*r-LdPDI*) which was of ~15 kDa molecular size and examined its effects on immunological responses in peripheral blood (PBMC) of human VL cases. For these studies, alanine was tested as an inhibitor and was used in parallel to all experiments. This protein was identified to have a direct correlation with parasite growth which significantly increased number of promastigotes as well as axenic amastigotes after 96 h of culture. Our experiments examining the immunological response against *r-LdPDI* also indicate the activation of pro-*L. donovani* dictated immunological responses in VL. The stimulation of PBMC with *r-LdPDI* induced lactate dehydrogenase (LDH) activities and up regulated interleukin-10 (IL-10) production but not the HLA-DR expression, Nitric oxide (NO) release and IFN-γ production indicating a pivoted role for *r-LdPDI* in causing a strong immunosuppression in a susceptible host. Further, we observed that an addition of alanine in *L. donovani* culture offers a significant inhibition in growth of parasite and helps in reconstitution of protective immune response in VL cases. Therefore, we demonstrate a future cross talk on use of alanine which can reduce the activities of PDI of *L. donovani*, eliminating the parasite induced immunosuppression and inducing collateral host protective response in VL.

1. Introduction

Visceral leishmaniasis (VL) commonly known as Kala-Azar is caused by *Leishmania donovani* and *Leishmania infantum* in the old world and *Leishmania chagassi* in the new world. Most of the infected individuals develop symptomatic disease due to spread of parasites in the spleen, liver, lymph node and other organs and becomes fatal without specific chemotherapy [14,12,46,35]. Previous studies have shown that the suppression of the specific Th1 kind of immune response as shown by decreasing production of IFN-γ and IL-12 in the patients promotes disease susceptibility and this suppression is critically regulated by interleukin (IL)-10, a pleiotropic cytokine secreted from different cell types including the macrophages [9]. Recombinant IL-10 was reported to have inhibitory effect towards nitric oxide (NO) mediated killing of *Leishmania* in human macrophages [49]. In the endemic areas, many individuals do not manifest clinical symptoms but show elevated Th-1 like immune response. This may be due to stimulation of protective immune response in them by *Leishmania* antigens [9]. Therefore, any intervention that helps the shift of immune response from Th-2 type towards Th-1 will have a major role in cure and prevention of VL. Such goal can be achieved through identifying the immunological mechanisms which the *Leishmanial* antigens may target during the metabolic pathways particularly those that underlie T cell unresponsiveness and accompanies the disease.

Such a unique enzyme target present in *Leishmania* can be protein disulfide isomerase (PDI) which remains highly abundant in the lumen of the endoplasmic reticulum [18]. It belongs to a member of the thioredoxin superfamily which can catalyse thiol disulfide interchange ensuring the proper folding and conformation of newly synthesized proteins and preventing cell toxicity due to ER stress [20,16,27,50,25]. In addition to its redox/isomerise activities, PDI was observed with chaperone activity which can increase the refolding of denatured lysozyme [40] and acid phospholipase A2 [51]. A typical PDI protein is composed of four

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consecutive thioredoxin-like domains (a–b–b′–a′) of which only two, a and a′ contain the characteristic CXXC active site motif [17] with two cysteine residues regulating the redox potential of the enzyme and its function as a thiol-disulfide reductase, oxidase or isomerise [16]. The domains of PDI are enveloped by an N-terminal signal peptide which helps in translocation of the protein into the ER and a C-terminus c domain that is rich in acidic amino acids and contains the KEDL retention signal [17]. The PDI of *L. donovani* is in contrast to other reported PDI because it lacks the ER retention signal KEDL and have only one active thioredoxin site instead of two.

The PDIs plays a pivoted role in a number of process involved in cell function and development during infectious diseases [23]. PDI proteins have been reported to help in accumulation of misfolded proteins triggered by oxidative stress in neurodegenerating disease [48]. In parasites such as Chlamydia, an obligate intracellular bacterial pathogen of eukaryotic cells, native PDI is required for effective attachment on the cell surface [11]. Likewise, it was reported to be involved in the adhesion of *Neospora caninum* techyzoites to host cells [37]. In addition, this protein is possibly involved in defence against protozoan parasites [31].

To date, little is known about the role of PDI in lower eukaryotes such as protozoan parasite, *L. donovani* and its relevance to host parasite interaction during visceral leishmaniasis. The precise mechanism, especially in relation to redox changes and its effects on immunological responses is also not completely understood [43,34,33,44]. The characterisation of PDIs obviously has important implication for the design of new drug or vaccine against *Leishmania* parasites. Two groups have reported a 15-kDa atypical PDI with only one catalysing site "CGHC" [38] and a 55-kDa PDI [25] in *L. donovani*. In this study, we expressed the enzyme encoding 15-kDa PDI protein of *L. donovani* and the potential function of recombinant *LdPDI* (*r-LdPDI*) on infection and immune response was assessed by growth analysis, Lactate dehydrogenase (LDH) and acid phosphatase (ACP) release, nitric oxide (NO) production, HLA-DR expression and cytokine interleukin-10 (IL-10) and IFN-gamma (IFN-γ) estimation in infected *Leishmania* patient. We show that protein of PDI promotes *L. donovani* infection, rendering macrophages more toxic which up regulates immunosuppressive factors such as IL-10 and causes retardation of anti-Leishmania activity of macrophage and that it also interrupts in the induction of protective T-cell response in VL patients. Moreover we also identify alanine as PDI modifying agent which can be a future strategy for the treatment of Kala-Azar (KA) cases.

2. Materials and methods

2.1. Samples from VL patients and control

A total of 20 subjects (aged between 17 and 40 years) of both sexes were studied after obtaining their informed consent. It included 10 patients with acute VL from endemic areas and 10 apparently healthy individuals who represented an endemic area. The study was conducted between September 2011 and March 2012. Measurement of body temperature, body weight, liver and spleen size, total and differential WBC count, haemoglobin, blood sugar, serum createnine and prothrombin, ECG and chest X-ray were performed in all cases. All the cases in VL group presented characteristic sign and symptom of VL infection and diagnosis was confirmed by presence of *L. donovani* in the Giesma stained spleen aspirate and by positive serology test (DAT and rk39). The healthy individuals in control group were negative for all disease symptoms and matched the patient group by sex and age (±2 years).

2.2. Parasite cultures

L. donovani parasites (AG83) were used in this study. The promastigote stains were maintained in Tobies biphasic medium and mass-cultured in RPMI-1640 medium (GIBCO, Invitrogen, USA) containing 20% heat inactivated foetal bovine serum (FBS; GIBCO, Invitrogen, USA) and 25 mM HEPES buffer (pH 7.2–7.4), 100 units/ml penicillin and 100 μg/ml streptomycin. Culture was set up at 2×10^6 parasites/ml and grown at 24 ± 1 °C in BOD incubator for 4–5 days before sub culturing (late log phase) which was harvested by centrifugation as described previously [13]. Axenic amastigote of *L. donovani* parasites (AG83) were generated from the promastigote forms at 37 °C in a CO_2 incubator using the media described above at pH 5.5 [8,45]. Cultures were maintained through serial sub-culturing for further studies.

2.3. PCR amplification and cloning of PDI from L. donovani

The *LdPDI* ORF was amplified from first strand cDNA (2 μl) with a sense (5′*GCG*GAATTCGGAGATTGTCGAGCTCAACC3′) and an antisense (5′*CGC*CTCGAGCTGCTTGTTGGCCGC3′) primers, where the EcoRI and xhol-sites are underlined and the translational initiation codons are italicised. These primers (Sigma, St. Louis, MO, USA) were designed to clone *Ld*PDI in vector pET22 b (+) plasmid with a histidine tag at the amino terminus. PCR was performed in a 50 μl reaction mixture containing 0.2 mM each dNTPs, 2.0 mM $MgCl_2$, 1.0 μM each primer, 1 μg *L. donovani* (Ag83) cDNA and 1.0 pfu DNA polymerase and 2 U Taq DNA polymerase with a Taq buffer ($+NH_2SO_4$). The conditions used for PCR was start at 95 °C for 5 min, denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, elongation at 72 °C for 2.5 min and subjected to 40 cycles with a final extension for 2 min at 72 °C. A ~380 bp PCR product was observed on 1% agarose gel electrophoresis. This PCR product was double digested with EcoRI and xhol, purified with gel extraction kit (Qiagen, Germany), and cloned into pET22 b (+) (Novagen Madison, WI, USA) in the same orientation as the T7 promoter. The ligation mixture was transformed in competent $DH5_\alpha$ cells (Novagen) which produced the pET22 b–LdPDI plasmid. The pET22 b–LdPDI construct was transformed into competent *Escherichia coli* BL21 DE 3 (Novagen, Madison, WI, USA) cells by heat shock at 42 °C for 45 s and the cells were grown at 37 °C on Luria Bertani (LB) agar medium in the presence of ampicillin (25 μg/ml) and Kanamycin (100 μg/ml) overnight at 37 °C under 200 rpm in shaker incubator for 4–5 h till the optical density reached (0.4).

2.4. Expression and purification of recombinant LdPDI protein

The recombinant protein was subsequently induced by 1.0 mM isopropyl-β-thiogalactoside (IPTG) overnight in shaker incubator at 22 °C and 200 rpm. The 5 ml overnight culture was inoculated into 500 ml fresh LB medium and cultured in shaker incubator at 22 °C and 200 rpm. Once the A600 reached between 0.5 and 0.6, 1 mM IPTG was added to induce expression of protein which followed further continuation of culture for 24 h at 22 °C. The bacterial cells were pelleted after harvesting of the cultured cells by centrifugation for 10 min at 5000 rpm and 4 °C and were then lysed in 30 ml cell lysing solution (50 mM Tris–HCl, pH 8.0, 300 mM NaCl, and 0.1% Triton X-100), 100 μg/ml lysozyme, and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min at 4 °C and further lysed by ultra-sonication at 85% amplitude and 0.5 s pulse for 5 min. The lysed cells were centrifuged at 14,000 rpm at 4 °C, and antigen recovered from supernatant was stored after addition of a cocktail of protease inhibitors (1 μl/ml).

For purification, supernatant was added to 1.5 ml slurry of nickel-nitrilotriacetic acid (Ni-NTA) and incubated for 3 h at 4 °C with gentle shaking. The resin was divided into three 10 ml disposable