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Case reports

Expression on hypoxia-inducible factor-1α in human tegumentary leishmaniosis caused by *Leishmania braziliensis*

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ABSTRACT. Several immune markers have been studied in controlling American tegumentary leishmaniosis based on mouse models. However, there is a lack of studies regarding human tegumentary leishmaniosis caused by *Leishmania braziliensis*. In this study, hypoxia-inducible factor-1α was found to be an important effector element in the localized control of human cutaneous and mucocutaneous lesions.

Key words: hypoxia-inducible factor-1α, *Leishmania braziliensis*, human leishmaniosis

Background

Hypoxia-inducible factor (HIF) is a transcription factor consisting of HIF-1α and HIF-1β. Under normoxic conditions, HIF-1α is hydroxylated into proline residues and degraded through the ubiquitin-proteasome pathway [1]. Under hypoxic conditions, hydroxylation is inhibited and nuclear translocation and transcription of HIF-dependent genes occur [2].

HIF-1α was originally identified as a regulator of the adaptive response to hypoxia. It accumulates in ischemic tissues and various tumors, and in situations of infectious diseases [2–4]. HIF-1α is involved in many aspects of cellular processes such as a promoter of glycolysis switch [5] and as one of the client proteins for the heat-shock protein 90 present in macrophages as well as *Leishmania* [6]. Heat shock protein 90 homeostasis controls stage differentiation in *Leishmania donovani* [7]. Recent studies have shown that HIF-1α is expressed in murine leishmanial lesions. It becomes stabilized in infected host cells and may be involved in the adaptation processes during infection [8–10].

There are a few studies on HIF-1α expression during human tegumentary leishmaniosis. Studies conducted so far [11–13] have shown that HIF-1α is expressed in skin biopsies of patients suffering from cutaneous leishmaniosis. However, these did not address the detection and identification of *Leishmania* species in such biopsies. In the present report, polymerase chain reaction (PCR) was used to identify and confirm *L. braziliensis* in tegumentary lesions, and immunohistochemical (IHC) assays were used to demonstrate the HIF-1α expression in skin biopsies from patients with active disease.

Case presentation

Two adult patients with active cutaneous and mucocutaneous ulcerating lesions characteristic of American tegumentary leishmaniosis (ATL), living

in the metropolitan region of Recife, State of Pernambuco, northeast of Brazil, were recruited in the study. All the study material was obtained in accordance with the recommendations of the Human Ethics Committee of the Health Sciences Center of the Federal University of Pernambuco (CAAE number: 51426415.0.0000.5208).

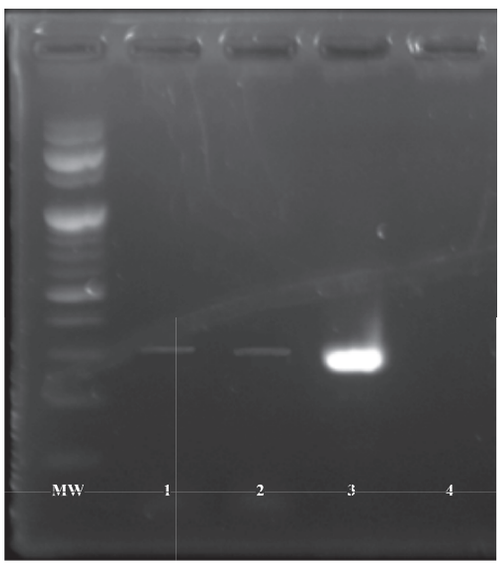

Fig. 1. Identification of *Leishmania braziliensis* in clinical samples from patients, using ITS1-PCR, viewed by means of electrophoresis on 1.5% agarose gel and stained with ethidium bromide. MW: molecular weight; 1. patient sample; 2. patient sample; 3. positive control; 4. negative control.

Paraffin-embedded skin biopsies were subjected to DNA extraction. First, the paraffin was removed by xylol solvent, and then the extraction was performed using a commercial genomic DNA purification kit (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany) and technical kit, following the manufacturer's recommendations. Extracted DNA was then quantified using Ultrospec 3000 UV/Visible spectrophotometer (Pharmacia Biotech, Cambridge, England) and analyzed using electrophoresis on 1.5% agarose gel in TAE buffer (40 mM of Tris-acetate, and 2 mM of EDTA). The product was stained with ethidium bromide or SYBR Safe to verify the integrity and quality of the sample.

PCR was performed using the primers LITSR (5'-CTGGATCATTTTCCGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3'), at a concentration of 50 Pmol, to amplify a fragment of the internal transcribed spacer 1 (ITS1) region [12]. The PCR was performed using the TopTaq™ Master Mix kit (Qiagen®, Hilden, Germany), with a 50 µl reaction. Positive controls used were extracted from the *L. braziliensis* reference strain MHOM/BZ/75/M2903 in Schneider's medium. These were obtained from the Parasitology Laboratory of the Aggeu Magalhães Research Center, Oswaldo Cruz Foundation (CPqAM-FIOCRUZ). Water without any added genetic material was also used as a negative control. Amplified products were viewed using electrophoresis on 1.5% agarose gel after staining with ethidium bromide by Transilluminator electrophoresis gel - L-PIX TOUCH.

To detect HIF-1α-positive inflammatory cells in cutaneous and mucocutaneous lesions, paraffinized sections (5 µm) were deparaffinized in xylene and rehydrated in baths of decreasing concentrations of ethanol and distilled water. Antigen retrieval was performed using citrate buffer. To block endogenous peroxidase and nonspecific binding sites, the sections were incubated with 5% hydrogen peroxide and 1% phosphate-buffered saline/bovine serum solution, respectively [9].

IHC analysis was carried out based on the immunoperoxidase technique, by using rabbit polyclonal anti-HIF-1α (NB100-479; diluted 1:100) as the primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG (Abcam 6721; diluted 1:100) as the secondary antibody. Each section was incubated overnight at 4°C and for 60 minutes at room temperature, respectively. After successive washing, the sections were incubated with diaminobenzidine (Sigma, Sigma-Aldrich, USA; 100 mg% in PBS and 0.1% hydrogen peroxide), which was used as a substrate solution. These sections were then counterstained with Erlich's hematoxylin and mounted using Entellan® (Merck, Germany). For each IHC assay, sections from normal human HIF-1α marker. For the negative control, the primary antibodies were replaced by nonimmune serum samples.

To count HIF-1α-positive inflammatory cells, a semi-quantitative method [11] was applied using scores from 0 to 3, such that 0 represented absence of labeling; 1, up to 25 labeled cells; 2, between 25 and 50 labeled cells; and 3, up to 50 labeled cells. The counting of labeled cells was carried out randomly in 10 microscopic fields.

All IHC images were captured using a digital imaging system, comprising an optical microscope (Eclipse E800, Nikon, Tokyo, Japan), a CoolSnap-

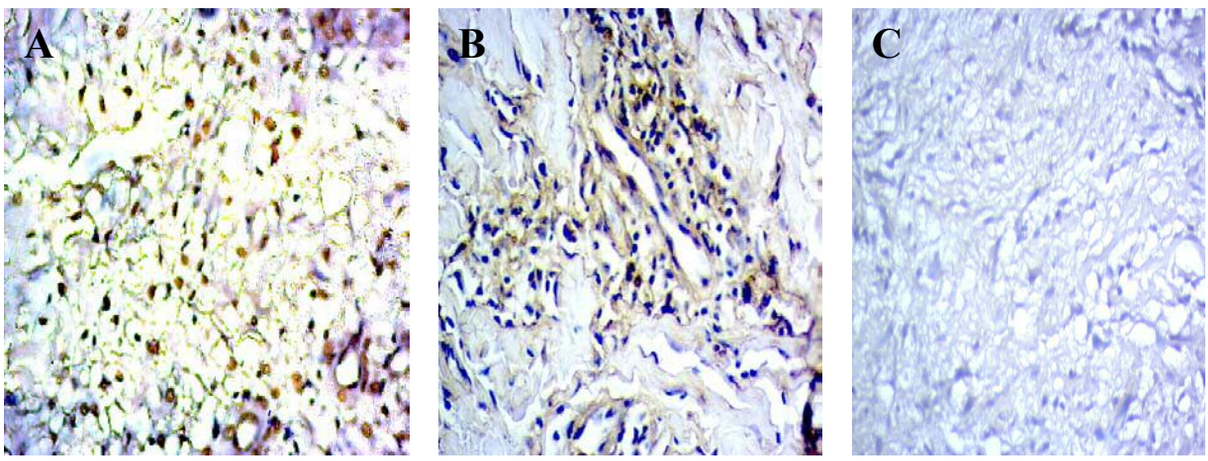

Fig. 2. HIF-1α expression in skin lesions due to cutaneous leishmaniasis caused by *L. braziliensis* (A and B). Arrows indicate HIF-1α-labeled mononuclear inflammatory cells and no immunoreactivity in normal human skin (C). Dermis contains higher expression of HIF-1α (score 3) in the nuclei of mononuclear inflammatory cells (A), while there is moderate expression of HIF-1α (score 2) in the cytoplasm of mononuclear inflammatory cells distributed around granulomatous dermatitis area (B). Sections A to C (x400) were stained with immunoperoxidase and counterstained with hematoxylin.

Pro color camera (Media Cybernetics, Inc., Silver Spring, MD, USA) and the Image-Pro Plus image capture software (Media Cybernetics, Inc., Silver Spring, MD, USA).

Skin biopsy specimens from these two patients recruited from the metropolitan region of Recife, Pernambuco, Brazil, were investigated for *L. braziliensis* using ITS1-PCR. *L. braziliensis* DNA was detected in both clinical specimens (Fig. 1). The sensitivity of ITS1-PCR was tested using the species *L. infantum* and *L. braziliensis*, where the PCR amplification showed sensitivity of up to 100 fg of DNA for both species tested, corresponding to one parasite per tube [15,16].

Both patients were confirmed to be infected with *L. braziliensis* but one of them presented small ulcerating cutaneous lesions on a lower limb (Case 1) while the other showed infiltrating and ulcerating mucocutaneous lesions on the upper lip (Case 2). In both cases, the inflammatory infiltrate was composed mainly of macrophages, Langerhans cells and lymphocytes. A nonspecific chronic ulcerating lesion was the main histopatological feature observed in Case 1 (Fig. 2A), whereas some parasite amastigotes and granulomatous dermatitis were observed in Case 2 only (Fig. 2B).

On investigating the presence of HIF-1α and its distribution within inflammatory cells, there were higher intensity levels of HIF-1α (score 3) in the nuclei of mononuclear inflammatory cells, particularly mononuclear cells such as macrophages and Langerhans cells. These were distributed randomly in the dermis of the patient with multiple cutaneous ulcerating lesions (Fig. 2A). On the other hand, a moderate level of intensity of HIF-1α (score 2) was observed in the cytoplasm of mononuclear inflammatory cells (macrophages and Langerhans cells). These were distributed around the granulomatous dermatitis of the case with mucosal infiltrating lesions (Fig. 2B). No HIF-1α staining was detected in normal human skin cells (Fig. 2C).

The findings of the present report are consistent with those of previously published in the literature [11–13], indicating that HIF-1α expression occurs during cutaneous and mucocutaneous leishmaniosis. This study can contribute to clarify the pathogenesis of human tegumentary leishmaniosis and perhaps the HIF-1α can be a useful marker to diagnostic patients that control the disease.

Conclusions

HIF-1α is an immune marker expressed in the skin inflammatory cells infected with *L. braziliensis*-infected patients. Although this can have a role in the management of cutaneous and mucocutaneous leishmaniosis, we still need more studies to confirm it. Since the clinical spectrum of leishmaniosis ranges from localized cutaneous ulcers to mutilating mucocutaneous disease and visceral infections, caused by several *Leishmania*

species, HIF-1α expression analyses needs to be performed simultaneously with parasite identification and histological assessment of the lesions.

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