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Expression of hypoxia-inducible factor-1α in the cutaneous lesions of BALB/c mice infected with *Leishmania amazonensis*

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Abstract

The hypoxia-inducible factor-1α (HIF-1α) is expressed in response to hypoxia and has been recently demonstrated in a variety of cells such as tumor cells and tumor-associated macrophages. Several characteristics of leishmanial lesions in humans and in animal models, such as microcirculation impairment, metabolic demand for leukocyte infiltration into infected tissue, parasite proliferation, and secondary bacterial infection, are strong indications of a hypoxic microenvironment in the lesions. We evaluated HIF-1α expression in the cutaneous lesions of BALB/c mice during *Leishmania amazonensis* infection. Immunohistochemical analyses of the lesions demonstrated, only in the later stages of infection when the lesion size is maximal and parasite burden is enormous and massive numbers of recruited macrophages and ulcers are observed, positive HIF-1α-infected cells throughout the lesions. HIF-1α is expressed mainly in the cytoplasm and around parasites inside the parasitophorous vacuoles of macrophages. This is the first evidence that macrophages in the microenvironment of lesions caused by a parasite produce a hypoxia-inducible factor.

Keywords: *Leishmania amazonensis*; Amastigotes; Murine leishmaniasis; Chronic cutaneous lesions; Parasitic diseases; Macrophage; Parasitophorous vacuoles; Immunohistochemistry studies; Hypoxia-inducible factor-1α; Hypoxia

Introduction

Leishmaniasis is an endemic parasitosis caused by several species of the genus *Leishmania*, an obligate intramacrophagic parasite. Several characteristics of leishmanial lesions in humans and in animal models, such as microcirculatory impairment, metabolic demand for leukocyte infiltration into infected tissues, parasite proliferation, and secondary bacterial infection (El-On et al., 1992; Grimaldi and Tesh, 1993; Giorgio et al., 1998; Kanan, 1975; McElrath et al., 1987), are strong indications of a hypoxic microenvironment in lesions that may play a role in the outcome of infection. Various studies have described hypoxia as a specific metabolic stimulus and signal that profoundly affects a broad range of macrophage properties such as cytokine secretion, expression of cell surface markers, migration, and adhesion (Lewis et al., 1999). Recently, we have demonstrated that experimental hypoxia did not depress *Leishmania amazonensis* in vitro phagocytosis by macrophages but induced these cells to reduce intracellular parasitism, suggesting that hypoxic modulation may be important during leishmaniasis (Colhone et al., 2004). Whether hypoxic areas are formed during the development of leishmanial lesions is not known.

Recent investigations have demonstrated the hypoxia-inducible transcription factor-1 (HIF-1) is expressed in response to hypoxia and activates expression of genes involved in erythropoiesis, angiogenesis, glycolysis, and modulation of vascular tone (Semenza, 2001; Shi and Fang, 2004). HIF-1 consists of a heterodimer of HIF-1α, the

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oxygen-responsive component, and HIF-1β (Wang and Semenza, 1995; Yu et al., 1998). HIF-1α is virtually undetectable under normoxia, but when cells are subjected to experimental hypoxia, levels of HIF-1α are rapidly expressed (Burke et al., 2002). In addition, immunohistochemical studies have shown that human macrophages in the hypoxic synovia of arthritic joints, myeloid cells, murine macrophages, and tumor-associated macrophages in avascular areas of various types of carcinomas express high levels of HIF-1α (Burke et al., 2002; Cramer et al., 2003; Hollander et al., 2001; Semenza, 2001). However, there is no previous investigation of expression of HIF-1α in lesions caused by a parasitic infection. In this study, we investigated the expression of HIF-1α by immunohistochemical analysis of the cutaneous lesions of a susceptible mouse strain (BALB/c) during the course of *L. amazonensis* infection.

Materials and methods

Parasite and infection

L. amazonensis (MHOM/BR/73/M2269) were maintained by regular passage in BALB/c mice. Amastigotes were purified from the footpad lesions of mice as previously described (Barbieri et al., 1993). Female BALB/c mice (6 weeks old) were obtained from Centro de Bioterismo-UNICAMP, Campinas, SP, Brazil, and injected subcutaneously in the right hind footpad with 10^5 amastigotes (Giorgio et al., 1998). The experimental protocols were approved by the Institute of Biology/UNICAMP Ethical Committee for Animal Research.

Evaluation of infection

The course of infection was monitored by measuring the increase in footpad thickness, compared with the contralateral uninfected footpad, with a dial caliper (Giorgio et al., 1998). To estimate parasite burden in the lesions, at designated periods, mice were sacrificed, the entire infected footpads were weighed, and amastigotes were recovered from the lesions as previously described (Barbieri et al., 1993) and counted. Three independent experiments involving 7–10 mice were performed to evaluate infection and for immunohistochemical study.

Immunohistochemistry studies

Foot tissues of BALB/c mice were obtained after animal perfusion fixation with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M PBS, pH 7.4. Tissues were processed for standard paraffin embedding and deparaffinized sections were dehydrated, treated with 0.3% hydrogen peroxide for 30 min, washed with PBS, and incubated with 1% bovine serum albumin for 30 min (Paffaro et al., 2003). Tissue sections were then incubated with rabbit polyclonal anti-HIF-1α antibody (H-206) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) developed with a secondary goat polyclonal anti-rabbit antibody conjugated with peroxidase (Sigma, St Louis, MO) and visualized with a peroxidase substrate solution containing 3,3′-diaminobenzidine and hydrogen peroxide (Yu et al., 1998). Control sections were incubated with normal rabbit serum as the primary antibody. Tissues sections were counterstained with hematoxylin, dehydrated in graded alcohol solutions, and mounted in cytoseal-60 mounting medium (Sigma) (Paffaro et al., 2003). *L. amazonensis* amastigotes were purified from lesions (Barbieri et al., 1993), washed with PBS, incubated with 1% bovine serum albumin, and immunostained with rabbit polyclonal anti-HIF-1α antibody as described for tissues. The images were recovered with a digital imaging system, a light microscope (Eclipse E800-Nikon/Japan), a Cool Snap-Pro Color camera (Media Cybernetics), and capture software (Image-pro plus-Media Cybernetics).

Results

L. amazonensis produces rapidly developing skin lesions in BALB/c mice as attested by the continuous increase in footpad thickness (Figs. 1A and 2). The presence of nonulcerating nodules and small-sized lesion was observed during the first 20 days after infection (Figs. 1A and 2). No sign of recovery was observed and after 70 days most of the mice had infected footpad skin ulcer sizes ranging from 7 to 10 mm (Figs. 1A and 2). At 140–150 days, necrotic areas with bacterial contamination were observed in the infected footpad and most of the animals had developed metastatic cutaneous lesions and visceralization of the parasites in the lymph nodes, spleen, and liver (Fig. 2, data not shown; El-On et al., 1992; Giorgio et al., 1998). The lesion parasite burden progressively increased with time, and by 150 days, the numbers of parasites were approximately 3000 and 250 higher than the numbers found in the lesions of mice at 3 and 20 days, respectively (Fig. 1B). All BALB/c mice had died after 160 days of infection.

To determine whether expression of HIF-1α is occurring during *Leishmania* infection, we analyzed the immunostaining with a polyclonal antibody to HIF-1α in mice footpads counterstained with hematoxylin (Fig. 3). Histological analyses revealed that the epidermis and glandular structures in footpads of infected BALB/c mice are conserved in lesioned footpads after 3 days of infection (Figs. 3a and b). Footpad tissue from normal mice showed no immunostaining with HIF-1α (Fig. 3A). Examination of cutaneous lesions at an early stage of infection also showed no immunostaining to HIF-1α (Fig. 3B). At 20 days, a mixed cellular population infiltrating the tissues and parasitized macrophages was observed in the lesioned footpads and no HIF-1α reactivity was detected in these tissues (Fig. 3c). Later in the infection, at 70 days, the lesions consistently