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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

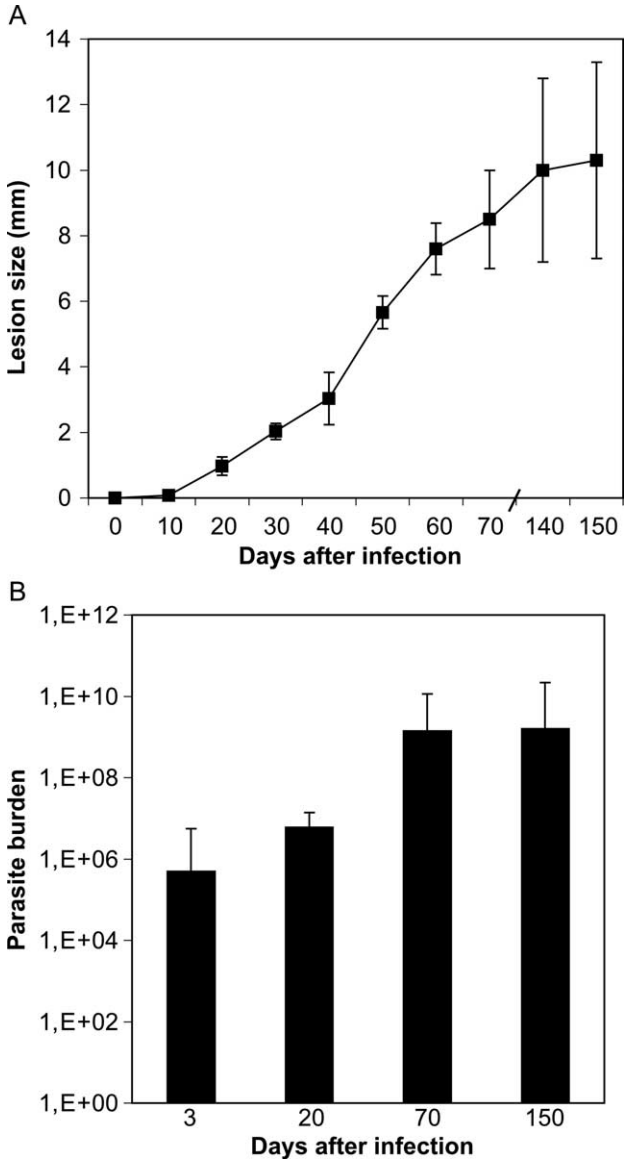

Fig. 1. Course of *L. amazonensis*-infected BALB/c mice. Animals were inoculated in the hind footpad with 10^5 amastigotes. (A) Lesion size is expressed as the difference in size between the infected and contralateral noninfected footpad. (B) At the times indicated, three mice were sacrificed and infected footpad collected and parasite numbers determined as described in Materials and methods.

showed macrophages containing numerous amastigotes within large parasitophorous vacuoles infiltrating subcutaneous fat and muscle and replacing normal tissues in various areas (Fig. 3d). The immunostaining revealed the presence of infected macrophages expressing HIF-1α. Staining of macrophages was observed in the cytoplasm, more specifically around amastigotes inside parasitophorous vacuoles (Fig. 3d). No immunostaining was observed in sections when antibody against HIF-1α was replaced with normal rabbit serum (Fig. 3e). At 150 days of infection, a similar pattern of histological picture was observed in footpads, that is, massive numbers of vacuolated and heavily parasitized macrophages replacing the normal tissue. Strong immunoreactivity for HIF-1α was observed in the parasitophorous vacuoles of macrophages and on the surface of the intracellular amastigotes (Fig. 3f). The immunostaining was observed in serial sections of this cutaneous lesion and in cutaneous lesions from other BALB/c mice at 70 and 150 days of infection with *L. amazonensis* (data not shown). Importantly, amastigotes purified from lesions of mice at later stages of infection were incubated with HIF-1α antibody and no staining could be detected on these preparations (data not shown), indicating that parasites themselves are not positive for HIF-1α antibody. Taken together, the results suggest that during the late stages of murine leishmaniasis, macrophages produce HIF-1α protein, which is localized in the cytoplasm and also accumulates in vacuoles containing parasites.

Discussion

Murine experimental leishmaniasis has become a useful model for studying lesions caused by intracellular pathogens (Solbach and Laskay, 2000). Mouse strains as BALB/c develop uncontrolled cutaneous lesions after *L. amazonensis* inoculation (Fig. 1) accompanied by microcirculation impairment, proliferating amastigotes, secondary bacterial infection, and increased metabolic demand (El-On et al., 1992; Grimaldi and Tesh, 1993; Giorgio et al., 1998; Kanan, 1975; McElrath et al., 1987), which are strong indications of a hypoxic microenvironment. The HIF-1α transcriptional

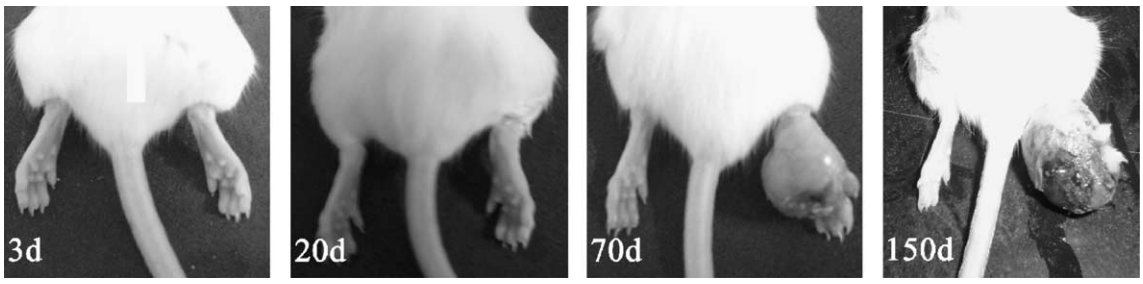

Fig. 2. Evolution of cutaneous *L. amazonensis* infection in BALB/c mice. Animals were inoculated in the hind footpad with 10^5 amastigotes. These photographs represent the typical evolution of cutaneous lesions illustrating reduced footpad inflammation and size at 3 and 20 days of infection. At 70 days, lesion became ulcerate, and 150 days after infection, an ulcerative lesion partially covered by crusts was observed.

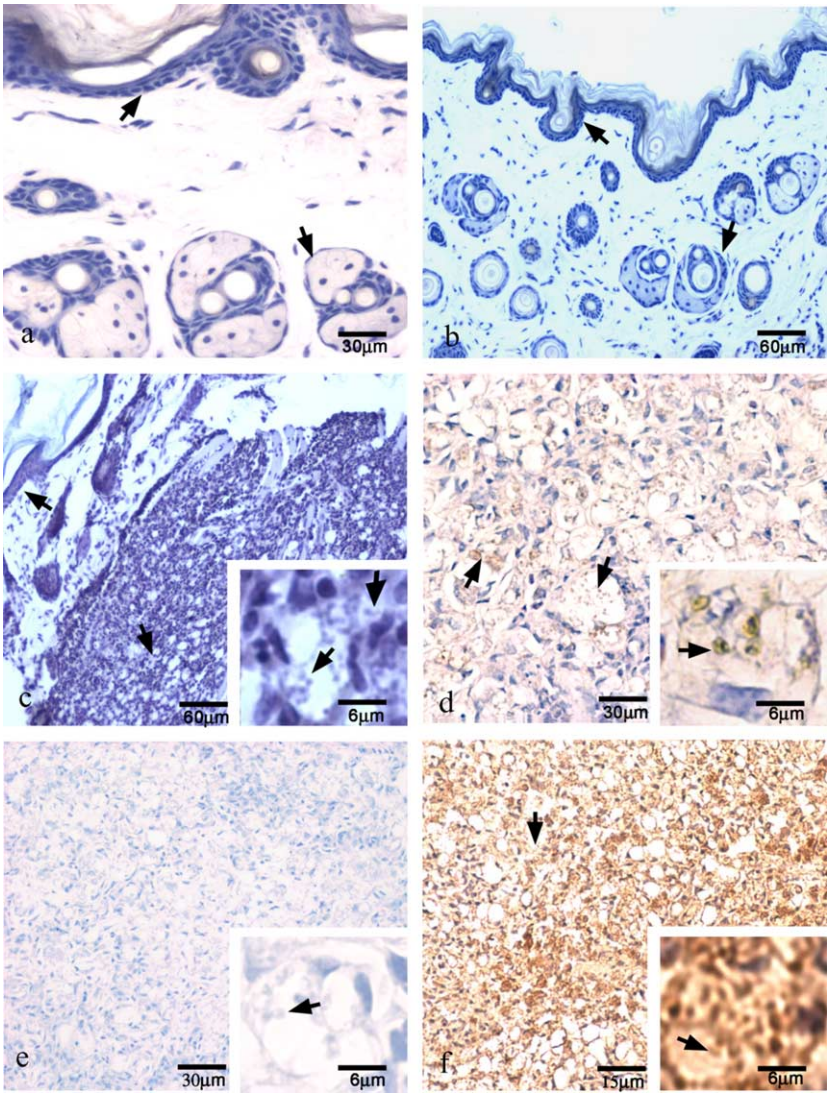

Fig. 3. Photomicrograph of BALB/c mouse footpad tissues. (a) Normal BALB/c mouse footpad tissue stained with HIF-1α polyclonal antibody and counterstained with hematoxylin. The top and bottom arrows indicate epidermis and glandular structure, respectively. No immunoreactivity was observed in the tissue. (b) BALB/c footpad lesion at 3 days of *L. amazonensis* infection, stained with HIF-1α polyclonal antibody and counterstained with hematoxylin. The top and bottom arrows indicate conserved epidermis and glandular structure, respectively. No immunoreactivity was observed in the tissue. (c) BALB/c footpad lesion at 20 days of infection, stained with HIF-1α polyclonal antibody and counterstained with hematoxylin. The top and bottom arrows indicate epidermis and cellular population infiltrating the tissue, respectively. Inset shows vacuoles of infected macrophages. No immunoreactivity was observed in the tissue. (d) BALB/c footpad lesion at 70 days of infection, stained with HIF-1α polyclonal antibody and counterstained with hematoxylin. The arrows indicate positively stained vacuolated infected macrophages. Inset shows infected macrophage with stained parasites. (e) BALB/c footpad lesion at 70 days of infection, stained with normal rabbit serum and counterstained with hematoxylin. No immunoreactivity was observed in the tissue. (f) BALB/c footpad lesion at 150 days of infection, stained with HIF-1α polyclonal antibody and counterstained with hematoxylin. The arrow indicates positively stained vacuolated infected macrophages. Inset shows infected macrophage with stained parasites.

protein is a prominent regulator of the genetic response to hypoxia and its expression is primarily controlled by oxygen tension (Wang and Semenza, 1995). A number of studies have demonstrated HIF-1α expression in tumors and ischemic tissues, and the immunohistochemical approach has been the method most extensively used to analyze expression and distribution of HIF-1α (Burke et al., 2002; Chavez and La Manna, 2002; Talks et al., 2000; Yu et al., 1998). The results shown in this report demonstrate that HIF-1α is expressed in the lesions of *L. amazonensis*-infected BALB/c mice. HIF-1α expression was not detected in the early stages of infection but could be demonstrated in the later stages (Fig. 3). After 70 days of infection, HIF-1α is mainly expressed in the cutaneous lesions in the cytoplasm and around parasites inside parasitophorous vacuoles of macrophages. Strong immunoreactivity for HIF-1α was observed in the macrophages and on the surface of the intracellular amastigotes in the lesions at 150 days of infection (Fig. 3f). Since parasites purified from the lesions did not react with antibody to HIF-1α, its reasonable to suppose that macrophages produce and express HIF-1α in the microenvironment of leishmanial lesions. No reports of the expression of HIF-

1α in cells infected with microorganisms are available for comparison with these results. Nevertheless, several studies have shown that tumor cells have predominantly nuclear HIF-1α accumulation, whereas tumor-associated macrophages and macrophage cell lines exposed to hypoxia express both nuclear and cytoplasmic HIF-1α (Burke et al., 2002; Crowther et al., 2001; Talks et al., 2000). Interestingly, HIF-1α was not detected in the nucleus of macrophages in leishmanial lesions (Figs. 3D and E). The reasons why HIF-1α, a transcription factor known to accumulate in the nuclei of hypoxic cells, was not localized in the nucleus of parasitized macrophages are not known. One explanation for this might be that HIF-1α binds to parasite factors, altering or blocking transport to the nucleus. Alternatively, ubiquitination, a prerequisite for proteosome degradation of HIF-1α, may occur in macrophages harboring parasites, causing HIF-1α to be rapidly shuttled out of the nucleus and to accumulate in the cytoplasm, as suggested by Burke et al. (2002) to explain the strong HIF-1α immunoreactivity of cytoplasm of tumor-associated macrophages from ovarian carcinomas.

Another interesting finding was the time-dependent expression of HIF-1α protein in cutaneous murine leishmaniasis. Only in the later stages of *L. amazonensis* infection (after 70 days), when lesion size is maximal, parasite burden is enormous, with ulcers, massive numbers of recruited macrophages, and intermittent vascular occlusion are observed, with positive HIF-1α-infected cells throughout the lesions (Figs. 1 and 3). Strong immunoreactivity for HIF-1α was detected at 150 days (Fig. 3f) when ulcerative and necrotic areas with bacterial contamination were observed in the infected footpad (El-On et al. 1992; Giorgio et al., 1998). Previous studies have shown that HIF-1α is upregulated in cultured cells at oxygen concentrations below of 2% (15 mm Hg) and in avascular areas of breast carcinomas (Burke et al., 2002; Jiang et al., 1996). Taken together, these observations suggest that leishmanial lesions become more hypoxic with time, reflecting the intense metabolic activity and oxygen demand of microorganisms and host cells. Although we can suggest that HIF-1α expression in macrophages from leishmanial lesions is a strong indication of a hypoxic microenvironment, we cannot exclude that other factors may upregulate HIF-1α. Previous studies had demonstrated that tumor necrosis factor, nitric oxide, and lipopolysaccharide stimulation induce HIF-1α expression in macrophages from healing wounds and in an alveolar macrophage-derived cell line (Albina et al. 2001; Blouin et al., 2004). The possibility that these and other factors may influence the expression of HIF-1α in these lesions cannot be ruled out. The physiological and pathological significance of HIF-1α expression in cutaneous leishmaniasis remains to be established. HIF-1α has been taken as a key factor in the regulation of gene transcription involved in cell- and tissue-hypoxia adaptations, such as angiogenesis, anti-apoptosis, and glycolysis (Lin et al., 2000; Minet et al., 2000; Shi and Fang, 2004). Whether the effects of HIF-1α in macrophages infected with *L. amazonensis* are protective for cell and parasite survival and are involved in metastases or on the contrary, are protective for cell host and are involved in relative control of parasite growth, as observed in an in vitro hypoxia cell system (Colhone et al., 2004), is not known. Future studies will examine the mechanism underlying HIF-1α induction and activity during leishmaniasis.

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