Immunohistochemical profile of HIF-1α, VEGF-A, VEGFR2 and MMP9 proteins in tegumentary leishmaniasis

Estudo da expressão imunohistoquímica das proteínas HIF-1α, VEGF-A, VEGFR2 e MMP9 em leishmaniose tegumentar americana

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Resumo: O presente estudo pode fornecer dados acerca do fenômeno hipóxia e da angiogênese em leishmaniose. We attempted to verify whether the HIF-1α protein expression may be associated to VEGF-A, VEGFR2 and MMP9 in leishmanial lesions.

Métodos: In this study, we gathered 54 paraffin blocks taken from skin lesions in patients from northern Minas Gerais, Brazil, with confirmed diagnosis of tegumentary leishmaniasis. Immunohistochemistry was used to evaluate the expression of the proteins. The expression of HIF-1α was categorized into two groups according to the median: HIF-1α lower and HIF-1α higher.

Resultados: Observamos aumento das expressões de VEGF e MMP9 no grupo HIF-1α acima da mediana. A análise de Spearman demonstrou correlação entre VEGF e MMP9.

Conclusão: Os dados aqui apresentados indicam uma alta expressão de proteínas HIF-1α em LTA. O grupo HIF-1α acima da mediana apresentou maior expressão das proteínas VEGF2 e MMP9. Foi demonstrada correlação entre as proteínas VEGF e MMP9, VEGFR2 e MMP9. Outros estudos in vitro e in vivo devem ser realizados a fim de esclarecer o mecanismo de ativação e resposta das proteínas HIF-1α, VEGF2 e MMP9 em leishmaniose tegumentar americana.

Palavras-chave: Indutores da angiogênese; Hipóxia celular; Leishmaniose

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INTRODUCTION

Leishmaniasis is one of the most important infectious diseases worldwide and unfortunately, it causes 60,000 deaths annually. Additionally, the number of cases of human leishmaniasis is increasing worldwide at a disturbing rate, estimated by the World Health Organization in 1998.\textsuperscript{1,2} Leishmania infections might result in a wide spectrum of clinical manifestations and its outcome is determined by complex host-parasite interactions.\textsuperscript{3} Leishmaniasis is an infectious disease caused by protozoa of the genus Leishmania that affect humans and different animal species and could manifest in different clinical forms.\textsuperscript{4} In Brazil, tegumentary leishmaniasis represents a public health problem and its incidence has increased significantly.\textsuperscript{4}

In leishmaniasis, it has been suggested that many characteristics of this lesions is associated to hypoxic events and it could have a role in the disease outcome.\textsuperscript{5-10} Furthermore, hypoxic events could affect cytokine secretion, expression of migration and adhesion cell surface markers of macrophages.\textsuperscript{3,11} Hypoxia still enhances angiogenic events, being highly dependent on the function of hypoxia inducible factor-1 alpha (HIF-1α). HIF-1 α associates with the HIF-1β subunit and this complex acts as a transcription factor of hypoxia-inducible genes such as vascular endothelial growth factor A (VEGF-A) and consequently its receptors, erythropoietin (Epo) and matrix metalloproteinases (MMPs), mainly MMP9. These genes code for proteins involved in several events such as embryogenesis, angiogenesis, cell proliferation, metastasis and during the cellular response to hypoxia.\textsuperscript{12-16}

Few studies about proteins associated to hypoxia have been performed in leishmaniasis and hypoxic areas formed in leishmanial lesions during the lesion development are unknown. Our study can provide more knowledge about angiogenic and hypoxic events in leishmaniasis by analyzing proteins involved in both phenomena. We attempted to verify whether the HIF-1 α protein expression may be associated to VEGF-A, VEGFR2 and MMP9 in leishmanial lesions. Besides understanding the pathway, we performed correlation of the VEGF-A, VEGFR2 and MMP9 proteins.

PATIENTS AND METHODS

In this study, we gathered 54 paraffin blocks taken from skin lesions in patients from northern Minas Gerais, Brazil, with confirmed diagnosis of tegumentary leishmaniasis. The diagnosis was confirmed in all cases through biopsy, direct parasitological examination and reaction of Montenegro. Although we did not have access to clinical data of lesions, all of them were cutaneous. All cases were of primary manifestations and visceral infections were discarded. These samples were collected from 54 patients aged between 6 and 90 years. Of them, 17 (31.5%) were females and 37 (68.5%) males. Referring to skin color, 33 (61.2%) were non-caucasian and 14 (25.9%) caucasian. The qualitative analyses can be shown as follows: in the epidermis, there was a presence of accentuated acanthosis, papillomatosis and hyperkeratosis; in the dermis, we observed predominance of intense exudative cellular reactions and the presence of mononuclear cells. All were treated with pentavalent antimonials and were clinically healed. Ethical approval for this study was obtained from the local ethics committees (Unimontes, CEP 1930/2010).

Immunohistochemistry staining

Each resected tissue specimen was fixed in formalin, embedded in paraffin, cut into 3-μm serial sections and mounted on organosilane-coated slides. The following primary mouse monoclonal antibodies were used: anti-HIF-1α (clone HIF-1α 67, Sigma-Aldrich, St. Louis, USA), anti-vascular endothelial growth factor A (clone 26503, Sigma-Aldrich, St. Louis, USA), anti-Flk-1 (VEGFR2) (clone A-5, Santa Cruz Biotechnology, CA, USA) and anti-MMP9 (clone 2C3, Santa Cruz Biotechnology, CA, USA).\textsuperscript{11} All monoclonal antibodies were incubated for 18 h at 4°C. Endogenous peroxidase was blocked by incubation with 0.03% H2O2 in ethanol for 30 min. For antigen retrieval, sections were heated in a steam cooker for 5 min at 125°C in Tris-EDTA buffer (1 mM Tris base, 1 mM EDTA and 0.05% Tween 20, pH 9.0). The primary antibodies against HIF-1α, VEGF-A, VEGFR2 and MMP9 were detected using the LSAB kit (LSAB-Kit Plus Peroxidase, Dako, California, USA). Signals were developed with 3’3-diaminobenzidine-tetrahydrochloride for 5 min and counter-stained with Mayer’s haematoxylin for 30 sec. Negative controls were performed by replacing the primary antibody with universal negative control mouse (Dako, Carpinteria, California, USA). In addition, in all the reactions of immunohistochemistry staining we took care to also verify the presence of internal positive control within each slide, ensuring thus the quality of the technique used.

Counting of immunostained samples

The immunohistochemical expression of biomarkers was evaluated using an Olympus® BH2 microscope (10× ocular and 40× objective lenses), and an ocular lattice (area 0.092 mm²) with 100 points composed of 10 horizontal and 10 vertical test lines was superimposed on the test field to be measured. A total area of 1.84 mm² was evaluated for each sample. Immunohistochemical analyses of all antigens investigated were performed by determining the percentage of positively stained cells (epithelial and leukocyte
cells) in all fields counted (10 fields for each specimen). Immunohistochemical expression data are expressed as mean ± standard error (mean ± sd) values.

**Statistical analysis**

Results of protein expressions are displayed as mean ± s.d. Data were analyzed by Mann-Whitney test. For analyses average duration of lesions, Kaplan-Meier test was performed and the variables were compared using the log-rank test. All statistical analyses were performed with the statistical pack SPSS® (SPSS Inc, Chicago, IL, USA), version 13.0 for Windows®. P values < 0.05 were considered significant.

**RESULTS**

The pattern expression of HIF-1α, VEGF-A, VEGFR2 and MMP9 proteins by immunohistochemistry is demonstrated in figure 1. HIF-1α and VEGF-A presented nuclear and cytoplasmic localization whereas VEGFR2 and MMP9 presented cytoplasmic localization. To determine whether increased levels of HIF-1α protein in leishmaniasis promoted increase of the other proteins, the patients were divided into two groups according to median values (94.70% in epithelial cells and 98.00% in leukocyte cells): HIF-1α lower and higher. Figure 2 shows mean and standard deviation of HIF-1α in leukocyte and epithelial cells. We observed increase of VEGFR2 and MMP9 protein expressions in HIF-1α higher group of epithelial cells (p=0.002 and 0.009, respectively) (Figure 3A). Considering leukocyte cells, we did not find any association between HIF-1α groups and protein expressions (Figure 3B). To understand the pathway, we then performed correlation of the VEGF-A, VEGFR2 and MMP9 proteins. Spearman analyses in epithelial cells showed correlation between VEGF-A and MMP9 protein expressions (r=+0.408 p=0.009) and VEGFR2 and MMP9 protein expression (r=+0.313 p=0.049). According to leukocyte cells, VEGF2 and MMP9 was correlated (r = -0.440 p=0.004). The average duration was 217.7 ± 194.7 days. There was no association between protein expressions and average duration (data not shown).

**DISCUSSION**

Angiogenesis is the process of vascular growth via pre-existing vessels. The process of vascularization occurs during embryonic development, growth and development of tissues, formation of corpus luteum and endometrium, regeneration and healing process of wounds. Abnormal angiogenesis is involved in many pathological processes including tumor growth, metastasis, diabetes and arthritis. Extracellular signals involved in this process are primarily paracrine secretion of factors and extracellular matrix components that usually carries specialized receptors and integrins. Angiogenesis is rapidly initiated in response to hypoxic, inflammation or ischemic conditions.

Hypoxia inducible transcription factor-1 (HIF-1) consists of a heterodimer of HIF-1α, the oxygen-responsive component, and HIF-1β. HIF-1α is held in response to hypoxia and activates expression of genes involved in erythropoiesis, glycolysis, modulation of vascular tone, and angiogenesis. In...
In a previous study, HIF-1α was not detected in the nucleus of macrophages in leishmanial lesions. The authors suggest that HIF-1α binds to parasite factors, altering or blocking their transport to the nucleus. Furthermore, HIF-1α reduces Staphylococcus aureus infections in mice and HIF-1α−/− cells, which reduce nitric oxide (NO) production, affect macrophage TNF-α production and bacterial killing. Besides, under hypoxia, the capacity of macrophages to phagocytose is HIF-1α-dependent. Although we found high expression of HIF-1α protein in both epithelial and leukocyte cells, in our study, we did not evaluate macrophage cells separately.

In angiogenesis event, it has been suggested that VEGF-A is a major player. VEGF-A induces vasodilation via endothelial NO production and its production is under control of HIF complex. Cell surface receptor tyrosine kinases for VEGF, constituting the VEGFR family, have been identified. VEGFR2 is known to be the most essential receptor in angiogenesis, mainly in regulation of capillary tube formation. Matrix metalloproteinases (MMPs) are secreted as zymogens and selectively degrade components of the extracellular matrix (ECM) and a variety of cells, including epithelial cells, fibroblasts, inflammatory cells, and endothelial cells can produce these endopeptidase. In particular, MMP9 protein acts downstream of Th2 cytokine signaling. We observed that only 11 patients presented MMP9 protein expression. However, MMP9 was increased in HIF-1α higher group. In our study, VEGF-A protein level correlated to MMP9. We demonstrated that VEGFR2 protein level was increased in HIF-1α higher group and correlated to MMP9 protein. These data support previous fin-

**Figure 2:** Mean and standard deviation of HIF-1α in leukocyte and epithelial cells.

**Figure 3:** Analysis of expression levels of VEGF-A, VEGFR2, and MMP9 in samples of leishmanial lesions. The VEGFR2 (a) and MMP9 (b) protein expression levels were significantly higher in HIF-1α higher group in epithelial cells (p=0.002 and p=0.009, respectively) (A). Considering leukocyte cells, we did not find any association between HIF-1α groups and protein expressions (B).
ings that show the expression of protein related to hypoxia can induce necessary elements to provide the establishment of angiogenesis in inflammation response.16,17,19-26. Taken together, HIF-1α protein could be involved in high expressions of VEGFR2 and MMP9 proteins. These findings together suggest that HIF-1α can enhance VEGFR2 and MMP9 proteins signals, helping the inflammatory cells response to specific sites in leishmanial lesions.

CONCLUSION

In conclusion, the data presented here indicate a deregulation in angiogenesis pathway and it is might be associated to increase of HIF-1α protein expression. HIF-1α higher group showed increase of VEGFR2 and MMP9 proteins. In epithelial cells, VEGF-A was correlated to MMP9 protein. Furthermore, considering leukocyte cells, VEGFR2 was negatively correlated to MMP9 protein levels. This pathway possibly prepares the cells for a higher activity in a hypoxic or an angiogenic microenvironment. The limitations of this study include lack of information about lesion clinical data as well as lack of information about the time of lesion evolution. In order to confirm our findings, in vitro and in vivo studies may clarify the mechanism underlying HIF-1α induction and activity in tegumentary leishmaniasis. □

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