Evaluation of myofibroblasts and its association with TGF-β and IFN-γ in lesions of patients with american tegumentary leishmaniasis *

Avaliação de miofibroblastos e sua associação com TGF-β e IFN-γ em lesões de pacientes com leishmaniose tegumentar americana

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Abstract: Background: Leishmaniasis is caused by protozoa of Leishmania spp. genus. It is transmitted by the bite of the sand fly insect. It is believed that 12 million people are infected with this disease and that its annual incidence is 2 million; this number is increasing.
Objectives: The present study aimed to evaluate the expression of myofibroblasts through alpha smooth muscle actin labeling, and to analyze their relationship with the expression of the cytokines Interferon gama (IFN-γ) and Transforming growth factor beta (TGF-β1) in lesions of American tegumentary leishmaniasis (ATL).
Methods: For this retrospective study, we gathered 28 patients diagnosed with ATL between 2002 and 2006. We verified α-SMA positivity and performed IFN-γ and TGF-β1 immunolabeling to identify the profile of these cytokines in both positive and negative cases for myofibroblasts, via immunohistochemistry, in order to assess the presence of myofibroblasts,.
Results: We observed that out of the 28 cases, 17 (60.71%) were positive for alpha smooth muscle actin, while 11 (39.29%) were negative, and IFN-γ was more expressed than TGF-β1 (p=0.007). The mean percentages of expression of IFN-γ and TGF-β1 in the group negative for alpha smooth muscle actin were different, with an increased expression of IFN-γ (p=0.047). However, in the group positive for alpha smooth muscle actin, there was no difference in cytokine labeling (p>0.05).
Conclusion: We verified the presence of positive α-SMA stromal cells in the majority of the cases of ATL, indicating the presence of myofibroblasts. In cases negative for alpha smooth muscle actin, an increased expression of IFN-γ compared to TGF-β1 was observed, revealing an inflammatory phase progressing to a healing process.
Keywords: Cytokines; Immunohistochemistry; Leishmaniasis; Leishmaniasis, cutaneous; Myofibroblasts

Resumo: Fundamentos: A leishmaniose é causada pelo protozoário do gênero Leishmania spp., sendo transmitida via picada do inseto flebotomíneo. Estima-se que 12 milhões de indivíduos estejam infectados com a doença, sendo a incidência anual de 2 milhões, número este que tende a aumentar.
Objetivos: Avaliar a expressão de miofibroblastos através da imunomarcação de actina de músculo liso alfa, e analisar sua relação com a expressão de citocinas IFN-γ e TGF-β1 nas lesões de pacientes com leishmaniose tegumentar americana.
Métodos: Trata-se de um estudo retrospectivo, em que foram avaliados 28 pacientes diagnosticados com leishmaniose tegumentar americana durante o período de 2002 a 2006. Na técnica de imuno-histoquímica avaliou-se a presença de miofibroblastos, através do marcador actina de músculo liso alfa, além da imunomarcação do IFN-γ e TGF-β1 para identificar o perfil dessas citocinas nos casos positivos e negativos para miofibroblastos.
Resultados: Observou-se que dos 28 casos, 17 (60,71%) foram positivos para actina de músculo liso alfa, enquanto 11 (39,29%) foram negativos. IFN-γ teve uma maior expressão do que TGF-β1 (p=0,007). A porcentagem média de expressão de IFN-γ e TGF-β1 no grupo negativo para actina de músculo liso alfa foi diferente, apresentando uma maior expressão de IFN-γ (p=0.047). Entretanto, o grupo positivo para actina de músculo liso alfa não apresentou uma diferença estatisticamente significativa (p>0,05).
Conclusão: Verificou-se uma expressão de actina de músculo liso alfa nos casos de leishmaniose tegumentar americana, indicando a presença de miofibroblastos. Nos casos negativos para actina de músculo liso alfa, observou-se uma maior expressão de IFN-γ comparando com TGF-β1, revelando que a fase inflamatória está envolvida no processo de cicatrização da lesão.
Palavras-chave: Citocinas; Imunoistoquímica; Leishmaniose; Leishmaniose cutânea; Miofibroblastos

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INTRODUCTION

Leishmaniasis is a disease caused by various species of protozoa of the genus *Leishmania*. Its transmission occurs as a result of the biting of female phlebotomine sand flies infected with the *Leishmania.* The clinical manifestations depend on the parasite species, the vector and the host's genetic and immunological constitution.[1,2] *Leishmania* is an intracellular and opportunistic parasite that affects all warmblooded animals including humans. According to the WHO, it is estimated that 12 million people are currently infected with leishmaniasis and two million new cases occur annually, of which 1.5 million are new cases of American tegumentary leishmaniasis (ATL).[3]

The overall framework of the histopathological changes of ATL lesions consists of an inflammatory infiltrate of mononuclear cells, neutrophils, eosinophils, lymphocytes, and plasma cells in the dermis.[4] In the epidermis, pseudoepitheliomatous hyperplasia can be found.[5]

The process of wound healing basically occurs over three phases that can occur in sequence or simultaneously. The inflammatory phase consists of the extravasation of plasma and cells, releasing platelets, fibroblasts, and cytokines, resulting in the destruction of the extracellular matrix. The proliferation phase is marked by angiogenesis and the deposition of extracellular matrix components such as collagen and granulation tissue, with the aim of repairing the damaged tissue. The remodeling phase occurs through the process of wound contraction, which facilitates interaction between the edges of the lesion. This phase is marked by the transdifferentiation of fibroblasts into myofibroblasts expressing alpha smooth muscle actin (α-SMA), promoting the interaction between actin and myosin, causing contraction.[6-8]

Myofibroblasts have an important role in tissue repair during the healing of the lesion. During the attachment of fibroblasts and their maturation phenotype to cells producing collagen, the process of wound contraction reaches its maximum efficiency. This is due to the change in phenotype of fibroblasts to myofibroblasts at the margins of the wound. Fibroblasts from these regions begin to show marginal functional characteristics similar to smooth muscle cells. Myofibroblasts are cells that are intermediate between smooth muscle and fibroblasts. Although their contractile mechanism is still to be elucidated, these cells are found in deposits aligned around the new extracellular matrix, making cell-to-cell unions and generating tensile strength. They also assist in the process of wound contraction by drying the wound's crust, which decreases in size during dehydration and drags the tissue acceded to it.[6,7]

In vitro studies showed the involvement of cytokines that modulate the transformation of fibroblasts into myofibroblasts; treatments with the transforming growth factor beta (TGFβ1) were found to be involved in the increased expression of α-SMA.[9-11] Despite this effect, the administration of interferon gama (IFN-γ) can inhibit the effect of TGFβ1, resulting in decreased α-SMA protein expression.[12-14] The present manuscript aimed to evaluate the expression of myofibroblasts through α-SMA labeling, and to analyze their relationship with the expression of the cytokines IFN-γ and TGF-β1 on ATL lesions in paraffin blocks.

MATERIALS AND METHODS

For this retrospective study, we gathered 28 patients diagnosed with American tegumentary leishmaniasis between 2002 and 2006. The selection was a convenience sample from lesions embedded in paraffin from cases with diagnosis of ATL from the Pathology service of the north of the state of Minas Gerais, Brazil. The diagnosis of ATL was confirmed by biopsy, direct parasitological examinations and/or Montenegro's skin test. All cases were of primary manifestations and visceral infections were discarded. These samples were collected from 28 patients whose age ranged from 6 to 90 years; 9 (32.1%) were female and 19 (67.9%) were male. Regarding skin color, 17 (58.7%) were non-Caucasians, 8 (28.6%) were Caucasians and in 3 (10.7%) it was unknown. The biopsies were performed in characteristic skin ulcers: 10 on the lower limbs, 11 on non-specified sites, 5 on the upper limbs, one on the face, and one on the trunk. All patients were treated with a pentavalent antimonial and they were completely and clinically healed. Ethical approval for this study was obtained from the local ethics committees (Unimontes, CEP 2494/2011).

Punch biopsy samples were taken from the skin lesions and fixed in formalin, embedded in paraffin, and serially sectioned (5 μm thickness). The sections were stained with hematoxylin and eosin (H&E) and evaluated under a conventional light microscope. The predominant histopathological pattern, obtained from the biopsies, was compounded by hyperkeratosis, acantosis, eosinophils, giant cells and eventual granulomas (Figure 1).

Immunohistochemical staining was performed using 3 mm sections of paraffin-embedded samples of all cases of ATL fixed in 10% buffered formalin. All reactions followed standard protocols. The sections were deparaffinized and submitted to 10% ammonia hydroxide in 95% ethanol for 10 min. After this, the slides were transferred to 1% H_2O_2 twice for 15 min each and incubated overnight with primary antibody α-SMA (1A4, Dako, Carpinteria, CA, USA), IFN-γ (H-

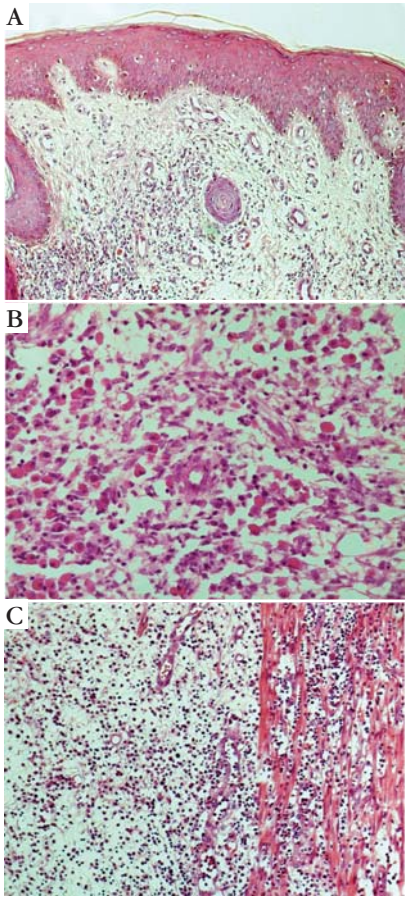

Figure 1: A. Epithelium showing the presence of hyperkeratosis, acanthosis and dyskeratosis (×200). **B.** Inflammatory phase with intense chronic inflammatory infiltrate in the connective tissue (×400). **C.** Proliferation phase is marked by angiogenesis and components such as collagen and granulation tissue (×200). Hematoxylin & Eosin stain

145, Santa Cruz Biotechnology, CA, USA), and TGF-β1 (DBS, Pleasanton, CA), all at the dilution factor of 1:100, followed by LSAB-HRP (LSAB-Kit Plus Peroxidase, Dako, California, USA). The reactions were carried out using 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma Sigma-Aldrich, St. Louis, USA) and were then counterstained with hematoxylin.[15] The positive controls for TGF-β and α-SMA immunostaining were placenta samples, whereas lymph nodes were used for IFN-γ. Negative controls for the reaction omitted the primary antibody staining but included the secondary antibody, DAB, and they were counterstained with hematoxylin.

The expression of myofibroblasts was evaluated via the positive detection of α-SMA, which was present or absent on fibroblastic and/or collagenous immunostaining. The immunohistochemical expression of TGF-β and IFN-γ was evaluated using an Olympus® BH2 microscope, ×400 magnification, using an ocular lattice with 100 points composed of 10 horizontal and 10 vertical test lines that were superimposed on the test field to be measured. Immunohistochemical analyses of the biomarkers were performed by obtaining the percentage of positively stained cytokines in all fields counted (10 fields for each specimen). Immunohistochemical expression data were expressed as mean ± standard deviation (mean ± sd) values.[15]

The data for the cytokine expression analysis and the presence or absence of immunostained α-SMA were analyzed by the Mann-Whitney test. All statistical analyses were performed using the statistical package SPSS® (SPSS Inc, Chicago, IL, USA), version 13.0 for Windows®; p values < 0.05 were considered significant.

RESULTS

In assessing the expression of myofibroblasts in the lesions of ATL, we observed that out of a total of 28 cases, 17 (60.71%) were positive for α-SMA, whereas 11 (39.29%) were negative (Figure 2A). All cases showed the labeling of internal muscles of blood vessels, but a positive evaluation was only considered for myofibroblasts marking the fibers and fibroblasts of the connective tissue (Figure 2B). These markings were found slightly deeper in the dermis, surrounded by an intense inflammatory process. We evaluated the phases which predominated in the lesions of ATL, and find that 12 (42.86%) were in the inflammatory phase and 16 (57.14%) in the proliferation phase (Figure 1B and Figure C).

The immunohistochemical expression patterns of IFN-γ and TGF-β are shown in figures 2C and figure 2D. In analyzing the mean percentage of the expression of positively immunostained cells, we noticed a significantly higher expression of IFN-γ compared to TGF-β ($p=0.007$) (Figure 3).

In order to evaluate the expression of cytokines on the regulation of transdifferentiation to myofibroblasts, the samples were divided into two groups: positive and negative for α-SMA. We observed that the α-SMA-negative group showed a greater expression of IFN-γ than TGF-β ($p=0.047$). However, we noticed no differences in the α-SMA-positive cases ($p>0.05$) (Figure 4).

DISCUSSION

Previous studies showed that several cytokines are involved in the healing of ATL lesions, where TGF-β has a decisive role, not only in scar remodeling, but also in all stages of wound healing.[7,8] TGF-β predominantly transmits the signals through cytoplasmic proteins called Smads, which are directly involved with the receptor complex of TGF-β, regulating the transcription and activating the cytokine.[8]

In an *in vivo* study with rats, the administration of an IFN-γ injection in the mucoperiosteal palate showed few cells expressing α-SMA, and a reduction in collagen type III was observed. There was also a more intense and prolonged inflammatory process, and a

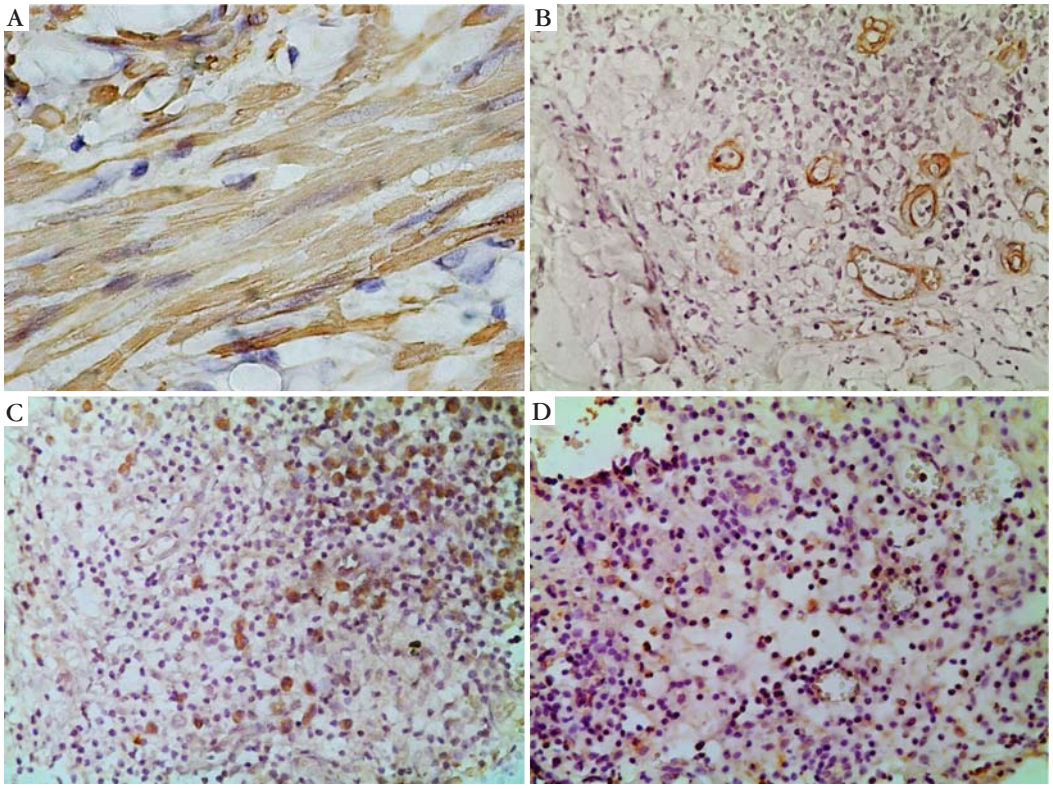

Figure 2: A. Positive α-SMA myofibroblasts on the dermis (×1000); **B.** Marking of the internal muscles of blood vessels. Expression of cytokines by immunohistochemistry; **C.** IFN-γ-positive cells; **D.** TGF-β1-positive cells (staining: DAB. Counterstained: Mayer's hematoxylin. Magnification: ×400)

delay in the formation of granulation tissue.[14] It is believed that myofibroblasts are responsible for skin contraction, and α-SMA positive myofibroblasts can cause this contraction. This study showed a significant increase in the expression of α-SMA after treatment with TGF-β1, *in vitro*. On the other hand, studies have shown that TGF-β can also induce the expression of SMA, suggesting that the stimulation of fibroblasts by TGF-β can induce their differentiation into myofibroblasts.[11]

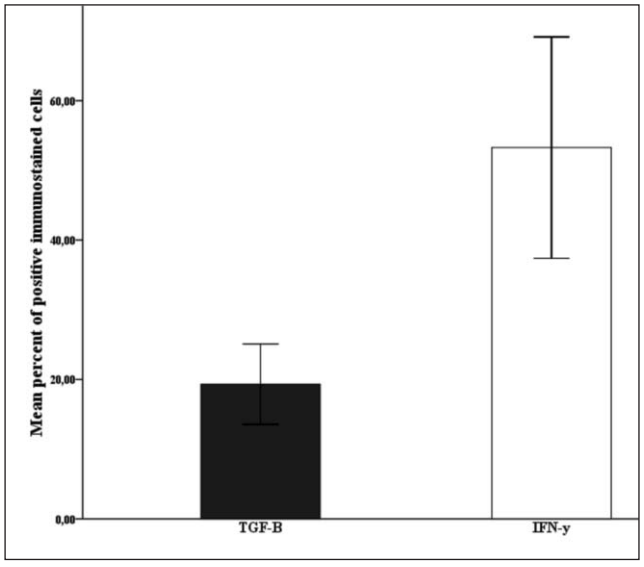

Figure 3: Analysis of the expression of cytokine-positive cells in all cases of ATL. There was a higher expression of IFN-γ compared to TGF-β1 (p=0.007)

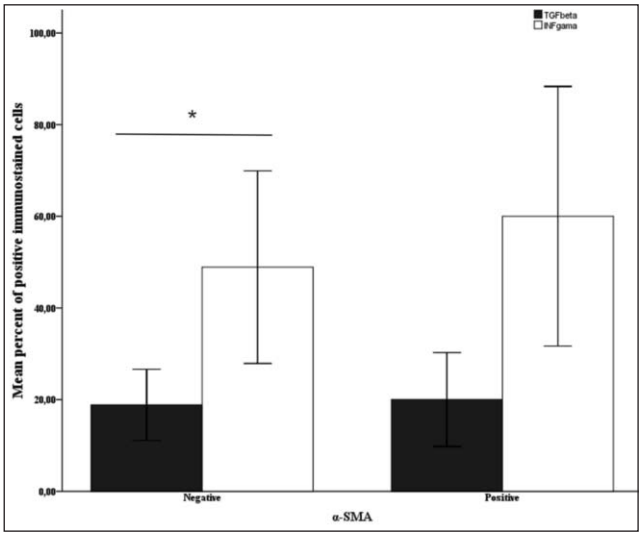

Figure 4: Analysis of immunostaining for the expression of cytokines in the α-SMA-negative group, showing a significant difference (p=0.047) between TGF-β1 and IFN-γ positivity. In the α-SMA-positive group, no significant difference (p>0.05) was found between these cytokines

In our study, we observed that the cases of ATL presented a higher level of expression of INF-γ than TGF-β1 in the α-SMA-negative group. When increasing concentrations of TGF-β1 were administered to cultured fibroblasts, a higher expression of α-SMA was observed; however, on the other hand, the addition of different levels of IFN-γ caused a decreased expression of α-SMA. IFN-γ blocks the TGF-β1-promoting changes in myofibroblasts, including α-SMA production.[9,10]

Interferon-γ can negatively modulate the wound healing process by suppressing the production and functional activity of TGF-β1. As TGF-β1 can inhibit IFN-γ production and the expression of its receptor, both cytokines can antagonize each other. Thus, the blockade of the IFN-γ signal transduction pathway can enhance TGF-β1 production and TGF-β1 signaling is a positive feedback factor and might be an important strategy for accelerating the healing process of skin wounds.[13] TGF-β1 also stimulates *in vivo* modulation of fibroblasts into α-SMA-expressing myofibroblasts. Other cytokines such as IL-1, PDGF, and TNF-α do not possess the capacity to induce α-SMA expression.[16] Wound healing myofibroblasts could be involved in neodermis formation and contraction, whereas fibroblasts could be involved in the stimulation of keratinocyte growth and neodermis formation.[17,18]

In conclusion, we found positive α-SMA stromal cells in ATL samples, indicating the presence of myofibroblasts in the majority of the cases (60.71%). Interferon-γ was detected more clearly in all cases than TGF-β1. In the α-SMA-negative cases, a higher expression of IFN-γ was observed compared to TGF-β1, revealing a delay in the healing process. ❑

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