Dual Role of Insulin-Like Growth Factor (IGF)-I in American Tegumentary Leishmaniasis

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1. Introduction

Leishmaniases are diseases caused by protozoan parasites of the genus Leishmania, endemic in around 88 countries. In Brazil, L. (Viannia) braziliensis is the main species for American tegumentary leishmaniasis (ATL), causing injuries ranging from benign cutaneous to disfiguring mucosal lesions [1]. After promastigote inoculation in the skin, these parasites interact primarily with innate host elements and growth factors present in this site. Once established the infection, the inflammatory infiltrate in the cutaneous leishmaniasis (CL) is characterized as chronic inflammation, with granuloma with or without necrosis, and the presence of macrophages, plasma cells, and lymphocytes [2]. The disease’s progression is not directly correlated to progressive parasite growth in the lesion site, with few parasites being detected mainly in chronic cases [3]. Instead, cell-mediated immunity has essential participation in CL pathogenesis. Chronic CL lesions are...
composed of an increased number of activated CD69⁺ T [4] and regulatory CD4⁺CD25⁺FOXP3⁺ IL-10-producing T cells [5, 6], granzyne A CD8⁺ cytotoxic T cells, or even proinflammatory CD4⁺ IFN-γ-producing T cells [6]. Further, in a recent transcriptomic study in skin samples of ATL patients, delayed or no cure was correlated to the higher expression of gene sets related to the cytolytic pathway [7]. These findings exemplify the complexity of CL immunopathogenesis.

In the skin, different innate elements and growth factors participate in the inoculation site of the parasite. Insulin-like growth factor (IGF)-I is one of them, and we have been studying its participation in Leishmania-vertebrate host interaction. It is a hormone that acts as an autocrine and/or paracrine element, being essential to maintain body homeostasis. It can be detected in the serum, but it has a widespread distribution in tissues [8]. Many cells, including macrophages, produce IGF-I [8–10]. Different IGF-I serum levels were associated with the pathogenesis of melanomas [11], HPV infection [12], and psoriasis [13]. In cutaneous tissues, IGF-I has a central role in wound healing [14]. In leishmaniasis, previous studies have demonstrated the effect of IGF-I in inducing in vitro proliferation of different species of Leishmania [15, 16]. In vivo, in mouse model of CL, an increase in lesion size and the number of viable parasites after infection with IGF-I preactivated promastigotes of L. amazonensis was observed [17]. IGF-I induces arginase activation, which in turn activates Leishmania promastigotes [18]. Moreover, infection with IGF-I-preactivated Leishmania leads to an increase in lesion size in mice, due to the expansion of the inflammatory infiltrate and parasite growth, suggesting that IGF-I may contribute to cell migration besides parasite proliferation [17]. However, in ATL patients with mucosal and disseminated forms, IGF-I serum levels were lower than in simple CL and healthy controls [19].

IGF-I can potentially interact with Leishmania parasites in the initial phase of infection. Nevertheless, it is still unknown whether IGF-I has any role in the later stages of infection and whether this growth factor may contribute to leishmaniasis’ pathogenesis. Therefore, we aimed to investigate whether IGF-I was present in the CL lesions and if this factor may influence the development of the lesion acting on parasite growth and/or on the inflammatory/healing process.

2. Subjects and Methods

2.1. Growth Curve of Leishmania spp. in the Presence of IGF-I. L. (V.) braziliensis (MHOM/BR/1975/M2903) promastigotes were maintained at 25°C, in Schneider’s insect medium (Gibco, Thermo Fisher Scientific Inc, MA, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco, USA) and 200 IU of penicillin per mL, and 200 μg of streptomycin (Sigma Chemical Company, St. Louis, Mo, USA) per mL, and grown until stationary phase before using in Leishmania growth curve analysis. The stationary phase promastigotes were distributed in triplicate into 24-well plates (5 x 10^⁵ parasites/well) in a final volume of 1.0 mL of Schneider’s insect medium (Gibco, USA) supplemented with 2% FCS and antibiotics, with or without 50 ng/mL recombinant human IGF-I (rIGF-I, R&D Systems, USA) and maintained during the experimental period.

2.2. Patients. The patient groups were composed of 37 men and 14 women, all of them living in endemic area of L. braziliensis transmission. The median age was 35 [18-57] years. The patients were classified according to the duration of illness at the moment of diagnosis as early group when the appearance of the lesions was less than 30 days before; intermediate, in the 30-60-day interval; late, more than 90 days. The patients were also grouped by having a good response (complete epithelization three months after the end of therapy) or poor response (no complete healing of lesions three months after the end of treatment or development of secondary lesions).

The following criteria were used for leishmaniases diagnosis: (i) type of lesion and epidemiological data compatible with ATL; (ii) positive delayed-type hypersensitivity reaction to leishmanial antigens; (iii) detection of serum anti-Leishmania antibodies; and (iv) detection of Leishmania parasites in lesion by microscopic examination of histological sections or by culture in NNN-modified medium. Patients were treated with pentavalent antimony (N-methyl-glucamine, Glucantime). All procedures were approved by the Ethical Committee of the Fundação Oswaldo Cruz, Ministério da Saúde, Rio de Janeiro, Brazil, and informed consent was obtained from each subject.

The skin fragment was obtained for diagnosis before treatment, and it was taken from the border of the cutaneous lesion. The fragment was divided into three parts: one was fixed for histopathology analysis, the second one was cryopreserved for immunohistochemistry analysis, and the last one was used for Leishmania DNA detection by PCR. Fragments of skin lesions were not taken sequentially after treatment for ethical reasons.

2.3. Immunohistochemistry. Skin fragments were frozen in OCT resin (Tissue Tek; Sakura Finetek, Torrance, CA, USA.), were cut into 3-4 μm thick sections and mounted on microscope slides (silanized slides; DakoCytomation, Carpinteria, CA, USA). To detect IGF-I expression, the slides were fixed in acetone : methanol : formalin (19 : 19 : 2) and rehydrated in Tris-Saline Buff (TBS) pH 7.6. The procedure was performed according to the Envision Double Staining kit (Dako, USA) manual. Briefly, endogenous enzymes were blocked with Endogenous Enzyme Block regent, and then, the slides were incubated with the first primary polyclonal rabbit anti-human IGF-I antibody (1 : 200; GroPep Limited–Adelaide, Australia) followed by incubation with the mouse and rabbit antibody conjugated to Polymer/HRP reagent. This first antibody was developed by DAB+ Chromogen. For the second part of immunostaining, beginning with the blocking step, the Doublestain Block reagent was used. The anti-Leishmania mouse serum was used as a second antibody and was added diluted 1:8000. This anti-Leishmania serum was obtained at two months of infection from BALB/c mice infected in the footpad with 10⁶ stationary phase L. amazonensis promastigotes [20]. Then, mouse and rabbit antibodies conjugated to Polymer/AP reagent were
2.3.1. Immunohistochemistry Analysis. Two independent observers analyzed the slides. All fragment area was analyzed, and the IGF-I and anti-\textit{Leishmania} staining were classified in only IGF-I detection, IGF-I and \textit{Leishmania} antigen coexpression, or only \textit{Leishmania} antigen detection. Twenty-five cases in which IGF-I was present were photographed. Five representative areas were photographed using Cool Snap-Pro Color (Media Cybernetics Inc, USA), and the photos were analyzed by Image-Pro Plus® Software (Media Cybernetics Inc, USA). The percentage of the positive area was measured for IGF-I and \textit{Leishmania} sp. antigen staining.

2.4. Statistical Analysis. Mann-Whitney test, Kruskal-Wallis test, and Spearman correlation were utilized (GraphPad Software; San Diego, CA, USA) using the software GraphPad Prism version 6. Results were considered statistically different when the $P$ value was $\leq 0.05$.

3. Results

3.1. \textit{Leishmania} (Viannia) braziliensis in vitro Growth upon IGF-I Stimulus. In a previous study, the effect of IGF-I on parasite growth in vitro \textit{L. braziliensis}-infected human monocytic cell line THP1 was inconclusive [19]; thus, we first investigated whether IGF-I can influence the \textit{L. braziliensis} growth in vitro. The addition of rIGF-I in promastigote cultures in physiological concentration increased the \textit{L. braziliensis} proliferation compared with controls without IGF-I (Figure 1).

3.2. IGF-I Was Present in Cutaneous Leishmaniasis Lesions. Next, we investigated the presence of IGF-I in fifty-one CL lesions by immunohistochemistry. IGF-I was present in 70.5% of cases ($n = 36$). Histologically, IGF-I was spread in the dermis and epidermal basal lamina (Figures 2(b) and 2(c)). In normal skin (control; $n = 3$), IGF-I was seen only in the basal lamina (Figure 2(a)).

Concomitantly with IGF-I immunostaining, we carried out the \textit{Leishmania} antigen detection. \textit{Leishmania} antigens were detected in 84.3% of cases ($n = 43$). Isolated \textit{Leishmania} antigen detection was found in 29.5% of cases ($n = 15$). \textit{Leishmania} antigens were observed in the extracellular matrix and cell foci in the dermis (Figure 2(d)). \textit{Leishmania} antigen detection decreased with chronicity: 92.8% of patients (13/14) in the early group; in 89.4% of patients (17/19) in the intermediate group; and in 72.2% of patients (13/18) in the late lesion group (Table 1). IGF-I and \textit{Leishmania} antigens were observed in the dermis of the same lesion area in 28 cases. However, IGF-I and \textit{Leishmania} antigens’ colocalization was not observed.

3.3. The Duration of Illness and Treatment Response in relation to the Area of Expression of IGF-I. To evaluate the relationship of the presence of IGF-I with clinical parameters, 36 IGF-I positive cases were grouped according to the duration of illness or treatment response. IGF-I seems to have a relationship to the chronicity of the lesions since the number of cases expressing IGF-I increases with the disease’s progression. The IGF-I was detected in 64.2% of patients (9/14) in the early lesion group, in 68.4% of patients (13/19) in the intermediate group, and in 77.7% of patients (14/18) in the late lesion group (Table 1). Moreover, percentage of positive area of IGF-I was also correlated with duration of illness (early lesion group: mean $= 1.52 \pm 1.12$, median $= 1.47\%$, $n = 7$; intermediate group: mean $= 2.32\% \pm 2.47$, median $= 1.5$, $n = 10$; late lesion group: mean $= 3.18\% \pm 1.9$, median $= 3.12$, $n = 8$; $r = 0.42$, $P = 0.023$) (Table 2).

To assess the relationship of IGF-I expression with treatment response ($n = 24$), we grouped patients in those having a good response (complete epithelization three months after the end of therapy) or having a poor response (no complete healing of lesions three months after the end of treatment or development of secondary lesions). IGF-I was detected in 75% of patients with good therapeutic response. Those with good response showed higher percentage of positive areas for IGF-I (good responder: mean $= 2.8\% \pm 2.1$, median $= 2.1\%$, $n = 18$) when compared with poor response (poor responder: mean: $1.3\% \pm 1.1$, median $= 1.05\%$, $n = 6$; $P = 0.03$) (Table 2).

4. Discussion

In this work, we initially showed the enhanced proliferation of \textit{L. braziliensis} promastigotes in the presence of IGF-I. Further, IGF-I was detected in human CL lesions, and for the first time, it was shown in lesions of an infectious cutaneous
disease. A limitation of this study was the unfeasibility to obtain sequential material from individual patients due to ethical issues, and further, we had patients who had a different time of development of the overt disease at the moment of diagnosis. However, we were able to analyze the histopathological alterations relating them to the duration of disease and also to response to treatment. Then, the percentage of patients presenting IGF-I detectable in the lesion was higher in chronic lesions. It was also higher among good responders for treatment.

IGF-I interacts with several *Leishmania* promastigote species inducing proliferation [15–17]. This effect did not
occur with IGF-II polypeptide, which shares 60% of similarity with IGF-I [15]. Here, we showed L. braziliensis promastigotes induced Leishmania antigens by immunohistochemistry. Leishmania antigens were detected mainly in recent lesions confirming previous observations [23]. In the mouse model, the infection with IGF-I reactivated L. amazonensis promastigotes induced larger lesions than non-pre-activated parasites [17]. The aggravation of mice CL lesions was due to an increase in the number of parasites along with intense cell migration. However, in the present study, we could not observe colocalization of IGF-I and Leishmania antigens in CL lesions suggesting another role for IGF-I in human ATL other than on parasite proliferation. In in vitro study, it was shown intrinsic IGF-I surrounding L. major within infected macrophages [24], but it could be due to the differences among Leishmania species and in this particular experimental conditions, very far from the lesion pathogenic process in the skin.

In this work, we analyzed the presence of IGF-I in CL lesions. It is the first data on the presence and influence of in situ IGF-I in human cutaneous infectious disease. In CL lesions, IGF-I was found spread throughout the dermis, basal lamina, and epidermis differing from normal skin where it was seen only in basal lamina. IGF-I’s capacity to continuously stimulate keratinocyte growth [9] may contribute to altering the tissue architecture and, at first glance, maybe connected to acanthosis often present in CL lesions [21]. In psoriasis, a cutaneous inflammatory disease, IGF-I contributes to lesion severity stimulating continuous keratinocyte growth [22]. Thus, we hypothesize that the presence of IGF-I in CL lesions may influence the disease outcome.

IGF-I presence in CL lesions with different distribution and a constitutive presence of IGF-I in normal skin leads us to ask whether this factor could act in different phases of Leishmania infection. To address this question, we have performed a double immunostain to IGF-I and Leishmania antigens to confirm previous observations [23]. In this particular experimental condition, we observed a different distribution of IGF-I and Leishmania antigens with IGF-I presence in CL lesions with different duration of illness and response to treatment.

Table 1: Insulin-like growth factor (IGF-I) or Leishmania antigen detection in lesions of cutaneous leishmaniasis patients according to the duration of illness.

<table>
<thead>
<tr>
<th>Groups</th>
<th>% of positive cases</th>
<th>Duration of illness</th>
<th>% detection (n/n_total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leishmanial antigens</td>
<td>84.3% (n = 43)</td>
<td>Early</td>
<td>92.8% (13/14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intermediate</td>
<td>89.4% (17/19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Late</td>
<td>72.2% (13/18)</td>
</tr>
<tr>
<td>IGF-I</td>
<td>70.5% (n = 36)</td>
<td>Early</td>
<td>64.2% (9/14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intermediate</td>
<td>68.4% (13/19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Late</td>
<td>77.7% (14/18)</td>
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</tbody>
</table>

Duration of illness: early = lesions with less than 30 days; intermediate = lesions with 30-60 days; late = lesions with more than 90 days.

Table 2: Area of expression of insulin-like growth factor (IGF-I) in the lesions of cutaneous leishmaniasis patients according to the duration of illness and response to treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>IGF-I-area %</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Duration of illness</td>
<td></td>
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</tr>
<tr>
<td>Early lesion (n = 7)</td>
<td>1.47</td>
<td>1.52 ± 1.12</td>
</tr>
<tr>
<td>Intermediate (n = 10)</td>
<td>1.50</td>
<td>2.32 ± 2.47</td>
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<tr>
<td></td>
<td></td>
<td>3.12 ± 1.90</td>
</tr>
<tr>
<td>Late lesion (n = 6)</td>
<td>3.12</td>
<td>3.18 ± 1.90</td>
</tr>
<tr>
<td>Treatment response</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good responder (n = 18)</td>
<td>2.10</td>
<td>2.80 ± 2.10</td>
</tr>
<tr>
<td>Poor response (n = 6)</td>
<td>1.05</td>
<td>1.30 ± 1.10</td>
</tr>
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</table>

**Spearman correlation, *Mann-Whitney test.**
action of IGF-I in wound repair in the reepithelialization [29]. Retarded wound healing in mice diabetic lesions was associated with a delay in IGF-I expression [30]. In humans, a lack of IGF-I expression in diabetes mellitus ulcer contributes to a retarded wound repair [31]. Besides, IGF-I treatment helped to restore the normal expression of both matrix metalloproteinase (MMP)-9 and tissue inhibitors of metalloproteinase (TIMP)-1 in diabetic rats with skin ulcers [32], indicating an important role of IGF-I in healing. Furthermore, in CL patients, MMP-2 was associated with a satisfactory response to antimonial therapy, in conjunction with moderate amounts of IFN-γ, IL-10, and TGF-β [33]. It is conceivable to argue that IGF-I present in CL lesions can act in this net of soluble factors comprising cytokines and hormones involved in the homeostatic process, thus influencing the healing process.

In the pathogenesis of lesion in cutaneous leishmaniasis, inflammatory cytokines [34] and cytotoxic mechanisms [7] are involved that are modulated during development to cure. In this context, the interplay between interferon-gamma (IFN-γ) and IGF-I may determine the outcome of the disease. IFN-γ decreases IGF-I expression through inhibition of transcription of IGF-I mRNA [35], and IFN-γ is a cytokine that is important for parasite control but implicated in the lesion pathogenesis. In patients with CL in Brazil, expression of IFN-γ tends to decrease during the time, mainly in subjects with good response to treatment [36]. Thus, we may speculate that it may result in higher expression of IGF-I in patients with chronic evolution and a good response to treatment.

Our results suggest that IGF-I can play a dual role in CL lesions. IGF-I was detected more in chronic ulcers where may act as an anti-inflammatory factor and in lesions of good responders to treatment where the contribution of this hormone on wound healing would prevail. IGF-I’s dual role in CL lesions could be explained by the complex pathogenesis of CL lesions, whereas in the same lesion, we can find areas of an intense inflammatory response and wound repair [34]. It is also observed regions with synthesis besides degradation of extracellular matrix granuloma, compatible with transient or reversible histopathological features occurring in CL lesions [37]. The interplay between IGF-I and other immune-inflammatory elements present in CL lesions may influence the disease outcome.

In the study of leishmaniasis’ immunity and pathogenesis, most approaches focus on the adaptive immune response that is undoubtedly relevant. However, the present data highlight the importance of searching nonspecific factors such as growth factors besides adaptive immune elements in the study of leishmaniasis’ pathogenesis.

Data Availability
The data used to support the findings of this study are included within the article.

Conflicts of Interest
There is no conflict of interest.

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