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Immunohistochemical evaluation of macrophage activity and its relationship with apoptotic cell death in the polar forms of leprosy

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ABSTRACT

The objective of the present study was to investigate the correlation between macrophage activity and apoptosis in the polar forms of leprosy because the immunopathological phenomena involved in these forms are still poorly understood. For this purpose, 29 skin biopsy samples obtained from patients with the polar forms of leprosy were analyzed. Macrophage activity and apoptosis were evaluated by immunohistochemistry using lysozyme, CD68, iNOS and caspase 3 as markers. The nonparametric Mann–Whitney test and Spearman's linear correlation test were used for statistical analysis. The results suggest that the apoptosis rate is under the direct influence of macrophage activity in lesions of patients with the tuberculoid form. In contrast, in lepromatous lesions other factors seem to induce programmed cell death, possibly TGF-β. Further studies are necessary to identify additional factors involved in the immunopathogenesis of leprosy.

1. Introduction

Leprosy, an infectious disease caused by the bacterium *Mycobacterium leprae*, is characterized by skin and neural lesions related to the death of Schwann cells and consequent demyelinization, in addition to alterations resulting from the action of the immune response. During the course of infection with *M. leprae*, the immune response pattern of the host determines the progression of the disease to one of the clinical forms (borderline, lepromatous and tuberculoid). The tuberculoid forms of the disease are characterized by expression of a Th1 cytokine profile, whereas expression of a Th2 cytokine profile is observed in the lepromatous forms. Various infectious agents are able to control the immune response through the induction of apoptosis of immune cells. Some studies have shown that *M. leprae* induces programmed cell death (apoptosis) of host cells, an event that plays an important role in the progression of the disease. Dysregulated apoptosis is involved in the physiopathology of various diseases such as autoimmune disorders, cancer and viral infections [1]. However, few studies have investigated the phenomenon in leprosy. During leprotic infection, cells apparently undergoing apoptosis can be identified in granulomas and lesions [2–4]. Similarly, apoptotic markers are detected in macrophages and T lymphocytes during infection [4]. In addition, patients with active leprosy present high rates of spontaneous apoptosis in isolated peripheral blood mononuclear cells, suggesting that the mycobacterium may facilitate apoptotic death. *In vitro* infection of monocyte-derived macrophages causes apoptosis accompanied by an elevated expression of pro-apoptotic TNF-α, Bax and Bak [5].

M. leprae can damage neurons by promoting their demyelinization; however, the role of apoptosis in this process is still unknown. Some studies have shown that the *M. leprae* can cause rapid demyelinization, apoptosis or an immune response in the tissue affected [6]. Another study has identified apoptosis of Schwann cells during the course of infection, with a 19-kD lipoprotein probably being involved in the triggering of programmed cell death of Schwann cells through the TLR-2 receptor [7].

M. leprae may cause apoptosis of T cells in an indirect manner. When infected, macrophages exhibit an elevated surface expression of FasL, a ligand inducing cell death. When Fas-expressing T lymphocytes bind to this ligand, it activates the extrinsic pathway of apoptosis, causing the death of the cell. Macrophages activated

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during infection with *M. leprae* are able to secrete a large amount of substances, such as inducible nitric oxide synthetase (iNOS), enzymes and TNF, which are potentially toxic to the cell and can induce apoptotic cell death [8]. These substances might be directly involved in the progression of deformities resulting from infection, with macrophage activity representing a factor associated with higher morbidity of the disease.

The objective of the present study was to investigate by immunohistochemistry the physiopathological aspects involved in the damage caused by macrophage activity and its implications in the occurrence of apoptosis in skin lesions of patients with the polar forms of leprosy.

2. Materials and methods

2.1. Characterization of the sample

Skin biopsies were obtained from 16 patients with the tuberculoid form (TT) and 13 patients with the lepromatous form (LL). The biopsies were collected after local anesthesia of the selected lesions with a No. 3 punch and embedded in paraffin. The material was cut into 5-μm thick sections with a microtome. The sections were stained with hematoxylin–eosin and Ziehl–Neelsen for morphological analysis and submitted to immunohistochemical staining using specific monoclonal antibodies against CD68 [9–11], iNOS [12,13] and lysozyme and against caspase 3 [14] for the evaluation of macrophage activity and apoptosis, respectively.

2.2. Immunohistochemistry

The streptavidin–biotin peroxidase (SABC) method [15] employing monoclonal antibodies were used for immunohistochemistry. Endogenous peroxidase was blocked by three incubations with 3% hydrogen peroxide for 10 min in a dark chamber. Next, the specimens were washed under running water and in distilled water for 5 min each and then stored in PBS, pH 7.4.

When necessary, antigen exposure was performed by incubation in a pressure cooker (Steam Cuisine 700 Hi Speed, T-FAL) with 10x concentrated target retrieval solution (Dako, code S1699) for 20 min after heating the buffer from 85 °C to 90 °C or by enzymatic digestion with 0.25% trypsin solution (Sigma Chemical Co., St. Louis, MO, USA; code T-8253). The slides were again washed under running water and in distilled water and PBS, pH 7.4, for an average of 5 min each. Nonspecific tissue proteins were blocked by incubation with 10% skim milk for 30 min at room temperature.

The specimens were then incubated with the primary antibodies diluted in 1% bovine albumin fraction V (Serva, code 11930) containing 0.1% sodium azide in PBS, pH 7.4, overnight at 4 °C. After two washes in PBS, pH 7.4, for 5 min each, the specimens were incubated with the secondary antibody directed against the primary antibody produced in goat for 30 min at 37 °C (goat anti-rabbit and goat anti-mouse immunoglobulin diluted 1:500 in PBS, pH 7.4, or rabbit anti-goat immunoglobulin diluted 1:500 in PBS, pH 7.4). The slides were again washed in PBS, pH 7.4, and incubated with the SABC complex diluted 1:500 in PBS, pH 7.4, for 30 min at 37 °C. After a new wash in PBS, pH 7.4, the reaction was developed with the chromogen solution consisting of 0.03% 3,3′-diaminobenzidine and 1.2 ml 3% hydrogen peroxide. The intensity of the color was monitored under a light microscope by comparison with positive controls included in each reaction. The specimens were then washed under running water for 10 min, counterstained with Harris hematoxylin for 20 s, again washed under running water, dehydrated in ethanol, cleared in xylene, and mounted in Permount resin.

2.3. Quantitative evaluation

The immunostained sections [15–17] were analyzed under a Nikon Eclipse 200 microscope. Positive immunostaining for the specific antibody was analyzed quantitatively in five fields of the lesion area at large magnification (400×) using a graded grid divided into 10×10 subdivisions and comprising an area of 0.0625 mm^2.

2.4. Statistical analysis

The results were stored in electronic spreadsheets using the Excel® program and analyzed with the BIOSTAT 4.0 program and are presented in the form of graphs. Differences in macrophage activity and apoptosis were determined using the nonparametric Mann–Whitney test. The correlation between these variables was analyzed using Spearman's linear correlation test. A level of significance of 0.05 was adopted.

3. Results

The patients, 16 with the tuberculoid form and 13 patients with the lepromatous form were from the Amazon region, State of Para, Brazil. The classification of the clinical presentation of the disease was made in accordance with the Ridley–Joplin. The histopathology from TT form lesion showing granulomatosus inflamatory infiltrate with giants cells Langhans-like. In the LL form, the lesion showing disperse granulomatosus inflammatory infiltrate, with multiples histiocytes in derma and sometimes Virchow cells. The immunohistochemistry showed to a more intense macrophage activity in TT forms of the infection and multiples apoptotic cells associated in LL forms. Overall comparison of the immunostaining pattern between the poles of the disease mostly showed no significant differences between groups, despite variations in the intensity of immunostaining. Mean macrophage activity as demonstrated by the immunoexpression of iNOS was 118.4 ± 69.79 cells/mm^2 in the TT group and 118.62 ± 49.56 cells/mm^2 in the LL group ($p = 0.496$). Mean CD68 positivity was 61.90 ± 28.83 cells/mm^2 in the TT group and 78.07 ± 69.99 cells/mm^2 in the LL group ($p = 0.949$). Similarly, evaluation of macrophage activity based on the immunoexpression of lysozyme showed no significant difference between the two groups (TT lesions: 68.16 ± 44.57 cells/mm^2 versus LL lesions: 73.95 ± 64.55 cells/mm^2, $p = 0.817$).

Analysis of apoptosis by immunostaining for caspase 3 revealed a mean of 0.37 ± 0.85 apoptosis/mm^2 in the TT group and of 5.83 ± 3.37 apoptosis/mm^2 in the LL group, with $p < 0.0001$ indicating a highly significant difference (Figs. 1–4).

Analysis of the correlation between the variables studied showed a negative linear correlation between CD68 and caspase 3 in the TT group ($r = -0.225$, $p = 0.0714$) (Fig. 5A). A significant positive linear correlation was observed between macrophage activity and apoptosis ($r = 0.3197$, $p = 0.0094$) when the immunoexpression of lysozyme was analyzed in relation to that of caspase 3, with the observation of an increase in the apoptosis rate with increasing macrophage activity especially in the cellular infiltrate of skin lesions of the TT group (Fig. 5B). Similarly, a positive linear correlation ($r = 0.226$) was observed between iNOS immunoexpression and apoptosis in the TT group, but the result was not statistically significant ($p = 0.095$) (Fig. 5C). No significant correlation with apoptosis was observed in any of the LL lesions when macrophage activity was evaluated by immunostaining for iNOS, CD68 or lysozyme (Fig. 5D, E and F).