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

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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.

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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 demyelination, 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 demyelination; however, the role of apoptosis in this process is still unknown. Some studies have shown that the M. leprae can cause rapid demyelination, 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
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 Zielh–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 mm².

2.4. Statistical analysis

The results were stored in electronic spreadsheets using the Excel® program and analyzed with the BIOMAT 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 of the TT form lesion showing granulomatous inflammatory infiltrate with giants cells Langhans-like. In the LL form, the lesion showing disperse granulomatous 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 immunoenexpression of iNOS was 118.4 ± 69.79 cells/mm² in the TT group and 118.62 ± 49.56 cells/mm² in the LL group (*p* = 0.496). Mean CD68 positivity was 61.90 ± 28.83 cells/mm² in the TT group and 78.07 ± 69.99 cells/mm² in the LL group (*p* = 0.949). Similarly, evaluation of macrophage activity based on the immunoenexpression of lysozyme showed no significant difference between the two groups (TT lesions: 68.16 ± 44.57 cells/mm² versus LL lesions: 73.95 ± 64.55 cells/mm², *p* = 0.817).

Analysis of apoptosis by immunostaining for caspase 3 revealed a mean of 0.37 ± 0.85 apoptosis/mm² in the TT group and of 5.83 ± 3.37 apoptosis/mm² 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 immunoenexpression 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 immunoenexpression 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).
4. Discussion

Leprosy is a chronic infectious disease whose clinical expression is strongly related to the immune response pattern of the host. Several studies have investigated the relationship of this immune response with the evolution of infection, systematically characterizing the phenotypic expression pattern of the cellular immune response and the expression of cytokines for each clinical form of the disease. Nevertheless, little is known about the function of macrophages and the role of apoptosis both as a mechanism of immune escape of the bacillus and as an induction factor of cell death of nerve endings in the disease. It is known that macrophages are the main cells involved in the formation of a granulomatous immune response and that their function is one of the key factors in the response to mycobacteria. On the other hand, different mechanisms are used by parasites for the control of the host immune response, with apoptosis playing an important role.

In the present study, quantitative analysis showed no significant difference in macrophage activity between the TT and LL groups. With respect to apoptosis, similar to other studies [2,4], we
observed differences in the occurrence of apoptosis between the TT and LL groups, with a clear predominance of apoptosis in patients with LL lesions. These results demonstrate that, despite the evident lack of significant quantitative differences between the groups studied, apoptosis seems to play an important role in the negative control of the immune response since it was observed in granulomas and in the group in which the progression to deformities is not as fast as in the TT group mainly because of the local immune response pattern. Similar results have been reported in previous studies. For example, apoptotic bodies scattered in lepromatous lesions [2] and an increased spontaneous apoptosis rate in peripheral blood mononuclear cells, especially CD8+ T lymphocytes and B lymphocytes, have been observed in patients with pauci- or multibacillary leprosy [18]. Another study showed that the addition of *M. leprae* to cultures promoted an increase in the expression of TNF-α and BAX and, at the same time, led to a dose-dependent increase in the rate of monocyte apoptosis in patients with the LL form [5]. Some investigators reported that in patients with multibacillary leprosy the expression of FasL on macrophages provides a privileged environment for maintenance of the bacillus...
Fig. 5. Linear correlation analysis between the variables studied. A) Negative correlation between CD68 and caspase 3 in the TT group ($r = -0.225$, $p = 0.0714$). B) Positive correlation between macrophage activity (lysozyme) and apoptosis (caspase 3) in the TT group ($r = -0.3197$, $p = 0.0094$). C) Positive linear correlation between iNOS immunoeexpression and apoptosis in the TT group ($r = 0.226$, $p = 0.095$). D) Correlation between CD68 and caspase 3 in the LL group ($r_s = -0.0509$, $p = 0.6542$). E) Correlation between macrophage activity (lysozyme) and apoptosis (caspase 3) in the TT group ($r_s = -0.0005$, $p = 0.9962$). F) Correlation between iNOS immunoeexpression and apoptosis in the TT group ($r = -0.226$, $p = 0.095$).
by inducing lymphocyte apoptosis during antigen presentation, thus favoring the persistence of infection [8].

The lack of differences in macrophage activity between the groups studied may reflect the poor sensitivity of the immuno-histochemical method in detecting peculiarities of each group, or, more likely, the fact that there is no difference in the intensity of macrophage activation but rather in the microbicidal activity of these cells. These data agree with the results of studies evaluating the importance of the NRAMP gene in patients with tuberculosis. This gene is responsible for the ability of macrophages to produce reactive oxygen and nitrogen radicals with microbicidal activity. In this respect, some investigators have reported that the production of nitrogen intermediates is reduced or absent in lesions of patients with LL and borderline-lepromatous leprosy [12,19,20]. This finding might be related to the presence of TGF-β in the inflammatory infiltrate of skin lesions. On the other hand, the increased expression of TGF-β in LL lesions may also explain the higher frequency of apoptosis in these lesions since this cytokine is also a strong inducer of apoptosis. This fact may explain, in part, the neural damage caused by the apoptotic death of Schwann cells observed in the polar form characterized by higher susceptibility [21,22]. However, other factors may influence the induction of apoptosis in the LL group and especially for the TT pole in which TGF-β is not expressed in large amounts.

Although the comparison of means between groups did not reveal significant differences, a positive linear correlation was observed between macrophage activity and apoptosis in TT lesions when lysozyme was used as a marker. In contrast, no correlation between the variables studied was observed in LL lesions. These results indicate a possible inter-relationship between macrophage activity and the phenomenon of apoptosis, a fact that may explain the occurrence of apoptosis in lesions of patients with the TT form. Finally, the observation of a low correlation between the variables analyzed indicates the importance of future studies evaluating in detail the influence of apoptosis as a control mechanism of the immune response and as an inducing factor of neural damage in leprosy. In addition, further studies are necessary to determine the functional aspects of these macrophages in each group of leprosy patients and their consequent relationship with disease progression.

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References


