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A Comparative Study of Apoptosis in Reticular and Erosive Oral Lichen Planus

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The oral lichen planus (OLP) is a chronic inflammatory disease, probably autoimmune, with different clinical forms. The most common types are the reticular and the erosive ones. Apoptosis participates in the destruction of basal keratinocytes, but its role in the perpetuation of the subepithelial lymphocytic infiltrates was not yet investigated. To evaluate the involvement of apoptosis in the epithelium and in subepithelial lymphocytic infiltrates, 15 samples of reticular and erosive OLP and 10 samples of healthy oral mucosa were collected and processed histologically. Apoptosis was quantified in the epithelium and in inflammatory cell infiltrates. TUNEL reaction was used to measure apoptosis in the infiltrates. Erosive OLP showed more intense epithelial apoptosis than reticular OLP and controls. In contrast, apoptosis in the inflammatory cell infiltrates was more frequent in reticular than in erosive OLP. Lymphocytes were the predominant cells within the inflammatory cell infiltrates and were more frequent in erosive OLP than in reticular type. These results suggest that different apoptotic levels are involved in the erosive/reticular switch in OLP, determining different clinical presentations. In conclusion, decreased apoptosis in inflammatory infiltrates may contribute to the persistence of T lymphocytes, worsening the attack to the epithelium in erosive OLP.

Key Words: oral lichen planus, apoptosis, inflammatory cell infiltrates, morphometry.

INTRODUCTION

Oral lichen planus (OLP) is a presumably autoimmune prevalent chronic disease that affects the tongue and oral mucosa with papule lesions or rashes (1). The pathogenesis of OLP remains unclear (2,3), but apoptosis has been reported in epidermal cells, indicating a role in epithelial destruction (4). Reticular pattern is the most frequent clinical presentation and appears in the form of a network of connections and overlapping white lines (5) combined with a few symptoms and reflecting a milder stage of the disease (6,7). Erosive/ulcerative OLP constitute the most destructive form and causes a great oral discomfort (5,7). The clinical differences between reticular and erosive forms appear to be a reflection of the biological variations found in these two types of OLP (6).

Although the cause of the OLP remains speculative, many findings are suggestive of a persistent immune disorder mediated by T lymphocytes (8). It is accepted that basal epithelial cells are targets for T lymphocytes (2,5). Although apoptosis is a mechanism in the destruction of the basal cell layer (9), its role in the subepithelial inflammatory cell infiltrates has not yet been investigated. There is also increasing evidence that deregulated apoptosis is a mechanism of immune evasion and that delayed apoptosis, resulting in prolonged inflammatory cell survival, is important in persistence of the inflammation in tissues (10).

The presence of intense lymphocytic infiltrates in OLP lesions is unquestionable. However, the mechanisms by which these cells remain in the focus

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of the lesion, whether by a permanent recruitment or by an escape of apoptosis, are not clear yet. Probably the two mechanisms may act simultaneously, but differently when the reticular and erosive types of OLP are compared. Since the pathogenesis of OLP is still not fully elucidated, the purpose of this study was to evaluate the involvement of apoptosis in lesions of reticular and erosive OLP, assessing this phenomenon in the epithelium and in the underlying inflammatory cell infiltrates.

MATERIAL AND METHODS

Tissue and Samples

Patients with OLP and controls were selected in the Clinics of Stomatology and Oral Surgery of the Pontifical Catholic University of Minas Gerais (PUC/MG). Biopsies were taken from all patients. Diagnosis in each case was established on the basis of clinical findings and confirmed by histopathological examination. Fifteen cases of each reticular and erosive OLP were included in this study, together with 10 samples of healthy mucosa obtained in surgical procedures for implant placement. Four-micrometer-thick paraffin embedded sections were stained with Shorr and the apoptotic cells were quantified morphometrically in the epithelium. TUNEL reaction was used to validate the morphological criteria employed for quantification of apoptosis in the epithelium and in the inflammatory cell infiltrates. Selected microscopic fields included those that had a higher inflammatory reaction. The project was approved by the institutional Ethics Committee of PUC-MG (CAAE 0038021300006). All participants or caregivers filled out a statement that the experiment was undertaken with their understanding and gave written consent.

In Situ Detection of DNA Fragmentation

TUNEL reaction (terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) was used for detection of *in situ* genome fragmentation and confirm apoptosis (11), using a commercial kit (TdT-FragEL DNA Fragmentation Detection Kit, Cat QIA33; Calbiochem, San Diego, CA, USA). Reactions were carried out as described by the manufacturer. Briefly, slides were incubated with 20 µg/mL of proteinase K (Cat # P5568; Sigma, St. Louis, MO, USA) and endogenous peroxidase was quenched three times with 3% H_2O_2 in methanol. Terminal deoxynucleotidyl transferase (TdT) and deoxynucleotides were applied and the slides were placed in a humid atmosphere at 37ºC for about 2-6 h. The reaction was stopped by a blocking buffer, and then the slides were treated with peroxidase streptavidin conjugate, placed in humid atmosphere at 37ºC for 30 min. Finally, they were washed and treated with diaminobenzidine and counterstained with light green or Harris hematoxylin.

Epithelial and Inflammatory Cell Infiltrate Apoptotic Indexes (AI) and Quantification of Lymphocytes

Apoptotic cells in the epithelium were identified using a morphological criterion (12): presence of spherical eosinophillic hyaline bodies, nuclear condensation sometimes surrounded by clear halo. Apoptotic cells in inflammatory infiltrates were identified using TUNEL reaction. Labeled cells were considered apoptotic and counted. Lymphocytes were identified through their typical morphological characteristics in TUNEL reaction when counterstained by methyl green. Apoptotic cells and lymphocytes were counted in a minimum of 20 fields at ×100 objective (13).

All morphometric procedures were performed using specific software (Kontron KS300; v.2.0; Kontron Elektronik, GmbH, München, Germany). The percentage of apoptotic cells (apoptotic index - AI) was the number of apoptotic cells divided by the total number of cells and multiplied by 100.

Statistical Analysis

Data were analyzed with GraphPad Prism 3.0 (GraphPad Inc., San Diego, CA, USA). The Kolmogorov-Smirnov test was used to check normal distribution and a Student's t-test to detect any differences between samples with Gaussian distribution. The correlations among epithelial apoptosis, infiltrate apoptosis and number of lymphocytes were evaluated using Pearson's correlation. A value of $p<0.05$ was considered significant. Results were presented as means ± standard error.

RESULTS

Apoptotic cells were found in basal and parabasal layers of epithelium in all Shorr-stained sections of OLP (Fig. 1A). These findings were confirmed by the TUNEL method (Fig. 1B). The AI of the epithelium in

the erosive group (57.27 ± 0.92) was higher than in the reticular group (26.85 ± 0.72), which was higher than in control group (4.87 ± 0.17), p<0.0001 (Fig. 2A).

Shrunken lymphocytes, with chromatin condensation and surrounded by a clear halo were observed in more numbers within the inflammatory infiltrate in the OLP reticular that on erosive (Fig. 3A and 3B). That morphology was suggestive of cells undergoing apoptosis, which was confirmed by TUNEL reaction (Fig. 3B). The AI of inflammatory infiltrates in the reticular type was higher (18.88 ± 0.81) than in erosive OLP (6.58 ± 0.39), with p<0.0001 (Fig. 2B). However, the number of lymphocytes in infiltrates was higher in erosive (73.24 ± 2.83) than in the reticular type (53.96 ± 1.68), with p<0.0001 (Fig. 2C).

On the other hand, correlations between apoptosis in the in the epithelium and in the inflammatory infiltrate (r= -0.6344; p<0.0001) (Fig. 4A) and between the number of lymphocytes and apoptosis in inflammatory infiltrate (r= -0.1833; p=0.0138) (Fig. 4B) were negative. Correlation between the number of lymphocytes and apoptosis in the epithelium (r=0.2167; p=0.0035) (Fig. 4C) was positive. All correlation results were statistically significant.

DISCUSSION

It has been suggested that basal and parabasal keratinocytes apoptosis in OLP are initiated by underlying inflammatory cells (6). The present study investigated apoptosis in reticular and erosive OLP. Here, erosive OLP presented a higher epithelial apoptosis than the reticular type and both clinical forms of OLP had a higher apoptotic index than the controls. These results confirmed that apoptosis plays a role in decreasing the thickness of epithelia, facilitating erosion and

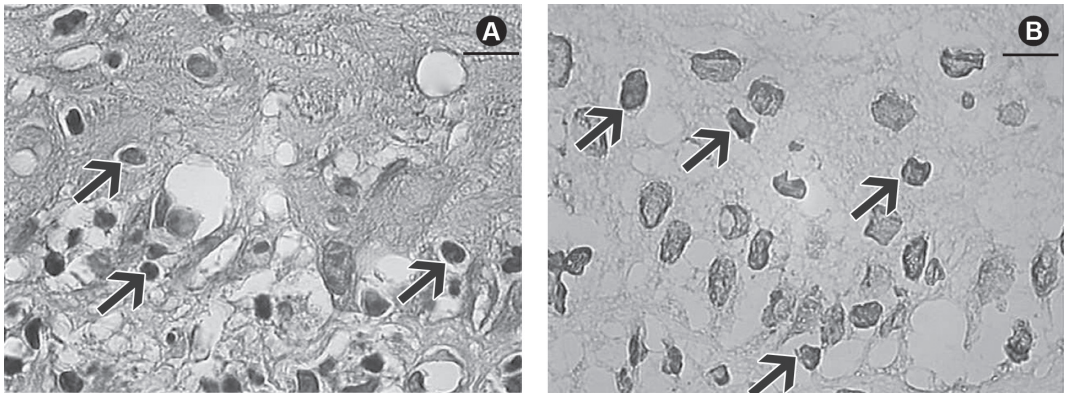

Figure 1. Photomicrographs of representative microscopical fields showing erosive oral lichen planus. A: Disorganization of the basal layer. Epithelial cells undergoing apoptosis, showing chromatin condensation and clear halo around (arrows). Subepithelial inflammatory infiltrate. Shorr (Bar=10 μm). B: Basal and suprabasal keratinocytes labeled with nuclear condensation and brownish clumps (arrows). TUNEL (Bar=10 μm).

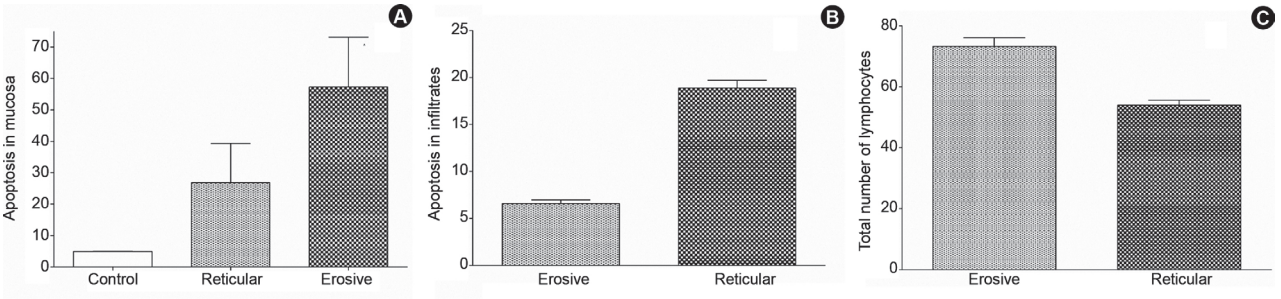

Figure 2. A: Graph of apoptotic indexes obtained in the epithelium of the control group and in erosive and reticular oral lichen planus lesions (OLPs). B: Graph of apoptotic indexes obtained in inflammatory infiltrates of erosive and reticular OLP. C: Graph of total lymphocytes in the inflammatory infiltrates in reticular and erosive OLPs.

ulceration, as previously reported (4). Other authors have described apoptosis in the epithelium of OLP (14,15), but evaluating different forms of lichen in a single group, without separating the clinical types.

Moreover, the studies on apoptosis in OLP have been concentrated especially on epithelium in basal and parabasal keratinocytes. Apoptosis in inflammatory infiltrate of OLP received less attention (14,16). However, apoptosis is involved in the resolution of inflammatory process and is responsible for the elimination of inflammatory cells when the process declines (17). In the present study, apoptosis within the inflammatory infiltrates in reticular OLP was higher than in the erosive form. These findings may explain the differences in clinical manifestations. A higher frequency of apoptosis in the reticular form means a higher elimination of inflammatory cells, decreasing the intensity of the reaction and of the symptoms. Other authors also evaluated apoptosis in inflammatory infiltrates in oral lichen (2,18,19), but considering all forms of OLP in a single group.

Apoptosis was more intense in the epithelium than in inflammatory infiltrates, according to the obtained apoptotic indexes. Neppelberg et al. (14) showed a similar result, with a high expression of complex FasR/FasL in both epithelium and inflammatory infiltrate in the OLP. It is possible that the signal produced by binding FasR/FasL to cell death via apoptosis is being blocked (14,16) by the activity of inflammatory cells, causing apoptosis escape by the expression of some anti-apoptotic molecules like Bcl-2, for example.

In contrast to the higher apoptosis within the inflammatory infiltrates in reticular OLP, the number of lymphocytes was higher in erosive than in reticular OLP, showing an evident negative correlation between the apoptotic and lymphocytic content of the infiltrates.

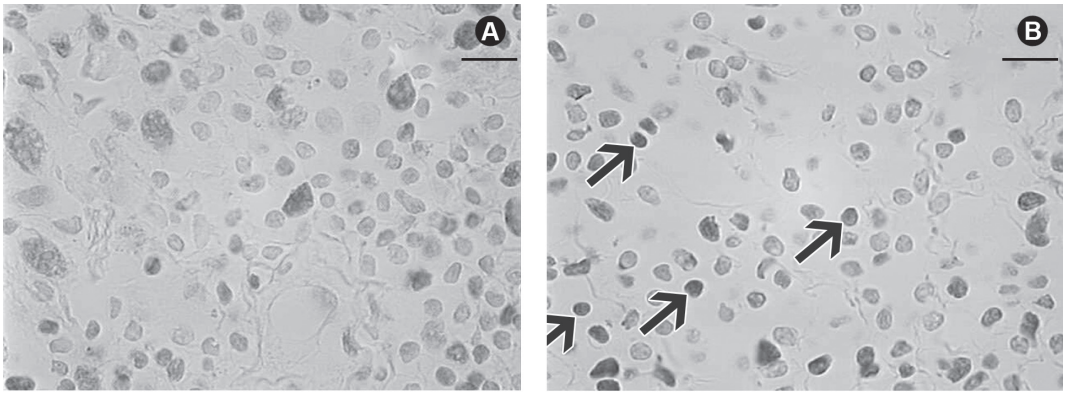

Figure 3. Photomicrographs of representative microscopical fields showing TUNEL reaction in inflammatory infiltrate of oral lichen planus (OLP). A: Erosive OLP. Intact lymphocytes. B: Reticular OLP. Apoptotic lymphocytes (arrows) TUNEL counterstained by methyl green (Bar=10 μm).

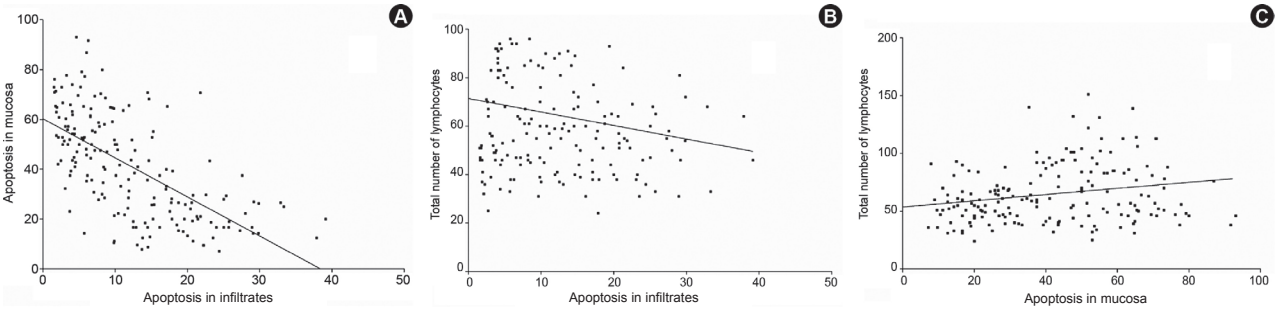

Figure 4. A: Negative correlation between the epithelial and inflammatory cell infiltrate apoptotic indexes. B: Negative correlation between infiltrates apoptotic index and number of lymphocytes in inflammatory infiltrates. C: Positive correlation between epithelial apoptotic index and number of lymphocytes in inflammatory infiltrates.

It is likely that the lower AI in erosive OLP contributes to persistence of lymphocytes in place, with persistent aggression to epithelium. Additionally, with the continuous inflammation, a permanent recruitment of cells is expected to occur.

A negative correlation was also found between apoptosis in inflammatory infiltrates and in the epithelium. More apoptosis within the infiltrates means less inflammation, which means less aggression to epithelial cells, less epithelial apoptosis and minor symptoms. Furthermore, the only positive correlation - between the number of lymphocytes in inflammatory infiltrates and apoptosis in the epithelium - shows a clear cause-effect association, which seems to confirm that lymphocytes within the infiltrates are responsible for the epithelial lesions in OLP.

In this study, apoptosis was quantified considering not only morphological criteria (12,17) but also TUNEL labeling of genomic fragmented cells (11). Labeled cells in TUNEL slides occurred mostly in the same regions as in sections stained with Shorr. Also, most of the labeled cells showed morphological characteristics of apoptosis. Few cells with morphology of apoptosis were not labeled as described elsewhere (20). TUNEL reaction is a technique that allows *in situ* detection of DNA fragmentation, which occurs in apoptosis (11,21) but also in necrosis (22). Despite this fact, it is a well-accepted laboratorial approach to apoptosis (23). Apoptotic cells show cellular and nuclear shrinkage, chromatin condensation, cellular and nuclear fragmentation and these morphological features are consistent enough to define apoptotic cell death in histological sections (24,25).

Taken together, the obtained results show for the first time some relationship between apoptosis in epithelia, subjacent inflammatory activity, apoptosis within inflammatory infiltrates, all evaluated in both forms of clinically distinct OLP. Other studies considering the different clinical forms are required for a better understanding of the pathogenesis of this disease. In conclusion, the results of this study suggest an important role for apoptosis in the control of inflammatory infiltrate in OLP lesions, contributing to the persistence of lymphocytes in the attack to epithelium.

RESUMO

O líquen plano oral (LPO) é uma doença crônica inflamatória, provavelmente auto-imune, com diferentes formas clínicas. Os tipos mais comuns são o reticular e o erosivo. A apoptose participa da destruição dos ceratinócitos basais, no entanto o seu papel na perpetuação do infiltrado linfocitário subepitelial ainda não foi investigado. Para avaliar o envolvimento da apoptose no epitélio e no infiltrado linfocitário subepitelial, quinze amostras de LPO reticular, quinze de LPO erosivo e dez amostras de mucosa oral saudável foram coletadas e processadas histologicamente. A apoptose foi quantificada no epitélio e nas células do infiltrado inflamatório. A reação de TUNEL foi usada para mensurar a apoptose no infiltrado. A intensidade da apoptose no epitélio mostrou ser maior no LPO erosivo que no LPO reticular e estes foram maiores que no controle. Em contraste, a apoptose nas células do infiltrado inflamatório foi mais freqüente no LPO reticular que no LPO erosivo. Os linfócitos foram as células predominantes dentro do infiltrado inflamatório e foram mais freqüentes no tipo erosivo de LPO que no tipo reticular. Estes resultados sugerem que diferentes níveis de apoptose estão envolvidos no tipo erosivo e reticular de LPO, determinando as diferenças nas apresentações clínicas. Em conclusão, a diminuição da apoptose no infiltrado inflamatório pode contribuir para a persistência dos linfócitos T, piorando o ataque ao epitélio no LPO erosivo.

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