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Identification of AgNORs and cytopathological changes in oral lichen planus lesions

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ABSTRACT

Objective: To evaluate cytopathological changes in epithelial cells of the oral mucosa of patients with oral lichen planus (OLP) compared with patients without OLP.
Subjects and methods: Swabs were collected from the oral mucosa of 20 patients with OLP (case group) and 20 patients without OLP (control group) using liquid-based cytology. After Papanicolaou staining, the smears were characterized based on Papanicolaou classification and degree of maturation. Nuclear area (NA) measurements, cytoplasmic area (CA) measurements, and the NA/CA ratio were determined from 50 epithelial cells per slide. For quantification of argyrophilic nucleolar organizer regions (AgNORs), the smears were stained with silver nitrate, and the number of AgNORs was counted in 100 cells.
Results: In both groups, there was a predominance of Papanicolaou Class I nucleated cells in the superficial layer. The average values of NA ($p > 0.05$) and CA ($p = 0.000$) were greater in the case group (NA = 521.6, CA = 22,750.3) compared with the control group (NA = 518.9, CA = 18,348.0). The NA/CA ratio was 0.025 for the case group and 0.031 for the control group ($p = 0.004$). There was no significant difference between the mean AgNORs values of both groups ($p > 0.05$).
Conclusion: The oral mucosa of patients with OLP exhibited significant cytomorphometric changes. However, there was no evidence of malignancy.

1. Introduction

Lichen planus (LP) is a chronic, inflammatory, mucocutaneous disease that belongs to the group of autoimmune diseases mediated by T cells (Scully and Carrozzo, 2008; Roopashree et al., 2010). LP affects 0.5–2.0% of the general population; however, the actual prevalence of oral lichen planus (OLP) is unknown (Schlosser, 2010). It is a disease of the middle age that predominantly affects females as compared to males, at a ratio of 3:1 (Scully et al., 2000; Eisen, 2002; Bermejo-Fenoll et al., 2009).

Clinically, 2 forms of LP most frequently affect the oral mucosa: reticular and erosive (Bermejo-Fenoll et al., 2009; Neville et al., 2009). Although clinical features may be sufficient for the diagnosis of OLP, a biopsy is necessary for diagnostic confirmation and to exclude the possibility of cellular atypia and signs of malignancy (Eisen, 2002; Sousa and Rosa, 2008).

Although the World Health Organization considers OLP to be a condition with malignant potential, this statement is the subject of much controversy (Cortés-Ramírez et al., 2009; Ben Slama, 2010). This issue is explained by the study of Gonzalez-Moles et al. (2008), which revealed (via a systematic review) a wide variation in the rate of malignant transformation (0 to 12.5%) in patients with OLP. Some authors believe that there are insufficient data to prove this association and that the interference of external risk factors may compromise the results of the studies (Bermejo-Fenoll et al., 2009; Laeijendecker et al., 2005). However, other authors state that patients with OLP have an increased risk of developing oral cancer (Ingafou et al., 2006; Liu et al., 2010).

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Exfoliative cytology is an excellent method of studying oral epithelial lesions, particularly those lesions with malignant potential (Cowpe et al., 1988; Ramaesh et al., 1998). This method predominantly uses suprabasal desquamated mucosal cells, which are stained and then analyzed based on morphological and morphometric criteria (Macluskey and Ogden, 2000; Alberti et al., 2003; Lima et al., 2007; Woyceichoski et al., 2008). Liquid-based cytology has been developed and poses a number of advantages over conventional cytology, including the following: better exposure of epithelial cells, and fewer overlapping cells (Digene do Brasil, 2002).

Papanicolaou staining allows for the observation of changes in epithelial cells based on the affinity of the dye for cytoplasmic constituents (Alberti et al., 2003; Williams et al., 1999). Using this stain, it is possible to observe changes that are indicative of malignancy, such as increased keratinization; alterations in the nuclear area (NA) and cytoplasmic area (CA); and changes in the NA/CA ratio (Cowpe et al., 1988; Ramaesh et al., 1998; Sugerman and Savage, 1996; McKee, 1997; Hande and Chaudhary, 2010).

Nucleolar organizer regions (NORs) are segments of DNA that aggregate in the nuclei, transcribe rRNA, and are visible within the nucleolus during interphase (Ruschoff et al., 1989). The NORs contain argyrophilia, as evident by their association with acidic, non-histone proteins that are selectively stained by the silver impregnation technique (Elengovan et al., 2008). When this technique is used, the argyrophilic nucleolar organizer regions (AgNORs) are visualized as intranuclear dark spots, which can then be quantified. The number of AgNORs per nucleus has been determined to correlate with cellular proliferation and with the degree of differentiation of various tumors, and thus, this marker is crucial to the study of the clinical course and aggressiveness of tumors (Caldeira et al., 2011).

Given the suspected malignant potential of OLP, periodic monitoring of the lesions is suggested; generally, these lesions are not static and exhibit periods of exacerbation and remission (Eisen, 2002; Neville et al., 2009). Furthermore, it is necessary to perform more studies for evidence that OLP can become malignant. To this end, the objective of the present study was to evaluate the cytopathological changes in epithelial cells of the oral mucosae of patients with OLP compared to patients without OLP.

1.1. Subjects and methods

The experimental protocol of this study was approved (no. 3824/10) by the Research Ethics Committee of Pontifícia Universidade Católica do Paraná (PUCPR). The patients were informed about the research objectives and signed a consent form after agreeing to participate. The present study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

1.2. Sample

For the selection of the case group, all female patients with histopathologic diagnosis of OLP in the registries of the Laboratory of Histopathology from PUCPR and Universidade Federal do Paraná (UFPR) were contacted. The purpose of the contact was to recruit the patients to perform a follow-up, although periodic monitoring of the lesions was conducted. Patients who attended the reevaluation were invited to participate in the study. Those who agreed to participate were included in the case group. The patients were contacted between January 2011 and March 2011, and the clinical reevaluations were performed between April 2011 and July 2011.

The case group consisted of 20 women who had histologically confirmed OLP and no other lesions in their oral mucosae. The following diagnostic criteria were established for the OLP case group: 1) Clinical features included whitish lesions in the form of interlacing lines (reticular) or plaques, possibly associated with eroded areas; the presence in more than 1 region of the oral mucosa, with periods of remission and exacerbation; no history of medication that could cause the exacerbation; and the absence of amalgam restorations in the area of the lesion, to exclude the possibility of a lichenoid reaction. 2) Histological features included jagged epithelial crests, which were arranged in a “saw-tooth” pattern; degeneration of the basal layer of the epithelium; the presence of Civatte bodies (degenerating keratinocytes); and an intense subepithelial inflammatory infiltrate band, which was predominantly composed of T lymphocytes (Neville et al., 2009; Schlosser, 2010). None of the 20 OLP cases showed evidence of dysplasia.

The control group selection was based on the following criteria: matched for age with case group, and absence of lesions in the oral mucosa. Female patients who attended the Dental Clinic at PUCPR and UFPR during the period of clinical reevaluation of case group (from April 2011 until July 2011), who showed the above mentioned criteria, were invited to participate. Those who agreed to participate were included in the control group. The control group consisted of 20 female patients.

Individuals who matched the following criteria were excluded from both groups: smokers; drinkers; and users of orthodontic appliances, illegal drugs, or alcohol-containing mouthwashes.

1.3. Cell collection

First, the patients were instructed to rinse their oral cavities with water to remove possible debris and bacteria that adhered to the mucosa. Cell collection was then performed using the liquid-based cytology technique, with a brush from the DNA-CITOLIQ® system kit (Digene, São Paulo/SP, Brazil) that was applied smoothly with rotating movements onto the oral mucosa of each patient. For patients in the case group, the reticular or plaque areas were chosen as collection sites, whereas the areas of erosion were avoided. After collecting the cells, the brush was dipped into a flask containing UCM (Universal Collection Medium) and remained immersed in the liquid while the bottle was capped and then refrigerated (2–8 °C) until laboratory processing (Woyceichoski et al., 2008; Digene do Brasil, 2002; Wandeur et al., 2011).

1.4. Laboratory processing

Initially, the contents of the flasks were homogenized for 30 s with the aid of a vortex (Model AP 56, Phoenix, USA). A 200 μL aliquot of each sample was filtered through a Filtrogene filter (Digene). The Prepgene metal press (Digene) was closed for 10 s, allowing the filtered material to be transferred to Lamigene glass slides (Digene). The smears were fixed in absolute ethyl alcohol solution for 20 min, and then, 2 slides were prepared. Papanicolaou staining (Lima et al., 2007; Woyceichoski et al., 2008; Wandeur et al., 2011) was used on 1 slide, and AgNOR staining was used on the other slide. The AgNOR staining method has been described by Ploton et al. (1986). The staining solution consisted of 2 parts of 50% silver nitrate ($AgNO_3$) and 1 part of 2% gelatin dissolved in 1% formic acid. The slides were then washed in deionized water and mounted in Canada balsam.

Prior to analysis, the identification numbers on the slides were covered to avoid bias. The examiner had been previously trained to recognize the presence or absence of cellular changes, identify the maturation criteria, quantify the CAs and NAs of the epithelial cells, and count the number of AgNORs.

1.5. Cytomorphological analysis

The slides that were stained using the Papanicolaou technique were analyzed by light microscopy using a binocular microscope

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