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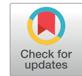
Mature dendritic cell density is affected by smoking habit, lesion size, and epithelial dysplasia in oral leukoplakia samples



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ABSTRACT

Objective: To compare the densities of CD1a + immature and CD83+ mature dendritic cells, and inflammatory infiltrate cells between smokers and non-smokers with oral leukoplakia. Parameters associated with malignant transformation were also evaluated.
Design: 21 smokers and 23 non-smokers diagnosed with oral leukoplakia were obtained. Densities of inflammatory infiltrate cells were calculated in H&E sections. Immunohistochemistry using anti-CD1a and anti-CD83 was performed and densities were calculated. Comparisons and statistical analyses were performed among the groups and parameters as gender, lesion size, site, and presence of cell dysplasia were analyzed.
Results: A lower density of CD83+ cells was observed in smokers compared to non-smokers ($P < 0.05$). For samples of smokers, a lower density of CD1a + cells, CD83+ cells, and inflammatory infiltrate cells was observed in samples with < 10 mm compared to samples ≥10 mm of diameter ($P < 0.05$), and a lower density of CD83+ cells was also observed between samples without dysplasia compared to samples with dysplasia ($P < 0.05$).
Conclusion: In oral leukoplakia samples, dendritic cell density decreases in the presence of smoking habit, and increases in larger lesions and with epithelial dysplasia. Smoking habit is an external factor that contribute to alteration of the anti-tumoral immune defense system in lesions of oral leucoplakia, reinforcing that smoking elimination is important to control the development of this disease.

1. Introduction

Oral leukoplakia (OL) is a common condition in the oral mucosa, with malignant transformation that ranges from 3% to 17% (Napier & Speight, 2008). For Van der Waal (2014), there are no reliable clinicopathological or molecular predicting factors of malignant transformation. Epidemiological studies demonstrate that smoking habit is the most common etiologic factor of OL (Holmstrup, Vedtofte, Reibel, & Stoltze, 2006; Pereira et al., 2011; Lima, Pinto, Sousa, & Correa, 2012; Van der Waal, 2014; Gheno et al., 2015; Warnakulasuriya & Ariyawardana, 2016). However, smoking habit is not considered a significant associated factor with malignant transformation, such as gender, lesion size, localization, etiology (idiopathic or tobacco-associated), presence, and severity of cell dysplasia (Holmstrup et al., 2006; Van der Waal, 2014; Pereira et al., 2011; Warnakulasuriya & Ariyawardana, 2016).

Studies have related the importance of the immune system in the prognosis of cancer (DeNardo et al., 2011; Nordfors et al., 2013). In an early phase of cell dysplasia or early tumor cell formation the immune system has the capacity to eliminate cells with DNA damage that may result in cancer (Zitvogel, Tesniere, & Kroemer, 2006). Dendritic cells (DCs) are important antigen-presenting cells of the immune system that capture antigens, migrate to regional lymph nodes, and evoke a T cell response (Banchereau et al., 2000). The relation between DCs and the prognosis of individuals with cancer has also been published (La Rocca et al., 2004; Tsuzuki et al., 2006; Esteban et al., 2012). It was observed that CD1a + DCs and T cells increase in tissue with epithelial dysplasia

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and dramatically increase in OSCC (Öhman, Magnusson, Telemo, Jontell, & Hasseus, 2012). Activation of T-cells demands interaction with DCs, and it was observed that presence of CD3-positive T-cells in oral premalignant leukoplakia indicates prevention of cancer transformation (Öhman et al., 2015). However, the role of DCs in the pathogenesis of both OSCC and OL remains unclear (Öhman et al., 2012).

Change in DCs quantity was proven in samples of pulmonary tissue in exposure to smoke (Robbins et al., 2004; Tsoumakidou et al., 2007). In thoracic lymph nodes of mice, cigarette smoke exposure reduces T-cell proliferation and DC maturation (Robbins, Franco, Mouded, Cernadas, & Shapiro, 2008). In prior studies, the present research group demonstrated that smoking is related to a decrease in CD1a + immature and mature CD83+ DCs densities in chronic gingivitis (Souto, Segundo, Costa, Aguiar, & Mesquita, 2011) or periodontitis (Souto, Queiroz-Junior, Costa, & Mesquita, 2014). In addition, Narwal and Saxena (2011) demonstrated that smokers diagnosed with OL and OSCC presented a decrease in CD1a + DCs in comparison with the normal mucosa of non-smokers. However, smokers diagnosed with OL were not compared with non-smokers with OL (Narwal & Saxena, 2011). Research evaluating DCs in tissues with epithelial dysplasia or OSCC did not consider the effect of smoking habit on DCs (Öhman et al., 2012). Our hypothesis was that the density of CD1a + immature and CD83+ mature DCs could be affected by smoking habit in samples of OL with or without epithelial cells dysplasia. Thus, the aim of this study was to compare the densities of CD1a + immature DCs, CD83+ mature DCs, and inflammatory infiltrate cells between samples of individuals diagnosed with OL and smokers (OLS); and OL and non-smokers (OLNS). Also, dendritic cell densities were evaluated in samples of OL considering clinical and histopathological characteristics.

2. Materials and methods

2.1. Ethical approval

This study was approved according to Brazilian law and the Helsinki Statement by the Ethics Committee in Research at the Universidade Federal Minas Gerais (UFMG), from Brazil, under protocol number 423/11.

2.2. Patients and samples

Paraffin-embedded biopsies from tissues previously collected, with a clinical diagnosis of OL and histopathological diagnosis of hyperkeratosis with or without epithelial dysplasia, were retrospectively retrieved from the files of the Oral Pathology Services at UFMG. Forty-four cases of OL, 21 OLS, and 23 OLNS were included in the study. Smokers were defined as those patients who smoked over 10 cigarettes per day over a 10-year period, and non-smokers were defined as those patients who never smoked (Cançado, Yurgel, & Filho, 2001).

Demographic and clinical data regarding gender, age, smoking habit, site, and lesion size in diameter were collected from patient records. Lesions located on the floor of the mouth, tongue, and palate were considered high-risk of malignant transformation. Other sites were classified as low-risk lesions (Waldron & Shafer, 1975). In respect to lesion size (diameter) (Holmstrup et al., 2006; Warnakulasuriya & Ariyawardana, 2016), the samples were grouped into percentiles and categorized according to median in two groups: samples of < 10 mm and ≥ 10 mm in diameter.

The histopathological diagnosis was confirmed by two independent oral pathologists by reviewing the sections stained with hematoxylin and eosin (H&E) retrieved from the files. All samples were graded according to the criteria of histopathological grading of leukoplakia defined by the World Health Organization (WHO) (2005) (Barnes, Everson, Reichart, & Sidransky, 2005). After, the samples were dichotomized in OL without (Fig. 1A and B) or with dysplasia (Fig. 1C and D) (Öhman et al., 2012).

2.3. Inflammatory assessment

The inflammatory infiltrate cells (IIC) were measured using H&E stained sections and the cell density (positive cell number/mm^2) was calculated on the lamina propria (LP) by a blinded examiner (GRS). The slices were digitized using a microscope (Axio Scoup A1, Zeiss, Göttingen, Germany) interfaced to a computer, at 200× magnification. The measure of inflammatory infiltrate density was determined using Image Tool software (version 3.0, University of Texas Health Science Center, San Antonio, TX, USA) (Souto et al., 2014).

2.4. Immunohistochemistry

Streptavidin-biotin protocol was used for immunohistochemistry reaction. Anti-CD1a is considered a marker for immature DCs (Caux et al., 1996) and anti-CD83 for mature DCs (Lechmann, Berchtold, Hauber, & Steinkasserer, 2002). The serial sections of 3 μm in thickness of paraffin-embedded tissues were performed. The serial sections were deparaffinized, dehydrated, and treated with anti-CD1a and anti-CD83. Antigen retrieval was carried out using a Dewaxing & Antigen Retrieval Buffer, pH 9.0 (Spring Bioscience, Pleasanton, CA, USA) for 12 min at 98 °C. The samples were incubated in two baths of 0.3% hydrogen peroxidase for 15 min each for block out endogenous peroxidase activity. The specimens were incubated with anti-CD1a at a dilution of 1:20 (clone MTB1, BioSB, Santa Barbara, CA, USA); and with anti-CD83 at a dilution of 1:100 (clone 1H4b, Abcam, Cambridge, UK). Anti-CD1a and anti-CD83 were incubated at room temperature for 1 h. Detection was performed using the Advanced HRP (Dako, Carpinteria, CA, USA) for anti-CD1a, and the Reveal System (Spring Bioscience, Pleasanton, CA, USA) for anti-CD83. The slides were subsequently exposed to 3,3-diaminobenzidine tetrahydrochloridechromogen (DAB, Sigma Chemical, St. Louis, USA, D5637). Mayer's hematoxylin was used for counterstaining.

2.5. Immunoexpression analysis and cell counts

For reaction analysis, the slices were digitized using a light microscope (AxioScoup A1, Zeiss, Göttingen, Germany) interfaced to a computer, at a magnification of 400×. Using a computer mouse and a software program (AxioVision, version 4.8, Zeiss, Oberkochen, Germany), the areas of cell counts were manually delineated in the epithelium (Ep) and LP. Sequential fields were digitized in all samples. Throughout the sections, the cells were counted by a blinded examiner (GRS). Positive cells for anti-CD1a (Fig. 1E) and anti-CD83 (Fig. 1F) were counted in the Ep, LP, and both (Ep + LP) regions. The cell density (positive cell number/mm^2) was calculated for both CD1a and CD83 (Souto, Nunes, Tanure, Gomez, & Mesquita, 2016).

2.6. Statistical analysis

Statistical analyses were performed with the SPSS Statistics software (SPSS Inc., version 17.0, Chicago, IL, USA). The densities of IIC, CD1a +, and CD83+ DCs were compared among individuals diagnosed with OLS and OLNS. Variables were grouped in demographic variables (age and gender), behavioral habits (smoking habit), clinical variables (site and lesion size in diameters), and histopathological variables (1 - criteria of the WHO; 2 - analysis dichotomized in OL with or without dysplasia) for individuals diagnosed with OLS, OLNS, or OLS + OLNS. Normal distribution of data was determined using the Shapiro-Wilk test. For comparisons between two groups, the Student *t*-test was applied in samples with a normal distribution. For comparisons among more than two groups, analysis of samples with abnormal distributions were performed using Kruskal–Wallis, after Mann-Whitney U tests. P values were 0.05, and were adjusted for multiple comparisons with Bonferroni's method. The Chi-square test was used to compare the distribution of gender. Reliability of the measurements was assessed by

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