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Does smoking habit affect dendritic cell expression in oral squamous cell carcinoma?

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Abstract: The aim of this study was to determine the presence of immature CD1a+ and mature CD83+ dendritic cells in oral squamous cell carcinoma, to compare immunoreactivity between smokers and nonsmokers, and to correlate the results with histopathological grading. In this observational study, twenty-eight paraffin-embedded biopsies of oral squamous cell carcinoma were retrospectively retrieved and submitted to immunohistochemistry for immature CD1a+ and mature CD83+. Descriptive and statistical analyses were performed. The sample consisted of 18 man (64.3%) and 10 women (35.7%), with a mean age of 64.6 years in the nonsmoker group and 53.2 years in the smoker group. The tongue (11 cases, 39.2%) was the most commonly affected anatomical site, followed by gingiva (6 cases, 21.4%). Histopathological grading revealed 7 low-grade and 7 high-grade malignancy cases in each group, and no correlation with the number of positive DCs. The number of immature CD1a+ was not significantly different between smoker and nonsmoker groups, while a lower number of mature CD83+ was detected in the smoker group ($p = 0.001$). Smoking changes the oral immune system and decreases the ability to activate and mature dendritic cells, which may influence the development and progression of oral squamous cell carcinoma.

Keywords: Dendritic Cells; Mouth Neoplasm; Tobacco; Immune System.

Introduction

Oral squamous cell carcinoma (OSCC) corresponds to almost 90% of all head and neck malignancies.[1] Approximately 300,000 new cases are reported annually, and although detection and treatment have improved over the last decades, more than 120,000 deaths per year can be attributed to OSCC as a result of poor prognosis.[2] In the majority of OSCC cases, the affected individuals are usually men older than 45 years who have been exposed to tobacco and alcohol.[3]

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.[4,5] Langerhans cells (LCs) are a type of DCs and reside in the epithelium of skin and mucosa. LCs play a role in initiating and regulating the immune response, especially by stimulating antigen-specific T cell proliferation.[6] When immature LCs

capture epithelial antigens, they migrate into the adjacent connective tissue as interstitial DCs and transfer the processed antigens to local lymph nodes, where they are presented to T cells. LCs become mature after antigen processing and presentation on the cell surface, losing the ability to capture new antigens.[5,7-9]

It is well known that the establishment of an immune response against malignant cells depends on the ability of DCs to transfer antigens to the lymph nodes,[10] and has been proposed that these cells play a role in immune surveillance against antigens associated with malignant transformation. Several studies have shown a correlation between the lack of LCs and malignant evolution in different tumors, such as colorectal, gastric, pancreatic, and breast cancers.[9-12]

A number of authors have demonstrated an altered antigen-presenting capacity in individuals exposed to cigarette smoke when compared to unexposed individuals.[8,13-16] Using immunohistochemistry (IHC) and RT-PCR, Liao et al.[16] detected increased numbers of immature DCs (CD1a+) in the lungs of cigarette smokers and patients with chronic obstructive pulmonary disease, and a concomitant reduction in mature cells. Campaner et al.[17] investigated the relationship between smoking habit and quantity of LCs in women with grade 3 cervical intraepithelial neoplasia using IHC for S100 protein, and found no significant difference between smokers, former smokers, or nonsmokers. However, nonsmokers had a higher median number of intraepithelial cells than the other two groups.[17]

Considering the relationship between DCs, smoking habits and malignant tumors, and the importance of determining and establishing this association in OSCC, the aim of the current study was to evaluate the presence of immature CD1a+ and mature CD83+ DCs in OSCC and to compare the immunoreactivity of smokers and nonsmokers, also correlating the results with histopathological grading. Our hypothesis is that OSCC from smoker patients will present low numbers of immunoexpression of immature CD1a+ and mature CD83+ DCs.

Methodology

Ethical approval

The present observational study was approved by the Research Ethics Committee of the Federal University of Pelotas (UFPel), Brazil, under protocol number 09/2014, in accordance to Brazilian law and the Helsinki Statement.

Patients and samples

A convenience sample was selected. Twenty-eight paraffin-embedded biopsies with a histopathological diagnosis of OSCC were retrospectively retrieved from the files of the Center of Oral Diseases of the Pelotas Dental School, UFPEL. OSCC included lesions affecting the tongue, floor of the mouth, palate, gingiva and buccal mucosa. Clinical information regarding sex, age, time of evolution, location, smoking habit and histopathological grading was obtained from patient records. Individuals who had smoked ≥ 100 cigarettes during their lifetime and those who smoked up to the time of the study were defined as smokers, while individuals who had not smoked ≥ 100 cigarettes in their lifetime, as classified by Tomar and Asma (2000), were defined as non-smokers.[18]

Histopathological grading

Two independent oral pathologists (AE and SB) confirmed the diagnosis of OSCC by reviewing the original 5-µm histopathological sections stained with hematoxylin and eosin (H&E). Histopathological grading was performed using Bryne's system.[1] Cases with a total score ranging from 4 to 8 were classified as low-grade malignancies and cases with a total score of 9 to 16 were classified as high-grade malignancies.

Immunohistochemistry (IHC)

The IHC reaction was performed according to the standard streptavidin-biotin protocol. Anti-CD1a and anti-CD83 are considered markers for immature and mature DCs, respectively. Also, only anti-CD1a is considered a marker for the LCs located in the epithelium.[19,20] Serial 3-µm thick sections of paraffin-embedded blocks were deparaffinized and rehydrated in a decreasing

ethanol series. Specimens were immersed in 10 mM antigen retrieval citrate buffer, pH = 6.0 (Lab Synth, Diadema, Brazil) for 20 minutes at 98°C, and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. The following monoclonal antibodies were used: anti-CD1a (clone O10 – Dako North America Inc., Carpinteria, USA, 1:100 dilution) and anti-CD83 (clone HB15e – Serotec, Oxford, UK, 1:150 dilution). Detection was performed using the biotin-streptavidin-peroxidase method and a 3.3'-diaminobenzidine tetrahydrochloride chromogen (Dako North America Inc., Carpinteria, USA). Mayer hematoxylin was used for counterstaining.

A case of OSCC that was not part of the sample studied and was shown to be positive for CD1a and CD83 served as the positive control for the immunohistochemical reactions; the negative control was obtained by omitting primary antibodies.

Immunoexpression analysis and cell counts

The density of immunolabeled cells, defined as cell number per square millimeter, was calculated for immature CD1a+and mature CD83+ DCs[21], and slides were fully digitized using a slide scanner (3D HISTECH, Budapest, Hungary). The OSCC area was delimited for each case, and positive cells were visually and manually counted by a calibrated and blinded examiner at 200x magnification using the Panoramic Viewer software (3D HISTECH, Budapest, Hungary). Cells that showed staining of the cytoplasm and clearly visible nuclei were considered positive for both CD1a and CD83. Immature CD1a+and mature CD83+ DCs were counted in both the stromal and parenchymal components of OSCC. The density of immunolabeled immature CD1a+ and mature CD83+ DCs was compared between the OCSS cases of smokers and nonsmokers patients and correlated with histopathological OCSS grading.

Statistical analyses

Statistical analyses were performed using Stata/IC 14.0 software (Stata Corp, College Station, USA). Normal distribution was tested using the Shapiro-Wilk test. Data regarding patient sex and age, time of evolution, location and histopathological grading of OSCC are presented as descriptive data. The nonparametric Mann-Whitney-Wilcoxon test was used to evaluate the association between the number of immature CD1a+ and mature CD83+ DCs and smoking habit and histopathological grading. The significance level was set at $p < 0.05$ in all analyses. The reliability of the measurements was assessed using the intraclass correlation coefficient (ICC), with an ICC value > 0.91 being considered to indicate a good correlation.

Results

Clinicopathological features

The clinical data of the patients are presented in Table 1. Twenty-eight cases of OSCC were selected for this study, equally distributed between smokers and nonsmokers. Males predominated among smokers, while among nonsmokers, there was no sex preponderance. Non-smokers were found to have a higher mean age (64.66 years) compared to smokers (53.26 years). The mean time of evolution was 3.95 months for both smokers and

Table 1. Clinical and histopathological profile of the smoking and non-smoking individuals with oral squamous cell carcinoma.

Groups	Sex		Age		Time of evolution	Location					Histopathological grading	
	M*	F*	Range	Mean	Mean (Months)	Gingiva	Tongue	Palate	Buccal mucosa	Floor	Low-grade	High-grade
Smokers patients	11	3	26-67	53.26	4.2	4	6	0	0	4	7	7
Non-smokers patients	7	7	53-83	64.66	3.7	2	5	1	5	1	7	7
Total	18	10		58.96	3.95	6	11	1	5	5	14	14

*M, Male; F, Female

nonsmokers. The most common site where the disease occurred was the tongue (11 cases, 39.28%), followed by the gingiva (6 cases, 21.43%). Regarding histopathological grading, each group had seven low-grade OSCC (Figure A) and seven high-grade OSCC (Figure B).

Immature CD1a+dendritic cells (DCs)

The number of immature CD1a+ DCs was not significantly different between smokers and nonsmokers (p = 0.462) (Table 2). IHC staining of immature CD1a+ CDs showed dendritic morphology in the epithelium (Figure C) and a round shape in the connective tissue.

Mature CD83+ dendritic cells (DCs)

A significant difference (p = 0.001) in the number of mature CD83+ CDs was found between smokers and nonsmokers, with a lower number of mature CD83+ CDs in the smoker group (Table 2). IHC staining of mature CD83+ CDs was observed in the epithelium and connective tissue as round or irregular brown structures (Figure D).

Histopathological grading

There was no correlation between the number of immunolabeled cells and the histopathological grading of OSCC, neither for immature CD1a+ DCs (p=0.073) nor for mature CD83+ DCs (p = 0.550) (Table 2).

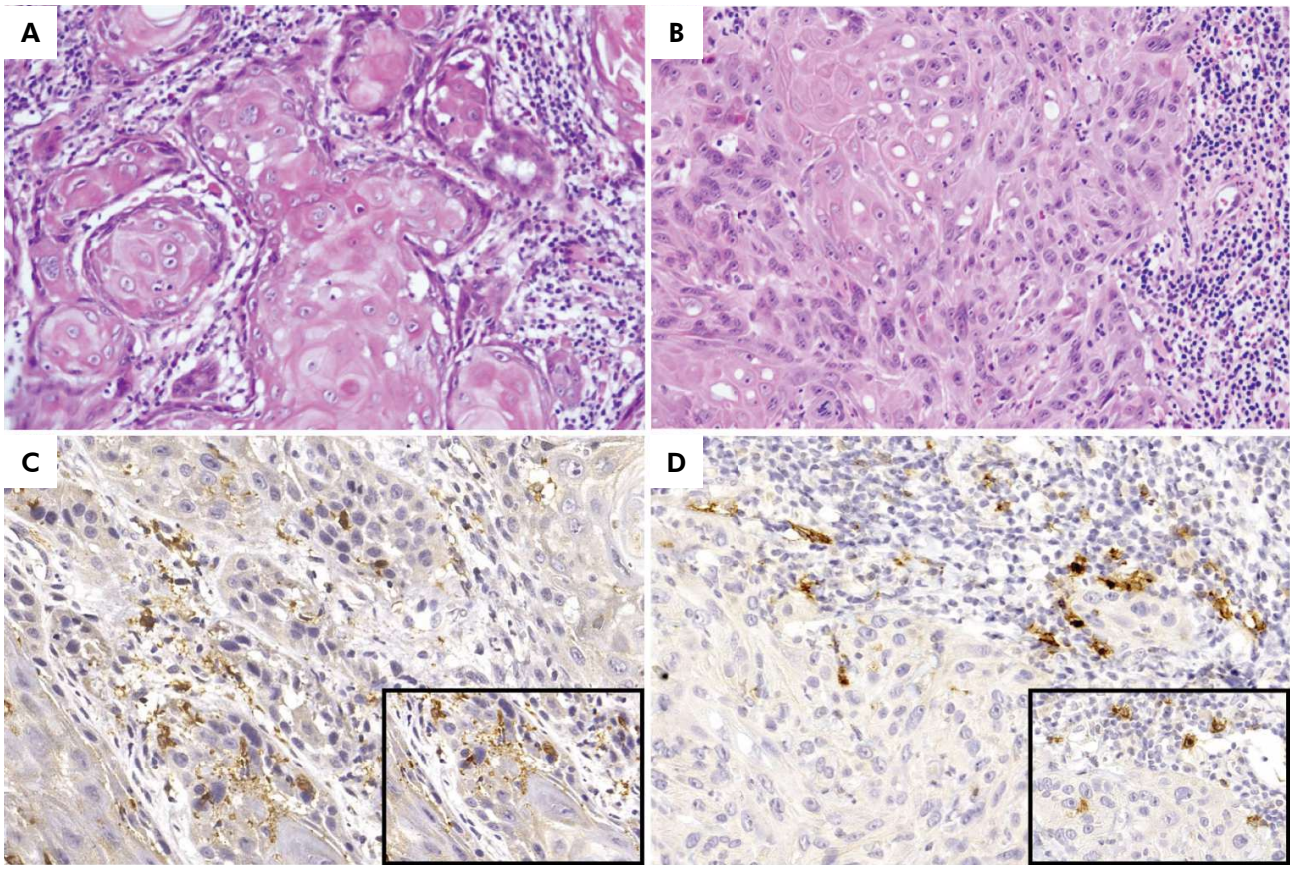

Figure. A - Low-grade oral squamous cell carcinoma (OSCC) is represented by sheets and islands of proliferative cells within connective tissue. The proliferative cells show discrete pleomorphism and hyperchromatic nuclei with individual keratinization (H&E, 200X magnification). **B** - High-grade OSCC is represented by sheets of proliferative cells within the connective tissue. The proliferative cells showed intense pleomorphism and hyperchromatic nuclei without individual keratinization (H&E, 200X magnification). **C** - Immunostaining for CD1a shows immature dendritic cells located predominantly within the OSSC and with dendritic morphology (box) (immunohistochemistry, 200X magnification). **D** - Immunostaining of mature CD83+ dendritic cells was observed predominantly in connective tissue in the form of rounded brown structures (box) (immunohistochemistry, 200X magnification).

Table 2. Mean number of CD1a+ immature and CD83+ mature dendritic cells per area in accordance to the smoking habit and histopathological grading of oral squamous cell carcinoma (OSSC).

Variable	CD1a+ immature dendritic cells			CD83+ mature dendritic cells		
	Mean	95%CI	p-value*	Mean	95%CI	p-value*
Smoking habit			0.462			0.001
Smokers patients	0.25	0.16–0.34		0.40	0.18–0.62	
Non-smokers patients	0.29	0.11–0.48		0.64	0.46–0.83	
Histopathological grading			0.073			0.550
Low-grade OSCC	0.19	0.10–0.27		0.51	0.34–0.67	
High-grade OSCC	0.36	0.19–0.53		0.54	0.28–0.79	

*Mann-Whitney ($p < 0.05$, 95%CI)

Discussion

The literature shows that tobacco modulates immunity by altering the functionality of immune cells, including DCs.[15,22,23] In an *in vivo* study in mice, Robbins et al.[13] demonstrated that smoker mice have a lower number of pulmonary DCs compared to nonsmoker mice. Although migration to lymph nodes was unaffected, the maturation was suppressed, thus altering T cells activation in smoker mice.[13] Culture of DCs in the presence of a cigarette extract resulted in silenced or dysfunctional cells during DC differentiation, demonstrating the effects of the extract on modulation of the immune response.[8]

In healthy smoker individuals, a lower number of mature CD83+ DCs in the lungs was associated with an increase in immature CD1a+ CDs compared to healthy nonsmoker individuals, as determined by flow cytometry and RT-PCR.[16] In addition, Souto et al.[20,21] demonstrated a lower number of mature CD83+ cells in the connective tissue of smokers with chronic gingivitis and chronic periodontitis compared to nonsmokers. Our findings of lower numbers of mature CD83+ cells in the smoker group of OSCC patients are consistent with previous *in vivo* and *in vitro* studies, suggesting diminished maturation ability and a less effective activation of the immune response in smokers.

An imbalance in the immune response may be responsible for the development of oral cancer.[9] Öhman et al.,[5] found an increased number of CD1a+ LCs in OSCC compared to oral leukoplakia with or without dysplasia using IHC. The authors attributed this result to the establishment of an immune response against the tumor.[5] Upadhyay et al.,[24] on the other hand, observed a lower number of immature CD1a+ CDs in the epithelium of dysplasia and OSCC patients compared to normal mucosal epithelium. In addition, no difference was found between cases of epithelial dysplasia and OSCC.[24] The authors stated that a dysplastic epithelium could inhibit the migration of LCs into the epithelial compartment, or that the interaction with dysplastic and inflammatory cells could lead to death of LCs.[24] In contrast, Costa et al.[25] observed an equal density of immature CD1a+ DCs in OSCC and in normal tissue, similar to the value reported by Souto et al.[14] when comparing the density of immature CD1a+ DCs between oral samples with and without epithelial dysplasia.

Regarding mature CD83+ CDs, Pellicioli et al.[26] - although they did not consider the smoking habit of the patients in their study - showed a significant decrease in the number in OSCC compared to oral epithelial dysplasia. The authors hypothesized that these cells may have migrated to the lymph nodes to present tumor antigens and to activate the immune system, or that cytokines secreted by the tumor microenvironment may have inhibited an adequate maturation of DCs.

Concerning histopathological grading, well-differentiated OSCC have been shown to have a higher density of CD1a+ LCs than poorly differentiated cases, a fact that may be directly related to tumor prognosis.[27] In the present study, the Byrne classification was used for the histopathological grading of the tumors as low-grade and high-grade. Our choice for Byrne classification was based on the study conducted by

Wagner et al.[1] The authors demonstrated that the Bryne's grading system is more effective in predicting OSCC survival.[1] The results showed that there was no correlation between histopathological grading and the density of mature CD83+ or immature CD1a+ DCs, which could be explained by the possible existence of a heterogeneous population of DCs involved in the development of OSCC, representing distinct cell lineages and the expression of variable immunophenotypes.[6]

Furthermore, according to a review by Tsoumakidou et al.,[28] a number of studies that examined the relationship between smoking habits and immature pulmonary CD1a+ DCs have shown no effect or an increase in the alveolar number of these cells in the presence of a chronic smoking habits, as also stated by others.[16,28] It is important to highlight that some studies used convenience sample for DC analysis, such as our data. Therefore, the findings need to be interpreted carefully. The present study did not show a statistically significant association between these cells and smoking habit, indicating an equal immunosurveillance capability for both smokers and nonsmokers. However, it is possible to infer that exposure to smoke does not affect the antigen uptake ability of immature CD1a+ DCs, but only interferes with the maturation process. Our findings reinforce the importance of future research with larger numbers of cases to confirm or not the association data. With a robust number of OSCC cases, significance will certainly be achieved.

In conclusion, OSCC is a multifactorial process, as demonstrated by a significant decrease of mature DCs in smokers compared to nonsmokers, with no significant difference in immature DCs between these groups. Although there was no correlation between the number of mature CD83+ CDs, immature CD1a+ CDs and OSCC histopathological grading, we suggest that suppression of mature CD83+ CDs in smokers can contribute to the development of intraoral cancer, demonstrating the importance of these cells in the immune response in this malignancy.

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