ORIGINAL RESEARCH
Oral Pathology

Impact of smoking on dendritic cells in patients with oral squamous cell carcinoma

Heron STÄHELIN[(a)]

Ana Lucia Noronha FRANCISCO[(b)]

Fernanda Viviane MARIANO[(c)]

Luiz Paulo KOWALSKI[(b)]

Rogério GONDAK[(d)]

[(a)]Universidade Federal de Santa Catarina – UFSC, Department of Dentistry, Florianópolis, SC, Brazil.

[(b)]AC Camargo Cancer Center, Department of Head & Neck, São Paulo, SP, Brazil.

[(c)]Universidade Estadual de Campinas – Unicamp, School of Medical Sciences, Department of Pathology, Campinas, SP, Brazil.

[(d)]Universidade Federal de Santa Catarina – UFSC, Department of Pathology, Florianópolis, SC, Brazil.

Abstract: Smoking has been shown to alter innate and adaptive immune responses and is directly associated with the onset of oral squamous cell carcinoma (OSCC). The purpose of this study was to evaluate the effect of cigarette smoke exposure on dendritic cells (DCs) from OSCC patients. CD1a and CD83 antibodies were used to identify immature and mature DCs, respectively, by immunohistochemistry in OSCC samples of 24 smokers and 24 non-smokers. Density of DCs was calculated in intra and peritumoral areas. Clinical and microscopic findings were reviewed and analyzed for all patients. Smokers with OSCC had a lower density of intra and peritumoral DCs when compared to non-smokers. Tumors classified as moderately/poorly differentiated had lower peritumoral CD1a+ DCs than well-differentiated tumors ($p < 0.001$). Smoking contributed to a depletion of immature and mature DCs in the OSCC.

Keywords: Mouth Neoplasms; Immunity; Cigarette Smoking.

Introduction

Cigarette smoking is the primary cause of the development of oral squamous cell carcinoma (OSCC) in Western countries.[1,2] This etiological factor has been linked to mutations in oral mucosa keratinocytes and invasion of connective tissue even though there is an antitumor immune response.[3] In the oral mucosa, various dendritic cells (DCs) subsets regulate and control the tumor-specific immune response exhibiting differential pro-tumorigenic and anti-tumorigenic functions.[4]

DCs provide fundamental signals for the activation of T-cells.[5] The recognition and absorption of the antigens are given by the immature CD1a+ DCs, which through chemokines, cytokines, and surface molecules begin their maturation and displacement to regional lymph nodes interacting with T cells.[6] After maturation, the mature CD83+ DCs play a role in the differentiation of T cells into cytotoxic T eliminating neoplastic cells.[6]

Smoking alters both adaptive and innate immune responses exerting a direct immunomodulatory effect on DCs.[7] Literature has diverged regarding the influence of smoking on the population of oral DCs[8,9,10] Although initiated by tobacco carcinogens, the progression of OSCC may be associated with the inability of DCs to act locally through antitumor responses. Hence, the purpose of this study was to evaluate the density

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Corresponding Author:
Rogério Gondak
E-mail: rogerio.gondak@ufsc.br

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of DCs in smoker and nonsmoker patients with OSCC as well as the interference of these cells in the clinicopathologic characteristics of the tumor.

Methodology

The Ethics Committee of the AC Camargo Cancer Center and the Federal University of Santa Catarina approved this study (#00741212.8.0000.5432).

Patient population

Surgical specimens of 48 patients diagnosed with OSSC affecting the tongue, collected from 2013 to 2019 at the Department of Head and Neck, AC Camargo Cancer Center, São Paulo, Brazil, and Department of Pathology, Federal University of Santa Catarina, Florianópolis, Brazil were used for the study. Twenty four patients were cigarette smokers and 24 were non-smokers matched for sex and age. The study excluded patients with autoimmune diseases, other habits as alcohol consumption, or other neoplasms than OSSC. Smokers were considered patients who smoked more than 10 cigarettes per day for at least 10 years while non-smokers were considered patients who never smoked.[11] Smoking habit information was collected from medical records and confirmed by face-to-face interviews or telephone contact.

Clinical and histopathological data

Clinical data including age, sex, primary tumor location, smoking habits, and staging of the disease were obtained from clinical charts. Histological grade, lymphovascular and perineural invasion were obtained from anatomopathological records and reviewed by two expert pathologists. Additionality, the inflammatory infiltrate and pattern of invasion were assessed based on criteria used by Affonso et al.[12] and Akhter et al.,[13] respectively.

Dendritic cells

OSSC specimens were fixed in 10% formalin for 24 hours. All fragments were embedded in paraffin and 3-µm thick histological sections were submitted for immunohistochemistry reactions.

The monoclonal antibodies CD1a (010, 1:200; Dako) and CD83 (1H4b, 1:40; Novocastra, Newcastle, UK) were used to identify immature and mature DCs from the oral mucosa, respectively. Normal skin tissue was used as a CD1a positive control and normal tonsil as a CD83 positive control, according to the manufacturer's recommendations. Antigen retrieval with citrate buffer (pH 6.0) at 96°C for 40 minutes was performed for both antibodies. The slides were incubated with secondary antibodies conjugated with biotin (LSAB, Dako, Glostrup; Denmark) for 30 min at 37°C, followed by streptavidin-HRP (Dako, Glostrup; Denmark) for another 30 min and developed with a chromogenic substrate (3,3 diaminobenzidine, Sigma, St Louis, MO, USA). The slides were counterstained with Harris hematoxylin. Negative controls were carried out for each reaction by omitting the primary antibodies.

The quantification of CD1a+ and CD83+ cells were obtained using ImageJ 1.53a software (National Institutes of Health, USA). The analysis was performed by two experienced oral pathologists, who were blinded (ICC=0.972; 95% CI 0.906-0.992). Five high-power fields in high magnification (400x) for each sample, were captured with a digital camera (Canon A620, Ōita, Japan) coupled to a light microscope (Axiostar Plus, Carl Zeiss, Oberkochen, Germany). Intra and peritumoral areas were analyzed and the results expressed as positive cells per square millimeter (+cells/ mm^2).

Statistical analysis

The comparison of immunostained DCs between the groups was performed by the Student t-test. Clinical and microscopic findings were analyzed by Pearson Chi-Square or Fisher's exact test when appropriate, for categorical variables, and by the Mann-Whitney or Kruskal-Wallis test for continuous variables. A p-value of <0.05 was regarded as statistically significant. The statistical software SPSS version 23.0 was used for the analyses.

Results

Patient population

The mean age of non-smoker and smoker patients with OSCC was 55.2 and 59.8 years, respectively. There was a predominance of female patients in both

groups. Stages I and II for OSCC were identified in 75% of the non-smokers and 66.7% of the smokers. Additional data are shown in Table 1.

Microscopic findings

Most smokers and non-smokers presented tumors classified as well-differentiated. Most cases in both groups had no lymphovascular invasion. Perineural invasion was identified in 75% of non-smokers and 58.3% of smokers.

Dendritic cells quantification

Non-smokers had a higher density of intratumoral CD1a+ DCs than smokers ($p < 0.001$). There was no difference in the density of intratumoral CD83+ DCs between groups (Table 2). Regarding the peritumoral compartment, non-smokers had a higher density of CD1a+ and CD83+ DCs than smokers ($p = 0.012$ and $p = 0.010$, respectively). Tumors classified as poorly differentiated had a lower density of peritumoral CD1a+ DCs than tumors classified as well-differentiated ($p < 0.001$) (Table 3). Additional data are shown in Figure.

Discussion

This study demonstrated the impact of chronic tobacco smoke exposure on the population of mature

Table 1. Clinical and microscopic findings in the different groups with oral squamous cell carcinoma.

Variables	Non-smokers with OSCC	Smokers with OSCC	p-value
	(n = 24)	(n = 24)	
Age	55.2 ± 22.4	59.8 ± 9.0	0.796[a]
Sex			
Male:Female	08:16	06:18	0.620[b]
Histological grading			
Well differentiated	20 (83.4%)	16 (66.7%)	0.318[c]
Moderately/poorly differentiated	4 (16.6%)	8 (33.3%)	
Lymphovascular invasion			
No	24 (100.0%)	22 (91.7)	0.500[b]
Yes	0 (0.0)	2 (8.3%)	
Perineural invasion			
No	18 (75.0%)	14 (58.3%)	0.667[b]
Yes	6 (25.0%)	10 (41.7%)	
Staging for OSSC			
Stage I-II	18 (75.0%)	16 (66.7%)	0.653[b]
Stage III-IV	6 (25.0%)	8 (33.3%)	
Pattern of invasion			
1	0 (0.0)	0 (0.0)	0.247[c]
2	9 (37.5%)	4 (16.7%)	
3	5 (20.8%)	8 (33.3%)	
4	10 (41.7%)	12 (50.0%)	
Inflammatory infiltrate			
Grade 0	0 (0.0)	0 (0.0)	0.370[c]
Grade 1	0 (0.0)	0 (0.0)	
Grade 2	5 (20.8%)	7 (29.2%)	
Grade 3	19 (79.2%)	17 (70.8)	

[a]Student t-test. [b]Fisher´s Exact Test. [c]Pearson Chi-Square.

Table 2. Quantification of intra and peritumoral CD1a+ and CD83+ cells (cells/mm^2 ± standard deviation) in the different groups with oral squamous cell carcinoma.

Variables	Non-smokers with OSCC	Smokers with OSCC	p-value
Intratumoral CD1a+ cells	1220.6 ± 367.0	679.6 ± 263.5	< 0.001[a]
Intratumoral CD83+ cells	296.8 ± 163.8	191.2 ± 187.9	0.161[a]
Peritumoral CD1a+ cells	612.1 ± 419.4	249.9 ± 233.2	0.012[a]
Peritumoral CD83+ cells	887.3 ± 502.9	280.9 ± 163.4	0.010[a]

OSCC: oral squamous cell carcinoma. [a] Student t-test.

Table 3. Comparison between clinicopathologic findings and the number of intra and peritumoral dendritic cells in oral squamous cell carcinoma patients (median and interquartile range).

Variables	Intratumoral CD1a+ cells/mm^2	p-value	Intratumoral CD83+ cells/mm^2	p-value	Peritumoral CD1a+ cells/mm^2	p-value	Peritumoral CD83+ cells/mm2	p-value
Histological grading*								
Well differentiated	882.5 (634.9)	0.505	203.1 (361.9)	0.935	393.6 (488.8)	0.001	336.5 (749.2)	0.684
Moderately/poorly differentiated	977.7 (571.4)		234.9 (247.6)		285.7 (493.6)		342.8 (746.0)	
Lymphovascular invasion*								
No	977.7 (634.9)	0.353	215.8 (292.0)	0.275	393.6 (457.1)	0.757	336.5 (768.2)	0.640
Yes	768.2 (0.0)		120.6 (0.0)		285.7 (0.0)		596.8 (0.0)	
Perineural invasion*								
No	1019.0 (488.8)	0.793	206.3 (292.0)	0.755	431.7 (507.9)	0.274	526.9 (819.0)	0.483
Yes	873.0 (457.1)		219.0 (209.5)		288.8 (158.7)		330.1 (336.5)	
Staging for OSSC*								
Stage I-II	1133.3 (623.8)	0.751	219.0 (328.5)	0.117	342.8 (461.9)	0.856	526.9 (873.0)	0.541
Stage III-IV	711.1 (304.7)		158.7 (157.1)		393.6 (252.3)		330.6 (258.7)	
Pattern of invasion**								
2	1050.7 (476.1)		142.8 (112.2)		469.8 (158.7)		746.0 (819.0)	
3	1111.1 (590.4)	0.076	234.9 (292.0)	0.161	241.2 (374.6)	0.112	317.4 (523.8)	0.321
4	768.2 (277.7)		158.7 (223.8)		342.8 (419.0)		466.6 (501.5)	
Inflammatory infiltrate*								
Grade 2	847.6 (515.8)	0.317	146.0 (277.7)	0.439	241.2 (382.5)	0.536	279.3 (234.9)	0.084
Grade 3	882.5 (819.0)		203.1 (209.5)		292.0 (317.4)		342.8 (634.9)	

*Mann-Whitney test. **Kruskal-Wallis Test. Results were expressed as median (interquartile range).

and immature DCs in OSCC. In agreement with previous studies in the lung, smoking impacts DCs having profound effects on immune responsiveness.[14,15] Although some studies have evaluated the number of DCs in oral cancer,[16,17] to the best of our knowledge, this is the first study to evaluate the population of mature and immature DCs in OSSC samples without tobacco interference.

Souto et al.[18] showed a lower number of immature and mature DCs in chronic gingivitis of smoking patients than in non-smoking patients suggesting there is a harmful tobacco effect on DCs. In our study, non-smoker patients had a higher number of mature and immature DCs in the peritumoral compartments than smokers, corroborating previous studies.[7,9,15]

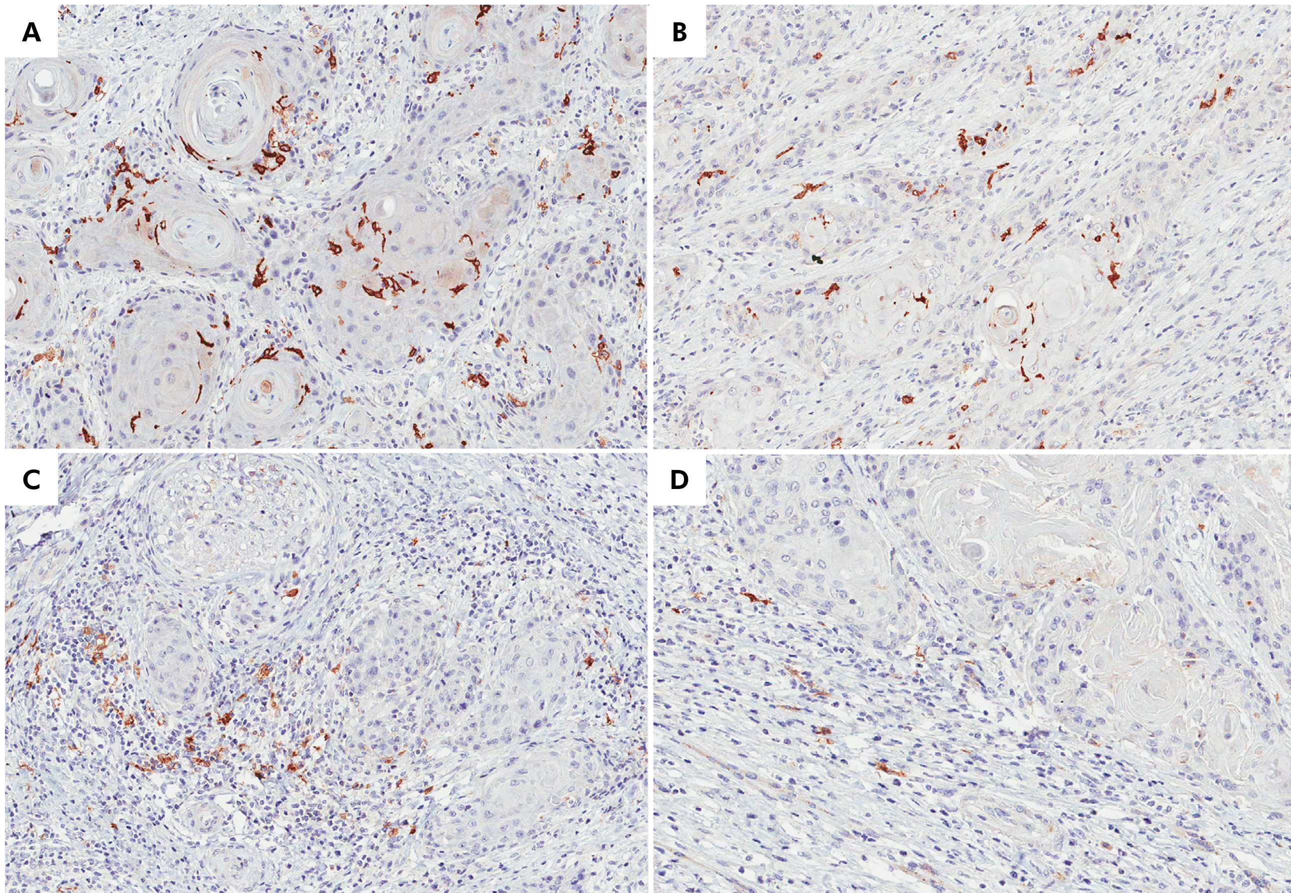

Figure. Immunohistochemical expression of CD1a+ and CD83+ cells in the tongue of non-smokers and smokers with oral squamous cell carcinoma. CD1a+ cells in non-smokers and smokers (A and B respectively). CD83+ cells in non-smokers and smokers (C and D respectively). High magnification (x400).

There are several methods of smoking assessment such as online questionnaire, paper-based questionnaire, and direct face-to-face interview. Some studies have shown face-to-face self-report of cigarette smoking status to be valid.[19,20] In addition, online assessment of cigarette smoking status and paper-based methods have been shown to have similar accuracy.[21] In this study, smoking habits were collected from medical records and confirmed by face-to-face interviews or telephone contact ensuring the reliability of the data obtained.

Depending on the state of maturation, DCs play an important role in the immune response.[22] Many different subtypes of immature DCs (CD1a+, CD207+, and S100+) and mature DCs (CD83+ and CD208+) have been described,[23,24,25,26] making it difficult to compare the studies. In the present study, we used the CD1a and CD83 markers for immature and mature DCs, respectively, since they are recognized and well-accepted DCs maturation markers in formalin fixed paraffin-embedded human tissues.[5,16,17]

Smoking has been shown to modulate the DC-mediated immune response by affecting both maturation and function of DCs.[9] Cigarette smoke alters the DCs maturation within the lymph nodes reducing cell surface expression of MHC and the costimulatory molecules CD80 and CD86.[13] On the other hand, cigarette smoke can be associated with tissue damage and induction of inflammation, activating differentiation of DCs.[27,28]

A high density of immature DCs was associated with decreased antigen presentation[29] and inhibition of T-cell proliferation and activity.[30] Some *in vitro*

studies using monocyte-derived DCs exposed to varying doses of nicotine[31,32] and cigarette smoke[33] have shown a negative effect on DCs functionality. Besides, tumor cells can escape immunologic response inducing DC disability and reduce lymphocytic function.[34] In the current study, non-smoker patients had a higher density of immature DCs in the intratumoral compartments. However, there was no difference in mature DC density between smokers and nonsmokers in this compartment. Possibly, the smoking habit does not interfere in the process of maturation of DCs inside the tumor islands.

In tumoral tissues, immature DCs migrate, recognize, and capture antigens in response to the production of chemokines acquiring a mature phenotype.[6] Mature DCs lose the ability to respond to the inflammatory chemokines but proceed to respond to the CCL19 and CCL21 chemokines migrating to the lymph nodes to present antigens to T cells.[6]

In a previous study, the number of Langerhans cells, a subpopulation of DCs, was considered an independent prognostic marker for head and neck squamous cell carcinomas (HNSCC).[35] The peritumoral density of CD1a+ DCs was associated with improved prognosis and reduced recurrence.[36] HNSCC patients who smoked had worse prognoses when fewer immature DCs were present in intra- and peritumoral compartments.[35] The peritumoral density of CD1a+ DCs was higher in OSCC patients with N0 disease.[37] However, the previous studies included samples of patients with OSCC in various oral cavity subsites and different stages of the disease that may influence DC density. For the current study, there was no difference in the density of DCs between the different stages of the disease. However, there was a significant decrease in the density of peritumoral CD1a+ DCs in poorly differentiated tumors. Thus, it is suggested that tumors with more aggressive behavior tend to have fewer peritumoral immature DCs.

Our results showed a predominance of females with OSCC who did not smoke cigarettes. Other studies found also a predominance of females among non-smokers with OSCC.[38,39] Additional etiological factors besides smoking may be associated with OSCC such as HPV infection, presence of synchronous cancer in the upper aerodigestive tract, infection by hepatitis A and C virus, race, immune deficiencies, or genetic alterations.[39,40]

The current study had some limitations. We focused on tobacco being the main etiological factor for cancer. However, other factors could affect the DC population, such as viral infections by EBV or HPV and nutritional factors. Moreover, this study was conducted to assess the interference of the population of DCs in tumors located exclusively on the tongue. Further studies are needed with samples from different anatomical regions of the oral cavity, since tobacco can interfere differently in these regions as well as subpopulations of DCs can express different responses.

DCs have a central role in controlling the tumor microenvironment and immune responses during OSCC development. Probably some subtypes of DCs are more susceptible to substances contained in cigarettes such as nicotine, which is recognized to have a suppressive effect on myeloid DCs. The knowledge of how smoking affects the recruitment of immune effector cells may elucidate the mechanisms involved during carcinogenesis. The literature regarding the role of DCs in OSCC is highly controversial, possibly due to varied research methodologies and patient populations. Hence, larger studies of inhomogeneous patient populations are needed to determine the role of DCs within and around tumors.

Conclusion

This study demonstrated the impact of chronic exposure to tobacco smoke on the population of DCs in OSCC patients possibly contributing to higher tumor aggressiveness.

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