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Effect of smoking on immunity in human chronic periodontitis

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ABSTRACT

Aim: Evaluate the effects of smoking on dendritic cells (DCs), cytokines, clinical periodontal parameters, and number of teeth in samples of human chronic periodontitis (CP).
Material and methods: Gingival samples were obtained from 24 smokers and 21 non-smokers with CP. Periodontal examination was carried out. Immunohistochemical staining was performed to identify Factor XIIIa+ immature, CD1a+ immature, and CD83+ mature DCs. The inflammatory infiltrate was counted, and IL-2, IL-10, IL-4, IL-6, IFN-γ, TNF-α, and IL-17A were measured using the cytometric bead array (CBA). Inflammatory infiltrate, DCs, cytokines, classification of CP, clinical periodontal parameters, number of teeth, smoking habit in years (SH/years), and number of cigarettes smoked per day (C/day) were correlated and compared.
Results: CD83+ mature DCs decreased in the smokers group. Negative correlations could be observed between the number of C/day with levels of IL-17A and number of teeth. Correlations between smoking, periodontal disease status, and other cytokines were not observed.
Conclusions: Smoking decreases mature DCs in chronic periodontitis. Moreover, a dose-dependent relation can be observed between C/day and number of teeth and levels of IL17A observed. Smokers show a different modulation of the CP immune response.

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Introduction

The response to periodontal pathogens is determined by the nature and control of both innate and adaptive immune responses. Host response factors, such as genetic and environmental factors, are essential in determining susceptibility to periodontal disease (Seymour, 1991; Seymour and Taylor, 2004). Smoking has proven to be a major environmental risk factor associated with common forms of human chronic periodontitis (CP) (Chambrone et al., 2013; Genco and Borgnakke, 2013). It has been reported that the smoking habit causes a reduction in the clinical signs of gingivitis (Kumar and Faizuddin, 2011). In a prior analysis, results demonstrated that smoking decreased both the inflammatory infiltrate and dendritic cells (DCs) in samples of chronic gingivitis (Souto et al., 2011). In addition, prior analysis also demonstrated that smoking decreased CC chemokine ligand (CCL)3 and CXC chemokine ligand (CXCL)8, while CC chemokine ligand (CCL)5 was increased in samples of CP (Souto et al., 2014). Srinivas et al. (2012) also identified a delayed neutrophil chemotaxis in smokers as compared to non-smokers on CP.

The host immune response begins when DCs capture microbes and their antigens while in the immature state and stimulate a T-cell response to these antigens in their mature state (Banchereau and Steinman, 1998; Cutler and Jotwani, 2004). Mature DCs are involved in the production of inflammatory cytokines and in the polarized pattern of Th1/Th2/Th17 responses in periodontal disease (Cutler and Jotwani, 2004; Allam et al., 2011). Moreover, DCs stimulate naive T cells to differentiate to effector T-cell subsets that may be actively involved in the immunopathogenesis of periodontal diseases (Cutler and Jotwani, 2004; Yanagita et al., 2012a,b).

In vitro studies demonstrate that DCs differentiated in the presence of nicotine and stimulated by lipopolysaccharide induced a differentiation of naive CD4 T cells into Th2 cells, whereas DCs

Abbreviations: CP, chronic periodontitis; DCs, dendritic cells; LPS, lipopolysaccharides; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; PD, probing depth; CAL, clinical attachment level; BOP, bleeding on probing; NS, non-smokers; S, smokers; SH/years, smoking habit in years; C/day, cigarette per day; H&E, hematoxylin and eosin; IHC, immunohistochemistry; CBA, cytometric bead array; LP, lamina propria; LC, Langerhans cells; DAB, diaminobenzidine tetrahydrochloridechromogen; OE, oral epithelium; SE, sulcular epithelium; APCs, antigen-presenting cells.

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differentiated without nicotine stimulated by lipopolysaccharides (LPS) induced Th1 immune responses (Yanagita et al., 2012a,b). Furthermore, studies evaluating venous blood samples (de Heens et al., 2009a,b) and gingival crevicular fluid (Tymkiw et al., 2011; Goutoudi et al., 2012) of individuals with CP verified that smoking increased the production of cytokines, such as IL-10 (de Heens et al., 2009a,b) and IL-1α, IL-1β, IL-6, IL-12 (p40), IL-8, MCP-1, MIP-1α, IL-2, IFN-γ, IL-3, IL-4, and IL-15 (Tymkiw et al., 2011). A more pronounced Th2 response in smoking periodontitis patients was linked to an increased severity of the disease samples (de Heens et al., 2009a,b). However, the effect of smoking on immature and mature DCs, cytokine production, clinical periodontal parameters, and number of teeth in gingival tissue samples of patients with CP needs to be better understood.

Therefore, the present study aimed to evaluate the effect of smoking on inflammatory infiltrate cells and immature and mature DCs, as well as in the production of pro-inflammatory (tumor necrosis factor (TNF)-α and interleukin (IL)-6); Th1 (IL-2 and interferon (IFN)-γ), Th2 (IL-4 and IL-10), and Th17 (IL-17A) cytokines; clinical periodontal parameters; and number of teeth in gingival tissue samples of individuals diagnosed with CP.

Material and methods

Patients and periodontal samples

The present study was approved by the Research Ethics Committee from Universidade Federal de Minas Gerais (UFMG), Brazil (423/11). Forty-five patients were recruited and provided written informed consent to participate in this study. All individuals received a full-mouth periodontal examination. Probing was performed in a circumferential mode, in four sites per tooth in all teeth, by a single trained examiner (GRS). The analyzed parameters included probing depth (PD), clinical attachment level (CAL), bleeding on probing (BOP), and the oral hygiene index were determined. Individuals presented proximal CAL $\geq$ 3 mm in $\geq$2 non-adjacent teeth were diagnosed as CP. Individuals presenting proximal CAL $\geq$ 5 mm in $\geq$30% of teeth were diagnosed as advanced CP, otherwise they were diagnosed as mild-moderate CP (Tonetti and Claffey, 2005). Gingival samples were obtained during tooth extractions for prosthetic or endodontic reasons. The patients evaluated in this study reported no presence of systemic diseases or immunologic abnormalities.

Regarding the characterization of smoking, individuals were classified according to the criteria of Tomar and Asma (Tomar and Asma, 2000; Demoor et al., 2009) in non-smokers (NS) and smokers (S). The individuals were asked about the time of their smoking habit in years (SH/years) and how many cigarettes they smoked per day (C/day).

Two gingival samples were collected for each individual. The first sample was set in 10% buffered formalin, histologically processed, sectioned, and either stained with hematoxylin and eosin (H&E) or subjected to immunohistochemistry (IHC). The second sample was stored in buffer (0.4 mM NaCl, 10 mM $NaPO_4$, pH 7.4) containing inhibitors of proteases (0.1 mM PMSF – phenylmethylsulfonyl fluoride – 0.1 mM benzethonium chloride, 10 mM EDTA, and 0.01 mg/mL aprotinin A) and Tween 20 (0.05%), pH 7.4, at a ratio of 1 ml solution per 100 mg tissue to perform the cytometric bead array (CBA). The evaluated cytokines comprised mediators present in the secreted form (tissue cytokines) and previously intracellular cytokines. Due to losses while processing the samples, 45 samples were used in CBA; 43 samples were used to analyze the inflammatory infiltrate cells, Factor XIIIa+ immature DCs, and CD83+ mature DCs; and 42 samples were used to analyze CD1a+ immature DCs.

Inflammatory assessment

The inflammatory infiltrate in gingival tissues was measured using H&E-stained sections (Fig. 1A). The sections were digitized using a microscope (AxioScoup A1, Zeiss, Göttingen, Germany) at a magnification of 400× and interfaced to a computer. Cell counts were taken during the sectioning by a blinded examiner (GRS). The mean inflammatory infiltrate was determined by counting the number of inflammatory cells on the lamina propria (LP) in consecutive fields of all gingival samples (field area: 0.04652 mm^2) using the software Image Tool, version 3.0 (University of Texas Health Science Center, San Antonio, TX). The mean number of cells per unit area (cells/mm^2) was obtained. The S and NS groups were compared according to inflammatory infiltrate density.

Immunohistochemistry and cell counts

An IHC reaction was performed using the streptavidin-biotin standard protocol. Anti-Factor XIIIa and anti-CD1a are considered markers for immature DCs. However, only anti-CD1a is considered a marker of Langerhans cells (LC) located in the epithelium (Caux et al., 1996). Anti-CD83 is considered a marker for mature DCs (Lechmann et al., 2002). Serial sections of 3 μm in thickness from paraffin-embedded blocks were deparaffinized and dehydrated. Antigen retrieval was carried out using a Dewaxing & Antigen Retrieval Buffer 4, pH 9.0 (Spring Bioscience, Pleasanton, CA, USA) for 12 min at 98 °C, for anti-CD1a and anti-CD83. Antigen retrieval was carried out with a 10-mM citrate buffer (Laboratory Synth, Diadema, SP, Brazil), pH = 6.0, for 20 min at 98 °C for anti-Factor XIIIa. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. Primary antibodies were incubated at room temperature for 1 h. The following monoclonal antibodies were used: anti-CD1a (clone MTB1; BioSB, Santa Barbara, CA, USA) at a dilution of 1:20; anti-Factor XIIIa (clone AC-1A1; BioSB, Santa Barbara, CA, USA) at a dilution of 1:500; and anti-CD83 (clone 1H4b; Abcam, Cambridge, UK) at a dilution of 1:100. Detection was performed using the Advance HRP (Dako, Carpinteria, CA, EUA) for anti-CD1a, the LSAB system (Dako, Carpinteria, CA, EUA) for anti-FatorXIIIa, and the Reveal system (Spring bioscience, Pleasanton, CA, USA) for anti-CD83. In addition, 3,3′-diaminobenzidine tetrahydrochloridechromogen (DAB, Sigma–Aldrich, St. Louis, MO) and Mayer hematoxylin was used for counter-staining.

Densities of immunolabeled cells (cells/mm^2) were calculated for anti-Factor XIIIa, anti-CD1a, and anti-CD83. Positive cell counts were restricted to immunolabeled cells that exhibited well-defined cell nuclei. The slices were digitized with a microscope (AxioScoup A1, Zeiss, Göttingen, Germany) at a magnification of 400× and interfaced to a computer. Cell counts were performed throughout the sections by a blinded examiner (GRS). Areas were delineated using a mouse and measured using the software AxioVision (version 4.8, Zeiss). Factor XIIIa+ immature, CD1a+ immature, and cells CD83+ mature DCs were counted in the oral epithelium (OE), sulcular epithelium (SE), and LP regions (Fig. 1A). The densities of Factor XIIIa+ immature, CD1a+ immature, and CD83+ mature DCs were compared between the S and NS groups.

Detection of tissue cytokines

Multiple gingival tissue cytokines (TNF-α, IL-6, IL-2, IFN-γ, IL-4, IL-10, and IL-17A) were simultaneously measured by flow cytometry using the CBA Human Th1/Th2/Th17 kit (BD Biosciences, San Jose, CA, USA) following manufacturer instructions. Acquisition was performed with a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). The instrument has been checked for

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