Human Immunology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Human Immunology

journal homepage: www.elsevier.com/locate/humimm

Overexpression of immunomodulatory mediators in oral precancerous lesions

Andréia Souza Gonçalves[a], Carla Mosconi[a], Filipe Jaeger[b], Isabela Jubé Wastowski[c], Maria Cássia Ferreira Aguiar[b], Tarcília Aparecida Silva[b], Rejane Faria Ribeiro-Rotta[d], Nádia Lago Costa[d], Aline Carvalho Batista[a,*]

[a] Department of Stomatology (Oral Pathology), Dental School, Federal University of Goiás, Goiânia, Brazil
[b] Department of Oral Surgery and Pathology, Dental School, Federal University of Minas Gerais, Belo Horizonte, Brazil
[c] Department of Biology, State University of Goiás, Morrinhos, Goiás, Brazil
[d] Department of Stomatology (Oral Medicine), Dental School, Federal University of Goiás, Goiânia, Brazil

ARTICLE INFO

Keywords:
Oral leucoplakia
Immune evasion
HLA-G
Programmed cell death 1 ligand 1
Oral cancer

ABSTRACT

Human leukocyte antigen (HLA) G and E, programmed cell death 1 ligand 1 (PD-L1), IL-10 and TGF-β are proteins involved in failure of the antitumor immune response. We investigated the expression of these immunomodulatory mediators in oral precancerous lesions (oral leukoplakia-OL; n = 80) and whether these molecules were related to the risk of malignant transformation. Samples of normal mucosa (n = 20) and oral squamous cells carcinoma (OSCC, n = 20) were included as controls. Tissue and saliva samples were analyzed by immunohistochemistry and ELISA respectively. Fifteen OL samples showed severe dysplasia (18.7%) and 40 samples (50%) presented combined high Ki-67/p53. Irrespective of the degree of epithelial dysplasia and the proliferation/apoptosis index of OL, the expression of HLA-G, -E, PD-L1, IL-10, TGF-β2 and -β3 was higher to control ($P < 0.05$) and similar to OSCC ($P > 0.05$). The number of granzyme B^+ cells in OL was similar to control ($P = 0.28$) and lower compared to OSCC ($P < 0.01$). Salivary concentrations of sHLA-G, IL-10 and TGF-β did not allow for a distinction between OL and healthy individuals. Overexpression of immunosuppressive mediators in the OL reflects the immune evasion potential of this lesion, which is apparently independent of at cytological and proliferation/apoptosis status.

1. Introduction

The frequency of the malignant transformation of oral leukoplakia (OL), the most common oral precancerous lesions (OPL), ranging from 0.13% to 34% (average 14.9%) [1,2]. Clinicians arbitrarily consider the clinical characteristics, size, location and severity of the dysplastic changes in the epithelium as parameters for predicting the risk of malignant transformation of these pathologies [1–3]. In addition, alterations in the proteins involved in cell proliferation and apoptosis have been pointed out in some studies as molecular parameters indicative of OL malignancy potential [4–6]. However, the mechanisms involved in the development and malignant transformation of the OL have not been clarified yet.

The malignant transformation of an OPL is a complex process called carcinogenesis, which culminates in the multiplication of a mutated or dysplastic cell, and thus, in the appearance of a malignant phenotype [7]. The immune system can prevent the process of malignant progression through the migration of antigen-presenting cells, cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells to the lesion site [7–11]. This process effectively occurs via the activation of these immune cells and consequent release of perforin and granzyme B (GB) [8]. Then, the escape of mutated cells from immunosurveillance is an

Abbreviations: HLA-G, human leukocyte antigen G; HLA-E, human leukocyte antigen E; PD-L1, programmed cell death 1 ligand 1; IL-10, interleukin-10; TGF-β, transforming growth factor beta; sHLA-G, soluble HLA-G; OL, oral leukoplakia; GB, granzyme B; OPL, oral precancerous lesions; CTLs, cytotoxic T lymphocytes; NK, natural killer; MHC, human major histocompatibility complex; PD-1, programmed cell death-1; OSCC, oral squamous cell carcinoma; SCC, squamous cell carcinoma; WHO, World Health Organization; IHC, immunohistochemistry; ELISA, enzyme-linked immunosorbent assay; UFG, Federal Universities of Goiás; UFMG, Federal Universities of Minas Gerais; H & E, hematoxylin-eosin; IRS, immunoreactive score; IQR, interquartile range; SPSS, Statistical Package for the Social Sciences

* Corresponding author at: Disciplina de Patologia Geral e Bucal, Faculdade de Odontologia, Universidade Federal de Goiás (FO/UFG), Praça Universitária S/N, Setor Leste Universitário, CEP: 74605-220, Brazil.

E-mail addresses: andreia.sg.odonto@gmail.com (A.S. Gonçalves), carlamosconi@gmail.com (C. Mosconi), filipejaeger@gmail.com (F. Jaeger), wastowski@gmail.com (I.J. Wastowski), cassiafaster@gmail.com (M.C.F. Aguiar), tarcilia@odonto.ufmg.br (T.A. Silva), rejanefrr@gmail.com (R.F. Ribeiro-Rotta), nadialago@hotmail.com (N.L. Costa), ali.caba@uol.com.br (A.C. Batista).

http://dx.doi.org/10.1016/j.humimm.2017.09.003
Received 30 April 2017; Received in revised form 5 September 2017; Accepted 19 September 2017

important factor in the development of a neoplasm [9,10].

Human leukocyte antigen G and E (HLA-G and -E), programmed cell death 1 ligand 1 (PD-L1), interleukin-10 (IL-10) and transforming growth factor beta (TGF-β) are immunoinhibitory molecules involved in the failure of the antitumor immune response [12–15]. These molecules can facilitate the proliferation of mutated cells, tumor progression and, consequently, to influence to tumor size, metastasis or reduced survival of patients affected by the cancer [9,10,16–20]. HLA-G and HLA-E are non-classical class I molecules of the human major histocompatibility complex (MHC) which inhibit the cytolytic activity of CTLs and NK, effector immune cells involved in immunity against tumors [12,13]. PD-L1, also known as B7-H1 or CD274, is a molecule which binds to the PD-1 receptor and negatively shapes the antitumor immune response by inhibiting CTL activity [15]. IL-10 and TGF-β are immunosuppressive and anti-inflammatory cytokines involved in tumor evasion and immunomodulation [14,21].

Another great advantage of investigating these molecules is that they have become direct or indirect targets of immunotherapy [22–25], with promising clinical outcomes for the treatment of oral squamous cell carcinoma (OSCC) [25–27]. Thus, considering the immunotherapeutic potential of these proteins and the scientific relevance of our previous findings on antitumor immunity and evasion mechanisms in OSCC [17,20,28–30], the present study was proposed with a view to contributing data to provide a better understanding of the immunoinhibitory mechanisms underlying the development of an OPL, lesions which could precede oral cancer.

Therefore, this study set out to evaluate the expression of HLA-G, -E, PD-L1, IL-10 and TGF-β in OL. Expression of each marker was related to the degree of epithelial dysplasia (WHO and binary grading system), the proliferation/apoptosis index and to the number of GB^+ cells.

2. Materials and methods

2.1. Patient selection

This cross-sectional study was approved by the Research Ethics Committees at the Federal Universities of Goiás (UFG) (CAAE 35868214.6.0000; 883.912 and 032/2011) and Minas Gerais (UFMG) (087/2007).

Patients with OL who underwent incisional biopsy between 2011 and 2016 in the Goiás Oral Disease Center and in the Oral Medicine Clinic of the Schools of Dentistry at UFG and UFMG were enrolled in the study. Out of a total of 80 samples of OL were included. Saliva was collected of 32 patients with OL. The control group (n = 20) consisted of saliva from healthy individuals and tissue from clinically and histologically normal oral mucosa obtained from biopsies of gingival mucosa associated with totally impacted third molars and the lateral border of the tongue derived from the area adjacent to oral pigmentation. All participants signed an informed consent form.

Patients with OL and controls underwent a detailed anamnesis and physical examination and the ones who used corticosteroids and non-steroidal anti-inflammatory drugs, with history of OSCC, signs of significant morbidity or active health problems such as autoimmune disease, HIV infection, abnormal renal function, congestive heart failure, active infection and hepatitis were excluded from the study.

Furthermore, OSCC samples (n = 20) were obtained from files in the Division of Anatomopathology and Cytopathology at Araújo Jorge Hospital, Goiás Cancer Combat Association, Goiânia, Brazil.

Clinical and demographic data such as age, gender, ethnicity, smoking and alcohol consumption habits were obtained of the all patients included in the study. Additionally, follow-up information including malignant transformation (for the samples of OL), metastasis, survival time and death data (for the samples of OSCC) were obtained in medical records.

2.2. Tissue samples

Microscopic characteristics were assessed by analyzing one 5 μm section of each sample stained with hematoxylin-eosin (H & E). OLs were assessed and graded based on the criteria described by the World Health Organization (WHO) (severe, moderate, mild epithelial dysplasia or no dysplasia) [31] and by the Kujan et al. [32] -binary system (high/low-risk of malignant transformation). OCSCC sections were graded according to the WHO classification of tumors [31]. The intensity of the inflammatory infiltrate (mononuclear immune-inflammatory cells/mm^2) was also evaluated. Two experienced oral pathologists independently graded epithelial dysplasia and performed the classification of each sample. Discordant cases were discussed in a face-to-face meeting using a microscope with one observer and four co-observers (AxioScope. A1, Carl Zeiss).

2.3. Immunohistochemistry (IHC)

Immunohistochemistry was performed as previously described by Gonçalves et al. [29,30] and Katsuya et al. [18] but with some modifications. The slides were incubated overnight with the following monoclonal mouse or rabbit anti-human primary antibodies: HLA-G (clone MEM-G/2, Exbio, Prague, Czech Republic, dilution 1:100), HLA-E (clone MEM-E/02, Exbio, dilution 1:50), PD-L1 (Clone E1L3N®, Cell Signaling Technology, Danvers, MA, USA, dilution 1:400), IL-10 (Abcam Inc., ab34843, Cambridge, MA, USA, dilution 1:300), TGF-β1 (clone sc-52893, Santa Cruz Biotechnology, Santa Cruz, CA, USA, dilution 1:50), TGF-β2 (clone sc-90, Santa Cruz Biotechnology, dilution 1:100), Ki-67 (clone MIB-1, Dako, Carpinteria, CA, USA, dilution 1:100), p53 (clone DO-7, Novocastra, Newcastle, UK, dilution 1:200), GB (clone GrB-7, Dako, dilution 1:100) and polyclonal rabbit anti-human TGF-β3 (clone sc-83, Santa Cruz Biotechnology, dilution 1:50). The Novolink™ Max Polymer Detection System was employed (Product No: RE7280-K, Novocastra, Newcastle, UK). Trophoblast samples were used as positive controls for HLA-G, HLA-E and PD-L1; OSCC samples were used for IL-10, OL samples were used for Ki-67 and p53; gastric cancer samples were used for TGF-β1, -β2 and -β3; lichen planus were used for GB (Supplementary file).

2.4. Evaluation of stained sections by IHC

HLA-G, HLA-E, PD-L1, IL-10, TGF-β1, -β2 and -β3 proteins were evaluated in epithelial cells (keratinocytes) and adjacent connective tissue cells (immune-inflammatory, endothelial cells and fibroblasts). In OSCC, these proteins were evaluated in the tumor invasion front and in the stroma. A semiquantitative method was used, according to Gonçalves et al. [17], where the immunoreactive score (IRS) was calculated by multiplying percentage of positive cells (PP) (stained 0–2) by staining intensity (SI) (stained 0–3). The PP was scored as follows: 0, no tumor cells stained; 1, < 25% of cells stained; and 2, ≥25% of cells stained and SI was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. In lesions with an IRS = 0, the expression of HLA-G, HLA-E, PD-L1, IL-10, TGF-β1, -β2 and -β3 was considered absent, while for an IRS ≤2 it was lower, and those lesions with an IRS > 2 showed higher expression. The SI was compared to the respective positive controls (trophoblast for HLAs and PD-L1; gastric cancer for TGF-β isoforms and OSCC for IL-10) (Supplementary file).

The number of the GB^+ cells and mononuclear immune-inflammatory cells (per mm^2) in the subepithelial region were evaluated. The morphometric analysis was undertaken in ten alternate microscopic fields (×400) using an integration graticule (4740680000000-Netzmikrometer ×12.5; Carl Zeiss) where, at that magnification, each field had an area of 0.0961 mm^2.

For evaluation of Ki-67 and mutated/wild type p53 proteins, 1000 cells (positive and negative) from the basal and parabasal layer were initially counted in five alternate microscopic fields (×400).