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Immunohistochemical expression of myofibroblasts, TGF-β1 and IFN-γ in oral fibrous lesions

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ABSTRACT

Objective: Analyze the presence of myofibroblasts (MFBs) in oral fibrous lesions and investigate TGF-β1 and IFN-γ expression by immunohistochemistry during their differentiation.
Design: Twenty giant cell fibromas (GCFs), 20 fibromas (FIBs), and 20 fibrous hyperplasias (FHs) were selected. To evaluate the presence of MFBs, anti-α-SMA-immunoreactive cells were quantified in connective tissue. TGF-β1 and IFN-γ expressions were evaluated in epithelial and connective tissue by determining the percentage of immunoreactive cells.
Results: Higher MFBs concentrations were observed in GCFs (median of 20.00), followed by FHs (15.00) and FIBs (14.00) ($P = 0.072$). No significant correlation between TGF-β1 or IFN-γ immunoexpression and the number of MFBs in oral fibrous lesions was observed ($P > 0.05$).
Conclusions: The higher density of MFBs found in GCFs, followed by FHs and FIBs, reaffirms the fibrogenic role of these cells, while the higher concentrations detected in GCFs, including evidence of giant MFBs, also suggest a role in the neoplastic behavior of these lesions. No correlation was observed between TGF-β1 and IFN-γ in the myofibroblastic transdifferentiation process of the analyzed lesions.

1. Introduction

Myofibroblasts (MFBs) are contractile cells that exhibit both fibroblast and smooth muscle cell features (Epivatianos, Andreadis, & Iordanidis, 2013). They can be distinguished according to specific cytoskeleton qualities, especially the expression of α-smooth muscle actin (α-SMA). MFBs assume a vital part in physiological remodeling, such as during dermal and wound healing (Hinz, 2016). Investigations propose that MFBs are the major producers of the extracellular matrix (ECM) in certain pathological situations, and increases in the amount of these cells has been described in different conditions, including in malignant tumors (Angadi, Kale, & Hallikerimath, 2011).

Particular growth components appear to drive the transdifferentiation of fibroblasts and other cell types into MFBs, and the role of transforming growth factor β1 (TGF-β1) as the main stimulator of this transdifferentiation has been reported (Thode, Jørgensen, Dabelsteen, Mackenzie, & Dabelsteen, 2011). On the other hand, interferon gamma (IFN-γ) inhibits this transformation, and its antagonistic action towards TGF-β1 decreases the amount of MFBs (Dooley et al., 2006; Hoffmann, Sturm, Stein, & Dignass, 2011).

Giant cell fibromas (GCF), or fibroblastomas, were first described by Weathers and Callihan (1974), and correspond to a benign neoplasm of mesenchymal origin, characterized by focal growth of the oral mucosa, with no association to chronic trauma, in which the presence of mono- or multinucleated giant cells is evidenced in connective tissue (Sonalika et al., 2014).

Fibroma (FIB) represents a tumor of a conjunctive nature originating from fibroblast proliferation, and is the most common soft tissue neoplasia in the oral cavity (Mishra, Khan, Ajaz, & Agarwal, 2017). Microscopically, it is composed of fibrous connective tissue, usually dense and collagenated. However, connective tissue of a looser nature can be observed in some cases, usually well delimited and free of

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inflammatory cells (Miguel, Andrade, Rocha, Freitas, & Souza, 2003; Patil, Rao, Sharath, & Agarwal, 2014).

Fibrous hyperplasia (FH) is considered a focal reactive growth that appears around the margins or borders of total or partially removable mal-adapted prosthetics and is related to chronic irritation caused by these and other oblique forces resulting from occlusal imbalances (Kiuchi et al., 2013; Mozzati, Mortellaro, Gallesio, Ruggiero, & Pol, 2015). Microscopically, it is characterized by a stratified, often hyperplastic, keratinized squamous epithelium, alternating hyperkeratosis and paraceratosis areas. The connective tissue varies according to the development stage of the lesion, presenting an aspect similar to a granulation reaction in young lesions and a dense and fibrous presentation in older lesions (Casian Romero, Trejo Quiroz, De León Torres, & Carmona Ruiz, 2011; Miguel et al., 2003).

In this context, the aim of the present study was to quantify MFBs in selected oral fibrous lesions by analyzing their α-SMA immunohistochemical expression and to evaluate TGF-β1 and IFN-γ expressions as MFBs stimulators and inhibitors, respectively, during cell transdifferentiation.

2. Material and methods

Sixty samples, comprising 20 GCFs, 20 FIBs, and 20 FHs, obtained from the Oral Pathology Department of the Federal University of Rio Grande do Norte (UFRN), were randomly selected for this study. This study was approved by the UFRN Research Ethics Committee, Natal, Brazil (Protocol No. 159/2010).

2.1. Immunohistochemistry

Histological sections (3 μm) were cut from paraffin-embedded tissue blocks. The tissue sections were deparaffinized and immersed in 3% hydrogen peroxide to block endogenous peroxidase activity. The sections were then washed in phosphate-buffered saline (PBS). Antigen retrieval, antibody dilution, and clone types for α-SMA, TGF-β1 and IFN-γ are displayed in Table 1. After preparation with normal serum, the sections were incubated with the primary antibodies in a moist chamber. Subsequently, the sections were washed twice in PBS and treated at room temperature with the labeled streptavidin biotin complex (LSAB + System-HRP; Dako, Carpinteria, CA, USA) for the anti-α-SMA antibody and with a polymer-based complex (Advance™ HRP; Dako) for the anti-TGF-β1 and anti-IFN-γ antibodies. Diaminobenzidine (Liquid DAB + Substrate; Dako) was used to visualize peroxidase activity, which resulted in a brown reaction product. Finally, the sections were counterstained with Mayer's hematoxylin and coverslipped. Sections of lobular capillary hemangioma were used as positive control for the anti-α-SMA antibody, and sections of human breast carcinoma and CCRF-CEM (human T-cell lymphoblast-like cell line) cell lysate served as positive controls for the anti-TGF-β1 and anti-IFN-γ antibodies, respectively. As a negative control (Fig. 1), the samples were treated as described above, except that the primary antibody was replaced with bovine serum albumin in PBS. To validate the antibodies used, normal oral mucosa fragments were included in our study.

2.2. Immunohistochemical analyses

Alpha-SMA-positive cells were evaluated according to the method proposed by Vered, Shohat, Buchner, and Dayan (2005). Tissue sections were inspected by light microscopy at ×100 magnification in order to recognize 5 fields in the superficial and 5 fields in the deep connective tissue of the oral fibrous lesions. In these fields, α-SMA-positive cells, excluding those from surrounding blood vessels, were counted at ×400 magnification. Subsequently, the total number of positive cells in all 10 fields examined per case was calculated to obtain the mean number of α-SMA-positive cells per field.

TGF-β1 and IFN-γ immunoexpressions were assessed in epithelial and connective tissue (superficial and deep) cells of all oral fibrous lesions. The amount of positive and negative cells was determined in each field at ×400 magnification and the percentage of TGF-β1- and IFN-γ-positive cells was calculated for each case. The percentage adopted herein followed the study conducted by Nonaka, Cavalcante, Nogueira, de Souza, and Pinto (2012): 0 (≤10% immunostained cells), 1 (11–25% immunostained cells), 2 (26–50% immunostained cells), 3 (51–75% immunostained cells), and 4 (> 75% immunostained cells).

2.3. Statistical analyses

The results were submitted to statistical analyses using the IBM SPSS Statistics program (version 20.0; IBM Corp., Armonk, NY, USA). The nonparametric Kruskal-Wallis (KW) test was used to compare the median number of MFBs and the percentage of TGF-β1- and IFN-γ-immunopositive cells between GCFs, FIBs and FHs. Spearman's correlation test was applied to verify possible correlations between the amount of MFBs and percentages of TGF-β1- and IFN-γ-immunopositive cells. A level of significance of 5% ($P < 0.05$) was adopted for all tests.

3. Results

Investigation of α-SMA expression by immunohistochemistry revealed the presence of MFBs in all GCFs, FIBs and FHs (Fig. 2). The median number of MFBs in all connective tissue was 20.00 in GCFs, 15.00 in FHs, and 14.00 in FIBs (Fig. 3). The median number of MFBs was higher in GCFs when compared to FHs and FBs ($P = 0.072$). In superficial connective tissue, the median number of MFBs was 11.00 in GCFs, 7.00 in FIBs and 4.00 in FHs. The median for MFBs in deep connective tissue in FHs was 7.00, 6.50 in GCFs and 6.00 in FIBs. The median number for MFBs in superficial tissue ($P = 0.033$) was higher in GCFs, while the median for deep connective tissue ($P = 0.637$) was higher in FHs (Table 2). Giant MFBs detected here in GCFs (Fig. 4).

All GCFs, FIBs and HFs displayed TGF-β1 immunoexpression in the epithelial component (Fig. 5). No statistically significant differences between groups ($P = 0.555$) were observed by the nonparametric KW test (Fig. 6). TGF-β1 in connective tissue was expressed in all groups (Fig. 5). No significant TGF-β1 expression in all connective tissue (Fig. 6) or in superficial or deep connective tissue (Table 3) was observed in GCFs compared to FIBs and FHs.

The epithelial component expressed IFN-γ in all GCFs, FIBs, and FHs (Fig. 7). FHs displayed significantly higher epithelial IFN-γ expression (Fig. 6) when compared to FHs and FIBs ($P = 0.001$). In connective

Table 1
Clone, specificity, company, dilution, antigen retrieval and incubation of the primary antibodies.

Clone	Specificity	Manufacturer	Dilution	Antigen retrieval	Incubation
α-sm1	α-SMA	Novocastra Laboratories, Benton Lane, NET	1:50	Citrate, pH 6.0 Pascal, 3 min	60 min
sc-146	TGF-β1	Santa Cruz Biotechnology, Santa Cruz, CA	1:4000	Pepsin, pH 1.8 Oven, 37 °C, 60 min	18 h
sc-8308	IFN-γ	Santa Cruz Biotechnology, Santa Cruz, CA	1:400	Pepsin, pH 1.8 Oven, 37 °C, 60 min	18 h