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Immunoexpression of PCNA and α-SMA in gingival overgrowth tissues

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Abstract

Purpose: This study aimed to investigate proliferating cell nuclear antigen (PCNA) and isoform-α of the smooth muscle actin (α-SMA) immunoexpression, as well as the epithelial thickness in drug-induced gingival overgrowth (DIGO) and idiopathic gingival fibromatosis (IGF) patients.

Methods: Gingival samples were obtained from 11 users of nifedipine and phenytoin, 6 of patients with IGF and 4 fibrous inflammatory hyperplasia. The specimens were submitted to immunohistochemical and morphological analysis.

Results: Our results show that PCNA-epithelial positive cells were slightly more common among IGF patients, but there were not statistically significant differences among the groups ($p>0.05$). Comparing the vessels counts with α-SMA-positive pericyte or smooth muscular cells, there was not statistical differences, although the control group presented a discrete higher count ($p>0.05$). The epithelial thickness analysis revealed that the DIGO group presented the higher mean, evidencing statistically significant differences in relation to the control group ($p=0,002$).

Conclusion: In conclusion, our results demonstrated similarities between DIGO and IGF according to PCNA and α-SMA immunoexpression, although the epithelial thickness was higher in DIGO group.

Key words: Gingival overgrowth; Phenytoin; Nifedipine; Gingival fibromatosis

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Imunoexpressão de PCNA e α-SMA em tecidos de crescimento gengival

Resumo

Objetivo: Este estudo avaliou a imunoexpressão do antígeno nuclear de proliferação celular (PCNA) e da isoforma-α da actina de músculo liso (α-SMA), bem como a espessura epitelial em pacientes com crescimento gengival induzido por drogas (DIGO) e fibromatose gengival idiopática (IGF).

Métodos: As amostras gengivais foram obtidas a partir de 11 usuários de nifedipina e fenitoína, 6 de pacientes com IGF e 4 com hiperplasia fibrosa inflamatória. Os espécimes foram submetidos à análise imuno-histoquímica e morfológica.

Resultados: Os resultados demonstraram que as células epiteliais PCNA-positivas foram ligeiramente mais comuns em pacientes com IGF, mas não houve diferenças estatisticamente significativas entre os grupos estudados ($p>0.05$). Comparando-se os vasos contados com pericitos ou células musculares lisas α-SMA-positivas, não houve diferenças estatísticas, embora o grupo controle tenha apresentado uma contagem discretamente superior ($p>0,05$). A análise da espessura epitelial revelou que o grupo DIGO apresentou a maior média, evidenciando diferenças estatisticamente significantes em relação ao grupo controle ($p=0,002$).

Conclusão: Em conclusão, os resultados demonstraram semelhanças entre DIGO e IGF de acordo com a imunoexpressão de PCNA e α-SMA; entretanto, a espessura epitelial foi maior no grupo DIGO.

Palavras-chave: Crescimento gengival; Fenitoína; Nifedipina; Fibromatose gengival

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Introduction

Gingival enlargement is a clinical condition induced by drugs, genetic diseases, or by inflammatory, neoplastic and idiopathic causes. Regarding the gingival enlargement caused by drugs, calcium antagonists, cyclosporine and anti-seizure drugs are largely known as responsible for the connective tissue overgrowth, with collagen deposition [1,2]. Calcium antagonists are widely used for the treatment of cardiovascular diseases in hypertensive or dialytic patients [1].

Nifedipine, a calcium channel blocker, is used as a long-acting vasodilator. However, one of its side effects is gingival overgrowth, characterized by an accumulation of collagen components within the gingival connective tissues and epithelial overgrowth [3,4]. The prevalence of clinically significant overgrowth associated with nifedipine is about 6% of users, affecting mainly males, being to linked inflammation [5].

Various degrees of gingival enlargement may also occur in phenytoin users, a drug used widely to treat epilepsy. The potential of phenytoin to induce gingival hyperplasia has been well established. High levels of dental plaque and calculus have been reported as critical co-factors for the development and severity of phenytoin-induced gingival enlargement. Phenytoin-induced gingival overgrowth is a very fibrotic tissue, meaning that it contains the highest proportion of fibroblastic cells and connective tissue fibers [6-8].

Another condition characterized by enlargement of the oral gingival tissues is the idiopathic gingival fibromatosis (IGF), a benign oral disorder that has no definite cause. Clinical examination does not reveal increased periodontal pocket depths, and plaque and gingival index scores are normal. The patients are systemically healthy and not subjected to medications, which could lead to gingival hyperplasia. Additionally, the clinical appearance of the lesions does not show any signs of trauma and, sometimes, generalized gingival overgrowth may occur in both arches at birth [9,10].

Histologically, in addition to fibrosis in the lamina propria,gingival overgrowth tissues can also be associated with thickening of epithelium and elongated rete pegs [2,11]. Proliferating cell nuclear antigen (PCNA), considered a good indicator of cell proliferation, is a 36-kDa acidic non-histone nuclear protein, with an important function in DNA synthesis [2,12]. The concentration of this marker is directly correlated with the proliferative state of the cell, increasing through G1 phase, peaking at the G1/S phase interface, decreasing through G2 phase, and reaching low levels in M-phase and interphase [2,13].

Myofibroblasts are mesenchymal cells that exhibit an intermediate hybrid phenotype between fibroblasts and smooth muscle cells [14]. These specialized cells have been demonstrated to play a role in synthesis and degradation of extracellular matrix components during inflammation, tissue repair and remodeling [15]. The expression of the specific isoform-α of the smooth muscle actin (α-SMA), a cytoskeletal protein that is the major constituent of the contractile system of smooth muscle cells, is considered as the myofibroblastic phenotype marker [16]. A previous report showed a significant increase in the expression of the myofibroblast marker α-SMA in cells from a family with hereditary gingival fibromatosis [17].

In this context, the aim of this study was to investigate the relationship between PCNA and α-SMA immunoexpression, as well as the epithelial thickness in DIGO and IGF patients.

Material and Methods

Gingival samples

Gingival tissue samples were taken from 11 users diagnosed with nifedipine- or phenytoin-induced gingival enlargement with at least with level two of overgrowth (6 men and 5 women; mean age, 44.65±9.36 years) (DIGO-Group I). Among them, six were users of nifedipine and five of phenytoin, exclusively. These samples of overgrown gingival tissue were obtained during gingivectomy procedures after oral care orientation, calculus removal and plaque control. Six individuals, with IGF (5 women and 1 man; mean age, 26.16±3.12 years), formed the Group II. In the Group II, patients did not use drugs associated with gingival enlargement and reported the history of ascendants and descendants with gingival enlargement. The most affected sites by gingival enlargement in this group were tuberosity (5 cases), and lingual gingiva (1 case). In all cases, there was not any relation between tissue enlargement and inflammatory processes. Tissue biopsies taken from lesions of four healthy participants affected by non-neoplastic proliferative processes were includes as control group (Group III) (three women and one man; mean age, 66.25±16.45 years). The histopathologic examination revealed in this group, 4 fibrous inflammatory hyperplasia affecting alveolar ridge. The Ethical Committee of State University of Montes Claros approved the study protocol.

Immunohistochemistry and Morphometry

Immunostaining was performed using 3-µm sections of paraffin-embedded of all gingival samples, fixed in 10% buffered formalin. All reactions followed standard protocols. Sections were deparaffinized and submitted to 10% ammonia hydroxide in 95% ethanol during ten minutes. Antigen retrieval was obtained by 10 mM citric acid digestion, pH 6.0, using 3 cycles of 6 min in a microwave. After that, the slides were transferred to 10V H2O2, twice for 15 minutes and incubated overnight with primary antibody for PCNA (Clone P-10, Dako – Carpenteria, CA, USA, 1:3,000), and α-SMA (clone1A4, Dako – Glostrup, Denmark, 1:200) followed by LSAB-HRP Dako – Carpenteria, CA, USA). Reactions were developed with 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma, St Louis, USA) and counterstained with hematoxylin. Cytoplasmic and membranous labeling was considered as positive. For analyzing PCNA immunoexpression, both positive and

negative stained cells were counted in five sampled high-power fields (X1000 magnification), located immediately below the epithelial basal stratum, including parabasal layer. The percentage of positive cells was calculated based on total number of counted cells [18]. The same methodology was employed to verify α-SMA positive fibroblasts dispersed in the sub-epithelial connective tissue. In the X400 magnification, the count of vessels positive to α-SMA pericyte cells was carried out, by using five fields per slide. Furthermore, the measure of epithelial thickness was also performed at 100X magnification, using three references in the borders of epithelial tissue in each slide. The counts and measures were performed using the software Nikon NIS-Elements-2.35 (Nikon Corporation – Melville, USA), using the square with 34.1% of magnification.

Statistical Analysis

The statistical analysis was performed using the non-parametrical Wilcoxon Signed Rank Test (SPSS 18.0, Software – Chicago, USA).

Results

In the immunohistochemical analysis, the data obtained from counts of positive PCNA cells, labeled vessels counted in the slides were tabulated, and the descriptive analysis is shown in Table 1. It was observed that PCNA-positive epithelial cells were slightly more common in the Group II, but there were not statistical differences in comparison with the other groups (Group I-Group II, p=0,715; Group I-Group III, p=0,173; and Group II-III, p=0,465). Furthermore, in all groups, the fibroblasts were negative to α-SMA labeling. Comparing the counts of vessels with α-SMA positive pericyte or smooth muscular cells, there were not statistical differences between the groups, although the Group III presented discrete higher counts (Group I-Group II, p=0,513; Group I-Group III, p=0,06; and Group II-III, p=0,107). The Figures 1 and 2 illustrate these findings. When the epithelial thickness analysis was performed, the Group I presented the higher mean comparing with other two groups with evident statistical differences (Group I-Group II, p=0,012; Group I-Group III, p=0,002; and Group II-III, p=0,182). The Table 2 shows these series of results.

Table 1. PCNA epithelial immunopositive cells and a-SMA labeling vessels according to groups.

Group	PCNA positive cells (%) mean	a-SMA vessels mean
I (DIGO)	48.5436[a], ±17.16060	8.6545[a], ±5.21472
II (IGF)	68.2633[a], ±18.35886	9.0333[a], ±4.05551
III (Control)	49.8275[a], ±18.06702	13.9500[a], ±6.17699

Numbers are the arithmetic means ± standard error means
[a]No statistically significant differences (p>0.05).

Table 2. Measures of mean of thickness according to groups

Group	Thickness mean
I (DIGO)	467.8270[a], ±120.96980
II (IGF)	393.5894[b], ±75.83236
III (Control)	296,0783[b], ±106.00089

Numbers are the arithmetic means ± standard error means
[a] No statistically significant differences (p>0.05).
[b] Statistically significant differences (p<0.001).

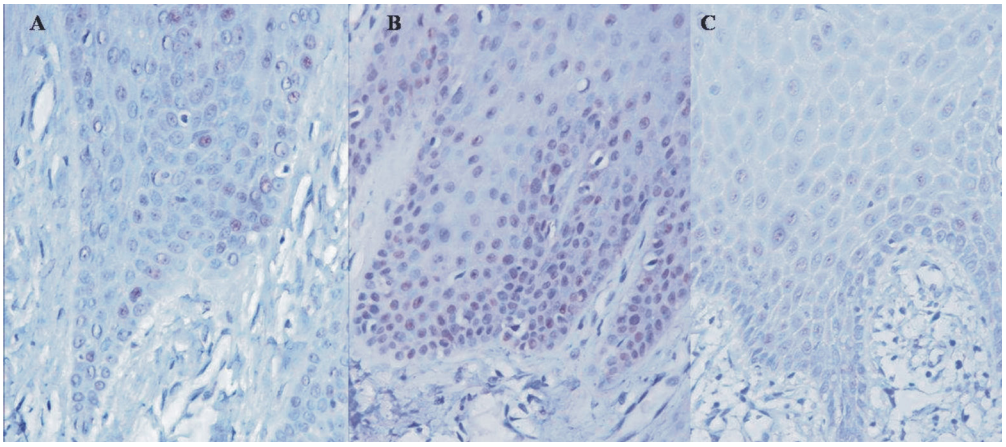

Figure 1. Representative photomicrographs of immunohistochemical staining of PCNA in epithelial tissue in Group I (DIGO) (A), II (IGF) (B), and III (control) (C).

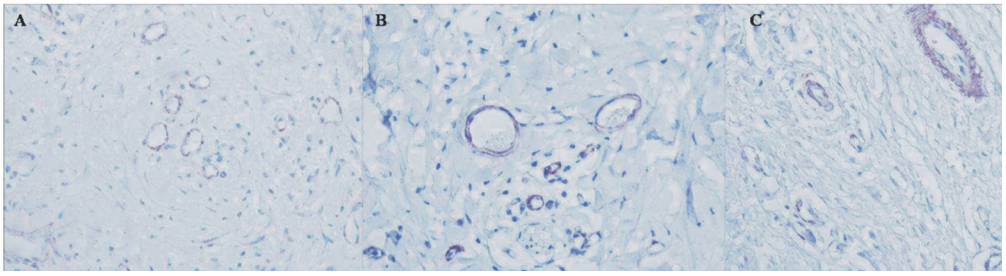

Figure 2. Representative photomicrographs of immunohistochemical staining of a-SMA in Group I (DIGO) (A), II (IGF) (B), and III (control) (C).

Discussion

The development of gingival overgrowth can be caused by three reasons: 1) inherited (recognized as hereditary gingival fibromatosis – HGF); 2) induced as a side effect of systemic drugs, such as phenytoin, cyclosporin A, or nifedipine; or 3) idiopathic (idiopathic gingival fibromatosis) [19]. Although over the past few years increasing efforts have been made to understand the pathogenesis of gingival overgrowth, these mechanisms remain unclear and more studies are needed. In this perspective, the present study evaluated PCNA and α-SMA immunoexpression, as well as the epithelial thickness in DIGO and IGF patients.

PCNA-positive cells counts revealed similarities among the three groups. In a previous study, which described PCNA immunoreactivity in the oral epithelium of cyclosporine users, there were no differences between the cyclosporin group and the control group (normal gingiva) regarding the immunoexpression of this marker and the epithelial thickness. However, the same study emphasized that inflammation may contribute to an increase of the proliferative activity in oral gingival epithelium [2]. In our study, control group specimens of inflammatory fibrous hyperplasia were used, an inflammatory tissue which may naturally show a higher percentage of proliferation than normal tissue.

Curiously, in our study, the DIGO group (I) presented, in mean, more evident epithelial thickening than the other groups. The mechanisms of DIGO are not entirely clear. As a common histological alteration, all forms of DIGO have the increase of connective tissue extracellular matrix, which could be either a result of an increased production of extracellular matrix proteins, mainly collagen, or a misbalance in the connective tissue turnover or both [20]. Moreover, it has been reported that increased epithelial thickness in DIGO patients might be related to the influence of connective tissue on epithelial architecture and cytodifferentation [21].

Kantarci et al. [7] previously showed that expression of CCN2, also known as connective tissue growth factor, correlates positively with the degree of gingival fibrosis, where phenytoin-induced gingival overgrowth specimens have the highest levels of both CCN2 and fibrosis. Furthermore, elevated levels of this marker were also seen in gingival epithelial cells, suggesting that CCN2 plays a role in both epithelial cell proliferation and in increased accumulation of collagen deposition and fibrosis in the connective tissue stroma. In a later study, Kantarci et al. [11] demonstrated that there are significantly higher numbers of basement membrane discontinuities in overgrowth tissues, sometimes containing epithelial-like cells. So, the authors suggested that these findings support the hypothesis that epithelial plasticity and epithelial to mesenchymal transition promote gingival overgrowth, resulting in compromised basal membrane structure and increased interactions between epithelial and connective tissue layers that contribute to fibrotic pathology.

Another important mechanism in the gingival overgrowth likely involves a reduction of the apoptotic index, as confirmed in relation to the fibroblasts. The inflammation led to an increase in apoptosis in 'non overgrowth' control gingiva, and the inflammation similarly appear to stimulate apoptosis within the context of gingival overgrowth, but to a lesser degree [7].

In an experimental study with rats, Cetinkaya et al. [22] suggested that cyclosporin A-induced gingival alterations are closely associated with increased epithelial proliferative activity, and dental plaque accumulation seems not to be essential, but it might aggravate the progression of the lesion. On the other hand, Castro et al. [23] indicated that the enlargement of epithelial rete pegs in patients undergoing chronic treatment with nifedipine does not occur either due to mitotic activity or because of inhibition of apoptosis in keratinocytes. Therefore, the authors suggested that the chronic use of this drug is not associated with subclinical changes in gingival tissue.

With respect to α-SMA immunoexpression, in both groups, fibroblasts were negative for this antibody, indicating the non-transdifferentiation to myofibroblast [24]. In a study with two families affected by HGF, α-SMA-positive cells were broadly detected in the gingival tissue samples from HGF patients of one of the families studied. In contrast, α-SMA expression by HGF cells was quite similar to normal gingival cells and no myofibroblasts were detected immunohistochemically in the specimens collected from individuals of another family. These findings might be explained due to the different biological mechanisms that may contribute for the gingival overgrowth [17]. *In vitro*, a previous report showed that TGF-β1 induced gingival fibroblast-myofibroblast transdifferentiation, whereas IFN-γ blocked this process [24]. On the other hand, connective tissue growth factor may also triggered this process but not in all cases. In DIGO and IGF, our report was the first in the literature that deal with α-SMA fibroblast immunostaining. It is necessary to perform new studies, even with *in vitro* approaches, to investigate if our findings are the general rule or if there are exceptions.

Hughes et al. [25] demonstrated that α-SMA immunohistochemistry labeling had been used to label mural cells and blood vessels. In this context, when blood vessels with positive α-SMA pericytes were counted, there were similarities in both studied groups and with the control. In fact, an inflammatory process was observed in these cases, even after oral care instructions and periodontal treatment in DIGO [7,8].

In conclusion, our results reveled similarities between DIGO and IGF patients according to PCNA and α-SMA immunoexpression, although the epithelial thickness was higher in DIGO group. The reasons for the differences between epithelial proliferation and apoptosis rates, in patients with gingival overgrowth, remain unclear. Therefore, we highlight the need for additional studies comparing DIGO and IGF patients.

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