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Original Article

Immunohistochemical Analysis of Cell Proliferation and Bcl-2 Expression in Drug-Induced Gingival Overgrowth

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Abstract

Objective: To compare the rate of cell proliferation and expression of antiapoptotic protein Bcl-2 between drug-induced gingival overgrowth (DIGO) and clinical healthy gingiva (CHG) and to establish associations with histopathological features. **Material and Methods:** Twenty specimens of DIGO and 20 CHG specimens were submitted to morphological and immunohistochemical analysis by light microscopy. Cell proliferation (Ki-67) and the expression of Bcl-2 were evaluated in epithelial cells and spindle-shaped mononuclear cells of the connective tissue by establishing the labeling index (LI). **Results:** In epithelial tissue, the mean LI for Ki-67 was 17.2% in DIGO and 21.71% in CHG ($p = 0.137$). The mean LIs for Bcl-2 in epithelial tissue were 14.67% and 10.24% in DIGO and CHG, respectively ($p = 0.026$). In connective tissue, DIGO and CHG specimens exhibited low LIs for Ki-67 and Bcl-2, with mean values of less than 0.5% in both groups. No significant differences in the LIs for Ki-67 or Bcl-2 in epithelial tissue were observed according to the degree of collagenization, degree of vascularization and intensity of inflammatory infiltration ($p > 0.05$). No significant correlations were observed between the LIs for Ki-67 and Bcl-2 ($p > 0.05$). **Conclusion:** The present results suggest that the pathogenesis of DIGO does not involve increased proliferation or decreased apoptosis of fibroblasts. On the other hand, the morphological pattern of elongated epithelial cristae observed in DIGO could mainly be due to the inhibition of keratinocyte apoptosis and not to increased proliferation of these cells.

Keywords: Gingival Overgrowth; Ki-67 Antigen; Genes, bcl-2; Immunohistochemistry.

Introduction

Drug-induced gingival overgrowth (DIGO) is an important side effect of the administration of certain drugs, particularly anticonvulsants, immunosuppressants and calcium channel blockers (CCBs) [1]. Phenytoin, cyclosporin and nifedipine are usually associated with the development of DIGO, although they continue to be the drugs of choice for the prevention of epileptic seizures, prevention of transplant rejection and treatment of arterial hypertension, respectively [1-4].

The mechanisms whereby drugs with different pharmacological actions induce different types of gingival overgrowth (GO) with relatively similar clinical and histopathological characteristics remain a matter of debate [2,5,6]. Although the etiopathogenesis of DIGO is not fully understood, studies suggest this disorder to be induced by the rupture of homeostasis between the synthesis and degradation of collagen and other extracellular matrix (ECM) components, as well as between cell proliferation and apoptosis involving the gingival epithelium and connective tissue [1,2,6-10].

The pathogenesis of DIGO has been suggested to be related to the presence of a genetically determined subpopulation of drug-sensitive fibroblasts, which may respond by increasing cell proliferation/survival or by altering the synthesis and remodeling of ECM [3,6,11-13]. In an attempt to better understand the pathogenesis of DIGO, the present study evaluated cell proliferation rates (Ki-67) and the immunoexpression of antiapoptotic protein Bcl-2 in this tissue growth and compared the findings with those observed for clinically healthy gingiva (CHG). Additionally, the associations with histopathological features were established.

Material and Methods

Forty gingival specimens, including 20 cases of DIGO and 20 cases of CHG, obtained from the Oral Pathology Departments of the State University of Paraiba and Federal University of Rio Grande do Norte, were conveniently selected for this study. The size of the sample was defined by the number of available institutional archival cases. All gingival specimens were obtained from patients who had undergone oral surgery due to esthetic and functional reasons prior to periodontal therapy. The patients with DIGO had been receiving treatment with DIGO-inducing drugs for at least 6 months. In the CHG group, none of the subjects had taken medication known to affect periodontal status during the 3 months prior to enrollment. Serial sections were taken from tissue blocks and processed for morphological and immunohistochemical analysis.

Histomorphological Study

The selected specimens were cut into 5-µm thick histological sections, which were mounted on glass slides and stained with hematoxylin and eosin. The tissue sections were analyzed in a blind fashion by two previously trained examiners under a Leica DM500 light microscope (Leica Microsystems Vertrieb GmbH, Wetzlar, Germany). Histomorphological characteristics of epithelial and connective tissues were assessed. The degree of collagenization, degree of vascularization, and

intensity of inflammatory infiltration in the specimens were classified as discrete, moderate or intense.

Immunohistochemistry

For immunohistochemical study, 3-μm thick tissue sections were deparaffinized, immersed in 3% hydrogen peroxide to block endogenous peroxidase activity, and washed in phosphate-buffered saline (PBS). Next, the sections were incubated in a moist chamber with the following primary antibodies: anti-Ki-67 (clone SP-6, Diagnostic Biosystems, Pleasanton, CA; antigen retrieval with citrate, pH 6.0, in a pressure cooker for 3 min; dilution 1:200; incubation 60 min) and anti-Bcl-2 (clone 124, Dako, Carpinteria, CA; antigen retrieval with citrate, pH 6.0, in a pressure cooker for 3 min; dilution 1:50; incubation overnight). The sections were then washed twice in PBS and treated with the labelled streptavidin biotin complex (LSAB + System-HRP, Dako) at room temperature to bind the primary antibodies. Peroxidase activity was visualized by immersing the tissue sections in diaminobenzidine (Liquid DAB+ Substrate, Dako), which resulted in a brown reaction product. Finally, the sections were counterstained with Mayer's hematoxylin and coverslipped. Sections of normal human lymph nodes served as positive control for anti-Ki-67 and anti-Bcl-2 antibodies. As a negative control, the samples were treated as described above, except that the primary antibody was replaced with a solution of bovine serum albumin in PBS.

Analysis of Immunostaining

Immunohistochemical analysis was performed by two previously trained examiners in a blind fashion under a Leica DM500 light microscope (Leica Microsystems Vertrieb GmbH). The immunoexpression of Ki-67 and Bcl-2 was evaluated both in the epithelial tissue and in the connective tissue of the specimens by establishing the labeling index (LI). In connective tissue, the expression of Ki-67 and Bcl-2 was analyzed in oval/spindle-shaped mononuclear cells, which were morphologically compatible with fibroblasts. Using ×100 magnification, five fields containing the largest number of immunostained cells were identified. Images of each field were acquired with a Leica ICC50HD camera (Leica Microsystems Vertrieb GmbH) at ×400 magnification. A total of 1,000 cells were counted in epithelial tissue and 500 cells in connective tissue using the ImageJ software (version 1.43u; National Institute of Mental Health, Bethesda, MD). The Ki-67 and Bcl-2 LIs are expressed as the percentage of immunostained cells in relation to the total number of cells counted.

Statistical Analysis

The results obtained were submitted to statistical analysis using the Statistical Package for the Social Sciences (version 17.0; SPSS, Inc., Chicago, IL). The Kolmogorov-Smirnov test revealed a normal distribution of Ki-67 and Bcl-2 LIs in epithelial tissue. Therefore, the parametric Student *t*-test was applied to compare mean Ki-67 and Bcl-2 LIs in epithelial tissue according to type of

specimen (DIGO versus CHG) and histomorphological characteristics. In connective tissue, statistical comparisons of the LIs for Ki-67 and Bcl-2 according to type of specimen (DIGO versus CHG) and histomorphological characteristics could not be performed due to the small number of immunopositive cases. Pearson's correlation test was used to determine possible correlations between Ki-67 and Bcl-2 LIs in epithelial tissue. The level of significance was set at 5% ($p < 0.05$) for all tests.

Ethical Aspects

The study was approved by the Research Ethics Committee of State University of Paraíba (0076.0.133.000-12).

Results

Morphological Analysis

Morphological analysis of the epithelial lining of CHG and DIGO specimens revealed areas of hyperplasia, acanthosis, hydropic degeneration, spongiosis and exocytosis that varied in extent and degree. Specifically in the case of DIGO, epithelial cristae exhibiting long extensions in the direction of the underlying connective tissue were identified in 11 (55.0%) of the 20 cases analyzed. In connective tissue, there was a higher frequency of cases exhibiting intense collagenization in DIGO (n = 18; 90.0%) compared to CHG (n = 12; 60.0%). Regarding vascularization, a higher proportion of CHG (n = 14; 70.0%) and DIGO (n = 11; 55.0%) specimens were classified as intense. Most DIGO cases (n = 13; 65.0%) exhibited an intense inflammatory infiltrate. On the other hand, a relatively similar distribution of the different degrees of inflammatory infiltration was observed in CHG (Table 1).

Table 1. Distribution of cases of CHG and DIGO according to histomorphological characteristics.

Characteristics	Groups	
	CHG n (%)	DIGO n (%)
Collagenization		
Discrete	0 (0.0)	0 (0.0)
Moderate	8 (40.0)	2 (10.0)
Intense	12 (60.0)	18 (90.0)
Vascularization		
Discrete	0 (0.0)	0 (0.0)
Moderate	6 (30.0)	9 (45.0)
Intense	14 (70.0)	11 (55.0)
Inflammatory infiltrate		
Discrete	7 (35.0)	4 (20.0)
Moderate	6 (30.0)	3 (15.0)
Intense	7 (35.0)	13 (65.0)

Immunohistochemical Analysis

All cases of DIGO and CHG were positive for Ki-67 and Bcl-2 in epithelial tissue. Immunostaining for Ki-67 was observed in the nucleus of cells of the basal layer and, occasionally, in

the nucleus of parabasal cells (Fig. 1A and 1B). With respect to Bcl-2, immunostaining was detected in the cytoplasm of cells of the basal layer and sometimes in the cytoplasm of parabasal cells (Fig. 1C and 1D).

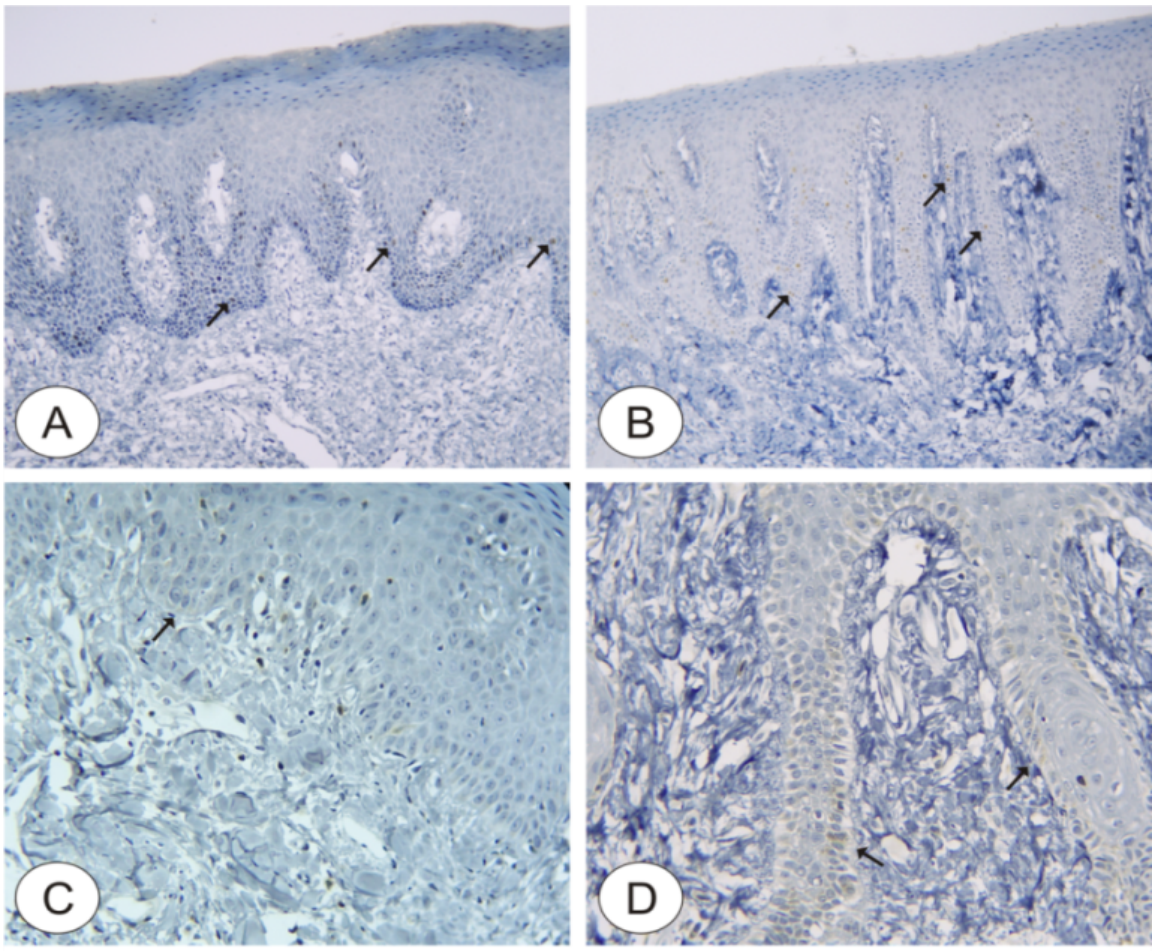

Figure 1. Epithelial immunostaining for Ki-67 in the nucleus of cells of the basal and parabasal layers (arrows) in CHG (A) and in DIGO (B) (LSAB, ´100). Immunoexpression of Bcl-2 in the cytoplasm of epithelial cells of the basal layer (arrows) in CHG (C) and in DIGO (D) (LSAB, ´400).

The mean LI for Ki-67 in epithelial tissue was 21.71% (range: 8.0% – 37.6%) in CHG and 17.2% (range: 0.4% – 36.6%) in DIGO ($p = 0.137$). With respect to Bcl-2, the mean LI was 10.24% (range: 0.8% – 19.3%) in CHG and 14.67% (range: 2.4% – 27.1%) in DIGO, with the observation of a significant difference between groups ($p = 0.026$). No significant differences in the LIs for Ki-67 or Bcl-2 in epithelial tissue were observed according to the degree of collagenization, degree of vascularization and intensity of inflammatory infiltration ($p > 0.05$) (Table 2).

Table 2. Number of cases, mean and range of LIs for Ki-67 and Bcl-2 in epithelial tissue and connective tissue, and their differences according to histomorphological characteristics.

Characteristics	n	Epithelial tissue			Connective tissue	
		Mean	Range	*p*	Mean	Range
Ki-67						
Collagenization						
Discrete/ Moderate	10	22.87	0.40 – 37.60	0.195	0.18	0.00 – 0.80
Intense	30	18.32	1.60 – 36.60		0.18	0.00 – 1.20
Vascularization						
Discrete/ Moderate	15	16.65	1.60 – 31.90	0.152	0.14	0.00 – 1.20
Intense	25	21.14	0.40 – 37.60		0.20	0.00 – 1.00

Inflammatory infiltrate						
Discrete/ Moderate	20	18.59	7.20 – 37.60	0.572	0.04	0.00 – 0.40
Intense	20	20.32	0.40 – 36.60		0.33	0.00 – 1.20
Bcl-2						
Collagenization						
Discrete/ Moderate	10	12.77	5.70 – 19.30	0.859	0.00	0.00 – 0.00
Intense	30	12.35	0.80 – 27.10		0.27	0.00 – 3.00
Vascularization						
Discrete/ Moderate	15	12.92	2.90 – 20.70	0.721	0.24	0.00 – 0.80
Intense	25	12.17	0.80 – 27.10		0.18	0.00 – 3.00
Inflammatory infiltrate						
Discrete/ moderate	20	13.25	2.90 – 20.40	0.434	0.15	0.00 – 0.80
Intense	20	11.65	0.80 – 27.10		0.26	0.00 – 3.00

In connective tissue, low Ki-67 and Bcl-2 immunostaining was observed in DIGO and CHG specimens (Fig. 2E and 2F). Only 7 (35.0%) of the CHG cases and 8 (40.0%) of the DIGO cases exhibited positive Ki-67 staining in oval/spindle-shaped mononuclear cells scattered in the ECM that were morphologically compatible with fibroblasts. For Bcl-2, 9 (45.0%) of the DIGO cases and 3 (15.0%) of the CHG cases exhibited positive staining in oval/spindle-shaped mononuclear cells scattered in the ECM.

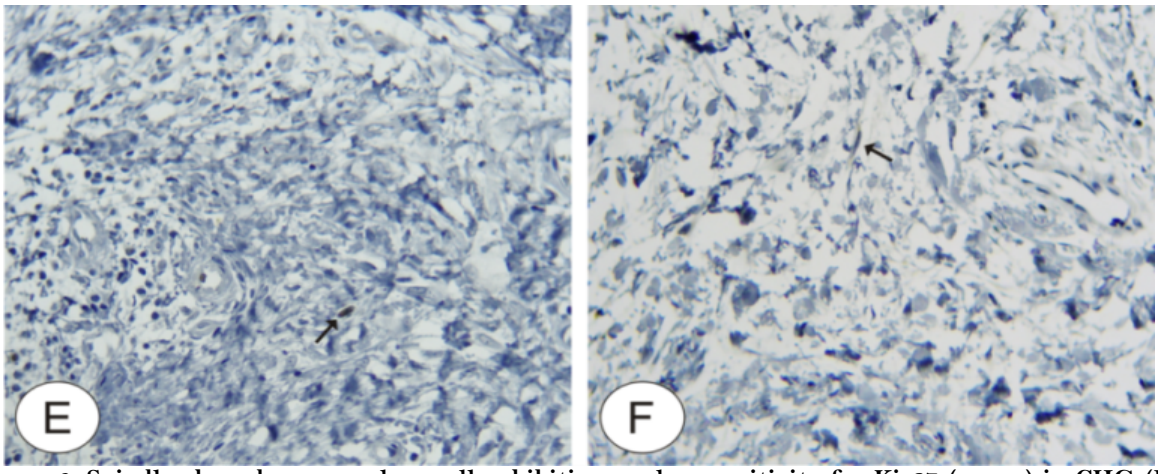

Figure 2. Spindle-shaped mononuclear cell exhibiting nuclear positivity for Ki-67 (arrow) in CHG (E) and cytoplasmic positivity for Bcl-2 (arrow) in DIGO (F) (LSAB, ´400).

Analysis of the LIs for Ki-67 in oval/spindle-shaped mononuclear cells of connective tissue revealed mean values of 0.15% (range: 0.0% – 1.0%) in CHG and 0.22% (range: 0.0% – 1.2%) in DIGO. The mean LIs for Bcl-2 were 0.06% (range: 0.0% – 0.6%) in CHG and 0.35% (range: 0.0% – 3.0%) in DIGO. Table 2 shows the LIs for Ki-67 and Bcl-2 in connective tissue of the specimens according to the degree of collagenization, degree of vascularization and intensity of inflammatory infiltration.

Pearson's correlation test revealed no significant correlation between Ki-67 and Bcl-2 LIs in the epithelial tissue of the specimens ($r = 0.067$; $p = 0.683$).

Discussion

DIGO is an adverse effect of different drug categories, including anticonvulsants, CCBs and immunosuppressants [3,4,13,14]. Although the pharmacological effect of each drug is unique and

the exact pathogenesis of GO is not fully understood, the clinical and histopathological characteristics of DIGO are similar, irrespective of the drug [3,14].

Several factors have been proposed in an attempt to explain the susceptibility of gingival tissues to the effects of drugs, including exposure to higher drug concentrations in view of greater blood circulation and spaces between epithelial cells [9]. Furthermore, variations in individual susceptibility have been suggested, with different tissue responses being influenced by pharmacological and demographic variables, oral conditions and/or genetic predisposition [3,14].

DIGO has been suggested to be the result of a combination of factors, including disturbances of the balance between the synthesis and degradation of ECM molecules, particularly collagen, interference with the fibroblast proliferation rate, and accumulation of fibroblasts or keratinocytes due to the inhibition of apoptosis, prolonging the survival of these cells [2,4,7,10,11,13,15,16].

In the present immunohistochemical study, no significant differences in the LIs for Ki-67 in epithelial tissue were observed between DIGO and CHG, suggesting that the pattern of elongated epithelial cristae observed in the former is not related to increased proliferation of keratinocytes. In line with this suggestion, other studies also found no significant differences in epithelial cell proliferation rates between DIGO and normal oral mucosa specimens [11,17]. However, human studies and studies using animal models demonstrated an increase in the number of Ki-67-positive epithelial cells in GO induced by different drugs, such as cyclosporine and nifedipine, suggesting that these drugs can induce increased proliferation of these cells [2,7,8,18].

Also in epithelial tissue, a higher Bcl-2 LI was observed in DIGO compared to CHG (p = 0.026), in agreement with previous findings [5,19]. Taken together with the results obtained for Ki-67, the higher expression of Bcl-2 in DIGO observed here suggests that the pattern of epithelial cristae in these lesions, forming long extensions towards the connective tissue, is mainly related to the inhibition of keratinocyte apoptosis and not to increased proliferation of these cells. In line with this suggestion, studies using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) technique, which is able to identify cells undergoing apoptosis, demonstrated lower rates of programmed cell death in the epithelial component of GO induced by nifedipine [11] and cyclosporine [9] when compared to CHG and inflamed gingiva.

Analysis of Ki-67 and Bcl-2 LIs in connective tissue of DIGO revealed a much smaller number of immunopositive oval/spindle-shaped mononuclear cells. In agreement with these results, in a study investigating nifedipine-induced GO in an animal model, Bcl-2 expression was restricted to the gingival epithelium [6]. Furthermore, some authors studying a sample of cases of GO induced by CCBs, reported the absence of Ki-67 staining in fibroblasts of the lamina propria [20]. Similarly, in a study on cyclosporine-induced GO, it was observed that gingival fibroblasts were negative for proliferating cell nuclear antigen (PCNA) [10]. These findings suggest that the pathogenesis of GO does not involve increased proliferation or decreased apoptosis of fibroblasts. Despite this suggestion, a previous study reported strong positive staining for Ki-67 in fibroblasts of cases of nifedipine-induced GO [18]. Furthermore, it was observed the inhibition of cell cycle arrest in G1 in

human gingival fibroblasts cultured in medium containing phenytoin, probably as a result of an increase in phosphorylated CDK2 and Rb levels and a reduction in p21 and p27 protein levels [21].

The high percentage of cases of DIGO exhibiting intense collagenization (90%) observed in the present study agrees with the results of other studies suggesting that the development GO involves alterations in fibroblast metabolism represented by an increased secretory activity of ECM components and/or a reduction in the secretion of matrix metalloproteinases [2,14,22]. Within this context, there is evidence indicating that the cellular and molecular characteristics of DIGO might vary as a function of the drug used [23]. Studies have shown that cases of GO induced by phenytoin express high levels of transforming growth factor beta (TGF-b) and connective tissue growth factor (CCN2), which induce the synthesis and accumulation of ECM [23,24]. On the other hand, Kim et al. [25] observed that nifedipine-induced GO expresses high levels of periostin, a protein that increases the biosynthesis of ECM and stimulates fibrosis. According to these authors, nifedipine would stimulate signaling through TGF-b and consequently increase the expression of periostin [25].

Despite the above considerations, Brown and Arany [4] recently proposed a unified hypothesis to explain the pathogenesis of DIGO. According to these authors, CCBs, anticonvulsants and calcineurin inhibitors would first inhibit cation influx which results in reduced influx of folate into gingival fibroblasts. This event, in turn, would lead to modifications in the metabolism of matrix metalloproteinases and failure to activate collagenases. The reduced availability of activated collagenases would reduce the degradation of connective tissue and its consequent accumulation in gingival tissues [4].

Taken together, the data reported in the literature and the results obtained here suggest that the pathogenesis of DIGO is mainly related to alterations in fibroblast metabolism. The cellular and molecular mechanisms involved in this process remain incompletely understood.

Conclusion

The present results suggest that the pathogenesis of DIGO does not involve increased proliferation or reduced apoptosis of fibroblasts. On the other hand, the morphological pattern of elongated epithelial cristae observed in DIGO could mainly be due to the inhibition of keratinocyte apoptosis and not to the increased proliferation of these cells.

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