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Immunohistochemical study of experimentally drug-induced gingival overgrowth

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Abstract

The increasing frequency of using in the medical practice drugs that have the potential to induce gingival overgrowth (GO) and the existence of many unknown aspects in GO etiopathogenesis have prompted us to carry out this immunohistochemical experimental animal study. We conducted a cell proliferation study by Ki67 immunostaining and a cytokeratin (CK) study using anti-pan-CK AE1/AE3 and anti-MNF116 antibodies, investigating the differences induced by different classes of drugs that are more frequently involved in the induction of GO. The results of our study indicate that CK AE1/AE3 plays an important role not only in normal cellular proliferation, but also in hypertrophic tissues, and can be considered a marker of the proliferative process occurring in GO. Immunostaining for the anti-MNF116 antibody was weaker and inconsistent in intensity compared to anti-CK AE1/AE3 antibody, its staining pattern appearing as diffuse or zonal.

Keywords: gingival hypertrophy, cell proliferation, cytokeratins.

Introduction

Gingival overgrowth (GO), depending on its extent, has multiple consequences on the dento-maxillary apparatus: functional disorders (altered speech), difficulty in mastication, aesthetic problems but can also cause important psychological problems [1–3]. In drug-induced GO, the most frequently incriminated drugs are: antihypertensive drugs, antiepileptic and immunosuppressive medication. Current studies on the pathogenic mechanism of gingival expansion associated with drug use focus on the direct and indirect effects of these drugs on the metabolism of gingival fibroblasts. Treatment is generally focused on substitution drugs and effective control of local inflammatory factors (bacterial plaque). When these measures prove to be ineffective, surgical interventions are recommended. Despite their effectiveness, they do not prevent recurrence. Therefore, the molecular approach associated with immunohistochemical (IHC) techniques is necessary to establish the pathogenesis of GO and to provide new information for the assessment of the preventive and therapeutic measures in the close future. New data support the idea that extracellular matrix (ECM) metabolism aspects are unique and can help explain the tissue specificity of GO.

Our motivation for choosing this theme is determined by the increasing use frequency of drugs that can induce GO; by the many unknown aspects still encountered in GO etiopathogenesis, despite the research already present in literature; as well as the continuation of an earlier experimental study, which, this time, we chose to perform with the aid of IHC techniques.

Materials and Methods

The studied material consisted of 25 three months old Wistar rats, with a body weight of 360–400 g, kept in the same environment conditions, at constant temperature of 20–24^{0}C. The rats received the same diet and drug administration was carried out for two months for all the animals.

The animals were divided into four groups:

▪ Group I: The rats were subcutaneously (s.c.) injected ×2/day with Phenytoin (PHT) sodium suspended in 0.5% Tween 80 solution (Richter Phenytoin 100 mg tablets). The doses were per kg/day and were increased every week for preventing the toxic effects, up to 120 mg/kg per day (1 mL/100 g) of PHT in the first week. For preventing lethal toxic values, the PHT amount was increased by 10 mg/kg every week. Every day, before the injection, the body weight was measured.

▪ Group II: Nifedipine solution was s.c. administered 250 mg/kg per day – Nifedipine (Terapia) 20 mg tablets [25 mg/mL obtained through the dissolution of Nifedipine powder into dimethyl sulfoxide (DMSO)].

▪ Group III: The rats in this group were also s.c. injected with Cyclosporine A (CsA) 30 mg/kg per day – Equoral solution 100 mg/mL.

▪ Group IV: Rats in this group were s.c. injected with CsA 30 mg/kg per day in the same daily dose administered in Group III plus Azithromycin in a dose of 60 mg/kg per day.

▪ Group V: This group represented our control group. The rats received the same diet, without any medication. They were s.c. injected with 9‰ sodium chloride solution (saline). Injections were performed daily.

The rats belonging to the five groups were weighed when the experiment begun and at the end of every week, for the administration of the correct drug dose, according to each rat's weight.

The rats belonging to the five groups were sacrificed under anesthesia three months after the start of the experiment. The mandibles were resected and buccal-lingual sectioned between the first and the second molar and fixed in 10% formalin solution buffered with calcium carbonate for 24–72 hours, depending on the size of the parts and then washed with distilled water. The fixation was performed at laboratory temperature. The pieces were then decalcified in 5% trichloroacetic acid solution for 21 days. IHC processing used Avidin–Biotin/Horse-radish peroxidase (ABC/HRP) complex technique. The antibodies used in this study are centralized in Table 1.

Table 1 – ***Antibodies used for the immunohistochemical study***

Antibody	Marker	Producer	Antigenic exposure	Dilution
Ki67	Nuclear cell proliferation factor	DAKO	Citrate, pH 6	1:100
AE1/AE3	Pan-CK	DAKO	Citrate, pH 6	1:100
MNF116	Pan-CK	DAKO	EDTA, pH 6	1:200

CK: Cytokeratin; EDTA: Ethylenediaminetetraacetic acid.

The experiment was approved by the Ethics and Academic Deontology Board of the University of Medicine and Pharmacy of Craiova, Romania (Approval No. 134/12.06.2015). We followed the principles of animal protection used for scientific purposes according to Directive No. 2010/63/UE of the European Parliament and Council on September 22, 2010; Act No. 43/2014 published in the Official Monitor, Part I No. 326/May 2014.

Results

Cellular proliferation study by Ki67 immunostaining

Ki67 or MKI67 is a protein encoded by the MKI67 gene. Ki67 antigen is a nuclear protein that is associated and may be required for cell proliferation. It is also associated with transcriptional ribonucleic acid (RNA) and the inactivation of the Ki67 antigen leads to inhibition of ribosomal RNA synthesis [4, 5]. The Ki67 antigen starts being expressed in the S phase of the cell cycle, progressively increasing from phase S to G2 phase and reaching a plateau at the time of mitosis. After cell division, the cells return to G1 with a Ki67 antigen stock, whose level falls rapidly during this phase [6, 7].

Cell proliferation is an important biological variable for some pathological situations, therefore identifying the percentage of cells expressing antigens of the mitotic activity growth cycle is a method of confirming the investigated clinical aspect.

The nuclear immunoreactivity for the Ki67 antigen was readily identifiable. The browning of the nuclei, irrespective of the color intensity, was interpreted as positive. This positive reaction was present, depending on the group, throughout the thickness of the epithelium. At the level of the lamina propria, Ki67 expression was present in fibroblasts in the gingival tissue presenting overgrowth. We emphasize that, on all sections, Ki67 immunoexpression was lower in the connective tissue compared to the epithelial detection for this marker.

The number of Ki67-positive cells in the epithelium, as well as the intensity of the immunoreaction, were variable, depending on the group. Our results showed that in Groups I, II and III, under PHT, Nifedipine and CsA medication, respectively, keratinocyte gestational mitotic activity, evidenced by Ki67-positive cells, was higher than in Group IV (in which the rats also received Azithromycin) and in which the Ki67 immunoassay was extremely low and only present at the basal layer of the epithelium. In the control group, the Ki67-positive cells were absent (Figure 1A). All normal oral mucosa samples belonging to the control group showed a slight Ki67 staining in the basal layer of the epithelium, which may be due to the physiological proliferative activity of the cells in this layer. Ki67-positive cells were more numerous on the sections from the PHT- and Nifedipine-treated groups (Figure 1, B and C), compared to those treated with CsA (Figure 1D). Increased immunoexpression for Ki67 on sections belonging to Groups I, II and III is due to high proliferative activity in the basal layer of the epithelium, secondary to the chronic drug-induced irritation associated with different degrees of inflammation and the inflammatory process at the level of the lamina propria.

The IHC study of cytokeratins (AE1/AE3, MNF116)

Cytokeratins (CKs) are epithelial cell-specific intermediate filaments and represent the most important biomarkers in histopathological diagnosis. In our study, the expression of CK AE1/AE3 was positive for all the groups examined. Immunomarker positivity was sometimes localized, especially in the basal layer (Figure 2A), other times it as present in all epithelial layers (Figure 2B). The most intense expression for CK AE1/AE3 was, however, in the basal layer, where the progenitor cells are located and an intense cellular multiplication process occurs. The immunostaining positivity shows the existence of a proliferating epithelium of the gingival mucosa, as we have highlighted in our experimental drug-induced hyper-growth. However, the immunomarker intensity showed small variations from one group to another (Figure 2, C and D). The results of our study indicate that CK AE1/AE3 plays an important role not only in normal cellular proliferation but also in hyperplastic tissues, thus being considered a marker of the proliferative process of GO.

Immunostaining for the anti-MNF116 antibody was weaker and inconsistent in intensity compared to immunostaining for anti-CK AE1/AE3 antibody. Its patterns can be described as diffuse (Figure 2E) or zonal (Figure 2F). There were sections belonging to the examined groups, and especially to the control group and to the Azithromycin-treated group, where the intensity of the immunostaining was very small, sometimes almost nonexistent.

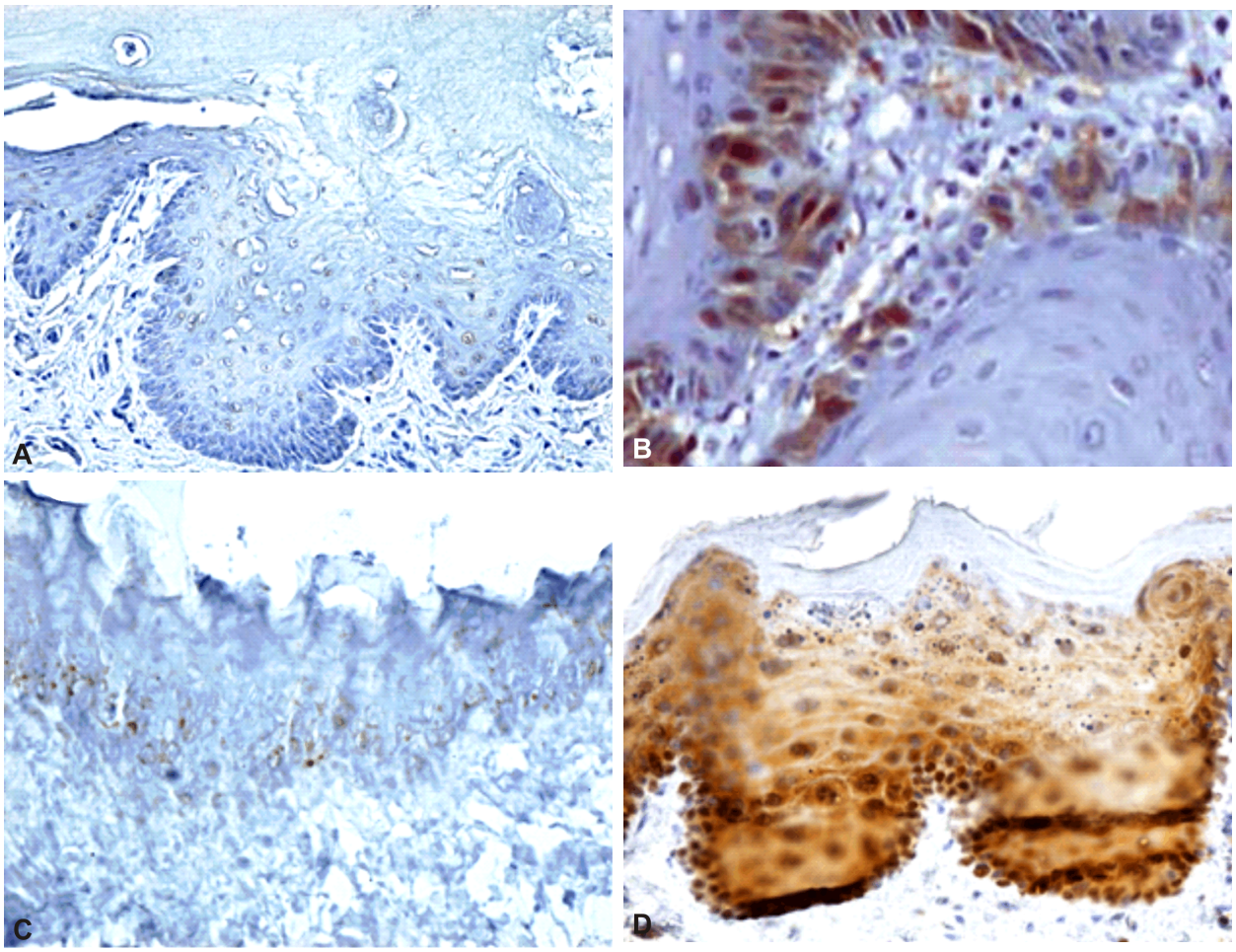

Figure 1 – ***Gingival mucosa, immunostaining for anti-Ki67 antibody: (A) Control group – very weak positive response in the basal layer of the epithelium (×100); (B) Positive reaction at epithelial level in the basal and intermediate layer and at the level of the fibroblasts of the lamina propria in PHT-induced GO (×200); (C) Positive response in the basal and intermediate layer of gingival epithelium in Nifedipine-induced GO (×100); (D) Positive response in all epithelial layers and at the level of the fibroblasts of the lamina propria in CsA-induced GO (×100). PHT: Phenytoin; GO: Gingival overgrowth; CsA: Cyclosporine A.***

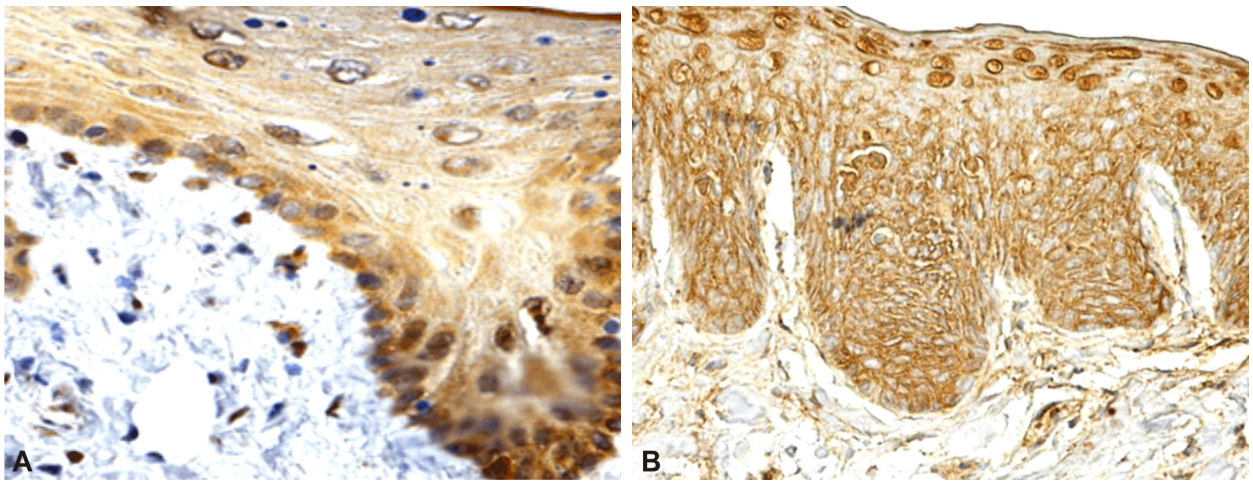

Figure 2 – ***Gingival mucosa, immunostaining for anti-CK AE1/AE3 antibody: (A) Intensely positive response in the basal layer of the epithelium and weak suprabasal response (×400); (B) Positive response throughout the epithelium (×200). CK: Cytokeratin.***

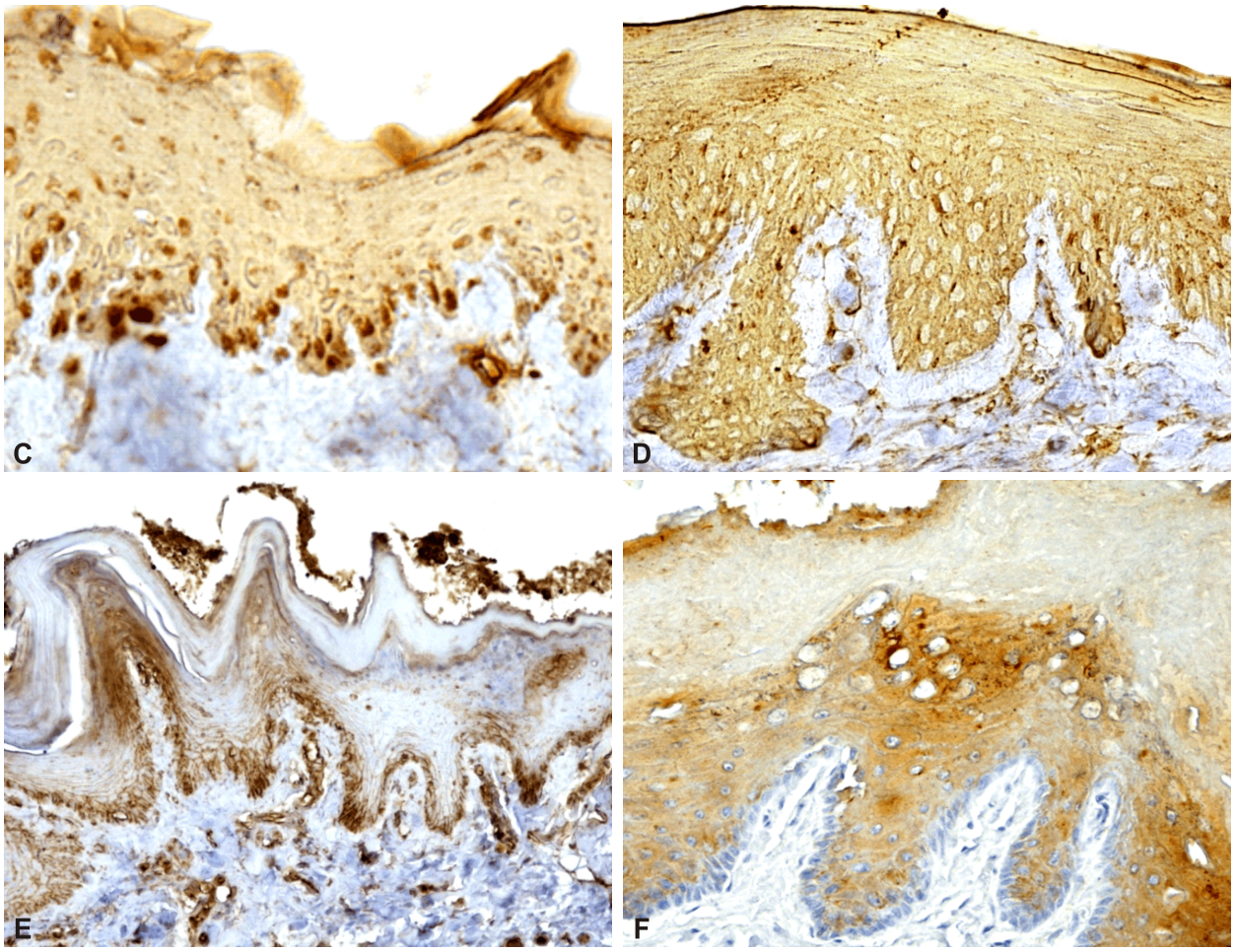

Figure 2 (continued) – ***Gingival mucosa, immunostaining for anti-CK AE1/AE3 antibody (C and D) and for anti-CK MNF116 antibody (E and F): (C) Positive response, of medium intensity, at epithelial level (×400); (D) Weak positive response, at epithelial level (×400); (E) Intense, diffuse, positive response (×400); (F) Intense but zonal positive response (×400). CK: Cytokeratin.***

Discussions

The etiology of drug-induced GO is multifactorial [8–10], the effects of pharmacological molecules being also modulated by the following factors: plaque index, which gender is directly correlated with, gender (males are three times more prone to developing plaque), age (inverse correlation), the daily dose of the drug (direct correlation). All these factors act synergistically for the development of GO. The pathogenic mechanisms involved in GO production are still incompletely clarified but include fibroblasts, cytokines and matrix metalloproteinases [11–13]. An IHC study has shown that the imbalance between cell proliferation and apoptosis contributes to the pathogenesis of hypercellularity seen in GO [14]. In conclusion, the mechanism underlying the development of GO is still unclear, but it has been suggested that it may reflect complex interactions between the drug, gum tissues and locally released mediators [15]. In GO, the remodeling of ECM involves two important aspects: synthesis and deposition of matrix components by active fibroblasts and their proteolytic degradation. Deregulation of ECM homeostasis is mainly due to increased collagen synthesis and secondary to the inhibition of its degradation [16].

According to studies, as inflammation becomes chronic, gingivitis evolves to periodontitis, which has two forms, chronic periodontitis, the most frequent, and aggressive periodontitis. Granular tissue rich in plasma cell infiltrate, which is the main contributor to inflammatory GO, replaces inflammatory tissue originally dominated by lymphocytes and polymorphonuclear cells [17].

Histologically, drug-induced GO is associated with epithelial thickening, fibrosis in the lamina propria and increased fibroblasts [18–20].

The oral epithelium in patients treated with Nifedipine was five to 10 times thicker than in healthy persons [21], similar to the overgrowth induced by CsA or PHT, compared to the non-medicated control groups [22]. The thickening of the gingival epithelium induced by Nifedipine [23] and CsA [24, 25] is caused by the thickening of the spinous (prickle cell) layer in the epithelium.

In vitro studies demonstrate that CsA inhibits the proliferation of keratinocytes obtained from various culture origins [26]. In opposition [27], the same studies demonstrated a heightened mitotic activity in the keratinocytes of the human hair follicle, in patients treated with CsA. Some authors demonstrated that Nifedipine and PHT stimulate the mitotic activity of the human

gingival keratinocytes *in vivo* [28]. Results obtained by clinical studies suggest that gingival inflammation augmentates the incidence and the severity of GO in patients treated with Nifedipine and/or CsA [29].

Several methods were employed for the study of cellular kinetics in proliferating tissues: the incorporation of tritiated thymidine followed by autoradiography, the incorporation of bromodeoxyuridine (BrdU), followed by anti-BrdU immunocytochesmistry, flow cytometry and immunocytochemistry using cellular cycle specific antibodies. The advantage of the last method is the preservation of tissular architecture. This method does not necessitate fresh tissue or radioactive substances and paraffin-included tissues may also be used. The monoclonal antibodies used for studying cellular kinetics are the proliferating cell nuclear antigen (PCNA) and the Ki67 antigen. The PCNA antigen is not expressed throughout the whole cellular cycle. Its first detection is in the G1 phase, it reaches a maximum in the S or G1/S phase, and it is impossible to detect through immunocytochemistry in the M phase. The Ki67 antigen is expressed in proliferating cells (G1, S, G2 and M phases), and absent in non-proliferating cells (G0 phase) [30]. The expression of the Ki67 antigen is strictly associated with cellular proliferation and is widely used as a proliferation marker, also serving as a marker for predicting tissular growth. The basal layer of the oral mucosal epithelium is the place for normal cellular proliferation, while suprabasal layers are only cell maturation sectors in which cell changes may present potential signs of dysplasia. The cellular proliferation markers are important for the evaluation of the proliferative capacities of epithelial cells. The basal layer is the only proliferative sector for the normal, healthy oral epithelium, while the other layers of the epithelium are maturation sectors only. Proliferative activity found in suprabasal layers is considered a warning sign.

Some authors [22] sustain the proliferative effect of CsA in gingival keratinocytes. A significant growth in the volume of the oral epithelium and the density of the epithelial crests has been found in GO induced by CsA, compared to the control study groups. The results found in literature suggest that oral epithelial overgrowth induced by medication (Nifedipine, CsA, PHT) is determined by the mitotic activity of the oral epithelium [31]. In marked sections, the average rate of Ki67-positive cells in PHT-induced gingival hyperplasia is more than 10%, which is comparable to that of the dysplastic oral mucosa. Some authors demonstrated that the proliferative rate is unmodified in patients with renal transplant and GO compared to a healthy control group [32]. The results of this study also showed the expression of the Ki67 antigen in fibroblasts found in the lamina propria, in all GO cases. Other authors found that long-term exposure to CsA may stimulate fibroblastic activity in the gingival epithelium [33, 34]. Some authors demonstrated that in all medication-induced gingival hyperplasia cases, there is also an increase in the proliferation of gingival fibroblasts [35, 36]. Furthermore, interesting remarks have been made regarding the bimodal effect of CsA over the proliferation of gingival fibroblasts – small doses of CsA stimulate proliferation while higher doses cause inhibition [37]. Clinically relevant doses of CsA together with bacterial products stimulate the proliferation of the gingival keratinocytes and GO by activating the cellular cycle and the replication of deoxyribonucleic acid (DNA) [38]. Some studies show that immunosuppressed patients in treatment with CsA may develop gingival neoplasms. The disorders regarding cellular proliferation and apoptosis are fundamental events in early carcinogenesis and may be useful in assessing healthy tissue compared to tissues that exhibit high histological risk for neoplastic development [39, 40].

Another animal study reported that treatment with CsA causes oral epithelial hyperplasia associated with an increase in PCNA expression [41–43]. The proliferating activity of keratinocytes could be explained by the fact that keratinocyte proliferation could be mediated by connective tissue mitogenic factors, such as keratinocyte growth factor and the dispersion factor [44, 45].

Our results are in agreement with the aspects mentioned by these authors, which further justifies the proliferative effect CsA has on keratinocytes *in vivo*. Ki67 is therefore a marker of activation for epithelial keratinocytes. We suggest that immunosuppressive medication may directly activate gingival keratinocytes. However, the role of inflammation cannot be ruled out. An association between CsA-induced GO and clinical inflammation has been reported in several studies [29, 36].

In our study, there was an increase in the mitotic activity of gingival keratinocytes, in all medication groups compared to the control group and the group in which antibiotic was administered (Azithromycin). We concluded that the epithelial overgrowth induced by PHT, Nifedipine and CsA was associated with increased mitotic activity. Other authors also observed an approximately two-fold increase in the percentage of Ki67-positive cells in Nifedipine-induced GO, compared with non-medicated cases [46].

The mean proliferation rate in epithelial cell cultures derived from the clinically uninflammed or slightly inflamed gums was much higher than the proliferation rate of cells derived from moderately and severely inflamed gums [47]. Subepithelial inflammation was mild compared to the inflammation detected beneath the sulcular epithelium. Therefore, the effect of inflammation in reducing the mitotic activity of keratinocytes in the sulcular epithelium may prevail over the effect of drugs, except in the case of the drug-induced immunosuppression group. In addition, the extension of the inflammatory cells infiltrate in the lamina propria found in the control and medication groups, justifies the assumption that drugs have an independent effect over mitotic activity.

Studies have shown that there is an increased proliferative activity of the oral gingival epithelium during the inflammation process compared to non-inflammatory lesions [48]. In view of these findings and considering the results of our histopathological and IHC study, we can say that increased epithelial proliferative activity can be considered a common response to inflammation.

Some authors found a negative significant correlation between the duration of drug administration and the percent of Ki67-positive cells in the oral epithelium in the group treated with Nifedipine. The negative correlation

encountered could partially explain the contradictory results regarding the relationship between GO and the dose, concentration and duration of Nifedipine administration [49]. The average number of epithelial cells presenting CsA deposit per square millimeter increased with the degree of GO and was positively correlated to the degree of the inflammatory cell infiltrate [50] in gingival biopsies obtained from patients with renal transplant. Authors also report that mean serum values for CsA did not have a correlation to the degree of GO. Differences found by authors between the mitotic activity in the oral and sulcular epithelium indicate that local gingival inflammation influences the drug-mediated modifications [31].

It seems that medication influences the growth and function of gingival fibroblasts and epithelial cells, both directly and indirectly. These processes are regulated by cytokines and growth factors [51]. Thickening of the oral epithelium in PHT-, Nifedipine- and CsA-induced GO is associated with increased cell proliferation rates and with the positive effect of inflammation over the proliferation of epithelial cells that increases with the thickness of the gingival epithelium [36].

The dynamic of the oral mucosa is well known for its defensive nature. Certain areas need protection when subjected to mechanical aggression. This is accomplished by a structural material called cytoskeleton, formed by intracellular protein filaments called CKs, found in the squamous epithelium of the oral mucosa [52, 53]. Knowledge of structural proteins, their expression, distribution and function, plays an important role in the possibility of their use as differentiation markers. Thus, they are an essential diagnostic aid and can be useful in future interventions through means of gene therapy.

There are two categories of CKs. The acid CKs (type I; CKs 9–20) are stored by the 17q chromosome and have a molecular weight ranging from 40 kDa (CK19) to 64 kDa (CK9). The basic or neutral CKs (type II; CKs 1–8) stored by the 12q chromosome have a molecular weight ranging from 52 kDa (CK8) to 67 kDa (CK18). They exist in pairs, where one is acidic and one is basic except for the CKs 15 and 19, which seem to be unpaired [54, 55]. High molecular weight CKs (CK 5/14) and the pan-CK cocktail (CKs 1/10, 4/13, 5/14, 6/16, 7/19, 2/3, 8/15) display increased immune staining in submucosal fibrosis.

The oral epithelium displays a regional diversity suited to its functional needs as it is exposed to various forms and levels of stress that directly affect epithelial cells. This requirement accomplished by forming intracytoplasmic filaments. The proteins that form the filaments belong to a protein group known as the intermediate filament family.

Mammalian keratins are subdivided into two distinct groups based on structure, function, and regulation. Cysteine-rich keratin is harder and forms the ‘hard’ keratin group found in hair and nails. The packs of keratin found in cytoplasm are called ‘soft’ keratins or CKs [55–57]. CKs are the basic structural proteins of epithelial cells. They are found in abundance in the epithelium of the oral cavity. Their distribution is specific and varies with location, the type of epithelium and its degree of differentiation. In the nonkeratinized and keratinized oral epithelium, CKs 5 and 14 are found in the basal layer. CKs 4 and 13 are found in the intermediate and superficial layers of the oral epithelium, more specifically in the nonkeratinized epithelium and CKs 1 and 10 in the keratinized epithelium. CKs 5, 13, 14 and 19 can be found in the gingival mucosa in the gingival sulcus; CKs 4, 5, 6, 13, 14, 16 and 19 can be found in the unattached gingiva and CKs 1, 2, 5, 6, 8, 10, 11, 14, 16, 18 and 19 in the attached gingiva [58].

CK AE1/AE3 is frequently used in IHC diagnosis. As name suggests, CK AE1/AE3 is a mix of two different clones of monoclonal anti-CK antibodies, the antibodies anti-AE1 and anti-AE3. Both individual clones detect certain keratins with high and respectively low molecular weights. Anti-AE1 antibodies detect high molecular weight, the CKs 10, 14, 15 and 16, as well as low molecular weight – CK19. The AE3 clones detect high molecular weight, the CKs 1, 2, 3, 4, 5, 6 and low molecular weight – CKs 7 and 8. A single reagent with a wide spectrum of reactivity results by combining these two subreagents, thus detecting both high and low molecular weight CKs. Reactivity to CK18 (along with CK8), one of the CKs found in simple epithelia and expressed in many carcinomas, is missing from this cocktail mix. Due to its wide reactivity spectrum, CK AE1/AE3 is also called pan-CK but, as it is unable to detect all CKs (CK17 and CK18), it is not a real pan-CK.

The anti-MNF116 antibody detects an epitope that is encountered in a wide range of epithelia, corresponding to CKs 5, 6, 8, 17 and, probably, 19 [55]. In normal, healthy tissue, it reacts with cells in simple glandular and stratified squamous epithelia.

The experiment we have performed has demonstrated that CK AE1/AE3 plays an important role not only in normal cell proliferation but also in hyperplastic tissues. This indicates that CK AE1/AE3 may be used as a marker of the proliferative process in medication-induced GO, compared to the immunostaining for the anti-MNF116 antibody, which was weaker and less consistent in intensity, with diffuse or zonal patterns.

Conclusions

We evaluated the proliferative activity of gingival epithelium and connective cells, comparing the expression of a proliferation marker, Ki67, commonly used in drug-induced GO. In our study, there was an increase in the mitotic activity of gingival keratinocytes in all medication groups, compared to the control group and the antibiotic group (Azithromycin). However, Ki67-positive cells were more abundant on the sections from the PHT- and Nifedipine-treated groups, compared to those treated with CsA and the control group. The result is that epithelial thickening in PHT-, Nifedipine- and CsA-induced GO is associated with an increased mitotic activity. The thickening of gingival epithelium in GO induced by PHT, Nifedipine and CsA is associated with increased proliferative cell activity and the positive effect of inflammation on epithelial cell proliferation that increases with the thickness of the gingival epithelium. By the results obtained, we can say that elevated Ki67 immunostaining shows an increased cell division and proliferation rate

that may be involved in drug-induced GO pathogenesis without neglecting the involvement of other factors: age, genetic predisposition, pharmacokinetic variables and induced inflammatory changes induced by bacterial plaque, that are important in the appearance and severity of the overgrowth. The heterogeneity of the molecules studied in the same group of animals shows that tissue disturbances have different intensities, probably due to individual peculiarities that cause GO to occur when administering drugs. The results of our study indicate that CK AE1/AE3 plays an important role not only in normal cellular proliferation but also in GO and can be considered a marker of the proliferative process of GO. Immunostaining for the anti-MNF116 antibody was weaker in intensity compared to that for anti-CK AE1/AE3 antibody. The results for anti-MNF116 antibody immunostaining were also inconsistent, with diffuse or zonal clotting patterns.

Conflict of interests

The authors declare that they have no conflict of interests.

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