PhD THESIS

ABSTRACT

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY OF THE ROLE PLAYED BY SOME DRUGS IN THE DEVELOPMENT OF GUM OVERGROWTH

PhD COORDINATOR
Professor MD PhD ȘTEFANIA CRĂIȚOIU

PhD STUDENT
ADELINA GABRIELA BOBIC

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Key-words: gum overgrowth, experimental model, oral mucosa, immunohistochemical markers, periodontium
INTRODUCTION

Gum overgrowth (GO) is a pathology that manifests through an abnormal, excessive enlargement of the extracellular matrix volume of the normal periodontal tissue, associated with a growth of the number of cells in the gingival mucosa (a process of hyperplasia) and with an increase in volume of these cells (a process of hypertrophy). Numerous etiological factors were incriminated. Gum overgrowth may be caused by a local inflammation due to the presence of bacterial plaque, by a certain medication (most frequently there is incriminated the medication for high blood pressure, epilepsy, immunosuppressors), of systemic or physiological causes (heredity, hormonal disbalance that appear during puberty or pregnancy) or pathological ones (leukemia), through the intervention of growth factors, while heredity seems to play an important role. The secondary effect of drug administration determines esthetic changes and clinical symptoms, including pain, sensitivity, bleeding, speech disorders, abnormal dental mobility, occlusion problems, increase of dental cavities and periodontal conditions (Brunet L, Miranda J, Farré M, 1996; Lin K, Guilhoto LM, Targas Yacubian EM, 2007). There are required approaches at a molecular level in order to establish the pathogenesis of gingival proliferation (Meller AT, Rumjanek VM, Sansone C, Allodi S, 2002; Andriani F, Margulis A, Lin N et al, 2003).

The main objective for research was represented by the performance of an experimental animal experiment that highlighted, through a histological and immunohistochemical study, the histological differences induced by various classes of drugs leading to GO. The rat model is more useful, due to the animal availability and small differences in drug reaction for different species, due to the reproduction of experimental results (Nishikawa S, Nagata T, Morisaki I, Oka T et al, 1996) and especially due to the fact that the oral epithelium is histologically similar to the human one (Gimenez-Conti IB, Shin DM, Bianchi AB et al, 1990; Santis H, Shklar G, Chauncey HH, 1964; Vairaktaris E, Spyridonidou S, Papakosta V, 2008; Gimenez-Conti IB, Slaga TJ, 1993). Also, we also performed a clinical study on the overgrown gingival mucosa from patients treated with three classes of drugs, the ones that were also used in the GO experimental induction.

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CHAPTER 1

PERIODONTIUM – HISTOLOGICAL AND HISTOPHYSIOLOGICAL CONSIDERATIONS

The odontium or dental organ is made of odontium (the tooth itself) and periodontium, the support and junction structure of the tooth in the jaw bones. The periodontium is made of all the tissues ensuring the teeth maintenance and support in the jaw bones: the cement, periodontal ligaments (periodontium), alveolar bone, part of the gingival mucosa. It comprises two compartments: the covering periodontium (superficial) made of the gingival f fibromucosa and the supraalveolar ligaments; the support periodontium (deep) made of the cement, alveolar bone, periodontium or desmodontium. It is also used the term of marginal periodontium, especially in clinical practice. The marginal periodontium represents all the tissues surrounding the tooth, except for the ones around the apex, also termed apical periodontium. The pathology affecting these areas presents a different etiopathogeneity: for the apical periodontium, the starting point is the endodontium, while for the marginal periodontium, the starting point is at the gingival mucosa and gingival groove (Dumitriiu HT, 2009).
CHAPTER 2
ETIOPATHOGENY OF DRUG-INDUCED GUM OVERGROWTH

Drug-induced GO is a secondary and unwanted effect of systemic medication, physically altering through the change of physionomy and functionally through the conditions emerged in the oral cavity, with repercussions on the patients’ mental state. Moderate and severe GO forms alter oral hygiene and may lead to a high accumulation of microorganisms. Oral infections determined by GO may alter the systemic health state of the patients (Casamassimo, 2000; Li X, Kolltveit KM, Tronstad L, Olsen I, 2000). Still, most frequently it may be present in three categories of drugs: calcium-channel blockers (Nifedipine, Diltiazem and Verapamil), drugs for epilepsy treatment (Phenytoin), immunosuppressors (Cyclosporine A) (Subramani T, Rathnavelu V, Alitheen NB, 2013). Not all the patients that received these drugs come to develop GO, this usually emerging three months after the medication started, in the form of an interdental papilla development, the process extending in all directions (Nishikawa S, Nagata T, Morisaki I, Oka T, Ishida H, 1996).

The GO pathogeny is multifactorial. There still are discussions regarding the role played by the bacterial plaque, some authors supporting the idea that this plays the part of triggering or aggravate GO (Micallef L, Vedrenne N, Billet F, 2012; Kessler D, Dethlefsen S, Haase I, 2001; Schild C, Trueb B, 2002). Other studies show that a good oral hygiene may reduce GO but it does not completely prevent its formation (Thomason JM, Seymour RA, Ellis JS. 2005). Dental plaque is considered a cofactor in the drug etiology of GO (Hood KA, 2002; Kataoka M, Kido J, Shinohara Y, Nagata T, 2005; Correa JD, Queiroz-Junior CM, Costa JE et al, 2011). Various factors like age, genetic predisposition, preexistence of bacterial plaque and gingival inflammation influence the relation between drugs and gingival tissues (Seymour RA, Thomason JM, Ellis JS, 1996).

The pathogenesis of drug-induced GO is not completely elucidated, studies suggesting the involvement of various factors: populations of fibroblasts, genetic predisposition, intracellular calcium metabolism, molecular mechanisms, collagen inactivity and bacteria-induced inflammation. In GO, more than the fibroblast proliferation, there was signaled a severe accumulation of extracellular matrix in the gingival conjunctive tissue, especially of the collagen component (Kataoka M, Kido JI, Shinohara Y, Nagata T, 2005). These discrepancies may be determined by various degrees of gingival inflammation in humans, because due to the production of inflammatory cytokine production, there is stimulated the proliferation of gingival fibroblasts.

In drug-induced GO, although the pharmacological effect of drugs is different and affects various primary tissues, they all have the same effect on a target tissue, namely on the gingival mucosa, thus causing common clinical and histopathological aspects (Academy Report, 2004).

Cyclosporine A determines a growth of glycosaminoglycans by the fibroblasts and Nifedipine and Phenytoin increase the heparin level (Newell J, Irwin CR, 1997). Other studies did not report any differences in vivo (Rocha LA, Martins RC, Werneck CC et al, 2000; Martins RC, Werneck CC, Rocha LA et al, 2003). More studies prove that these drugs inhibit the production of extracellular matrix by the gingival fibroblasts and/ or in vitro cellular proliferation (McKevitt KM, Irwin CR, 1995; edlich M, Greenfeld Z, Cooperman H et al, 1997), this fact being in contradiction with the in vivo aspects of drug-induced GO.
The studied material was represented by 25 Wistar rats of the same age (3 months old), with a weight of 360-400 g, kept in the same environment conditions, with a constant temperature of 20°-24°C, receiving the same diet. Drug administration was made in all animals for 2 months. The animals were divided into five groups:

- **Group I** – Sc injected x 2/day with sodic Phenytoin suspended in 0.5% Tween 80 solution (Richter Fenitoin 100 mg tablets). Te doses were per/kg/day and increased weekly for preventing toxic effects. The initial dose was 120 mg / kg per day (1 ml/100 g) of PHT in the first week and increased by 10 mg/kg every week. The body weight was measured daily, before injection.

- **Group II** – It was administered per bone a Nifedipine solution 250 mg/kg/day NIFEDIPINE Retard Terapia 20 mg tablets with long-time release (125 mg/ml obtained by dissolving the Nifedipine powder in dimethyl sulfoxide (DMSO)).

- **Group III** – Sc injected with Cyclosporine 30 mg/kg/day - Equoral Teva 50 mg soft capsules.

- **Group IV** – Sc injected with Cyclosporine 30 mg/kg/day in the same daily dose administered in group III plus Azithromycin in a dose of 60 mg/kg/day.

- **Group V** - the witness group received the same diet, without medication, the rats being sc injected with NaCl every day.

The material processing was performed by the paraffin inclusion technique, the sections obtained were stained in Hemtoxylin-Eosin and Goldner-Szekelly trichromic.

There were differences of the gingival mucosa aspect in the experimental groups. In the witness group, there was no significant tissue overgrowth. The tissue sampled from the group treated with Phenytoin presented obvious microscopic changes, characterized by an extended gingival overgrowth. Acanthosis was present in different stages, determined by the spinous stratum hyperplasia of the gingival mucosa epithelium. Sometimes, on certain subepithelial sections a high number of capillaries. There were present chronic inflammatory cells, associated to the collagen fibrillary component, especially lymphocytes and plasmocytes, macrophages, arranged in many areas, especially perivascularly (Fig. 3.1, Fig. 3.2).

On the samples obtained from the group where Nifedipine was administered, the identified histological changes were epithelial hyperplasia, increase of lamina propria thickness without any subepithelial collagen deposit, reduced cellularity and rich vascularization (Fig. 3.3, Fig. 3.4).

The examination of the sections from group III indicates an overgrowth of the epithelial tissue, in the form of epithelial prolongations proliferated towards lamina propria. Epithelial hyperplasia is sometimes irregular, existing areas where the normal thickness epithelium alternates with areas where the epithelium presents deep apices, “glove finger”-like. At conjunctive tissue level, the collagen fibers are numerous, arranged in perpendicular fascicles, among them being present a chronic, lymph plasmocyte inflammatory infiltrate. Vascularization is high, with a predominantly subepithelial localization (Fig. 3.5, Fig. 3.6).
In group IV there was administered an antibiotic, Azithromycin together with Cyclosporine. The epithelium thickness did not present any significant differences in comparison to the other groups where there were administered other classes of drugs. There was observed a reduction of collagen fibers on the sections coming from the rats treated with Azithromycin, in comparison with group III where there was administered only Cyclosporine. Fibroblasts and inflammatory cells were more reduced, which indicates a diminishing of the inflammatory process. Vascularization was less developed (Fig. 3.7, Fig. 3.8).

Fig. 3.1 Parakeratosis, mild acantholysis. In the lamina propria, there are collagen fibers arranged in fascicles, moderate vascularization. HE staining X 100

Fig. 3.2 Epithelial apices with a "glove finger"-like aspect, in the lamina propria, the increase of collagen component. HE staining X 100

Fig. 3.3 Epithelium with parakeratinization, moderate chronic inflammatory infiltrate, interdigitations in the epithelial-conjunctival interface. HE staining X 100.

Fig. 3.4 Lymph plasmocyte and macrophage reduced inflammatory infiltrate in the lamina propria. HE staining X 100.

Fig. 3.5 Deep epithelial apices, high cellularity in the lamina propria and subepithelial collagen deposit. HE staining X 200

Fig. 3.6 Epithelial proliferation, balloon-like cells, acanthosis, inflammatory infiltrate, intense vascularization of the lamina propria. HE staining X 200
In all the studied groups, there was a gingival overgrowth associated with a gingival inflammation measured on a scale from 1 to 3. There predominated the lymph plasmocyte infiltrate associated with a high vascularization, represented by numerous typical capillaries, but also young neof ormation capillaries. The morphological analysis referred to the inflammatory process, stage of vascularization and representation of the collagen fibrillary component (Table no. 3.1).

Table no. 3.1 Quantification of the histopathological change intensity

<table>
<thead>
<tr>
<th>STRUCTURE ELEMENTS</th>
<th>GROUP I (Phenytoin)</th>
<th>GROUP II (Nifedipine)</th>
<th>GROUP III (Cyclosporine)</th>
<th>GROUP IV (Cs + Azt)</th>
<th>GROUP V (Witness)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIBROSIS (Density of collagen fibers)</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>FIBROBLASTS</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+_</td>
</tr>
<tr>
<td>INFLAMMATORY CELLS</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>VESSELS</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+_</td>
</tr>
</tbody>
</table>

CHAP TER 4
IMMUNOHISTOCHEMICAL STUDY OF THE EXPERIMENTAL DRUG-INDUCED GINGIVAL OVERGROWTH

For the immunohistochemical study, the researched material came from the rats in the five groups described in Chapter 3. The immunohistochemical diagnosis algorithm used for the experimental study was centered on the investigation of the following aspects:

➤ Study of cellular proliferation by immunomarking with Ki 67;
➤ Study of cytokeratines with: p ancitokeratin AE1-AE3 and with MNF116;
➤ Immunohistochemical study of blood vessels and angiogenesis with: F VIII or von Willebrand (FVW) factor and STL (Solanum tuberosum lectin).

The high immunoe xpression for Ki-67 on the sections taken from groups I, II, III is the result of a high proliferative activity in the basal stratum secondary to the chronic irritation
caused by medication, to which there also associates the inflammatory process present in the lamina propria (Fig. 4.1, Fig. 4.2, Fig. 4.3). Cytokeratin AE1/AE3 was positive in all the examined groups, presenting low intensity variations from one group to another (Fig. 4.4, Fig. 4.5). The MNF 116 immunomarking was low in intensity in comparison to the immunomarking for Cytokeratin AE1/AE3 and also irregular, presenting a diffuse or zonal aspect (Fig. 4.6). The most intense vascularization and the presence of the angiogenesis process were observed in group III, treated with Cyclosporine, and the lowest was in the group treated with Phenytoin and the one with antibiotic treatment, namely Azithromycin. The blood vessels had mainly a subepithelial localization, this localization being motivated by the high requirements of the proliferation epithelium. (Fig. 4.7 - Fig. 4.12).
CHAPTER 5
HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY OF DRUG-INDUCED GUM OVERGROWTH IN HUMANS

The material used in the study was represented by fragments of gingival mucosa that came from 10 patients who presented drug-induced gum overgrowth: 4 patients were treated with Phenytoin; 4 patients received a treatment with Nifedipine; 2 patients received a treatment with Cyclosporine A for systemic conditions. We followed the confirmation of the histological aspects highlighted during the experiment on animals and the performance of an immunohistochemical study of the inflammatory process, taking into consideration that this was present in all the three groups of patients selected for the morphological study.

The histological study was performed on the fragments that came from the three groups of patients, which were processed by the histological technique of paraffin inclusion, by performing the following stainings: Hematoxylin- eosin, Masson Trichrome and Goldner Szekely trichrome. For the immunohistochemical study there were used antibodies needed for the study of the inflammatory process (Table 5.1).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Epitope / marker</th>
<th>Manufacturer</th>
<th>Antigen demasking</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD20</td>
<td>Lymphocytes B</td>
<td>DAKO</td>
<td>Buffering cytrate pH=6</td>
<td>1:50</td>
</tr>
<tr>
<td>CD79 alpha</td>
<td>Plasmocytes</td>
<td>DAKO</td>
<td>Buffering cytrate pH=6</td>
<td>1:100</td>
</tr>
<tr>
<td>CD45</td>
<td>T lymphocytes</td>
<td>DAKO</td>
<td>Buffering cytrate pH=6</td>
<td>1:100</td>
</tr>
<tr>
<td>CD3</td>
<td>T lymphocytes</td>
<td>DAKO</td>
<td>Buffering cytrate pH=6</td>
<td>1:100</td>
</tr>
<tr>
<td>CD68</td>
<td>Macrophages</td>
<td>DAKO</td>
<td>Buffering cytrate pH=6</td>
<td>1:50</td>
</tr>
<tr>
<td>CD34</td>
<td>Vessels</td>
<td>DAKO</td>
<td>Buffering cytrate pH=6</td>
<td>1:50</td>
</tr>
</tbody>
</table>

Generally speaking, GO was performed by the epithelial hyperplasia and by the lamina propria overgrowth. The difference between the three categories was made by the lamina propria change, epithelial hyperplasia being present in the quasi-equal parameters in all the investigated patients. Gum overgrowth was mostly present in the patients treated with Cyclosporine A, followed by the ones treated with Phenytoin and the last were the patients treated with Nifedipine (Fig. 5.4 - Fig. 5.6).

The epithelial and lamina propria changes were present in all gorups of patients, but the investigated parameters (inflammatory and non-inflammatory cellularity, vascularization and
aspect of collagen fibers) presented variations of intensity according to the administered medication.

In drug-induce GO with Phenytoin, Nifedipine and Cyclosporine A there is a fibrilogen process associated with an inflammatory one, which determined us to immunohistochemically highlight the involved cell populations.

The most numerous cells were lymphocytes, namely positive T CD3 and CD45 lymphocytes, in comparison to positive B CD20 lymphocytes, indicating the presence of an immune defense process, with a prevalence of cell-type immune defence. Also, there were present numerous macrophages and a different stage vascularization, according to the type of medication (Fig. 5.7- Fig. 5.12).
GENERAL CONCLUSIONS

Gum overgrowth, with its esthetic and functional implications, represents an important problem both for the patients and the clinicians, which justifies the necessity of performing a thorough morphological study, on animal models.

The most important overgrowth was presented by the gingival mucosa from group III treated with Cyclosporine, followed by the one in group I treated with Phenytoin and followed by the gingival mucosa in group II treated with Nifedipine, while in group IV where there were administered both Cyclosporine and an antibiotic, Azithromycin, there was recorded a reduction of mucosa thickness, especially due to lamina propria.

In our study, there existed an overgrowth of the mycotic activity of gingival keratinocytes in all groups of medication, in comparison to the witness group and the group where there was administered an antibiotic (Azithromycin). Still, the positive Ki 67 cells were more numerous on the sections that came from the groups treated with Phenytoin and Nifedipine, in comparison to those treated with CsA and the witness group.

For the immunohistochemical highlighting and analysis of vessels we used the anti FVIII and the anti STL antibodies. These are less frequently used in practice and, according to our knowledge, they have not been used before in the investigation of this pathology, this representing a first performance until now.

In drug-induced OG with Phenytoin, Nifedipine and Cyclosporine A there is a fibrilogenetic process associated with an inflammatory one. The most numerous cells were lymphocytes and, among these, positive T CD3 and CD45 lymphocytes, in comparison to positive CD20 B lymphocytes, which indicates the presence of an immune defence process, with the prevalence of cell immune defence.

The obtained results give the opportunity to understand certain aspects connected to the pathogeny of this conditions and also indicate the possibility of developing a strategy of non-surgical reduction of gum overgrowth, by inducing the fibroblast apoptosis, associated with an anti-inflammatory medication, which, in its turn, may stimulate apoptosis.


