PhD THESIS

IMMUNOHISTOCHEMICAL AND BIOCHEMICAL STUDY ON THE PATHOGENIC PATHWAYS INVOLVED IN DRUG-INDUCED GINGIVAL OVERGROWTH

- ABSTRACT -

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2011
CONTENTS OF THE PhD THESIS

ABBREVIATIONS

INTRODUCTION

REVIEW OF THE LITERATURE
1. MORPHOLOGY AND HISTOPHYSIOLOGY OF THE GINGIVA
2. DRUG INDUCED GINGIVAL OVERGROWTH
   2.1. The concept of gingival overgrowth
   2.2. Risk factors for drug induced gingival overgrowth
   2.3. Clinical importance of drug induced gingival overgrowth
   2.4. Mediators in the pathogenic mechanisms of drug induced gingival overgrowth
      2.4.1. Matrix metalloproteinases and their natural tissue inhibitors
      2.4.2. Growth factors and cytokines
      2.4.3. Effectors of the epithelial to mesenchymal transition
      2.4.4. Toll-like receptors

PERSONAL STUDY
3. MOTIVATION AND WORK HYPOTHESES
4. AIMS OF THE THESIS
5. MATERIAL AND METHODS
   5.1. Materials
      5.1.1. Reagents
      5.1.2. Biological materials
   5.2. Methods
      5.2.1. Histological methods
      5.2.2. Immunohistochemical methods
      5.2.3. Enzyme linked immunosorbent assay (ELISA)
      5.2.4. RNA isolation and RT-PCR
      5.2.5. Statistical analysis
6. RESULTS
   6.1. Effects of phenytoin and dihydropyridine Ca-channel blockers on gingival mucosa morphology and composition
   6.2. Effects of phenytoin and dihydropyridine Ca-channel blockers on gingival fibroblasts phenotype
   6.3. Effects of phenytoin and dihydropyridine Ca-channel blockers on the imbalance of extracellular matrix synthesis/breakdown
   6.4. Effects of phenytoin and dihydropyridine Ca-channel blockers on the expression of some mediators of the epithelial to mesenchymal transition
   6.5. Effects of phenytoin and dihydropyridine Ca-channel blockers on the expression of the innate immune receptors Toll-like (TLR)
   6.6. Effects of phenytoin and dihydropyridine Ca-channel blockers on the genic expression of TGFβ1, Smad, TLR-2 and TLR-4
   6.7. Effects of phenytoin and dihydropyridine Ca-channel blockers on the salivary levels of some mediators of the collagen synthesis/breakdown
7. DISCUSSION
   7.1. Imbalance of ECM synthesis/breakdown due to the influence of several profibrogenic growth factors on the MMP/TIMP balance and on gingival fibroblast heterogeneity in phenytoin and dihydropyridine Ca-channel blockers induced gingival overgrowth
   7.2. Modulation of the epithelial to mesenchymal transition in phenytoin and Ca-channel blockers dihydropyridine induced gingival overgrowth
   7.3. Contribution of the receptors of the innate immune response, TLR, to the interrelation between inflammation-epithelial to mesenchymal transition-gingival fibrosis in phenytoin and dihydropyridine Ca-channel blockers induced gingival overgrowth
8. CONCLUSIONS
9. REFERENCES
10. PUBLISHED WORKS RELATED TO THE TOPICS OF THIS THESIS
**INTRODUCTION**

Currently, more than 20 prescription medications are associated with gingival enlargement [Academy Report, 2004]. “Gingival enlargement” or “gingival overgrowth” (GO) is the preferred term for all medication-related gingival lesions previously termed “gingival hyperplasia” or “gingival hypertrophy.” These earlier terms did not accurately reflect the histologic composition of the pharmacologically modified gingiva. Drugs inducing gingival overgrowth (DIGO) can be broadly divided into three categories: anticonvulsivants, Ca-channel blockers, and immunosuppressants. Although the pharmacological effect of each of these drugs is different and directed toward various primary target tissues, all of them seem to act similarly on the gingival mucosa as a secondary target.

Clinical and histopathological findings were intensively studied [Marshall and Bartold, 1999; Seymour et al., 2000; Kataoka et al., 2005; DeAngelo et al., 2007; Baniţă et al., 2008; Pisoschi et al., 2012]. These studies revealed that irrespective the risk factor histological changes are unspecific (the epithelial enlargement, connective tissue accumulation and different degrees of inflammation in the lamina propria).

Molecular factors controlling collagen turnover in gingival overgrowth act on two pathogenic pathways: (i) accumulation due to an excessive synthesis; (ii) inhibition of extracellular matrix (ECM) breakdown. Both pathways involved the active „dialogue” between fibroblasts and their cellular partners (epithelial, endothelial and proinflammatory cells).

This paper concerns on new research regarding the mechanisms through which the pharmacological agents, such an anticonvulsivant (phenytoin) and some dihydropyridine Ca-channel blockers (nifedipine and amlodipine) acts on the gingival tissue as a secondary target, focusing on the link between the growth factors controlling fibrosis and the epithelial-mesenchymal transition and disregulation of the innate immune systems as pathogenic pathways involved.

Since in the last few years analysis of saliva gained a great research interest in order to find less invasive tools for diagnosis of systemic and local diseases, many studies revealed the possible relation between salivary levels of several cytokines and their tissue expression depending the presence of a risk factor and proposed some of these molecules as possible biomarkers for gingival overgrowth [Buduneli et al., 2001; Wright et al., 2001; Ruhl et al., 2004; Gurkan et al., 2008; Pisoschi et al., 2010].

Such a fundamental research could have as an immediate impact the approach of new directions in this field and, as a long-term perspective, the statement of new non-invasive methods of analysis and therapeutic strategies with topic drugs used to interfere in the progression of gingival fibrosis.

**REVIEW OF THE LITERATURE**

Chapter 1. ”Morphology and histophysiology of the gingiva” describes the main histological, biochemical and physiological features of the two tissues from the gingival mucosa – epithelium and lamina propria.

Chapter 2. ”Drug induced-gingival overgrowth” describes the concept and the risk factors of gingival overgrowth, data regarding the prevalence and the clinical consequences of drug induced gingival overgrowth, the mechanisms of phenytoin and dihydropyridine Ca-channel blockers-induced biotransformation and some important structural and functional features of the main mediators involved in ECM remodeling: growth factors and cytokines, matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), effectors and markers of the epithelial to mesenchymal transition (EMT), Toll-like receptors (TLR), as receptors involved in the innate immune system.
- PERSONAL STUDY

- AIMS OF THE STUDY

The specific working objectives were related to:

1. Investigation of the imbalance between gingival connective tissue elements as a side effect of the pharmacodynamic action of phenytoin and dihydropyridine Ca-channel blockers (nifedipine and amlodipine).

2. Investigation of cell proliferation and fibroblasts phenotype heterogeneity in the connective tissue as a side effect of the pharmacodynamic action of phenytoin and dihydropyridine Ca-channel blockers (nifedipine and amlodipine).

3. Investigation of the gingival expression of some cellular and molecular markers (growth factors – CTGF, cytokines - TGFβ1, MMPs-MMP-1 and MMP-2, TIMPs-TIMP-1 and TIMP-2) involved in connective tissue imbalance as a side effect of the pharmacodynamic action of phenytoin and dihydropyridine Ca-channel blockers (nifedipine and amlodipine).

4. Investigation of the hypothesis that side effects of phenytoin and dihydropyridine Ca-channel blockers (nifedipine and amlodipine) on gingival ECM homeostasis involve the epithelial to mesenchymal transition as a pathogenic pathway according to the expression of E-cadherin, FSP1, Smad3 and Snail.

5. Investigation of gene expression and localization of TLR2 and TLR4 in the gingival mucosa in phenytoin and dihydropyridine Ca-channel blockers-induced gingival overgrowth.

6. Testing the hypothesis that in saliva could be identified specific biomarkers related to the imbalance of collagen homeostasis in DIGO and validation of the correlation between their tissue and salivary levels.

- MATERIAL AND METHODS

This chapter describes the materials (reagents, samples) and methods used for the research.

Part of the experiments discussed in this thesis were conducted in a research project funded by the Ministry of Education, Program Ideas of the PNCDI II carried out between 2008 and 2011 in which I was part of the research team.

The experiments used gingival tissue and whole saliva from 43 subjects both men and women, obtained in the Clinic of Oro-Maxillo-Facial Surgery, County Emergency Hospital Craiova or available from the pool of biological samples formed for the project ID563 between 2008-2011. Samples were distributed in several batches: I<sup>st</sup> batch (normal control) – samples without GO collected after the orthodontic treatment; II<sup>nd</sup> batch – samples with GO as a side effect of dihydropyridine Ca-channel blockers (IIa) and phenytoin (IIb); III<sup>rd</sup> batch (used as pathologic control) - samples with GO due to inflammation or with gingival fibromatosis. Clinical examination evaluated all the usual clinical parameters for GO (probing depth, bleeding on probing, gingival attachment loss). Were excluded samples of GO due to systemic disease and those collected from subjects treated with corticosteroids, oral contraceptives and antibiotics in the last 6 months.

For the histological study we used several methods, namely the usual staining techniques (hematoxylin-eosin for the general morphological evaluation, Masson trichrome, silver impregnation and PAS-Alcian for connective tissue components) and several immunohistochemical techniques (ABC, LSAB, EnVision) to highlight specific markers devoted to each pathogenic pathway (Table 1).

For the biochemical study we used ELISA techniques to quantify salivary level of growth factors (CTGF and TGF-β1) and RT-PCR to analyze gene expression of TGF-β1, Smad3, TLR2 and TLR4.

Statistical analysis. For numeric parameters the following statistical indicators were calculated: minimum value, maximum value, means, standard deviation (SD). We used Mann-Whitney and Kruskal-Wallis tests, at 5% level of significance, to compare data and Graphpad Prism5.04 to generate the graphic charts.
**Table 1 Antibodies used for the immunohistochemical labeling**

<table>
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<th>Anticorp</th>
<th>Dilution</th>
<th>Code</th>
<th>Method</th>
</tr>
</thead>
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<td>Monoclonal mouse anti-human α-smooth muscle actin (1A4)</td>
<td>1:100</td>
<td>M0851</td>
<td>EnVision</td>
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<td>Monoclonal mouse anti-human vimentin (V9)</td>
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<td>Monoclonal mouse anti-Ki67 (MIB1)</td>
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<td>M7020</td>
<td>ABC</td>
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<tr>
<td>Monoclonal mouse anti-human TGF-β1, (TB21)</td>
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<td>sc 52893</td>
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<td>Monoclonal mouse anti-human MMP-1 (3B6)</td>
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<td>Monoclonal mouse anti-human E-cadherin (NCH38)</td>
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<td>Rabbit polyclonal to TLR2</td>
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<td>Polyclonal rabbit to TLR4</td>
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<td>ABC</td>
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<td>Rabbit polyclonal to Smad3</td>
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<td>Rabbit polyclonal to SNAIL+SLUG</td>
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**RESULTS AND DISCUSSION**

Results obtained in this work are integrated in relation to their involvement in various pathogenic pathways postulated for phenytoin and Ca-channel blockers dihydropyridine-induced gingival overgrowth: (i) imbalance of extracellular matrix synthesis/breakdown due to the influence of several profibrogenic growth factors (TGF-β1 and CTGF) on MMP/TIMP balance and on fibroblasts phenotype heterogeneity; (ii) modulation of the epithelial to mesenchymal transition; (iii) mediatory involvement of Toll-like receptors of the innate immune response system in the interrelation between epithelial to mesenchymal transition and gingival fibrosis.

(i) Effect of phenytoin and dihydropyridine Ca-channel blockers on gingival mucosa morphology and composition revealed nonspecific morphological changes represented by epithelial enlargement and connective tissue accumulation in the lamina propria with various degree of inflammation and changes in the collagenous/noncollagenous ECM components, phenytoin GO being more fibrotic.

Disruption of ECM homeostasis was due mainly to the up regulation of collagen synthesis, and, secondary to down regulation of its breakdown.

A lot of consistent works argue that fibroblasts metabolism is essential for collagen turnover, their proliferation and differentiation, and collagen synthesis being controlled by cytokines that initiates signaling cascades mediated by specific receptors [Gressner *et al*., 2002; Trackman and Kantarci, 2004; Bataller and Brenner, 2005].

In all types of DIGO we noticed fibroblast phenotype heterogeneity (affirmation sustained by the identification of three types of fibroblasts: some labeled with vimentin, few number of cells labeled with α-smooth muscle actin, α-SMA, and an increased number of cells labeled with fibroblast specific protein-1, FSP-1) which suggests their participation in conjunction to GO development. Activation mechanisms are needed to determine „the recruitment” of susceptible fibroblasts to respond particularly to drug action by excessive collagen synthesis.

Cells of the gingival mucosa expressed a combination between MMP and TIMP dependent on risk factor; complex mechanisms control this pattern and their disruption represents another pathogenic pathway of ECM accumulation.

Correlations between TGF-β1 and CTGF expression argue for the essential role of TGF-β1 in drug induced fibrogenesis not only by controlling MMP/TIMP ratio but by increasing CTGF action.
Lack of correlation between TGF-β1 expression, the degree of fibrosis and the presence of α-SMA labeled cells, characterized by a high rate of collagen synthesis, suggests that reducing the balance MMP/TIMP and activation of myofibroblasts are not the only pathogenic pathways for the cellular action of phenytoin and dihydropyridine Ca-channel blockers on the lamina propria.

Because levels of TGF-β1 and CTGF in whole saliva and the degree of their expression in the gingival mucosa were not significantly correlated, additional measurements on higher groups of samples are needed in order to validate these biomarkers.

(ii) Abnormal TGF-β1 and CTGF cytokines expression profile as a result of drug action on the cellular immune system leads to the activation of several pathogenic pathways by which they stimulate and maintain changes of the ECM in phenytoin and dihydropyridine gingival overgrowth.

Differences between the expression of E-cadherin, FSP-1 and Snail (Slug) comparing to normal gingival tissues argue the hypothesis that TGF-β1 mediated EMT can be one of the pathogenic mechanisms of phenytoin and dihydropyridine induced GO by activating Smad-Snail signaling pathway.

Precise temporal relationship between EMT mediators is difficult to reveal in vivo since most of them are early markers of this phenomenon.

(iii) We evaluated the expression of TLR2 and TLR4 in overgrown gingival mucosa in order to integrate their expression in the interrelation EMT-gingival fibrosis. Drug induced GO is accompanied by an abnormal cytokine profile secondary to drug or drug metabolites action on the innate or acquired immune response systems.

Gingival overgrowth contains various subpopulations of macrophages and other inflammatory cells dependent on the drug administrated and different comparing the normal tissue [Trackman and Kantarci, 2004]. Recent studies revealed that macrophages are activated by Th1 and Th2 cytokines mediators and gain various phenotypes, the most important being that involved in fibrosis that follows the inflammation [Meneghin and Hogaboam, 2007].

Proinflammatory cells, fibroblasts and epithelial cells from the gingival mucosa expressed TLR2 and TLR4 irrespective the risk factor involved. We noticed an increased expression of these receptors in phenytoin and to a lesser extent in dihydropyridine induced GO comparing to the normal or inflamed mucosa.

This pattern can be associated not only to their action in recognizing PAMPs produced in the oral cavity even in normal conditions but also to recognize protein adducts formed during the interaction with drug metabolites. Recently was proved that oxidized and nitrated proteins released from damaged cells could act as PAMPs for TLR [Rifkin et al., 2005].

As a consequence, the insufficiency of pathogens scavenge is a permanent source of cell injury and chronic inflammation. As an unexpected final of this overactivated innate immune system fibrosis occurs.

Because TGF-β1 is a key member of all signaling pathways controlling inflammation, EMT and fibrosis, directly or through is down stream profibrotic effector, CTGF, we can conclude that these three events concur to mediate the alterations induced by these drugs on gingival mucosa.

- CONCLUSIONS -

⇒ Continuous dialogue between the two main types of cells from the gingival mucosa – epithelial and mesenchymal cells is responsible for the peculiar response of the gingival mucosa in the presence of risk factors for gingival overgrowth.

⇒ In phenytoin and dihydropyridine-induced gingival overgrowth, disruption of ECM homeostasis is due mainly to the up regulation of collagen synthesis, and, secondary to the down regulation of its breakdown.

⇒ TGF-β1 has an essential role in the pathogenesis of gingival fibrosis induced as a side effect of phenytoin and dihydropyridine Ca-channel blockers acting through several mechanisms: regulation of MMP/TIMP balance, increase of the profibrogenic action of CTGF, control of fibroblasts phenotype changes, mediation of the epithelial-mesenchymal transition and inflammation.
TGF-β induced epithelial to mesenchymal transition is a new pathogenic pathway that function as a point of convergence between the innate immune response and fibrosis in phenytoin and dihydropyridine induced gingival overgrowth.

Altered ECM turnover in drug induced gingival overgrowth need a cooperation between direct action of drug or of their metabolites with coexistent inflammation.

Correlation between the expression of TGF-β1 and CTGF and the degree of gingival fibrosis due to drug action are encouraging but validation of salivary biomarkers for fibrosis reflecting the precise cellular ambient still remains a desiderate for future research.

**REFERENCES**


**LIST OF PAPERS PUBLISHED OR PRESENTED RELATED TO THE TOPICS OF THIS PhD THESIS**

1. Growth Factors and Connective Tissue Homeostasis in Periodontal Disease, Cătălina Pisoschi, Camelia Stănciulescu, Monica Baniţă. Chapter in the book: *Pathogenesis and Treatment of Periodontitis*, InTech Open Acces Publisher (published online from January 2012)
2. Evidence for the epithelial mesenchymal transition as a pathogenic mechanism of phenytoin induced gingival overgrowth, C Pisoschi, C Stănciulescu, C Munteanu, A M Fusaru, M Baniţă, *Farmacia (in press)*, ISSN 0014-8237


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Dates/Title of qualification awarded  1993-1999 – PhD
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<th>Organization providing education</th>
<th>Faculty of Chemistry, Specialization – Biochemistry, University of Bucharest, Romania</th>
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<td>Dates/Title of qualification awarded</td>
<td>2002 – Research grant financed by the Universita degli Study, Ancona 2000-2001-Fellowship awarded by the Romanian Government Specialization in techniques of photonic and electronic microscopy, immunohistochemistry</td>
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<th>Institute of Normal Human Morphology, Universita degli Studi, Ancona, Italy</th>
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<td>2000 - Specialization in techniques of molecular biology Advanced studies “From cell biology to molecular medicine”</td>
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<td>Dates/Title of qualification awarded</td>
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<tr>
<th>Organization providing education</th>
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<th>Organization providing education</th>
<th>Hellenic Proteomics Society, Faculty of Pharmacy, University of Patras, Greece</th>
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**Additional information**

**Collaboration on Research Grants:**
8 grants financed by the Romanian Academy, the Ministry of Education and Research and Universita Politecnica delle Marche

**Published works:**
8 books and 2 chapters in international books
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