EVIDENCE FOR THE EPITHELIAL-MESENCHYMAL TRANSITION AS A PATHOGENIC MECHANISM OF PHENYTOIN-INDUCED GINGIVAL OVERGROWTH

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Abstract

Gingival overgrowth was reported as a side effect of more than twenty therapeutic agents. Despite the different pharmacological effect of each of these drugs, all seem to act on the gingiva as a secondary target having in common the enlargement of the gingival tissue due to an extracellular matrix accumulation. Previous studies indicated the imbalance of collagen synthesis and breakdown as the main biochemical change, but the precise pathogenic mechanisms are incompletely understood. We investigated the hypothesis that Transforming Growth Factor β1 (TGF-β1) mediated epithelial-mesenchymal transition could be involved in the development of phenytoin-induced gingival overgrowth. Immunohistochemistry disclosed an elevated response for TGF-β1 in gingival mucosa associated with a decreased expression of the epithelial marker E-cadherin and an increased number of cells expressing the fibroblast specific protein-1, also known as S100A4, and the transcriptional factors Smad and Snail. Taken together, these data suggest that TGF-β1/Smad/Snail signaling pathway plays an important role in the epithelial-mesenchymal transition in phenytoin-induced gingival overgrowth.

Rezumat

Hipercreşterea gingivală a fost raportată ca reacţie adversă care apare la administrarea unui număr mare de medicamente din grupe farmacoterapeutice diferite. În ciuda efectului farmacologic diferit, toate par să aibă mucoasa gingivală ca ţintă secundară a modificărilor histologice, trăsătura comună fiind îngroşarea acesteia ca rezultat al acumulării excesive de matrice extracelulară. Studii anterioare au arătat dezechilibrul balanţei sinteză/degradare a colagenului ca principala modificare biochimică, mecanismele etio-patogenice fiind incomplet cunoscute. În acest studiu, a fost evaluată ipoteza că tranziţia epitelio-mezenchimală mediată de factorul de creştere transformată β1 (TGF-β1) ar putea fi unul dintre mecanismele implicate în hipercreşterea gingivală indusă de fenitoină. Studiul imunohistochimic a arătat o supraexprimare a TGF-β1 asociată cu reducerea exprimării markerului epitelial E-caderină şi creşterea numărului de celule pozitive pentru proteina specifică fibroblastelor-1, cunoscută şi ca S100A4, precum şi factorii de transcripţie Smad şi Snail. Rezultatele obţinute sugerează că semnalizarea pe

calea TGF-β1/Smad/Snail are un rol important în tranziţia epitelio-mezenchimală implicată în hipercreşterea gingivală indusă de fenitoină.

Keywords: gingival overgrowth, phenytoin, epithelial-mesenchymal transition, TGF-β1, immunohistochemistry.

Introduction

Gingival overgrowth is the preferred term for all medication-related gingival lesions previously termed "gingival hyperplasia" or "gingival hypertrophy" because it better reflects the histological changes of the gingival mucosa in the presence of various risk factors. Currently, more than twenty prescription therapeutic agents were reported to induce gingival overgrowth, the anti-seizure drug phenytoin being one of the most incriminated [5,9,19]. Although the pharmacological effect of each of these drugs is different, all of them seem to have the same side effect on the gingival mucosa as a secondary target.

Histological analysis showed that irrespectively the etiological factor involved, the changes of the mucosa in gingival overgrowth refer both to the gingival epithelium and *lamina propria* [1,2,4]. There is now a general agreement that all gingival overgrowth lesions contain fibrotic or expanded connective tissue with various levels of inflammation and an enlarged gingival epithelium. Previous studies revealed that phenytoin-induced gingival overgrowth is more fibrotic then inflamed [2,18]. The precise pathogenic mechanisms involved in the imbalance of collagen synthesis/degradation in fibrotic drug-induced gingival overgrowth are still investigated.

The most important cytokine controlling collagen homeostasis in fibrotic diseases is the Transforming Growth Factor-β, mainly its TGF-β1 isoform, able to selectively stimulate the production of extracellular matrix molecules [6,7]. In the same time, TGF-β is greatly involved in inflammation [16] and represents a key mediator of the epithelial-mesenchymal transition [23, 24].

Epithelial-mesenchymal transition (EMT) is a well-defined concept that occurs normally in the development process. In pathology, EMT is involved in fibrosis, cancer progression and metastasis. During EMT epithelial cell–cell and cell–extracellular matrix interactions are weakened as epithelial cells transdifferentiate into fibrogenic fibroblast-like cells [17]. EMT involves changes in gene expression that induce the loss of proteins associated with the epithelial phenotype (such as, E-cadherin, ZO-1) and increase the expression of proteins associated with a mesenchymal and migratory cell phenotype (vimentin, fibroblast specific protein-1, α-smooth

muscle actin, N-cadherin) with concomitant alterations in cytoskeletal organization, cell adhesion and production of extracellular matrix [8,24]. The TGF-β/Smad/Snail is a key signaling pathway involved in different subtypes of EMT [8,25].

The present immunohistochemical study investigated the hypothesis that TGF-β1/Smad/Snail pathway is involved in the progression of phenytoin-induced gingival overgrowth.

Material and Methods

Reagents. Masson stain kit was purchased from Bio-Optica, Italy. Normal swine serum, mouse monoclonal anti–human E-cadherin, rabbit polyclonal anti-S100A4 and polyclonal swine >>Multi Link<< were purchased from Dako, USA. Mouse monoclonal anti-TGF-β1 was purchased from Santa Cruz Biotechnology Inc., rabbit polyclonal to Smad3 and rabbit polyclonal to Snail+Slug from Abcam, UK. Vectastain kit (Vector Laboratories, USA) was used to amplify the immune reactions and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Co., USA) and hydrogen peroxide as developers. The anhydrous mounting medium (Neo-Mount), hydrogen peroxide and buffers were purchased Merck KGaA, Germany.

*Tissue samples***.** Gingival tissue samples were collected from subjects undergoing periodontal surgery in the Department of Oro-Maxillo-Facial Surgery of the University of Medicine and Pharmacy Craiova, Romania and the Clinic of Oro-Maxillo-Facial Surgery of the Emergency County Hospital of Craiova. All gingival fragments were obtained after the informed consent of patients were signed. We used samples from four donors with phenytoin-induced gingival overgrowth and two without gingival overgrowth. Previously, the experimental protocol was approved by the Ethical Committee of the University of Medicine and Pharmacy, Craiova, Romania. None of the donors had received periodontal treatment in the last years and all gingival biopsies were obtained prior to the periodontal therapy. Age, gender and clinical inflammation (gingival index and bleeding on probing) were recorded for each patient before surgical procedures. Tissues were washed with physiologic saline solution and processed for the morphological study.

Histological analysis. Tissue samples were fixed in 4% buffered paraformaldehyde at 4°C for 48 hours and then processed for paraffin embedding. Blocks of paraffin-embedded tissues were cut at 3 μm

thickness, dewaxed, rehydrated and colored with modified Masson trichrome stain.

Immunohistochemistry. Serial sections of 3 μm were dewaxed in xylene and rehydrated *via* graded alcohols. Antigen retrieval was performed after microwave incubation of sections in citrate buffer, pH=6. Endogenous peroxidase activity was blocked with methanol and 0.3% hydrogen peroxide solution. Sections were treated with normal swine serum in order to block unspecific binding and then were incubated with one of the primary antibodies mentioned bellow:

Antibody	Dilution	Vendor Code
Mouse monoclonal anti-human TGF-β1	1:200	sc 52893
Mouse monoclonal anti-human E-cadherin	1:100	M3612
Rabbit polyclonal anti-human S100A4	1:200	A5114
Rabbit polyclonal to Smad3	1:400	ab51451
Rabbit polyclonal to Snail+Slug	1:1000	ab85936

The next day, sections were processed for the amplification of the immune signal using the polyclonal "Multi-Link" and the avidin-biotin complex. 3,3'-Diaminobenzidine tetrahydrochloride and hydrogen peroxide were used for color development and Mayer hematoxylin for nuclear counterstaining.

Evaluation. Slides were observed and registered with a Nikon Eclipse microscope coupled to a digital camera. Images were finally processed using the Microsoft Office Picture Manager. The evaluation of the immunohistochemical reactions was performed by two different observers according to the following: immunohistochemical reactions (brown deposits in labeled structures) were graded as absent (negative or diffuse weak signal) or present (moderate or strong intensity of the signal), involving an evaluation of the mean signal in all microscopic fields from the whole slide. Analysis of nuclear immunostaining for Snail was performed by counting the total number of positive nuclei in a given area and normalizing to the total number of cells, results being expressed as the percent of positive stained cells. For each antibody tested, it was performed a negative control in which the primary antibody was replaced by phosphate buffer saline, pH 7.4-7.6.

Results and Discussion

Histological stains showed unspecific changes: enlargement of the gingival mucosa, with a thick stratified squamous epithelium bending deeply into the underlining connective stroma to form the so called "rete

pegs" and various degrees of collagen fibers accumulation intricate with islands of pro-inflammatory cells (Fig.1A).

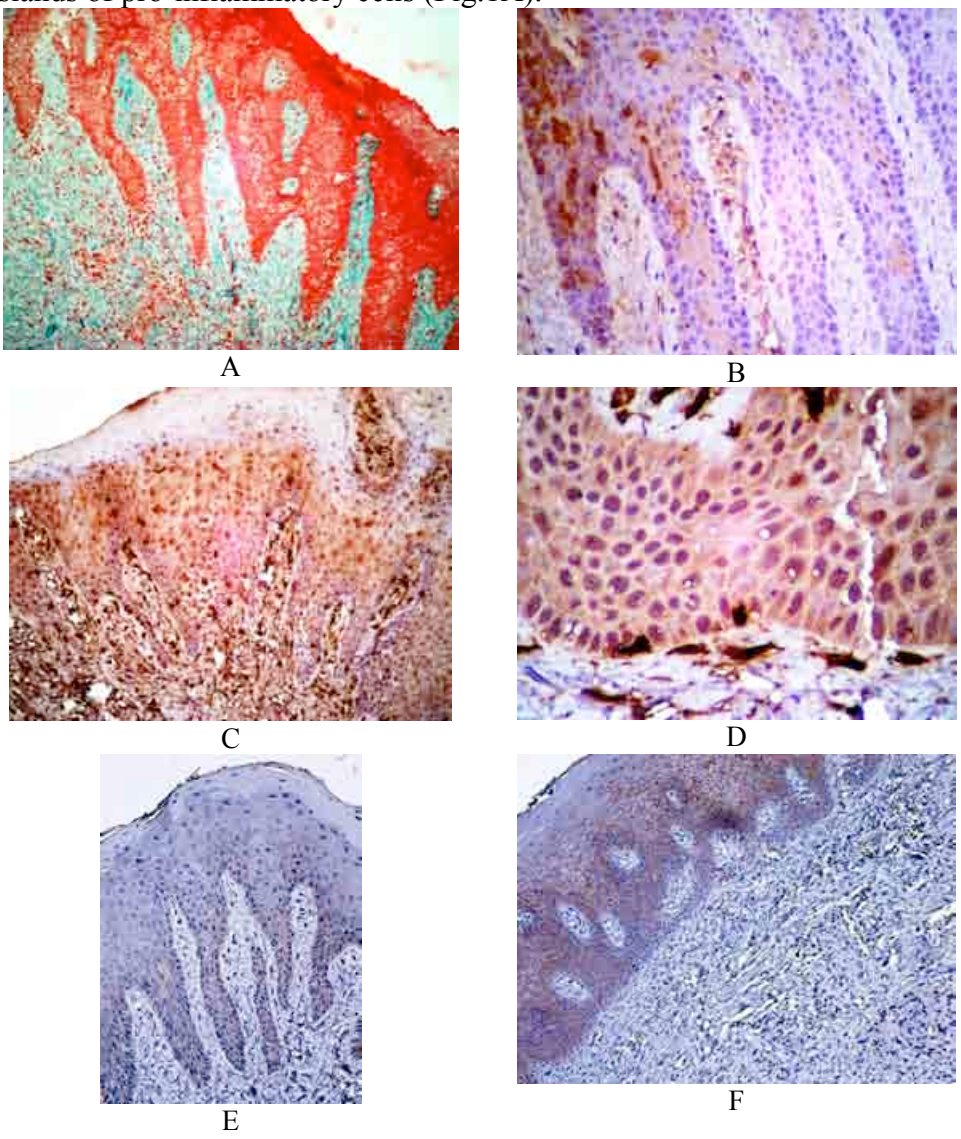
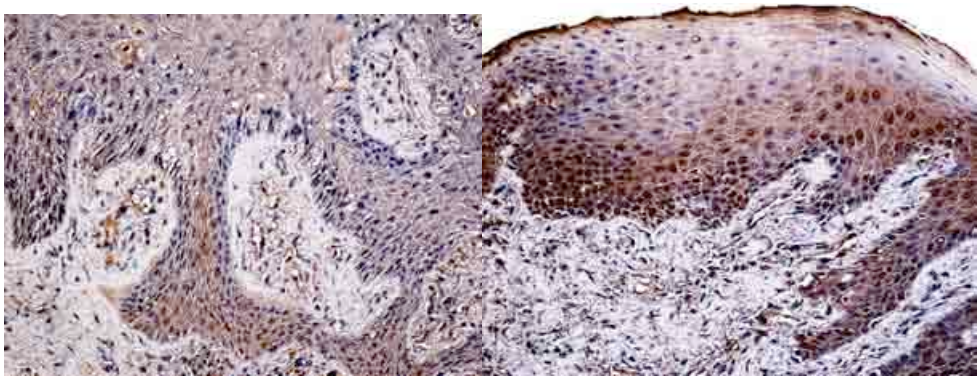


Figure 1

Morphological features of gingival overgrowth (GO) samples. (A) Enlargement of the gingival epithelium and collagen accumulation in lamina propria in phenytoin induced GO, Masson staining (x4); (B) Immunostaining for TGF-β1 in basal and parabasal epithelial layers of phenytoin induced GO mucosa (x10); (C) Immunostaining for S100A4 – general view (x10); (D) Positive S100A4 cells in the basal layer and lining the epithelium in lamina propria of phenytoin induced GO (x40); (E) Immunostaining of E-cadherin in phenytoin induced GO. Epithelial layers are almost negative (x10); (F) Positive reaction for E-cadherin in control mucosa without GO (x4).

The positive reaction for TGF-β1 in phenytoin-induced gingival overgrowth samples was observed in extended areas of the basal and parabasal epithelial layers and in some mesenchymal cells lining the epithelium (Fig.1B). As we previously observed, TGF-β1 had an increased expression in many pro-inflammatory cells also [2]. Figure 1C represents a general view of the overgrown gingival mucosa where one can notice an increased number of S100A4 positive cells both in the epithelium and lamina propria. With a higher magnification we detected the distribution of these S100A4 positive cells mainly in the basal epithelial layer nearby the basement membrane and in the connective tissue close to the epithelium (Fig.1D). Tissues with phenytoin-induced gingival overgrowth showed significant reduction of E-cadherin expression in the gingival epithelium compared with tissues from subjects without overgrowth where E-cadherin had a constant presence in the adherent junctions between keratinocytes (Fig.1E,F).

As the figures 2A and 2B reveal, we found an up-regulation of the transcription factors Smad3 and Snail in cells from profound layers of the gingival epithelium. Often these positive cells were round or elongated, surrounded by a clear halo proving that they are losing adhesion contacts invading through the extracellular matrix. We observed mainly nuclear Snail positive reaction proving the existence of the active form of this transcription factor in 58% of the epithelial cells.



A B

Figure 2

Detection of EMT regulators in phenytoin induced gingival overgrowth (GO) samples. The expression of the transcriptional factors Smad 3 and Snail were analyzed. Weak nuclear staining for Smad 3 (A) and strong nuclear and cytoplasmic staining for Snail (B) in areas of epithelial remodeling (x10).

The histological assessment showed that in drug-induced gingival overgrowth, although the pharmacological effect of the drug is different, the changes of the mucosa refer both to a fibrotic or expanded connective tissue with different levels of inflammation and an enlarged gingival epithelium, a significant difference being represented by the degree of fibrosis or inflammation [2].

Because not all the patients taking phenytoin develop gingival overgrowth, such individualized drug response is related to the drug dose, the duration of administration and genetic variations of cytochrome P450 isoforms and other enzymes involved in its metabolism [14,20]. 5-(parahydroxyphenyl)-5-phenyl-hydantoin, the major metabolite of phenytoin, could react with a phenotipically distinct subpopulation of gingival fibroblasts [18]. We presume that this cell subpopulation could be represented by fibroblasts trans-differentiated from the epithelial cells undergoing EMT.

Many research studies have been shown that TGF-β1 signaling plays an important role in regulating epithelial plasticity and is one of the most significant lines of communication between stroma and epithelium [6,24]. TGF-β1 is a pro-fibrogenetic cytokine acting through the well-orchestrated transcription program of EMT which stimulates the loss of the epithelial features and gain of the mesenchymal ones, cells undergoing this program being actively involved in collagen synthesis. The cells undergoing this mechanism express a specific marker, the fibroblast specific protein-1 (FSP-1), also named S100A4 [12, 21].

Our study revealed some S100A4 positive cells in the epithelium, mainly in the profound layers, and a great number in the connective tissue. In parallel to the increased S100A4 expression, a substantial reduction of E-cadherin reaction was observed in the epithelium. Loss of this transmembrane adhesion protein is considered a hallmark event of the TGF-β induced EMT.

Two possible signaling pathways are recognized as mediators of TGF-β induced EMT. The first involves Smad proteins [16]. Activation of TGF-β1 results in the stimulation of a signal transduction pathway to induce EMT *via* the Alk5 receptor [10,15]. TGF-β induced activation of the receptor complex leads to activation of Smad2 and Smad3 through direct C-terminal phosphorylation. These form then trimers with Smad4 and translocate into the nucleus and cooperate with other DNA binding transcription factors to regulate target genes transcription [24]. Snail is one of the three families of transcription factors activated by Smad in response to TGF-β able to induce changes that facilitate mobility. Snail represses the

expression of E-cadherin, resulting in diminished cell junctions and disrupting intercellular adhesion between cells, and activates the expression of vimentin, fibronectin, N-cadherin leading to a full EMT phenotype [3,11,13].

In our study, we didn't find a correlation between Smad3 and Snail expression. Because gingival samples assessed presented advanced chronic lesions, it is possible that the presence of the activated form of several proteins involved in the TGF-β signaling pathway may not be revealed in an immunohistochemical assay. Other authors [22] had the same difficulty to reveal the *in vivo* expression of some transcription factors (Smad and Slug) and suggested that it is required an animal model to establish temporal relationships for changes of expression of EMT markers as gingival overgrowth develops.

Conclusions

In phenytoin-induced gingival overgrowth is TGF-β1 overexpressed and drives several pathogenic pathways to promote fibrosis, one of these being the orchestration of those cellular events that lead to the trans-differentiation of epithelial cells into fibroblast-like cells through epithelial-mesenchymal transition.

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