Role of transforming growth factor β–connective tissue growth factor pathway in dihydropyridine calcium channel blockers-induced gingival overgrowth

CÂTÂLINA GABRIELA PISOSCHI1), CAMELIA ELENA STÂNCIULESCU1), ANA MARINA ANDREI1), ANCA BERBECARU-IOVAN1), CRISTINA MUNTEANU2), FLORICA POPESCU1), ILEANA MONICA BANIŢĂ3)

1) Department of Pharmacy, University of Medicine and Pharmacy of Craiova, Romania
2) Department of Dentistry, University of Medicine and Pharmacy of Craiova, Romania
3) Department of Morphological Sciences, University of Medicine and Pharmacy of Craiova, Romania

Abstract
Background: Gingival overgrowth was reported as a side effect after chronic administration of several drugs, which, despite their different pharmacological effect, seem to have the gingival mucosa as a secondary target. The thickness of the gingival epithelium and fibrosis in the lamina propria are unspecific changes that together determine the enlargement of the gingival mucosa, but the molecular mechanisms responsible for the imbalance of collagen synthesis/breakdown are still uncertain. The aim of our study was to assess the role of TGF-β1–CTGF pathway in the activation of cells with a fibroblastic phenotype responsible for the gingival fibrosis developed after chronic administration of dihydropyridine calcium channel blockers. Materials and Methods: Fragments of gingival tissue collected from patients clinically diagnosed with gingival overgrowth after chronic administration of nifedipine and amlopidine were processed for paraffin embedding. Serial sections were used for routine staining Masson and Gömöri’s silver impregnation in order to reveal collagen accumulation and for immunohistochemical reactions to label TGF-β, CTGF, Ki67 and α-SMA. Results: Routine histological staining for collagen revealed the presence of gingival fibrosis and a change between type I collagen/type III collagen ratio. Regardless of the drug involved, many slides showed extended TGF-β1 positive areas, mainly in the profound – spinous and basal – layers, but also in some cells from the subjacent connective tissue. CTGF exposed intense positive reaction in the basal and parabasal layers, but also in resident cells from the connective tissue. Ki67 immunolabeling did not reveal an increased fibroblast proliferation in the lamina propria. We noticed the presence of a small number of myofibroblasts in the lamina propria. Conclusions: These findings suggest that TGF-β1–CTGF axis is activated in dihydropyridine calcium channel blockers-induced gingival overgrowth and exerts a different control on the activation of fibroblasts with a synthetic phenotype. These results also have implications for better understanding mechanisms of fibrosis and the future use of this pathogenic pathway as a therapeutic target in order to limit gingival fibrosis.

Keywords: calcium channel blockers-induced gingival overgrowth, dihydropyridines, TGF-β1, CTGF, immunohistochemistry.

Introduction
Gingival overgrowth (GO) or enlargement is the term now used to replace the former gingival hyperplasia or hypertrophy, inappropriate terms because overgrowth is not the result of an increase in cell number or their size but rather an increase in extracellular tissue volume [1, 2]. Besides its involvement as an unspecific change induced by inflammatory triggers, GO could arise most commonly as a result of two major conditions: (i) the long-term ingestion of various medications; (ii) gingival fibromatosis, generally of an unknown etiology, that appears to have a strong genetic background [3–5]. Currently, more than 20 prescription medications are associated with GO [2]. Main drugs associated with GO can be divided into three categories: anticonvulsants (such as phenytoin), calcium channel blockers (CCB) (diltiazem, verapamil, nifedipine) and immunosuppressants (such as cyclosporine A) [1]. There are also case reports on GO associated with antibiotics and sulphonamides [6–8]. Although each drug has a different pharmacologic effect and targets various target tissues, all of them seem to act similarly on the gingival connective tissue as a secondary target, leading to common clinical and histopathological changes. Clinical manifestation of GO frequently appears within one to three months after initiation of treatment with the incriminated medication [9]. Gradually, gingival lobules are formed and they may appear inflamed or more fibrotic depending on the degree of local inflammation. The enlargement is confined to the attached gingiva but sometimes may extend coronally. GO produces aesthetic changes and its clinical symptoms include tenderness, bleeding, speech disturbances, dental occlusion problems, and enhanced susceptibility to periodontal diseases [8, 10]. Histologically, drug-induced GO is virtually indistinguishable from other types having in common the enlargement of the gingival tissue due to an extracellular matrix (ECM) accumulation, although the pathogenesis remains multifactorial and seems to be strongly dependent on growth factors, etc. [12].
Calcium channel blockers-induced GO (CCB-GO) is an unwanted outcome of systemic medications reported since the 1980s for all types of CCB prescribed for the treatment of various cardiovascular diseases: phenylalkylamines (such as verapamil), benzothiazepines (diltiazem) and dihydropyridines (nifedipine) [1]. Rare cases of this side effect were also described for the second (felodipine) and third generation (amlodipine) dihydropyridines [8, 13]. The precise mechanism by which drug-induced gingival enlargement occurs is still not completely understood, although a number of hypotheses have been suggested. One of these relates to the fact that CCB affect cellular calcium metabolism and because the matrix metalloproteinases involved in collagen breakdown are modulated by calcium influx, fibroblasts from patients treated with these drugs fail to reduce active collagenases, being responsible for an increase in ECM [1].

Gingival tissues are generally in a state of injury and repair that involves repetitive cycles of production of chemotactic factors, inflammatory cell recruitment and connective tissue remodeling. Connective tissue turnover are largely controlled by cytokines and chemokines secreted by inflammatory cells and, to a lesser extent, by fibroblasts [14]. Drugs have separate impacts on the range of cytokines and growth factors involved in connective tissue metabolism [15]. One of the most important cytokine controlling collagen metabolism is the Transforming Growth Factor-β (TGF-β), protein involved in normal and pathological processes of the oro-facial tissues [16]. TGF-β1 isoform is chemotactic for fibroblasts and selectively stimulates the production of some ECM components (type I collagen, tenascin, fibronectin) being implicated in several fibrotic diseases associated with ECM accumulation [16–18]. Connective Tissue Growth Factor (CTGF), a multifunctional matricellular protein, was found to occur at elevated levels in a variety of fibrotic pathologies and act as a possible downstream mediator of the fibrictic effects of TGF-β1 without interference in other functions of this cytokine [19].

The aim of our study was to assess the role of TGF-β1–CTGF pathway in the activation of cells with a fibroigenetic phenotype responsible for the fibrosis developed after chronic administration of dihydropyridine calcium channel blockers.

Materials and Methods

Reagents

Masson and silver impregnation staining kits were purchased from Bio-Optica, Italy. Normal swine serum, mouse monoclonal anti-human α-smooth muscle actin (α-SMA), mouse monoclonal anti-Ki67 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 CTGF 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 developpers. The anhydrous mounting medium (Neo-Mount), hydrogen peroxide and buffers were purchased by 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 of Craiova and the Clinic of Oro-Maxillo-Facial Surgery of the Emergency County Hospital of Craiova, Romania. All gingival fragments were obtained after the informed consent of patients. We used samples from eight donors with nifedipine and amlodipine-induced GO and three untreated patients without gingival enlargement. Previously, the experimental protocol was approved by the Ethical Committee of the University of Medicine and Pharmacy of Craiova. 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 histological 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 were cut at 3 μm thickness, dewaxed, rehydrated and stained with modified Masson trichrome and Gomori’s silver impregnation staining.

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 (Table 1).

Table 1 – Antibodies used for immunohistochemistry analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Vendor Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse monoclonal anti-human Ki67</td>
<td>1:50</td>
<td>MIB-1</td>
</tr>
<tr>
<td>Mouse monoclonal anti-human TGF-β1</td>
<td>1:200</td>
<td>sc 52893</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-human CTGF</td>
<td>1:300</td>
<td>ab</td>
</tr>
<tr>
<td>Mouse monoclonal anti-human α-SMA</td>
<td>1:100</td>
<td>A5114</td>
</tr>
</tbody>
</table>

The next day, sections were processed for the amplification of the immune signal using the polyclonal swine >>Multi-Link<< and the Avidin–Biotin mix. 3,3′-Diaminobenzidine tetrahydrochloride and hydrogen peroxide were used for color development and Mayer’s 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 done by two different observers according to the following: immunohistochemical reactions (brown deposits in labeled structures) were graded as absent
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(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. For each antibody tested, was performed a negative control replacing the primary antibody with phosphate buffer saline, pH 7.4–7.6.

Results

Routine Masson stain revealed the enlargement of the gingival epithelium and a fibrotic expanded connective tissue as the main histopathological features of GO regardless of the dihydropyridine CCB involved, nifedipine or amlodipine (Figure 1a). We noticed changes of the type I/type III collagen ratio, Gömöri’s silver impregnation stain displaying an increased amount of type I collagen in the lamina propria (Figure 1b).

Although in the epithelium of samples without GO the immunoreaction for TGF-β1 was absent, we noticed a positive reaction for this protein in keratinocytes from the spinous and basal epithelial layers both in amlodipine and nifedipine-induced GO samples (Figure 2). The positive reactions for TGF-β1 in dihydropyridine induced GO extended to cells and ECM from the inflamed areas of the lamina propria (Figure 2).

Regarding the expression of CTGF, we noticed the same distribution in both types of CCB-GO: an intense CTGF positive reaction in the basal and parabasal epithelial layers and in many cells spread evenly in the lamina propria (Figure 3). No positive reaction for CTGF was observed in gingival samples without GO.

Ki67 immunolabeling to assess cell proliferation revealed an increased number of positive cells in the profound epithelial layers but no fibroblast proliferation in the lamina propria although the number of resident connective tissue cells was increased (Figure 4). Immunohistochemical reactions for α-SMA revealed the presence of few positive fibroblasts in the lamina propria, their incidence being correlated with TGF-β1 expression but not with collagen accumulation (Figure 5).

![Figure 1](image1.png) Morphological features of gingiva in CCB-GO: (a) Epithelial thickness and connective tissue accumulation in the lamina propria, Masson staining, ×100; (b) Increased amount of type I collagen (yellow) in the lamina propria, Gömöri’s silver impregnation, ×200.

![Figure 2](image2.png) Immunohistochemical evaluation of TGF-β1 expression. Negative reaction in the gingival epithelium without GO (a, ×100); Positive cells in the spinous and basal layers (b, ×100) and in the ECM (d, ×200) in amlodipine-induced GO; Positive cells in the epithelium (c, ×100) and in the lamina propria (e, ×400) in nifedipine-induced GO.
Main histological features of clinically-diagnosed dihydropyridine CCB-induced GO (nifedipine and amlo- dipine) include hypertrophy of keratinized epithelium, excessive connective tissue accumulation and various degrees of inflammation in the lamina propria. In our previous studies we noticed that the difference between this type of GO and those induced by other risk factors (inflammation due to local conditions or diabetes mellitus, hereditary fibromatosis or phenytoin administration) refers only to the ratio between inflammation and fibrosis finding in accordance with other reports [3, 20]. This type of GO seems to be less fibrotic and more inflamed than that induced by phenytoin or gingival fibromatosis.

Fibrosis usually results from chronic inflammation when the inflammation occurs simultaneously with tissue remodeling and repair processes. A persistent trigger that can be infectious, chemical, physical or mechanical induces fibrosis. The repair process involves a regenerative phase and a second one – fibrosis – when connective tissue replaces normal parenchyma. This phase can become harmful if not properly controlled and the result will be an excessive accumulation of ECM [21].
The pathogenic mechanisms that trigger fibrosis in drug-induced GO are not definitively understood and, although literature data are extensive, some aspects are contradictory. Studies of Modéer et al., cited in [22], demonstrated that some drugs are able to inhibit ECM production by gingival fibroblasts and cell proliferation in vitro. In contrast, others studies showed that the accumulation of proteins, particularly collagen, in ECM, might occur due to an imbalance of its turnover in overgrown gingival connective tissue [23]. The results suggest that the imbalance that leads to GO might be related to decreased collagen degradation, but not to an increase of its synthesis. Collagen production from gingival fibroblasts is controlled by the coordination of transcription and post-translation regulatory mechanisms, including its intra-cellular degradation. Collagen phagocytosis by fibroblasts is one of the pathways by which collagen fibers are degraded, the other being extracellular where collagen degradation is controlled by collagenases, a subgroup of matrix metalloproteinases (MMP). Several growth factor and cytokines and tissue inhibitors of metalloproteinases (TIMPs) regulate MMP activity. Previous reports sustain that, drug-induced gingival overgrowth is associated with reduced activity and expression of MMP, unlike periodontitis where their activity is increased [3, 11].

Several hypotheses were proposed to explain the mechanisms through which CCB, like other drugs, induce the imbalance of connective tissue homeostasis in gingival overgrowth. Because gingival overgrowth does not develop in all the patients treated with these medications, it has been hypothesized that the individuals affected have fibroblasts with an abnormal susceptibility to the drug. In support of this hypothesis, it has been shown that gingival fibroblasts present a functional heterogeneity in response to various stimuli and was further demonstrated that some drugs could react with a phenotypically distinct subpopulation of gingival fibroblasts to enhance protein synthesis [24, 25]. The drugs induce a direct effect on a subgroup of fibroblasts, named “responders”, that are apparently genetically determined to be sensitive to the drug causing gingival overgrowth; they affect calcium metabolism and induce a decrease in the Ca^{2+} influx, which causes a decrease in cellular folic acid uptake and thus limits the production of the active form of collagenase [2].

Important elements directly responsible for the events of drug-induced gingival overgrowth are growth factors and cytokines. It has been shown that phenytoin, nifedipine, and cyclosporine may regulate the expression of cytokines and growth factors in gingival tissues [26]. Two of them, TGF-β and CTGF, were found in higher levels in fibrotic tissues and, as we observed in this study, dihydropyridine CCB induce their release.

TGF-β is a cytokine secreted by several cell types, including macrophages, and has an important role in regulating fibroblast proliferation, collagen and ECM synthesis, activation of TIMPs, inhibition of MMPs, characteristic processes observed in fibrotic lesions [16]. However, the effects of TGF-β on gingival tissues are surprisingly low if compared with other connective tissues. CTGF could mediate the effects of TGF-β in the metabolism of ECM [27]. CTGF/CCN2 is a member of the CCN family of factors whose members contain conserved cysteine-rich domains and have a variety of biological activities, being important to stimulate proliferation of diverse cell types and to promote fibrosis [28]. CTGF/CCN2 is highly expressed in a wide variety of fibrotic lesions and was already demonstrated that CTGF levels are highest in gingival tissues from phenytoin-induced lesions, intermediate in nifedipine-induced lesions, and nearly absent from cyclosporine A-induced overgrowth [29]. CTGF/CCN2 levels correlate positively with fibrosis, consistent with the role of CTGF/CCN2 in promoting and maintaining fibrosis [30]. It has been reported that CTGF stimulates fibroblast proliferation and ECM synthesis. As demonstrated, there is a requirement to use simultaneously CTGF and TGF-β to induce fibrosis in other organs [27]. Thus, neither of the two factors alone was able to initiate the fibrotic processes. CTGF is rapidly and potently induced by TGF-β and contributes to the regulation of some genes in the ECM [31]. Although CTGF is continuously present in the tissue, TGF-β is found only in the initial periods, indicating that there may be a cascade effect [30]. The mechanism by which CTGF promotes fibrosis has been investigated and a possible interaction with integrins has been proposed [14].

Because our results regarding the reaction for Ki67 did not prove an increased fibroblast proliferation despite an increased number of resident connective tissue cells, we presume that the active fibroblasts for ECM accumulation could be due, in part, to a diminished programmed fibroblast death in the gingival tissue as Kantarci et al. demonstrated for the phenytoin-induced fibrosis [32].

The second interesting event linking CCB administration to gingival fibrosis is the presence of myofibroblasts. Myofibroblasts are differentiated cells able to synthesize large quantity of collagen being involved in the tissue repair process [21]. The presence and role of myofibroblasts in other fibrotic diseases were already known [21]. In dihydropyridines CCB-induced GO, we noticed few resident fibroblast positive for α-SMA, in contrast with their increased number in phenytoin-induced GO [3, 33].

Recent studies shows a possible association between GO and epithelial mesenchymal transition (EMT) [34, 35]. EMT is a process in which epithelial cells trans-differentiate into fibroblast-like cells. TGF-β1 is a potent inducer of EMT in a variety of tissues and CTGF expression is increased in cells undergoing EMT [34]. Then, further studies are requested to prove the observation that elevated levels of some EMT markers in the dihydropyridines CCB-induced GO lesions could suggest the involvement of EMT as a pathogenic mechanism of GO and fibrosis.

Conclusions

Based on these findings, it is possible to conclude that the process leading to dihydropyridine CCB-induced gingival overgrowth involves a fine regulation of growth factor and suggest that TGF-β1–CTGF axis is activated in chronic administration and differentially mediate the...
activation of fibroblasts with a synthetic phenotype. The results also have implications for understanding pathogenic mechanisms of fibrosis and the perspective of using this pathway as a therapeutic target for limiting gingival fibrosis.

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References


Corresponding author
Camelia Elena Stânicăulescu, Lecturer, Pharm, PhD, Department of Pharmacy, University of Medicine and Pharmacy of Craiova, 2 Petru Rareş Street, 200349 Craiova, Romania; Phone/Fax +40251–523 939, e-mail: camiparsot@yahoo.com

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