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Chapter 26

Epithelial-Mesenchymal Transition — A Possible Pathogenic Pathway of Fibrotic Gingival Overgrowth

Ileana Monica Baniță, Cristina Munteanu,
Anca Berbecaru-Iovan, Camelia Elena Stănciulescu,
Ana Marina Andrei and Cătălina Gabriela Pisoschi

Additional information is available at the end of the chapter

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1. Introduction

Gingival overgrowth (GO) or gingival enlargement refers to important changes of gums aspect and function. Even it seems an issue of little significance, health of gums is a prerequisite condition for a psychological and physical comfort because severe GO affects speech, mastication, and nutrition, causes aesthetic concerns and increases susceptibility for periodontal and systemic diseases. The treatment of severe cases needs gingivectomy that may be repeated if is necessary.

At clinical endo-oral examination, GO is characterized by increased gums volume, swollen and deepening of gingival sulcus. Thickening of soft tissues covering alveolar ridges is more than 1 mm comprising both the mobile and attached gums. The degree of overgrowth can be variable from the interdentally papilla to cover the entire tooth crown. Enlargement is painless, slowly progressive and depends to a great extend on the oral hygiene [1-6].

Usually, GO is classified according the clinical appearance and the etiological factor. Histological and cell molecular studies have uncovered some of the pathogenic pathways and cellular alterations associated with GO but still remain unknown aspects.

In this chapter we describe recent insights into the pathogenic mechanisms of GO overgrowth discussing in detail the role of epithelial-mesenchymal transition (EMT) in gingival fibrotic diseases.

2. Terms definition, classification and risk factors for gingival overgrowth

Gingival overgrowth (GO), often named gingival hyperplasia or hypertrophy, is classified according the clinical appearance and the etiological factors, if these are known. If clinical examination displays rather an inflammatory aspect (gingivitis and periodontitis) the gums are red, soft, shiny, and bleed easily. Inflammatory gingivitis is induced frequently by poor dental hygiene resulting in bacterial plaque and causes reactive GO, named also focal reactive GO, inflammatory hyperplasia or epulis. Generally, the epulides are pedunculated or sessile lesions of gums; because this term, considered unsuitable, is clinico-topographical, without a histological description of the lesion, nowadays the preferred term is gums reactive lesion [7, 8]. Smoking, systemic diseases (diabetes mellitus, HIV infection) determine also inflammatory gums lesions.

Non inflamed gingival enlargement tends to have a darker red or purple color, is either firm or soft, when bleeds easily. Determinative causes are extremely polymorphous: (i) subjects with poor dental hygiene; (ii) specific hormonal states-puberty, pregnancy; (iii) nutritional deficiency, such as scurvy; (iv) blood conditions, such as acute leukemia, lymphoma or aplastic anemia; (v) genetic conditions – epulis or Neumann tumor; (vi) drug-induced GO (named also fibrotic gingival hyperplasia) appeared after administration of some anticonvulsivants (phenytoin), immunosupressants (cyclosporin A, CsA) and antihypertensive calcium channel blockers (verapamil, diltiazem, nifedipine); (vii) systemic diseases such as sarcoidosis, Crohn disease, acromegaly, primary amyloidosis or type I neurofibromatosis [6,9,10].

Gingival fibromatosis (GF) is the term frequently used for any GO when suspect a hereditary pattern (hereditary gingival fibromatosis, HGF), as part of a more extensive syndrome (Table 1) or the etiologic factor remains unknown-idiopathic gingival fibromatosis (IGF). Specialty literature is sometimes confuse or redundant regarding the relation between definition and the etiological factors. For the beginning, we tried to present a brief synthesis of these definitions.

Hereditary gingival fibromatosis (HGF), previously known as gingival elephantiasis, idiopathic gingival fibromatosis, hereditary gingival hyperplasia, non-bacterial plaque gingival lesion, gingival gigantism or just hypertrophic gums [11,12] can be classified as follows:

i. Hereditary or isolated GF named also non-syndromic or type I GF seems to be determined by the mutation of SOS1 (*Sun of sevenless-1)* gene on chromosome 2. For the first time, this mutation was described in a large Brazilian family [13]. SOS1 is an oncogene involved in cell growth. This mutation was designated GINGF1 (Mendelian Inheritance in Man classification MIM135300) [10].

Recently a type 2 HGF, GINGF2 (MIM605544), was described in association to a mutation mapped on chromosome 5 but the specific gene involved has not yet been identified [10,14-16]. The presence of teeth in alveoli seems to be a condition for hereditary GO development as it disappears or reduces after tooth extraction. Some authors consider HGF an atypical pathology of childhood because it is present mainly during the mixed dentition stage [11]. Another type of HGF with family aggregation, GINGF3 (MIM 609955), a mutation mapped on chromosome

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2 but not to the SOS 1 gene in which clinical signs appear earlier during the primary dentition stage was described by [17].

ii. Syndromic GF is associated with several clinical signs in some syndromes (Table 1). In syndromic GF, gingival events are caused by chromosomal abnormalities (duplications, deletions) of chromosomes 2p12-16 [18,19], 4q (MIM252500), 8 (MIM266270), 14q [20], 19p (MIM266200), 19q (MIM248500) and Xq [8,9,13,21-26].

Syndrome	Clinical signs
Zimmerman-Laband Syndrome	GF and facial deformability, changes of nose and ears, nail dystrophy, hypoplasia, epilepsy, hepato-splenomegaly, deafness, mental retardation
Rutherford Syndrome	GF and corneal dystrophy, aggressive behavior, mental retardation
Jones Syndrome	GF and progressive deafness, maxillary odontogenic cysts
Cross Syndrome	Gingival hypertrophy and microphthalmia, mental retardation, hypopigmentation
Murray-Puretic-Drescher Syndrome	GF and bone, cartilage, skin and muscle diseases
Ramon Syndrome	GF and cherubism, hypertrichosis, mental retardation, convulsions, growth retardation, juvenile rheumatoid arthritis
Cowden Syndrome	Localized GF with multiple hamartomas

Table 1. Syndromic gingival fibromatosis [adapted after 6,15,16,27]

Recently, in [28] is described a new syndrome that includes generalized thin *hypoplastic amelogenesis imperfecta* found in a family with multiple consanguineous marriages, this type having clinical and histological similarities with GINGF1 and GINGF3.

Genetic and syndromic fibromatosis are sometimes termed IGF [12,27,29-32].

Lacking specific immunohistochemical markers, the diagnosis of HGF is based exclusively on clinical examination, patient medical history and family pedigree.

It was recommended to use the term „idiopathic fibromatosis" only for GF that doesn't incriminate genetic and hereditary causes mentioned above, in order to avoid these confusions of classification [6,9].

GO incidence varies according the socioeconomic status and the risk factors involved being reported a rate of 1/9000 adults; the most numerous GO are inflammatory or induced by drugs-phenytoin increases gingival volume in 57% of cases, CsA in 30-46% and calcium channel blockers in 10% [3,33,34]. HGF is the most rare type of GO and estimated to affect 1/750,000 people with the same incidence in both sexes [2,6,10,35,36].

Under the influence of such risk factors, clinical increase of gums volume is due to the enlargement of both epithelial and connective tissue. Microscopically examination displays the coexistence of tissue hypertrophy and cellular hyperplasia which imposed the generic term

of GO [2]. Irrespective the risk factor, presence of bacterial plaque and hereditary predisposition are constantly incriminated as etiological cofactors mainly for drug-induced GO [2]. To sustain this association, it was revealed that patients with inflammatory GO before the onset of treatment with CsA developed more severe forms of GO [37] and suggested that patients carriers of a genetic polymorphism related to IL-1A expression often develop GO after CsA treatment [38]; specialty literature reports cases of IGF or HGF associated with chronic or aggressive periodontitis [39-41].

3. Histological aspects of gingival fibromatosis

Histological studies that we performed on samples of fibrotic gingival tissues revealed common, non-specific aspects despite the numerous risk factors, generally characterized by an increase of gums volume to which contribute both the epithelium (cellular hyperplasia) and lamina propria (accumulation of extracellular matrix, ECM, and cells) (Figure 1). Various types of GF are characterized by different incidence of pro-inflammatory cells.

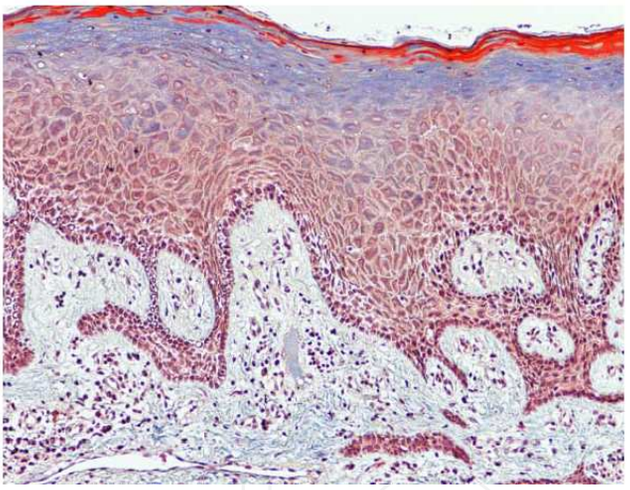

Figure 1. General view of a sample with gingival overgrowth (trichrome staining, x100)

In drug-induced GO, connective tissue is more rich in pro-inflammatory cells than in HGF or IGF. An exception is phenytoin-induced GO characterized mainly by fibrotic lesions unlike CsA or nifedipine-induced GO which determine important inflammatory reactions [23,42-45]. Due to its clinical and histological features, phenytoin-induced GO is often included in the category of fibromatous GO [11,15,23] which yield some confusions.

Histological changes of syndromic GO, HGF and phenytoin-induced GO are similar: epithelial hyperplasia with hyperkeratosis and elongated papillae, thickening of collagen bundles, increase of tissue differentiation and fluctuating number of fibroblasts (Figure 2a).

Enlargement and acanthosis of gingival epithelium with deep epithelial ridges was reported [46, 47]. Epithelial hyperplasia results from acanthosis but appears only in the areas of chronic

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inflammation [46-48]. In many areas epithelial hyperkeratosis was observed [17,26,47,49]. Regarding the sulcular epithelium we noted many signs of considerable degeneration, subpepithelial edema and extensive inflammatory cell infiltration (Figure 2b). Thick, densely wrapped collagen bundles with scattered resident cells of connective tissue were observed in lamina propria.

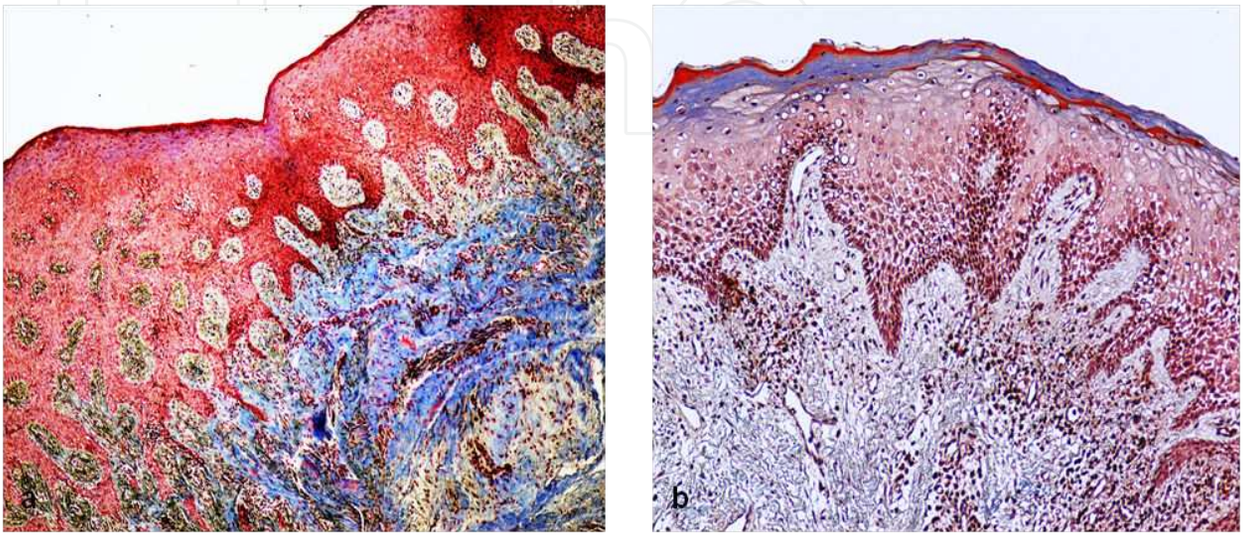

Figure 2. General view of syndromic GO: a) masticatory gingival mucosa; b) sulcular gingival mucosa (trichrome staining, x100)

The incidence of fibroblast is disputed; some authors reported numerous fibroblasts [16,31,45,50,51] while others claimed on the contrary a decreased number [17,41,43,52]. This variable number of fibroblasts even within HGF pointed attention to the different molecular mechanism underlying gingival fibrotic processes.

4. Pathogenic pathways of gingival overgrowth

Histological and cell molecular studies have uncovered some of the pathogenic pathways and cellular alterations associated with GO but still remain unknown aspects.

Studies revealed that the same molecules and biological events are involved in inflammation, wound repair and fibrosis. Theories and previous investigations on the morphology and molecular mechanisms by which the fibrotic deposition occurs have been widely published. Integrating these findings, Bartold and Narayanan state in [53] that fibrosis can evolve as a response to the action of a single factor or of a combination of various factors such as: (i) abnormal release of inflammatory mediators; synthesis of some molecules frees others and their crosstalk could have synergic, cumulative or antagonist effects; (ii) persistence of abnormal changes in the action of growth factors and cytokines; even the intensity of cell response to this stimulation is not so great the long lasting effect is cumulative and increased;

(iii) establishment of a pro-fibrotic cell phenotype; aberrant interaction of normal cell phenotypes with peptide mediators could induce the recruitment of abnormal cells.

These cell interactions determine the accumulation of gingival tissue through two main pathogenic pathways: (i) excessive synthesis of ECM and (ii) decrease of its breakdown [15,23,43,53-55]. Each of these pathways is initiated and sustained by growth factors, cytokines, molecules involved in ECM breakdown, matrix metalloproteinases – MMP, and their tissue inhibitors (TIMP) released by cellular elements that belongs both to the epithelium and gingival chorion. Recently, epithelial to mesenchymal transition (EMT) has been proposed as another pathogenic pathway promoting gingival fibrosis.

5. Role of epithelium in extracellular matrix accumulation — The epithelial-mesenchymal transition

Development of fibrotic lesions is indirectly related to the presence and histophysiology of epithelial cells. The interference of oral epithelial cells in ECM storage is sustained by the results of many studies reporting epithelial morphological changes besides the accumulation of connective tissue. In the same time, epithelial keratinocytes or inflammatory cells infiltrating the epithelium synthesize several biomolecules (growth factors, cytokines, MMPs and TIMPs) which alter collagen metabolism and ECM synthesis in the lamina propria. In a recent study, Menga and coworkers in [56] showed an intense expression of type 1 collagen and TIMP-1 in fibroblasts from mixed cultures of keratinocytes and fibroblasts obtained from patients with GF, in parallel to an increased rough endoplasmic reticulum. The authors suggested that keratinocytes play an important role in the pathogenesis of GF through increase of ECM storage. The epithelium suffers acanthosis and hyperkeratosis, increases the number of epithelial cells, and of many inflammatory cells infiltrating its deep layers. The increase of keratinocytes number determines not only the epithelial enlargement mainly in the spindle layer but also the appearance of many epithelial ridges ascending deep in the lamina propria. These epithelial ridges often branch and adhere one to another (Figure 1, and 2a, b).

These findings are constantly accompanied by the increase of keratinocyte mitotic activity proved by Ki-67 or PCNA immunostaining in [45,57-60]. In a recent study, using immunohistochemistry, [61] reported that Mcm-2 and Mcm-5 (members of minichromosome maintenance protein family), considered a novel class of proliferation markers, and geminin, also a proliferation marker according to [62], showed various expression in samples from three different families with GF. No differences between the expression of apoptotic markers Bcl-2 and Bax were observed among the group. Thus the authors concluded that an important heterogeneity of gingival fibrosis occurs. Epithelial cells proliferation is stimulated by pro-inflammatory cytokines and growth factors, such as KGF (Keratinocyte Growth Factor) or EGF (Epidermal Growth Factor). EGF and its receptors (EGfr) are positively correlated with the proliferative potential of the cells from rete pegs [63,64]. In a previous study, we observed that epithelial cells have an increased mitotic index in cases of GF highly infiltrated with inflammatory cells (Figure 3) (*unpublished data*).

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Epithelial proliferation seems to have at least two functions in GO. First it ensure a continuous regeneration of keratinocytes, the regenerative capacity of the epithelium being compulsory when continuous desquamation of the superficial cells prevents bacterial colonization of the mucosa; second, epithelial proliferation could contribute to fibrosis by maintaining a cell pool to replace those cells involved in EMT and transformed in fibroblasts.

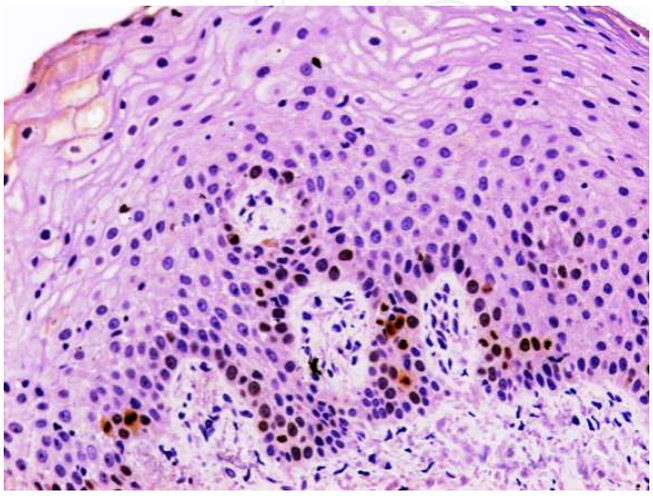

Figure 3. Gingival fibrosis. Increased number of Ki-67 positive cells in the basal epithelial layer (IHC, x200)

5.1. Concept of epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a concept first defined "epithelial–mesenchymal transformation" by G. Greenburg and E. Hay to characterize the conversion of epithelial cells to mesenchyme (EMT) and vice versa (mesenchymal-epithelial transition, MET) during chick embryonic development. This well-defined concept refers to a form of inherent plasticity of the epithelial phenotype that occurs normally in the developmental process. During EMT cells undergo a switch from a uniform, polarized epithelial phenotype to a motile mesenchymal phenotype. Current interest in this process stems from its importance in embryonic development and involvement in several pathologies (wound healing, fibrosis, cancer progression and metastasis) and has been extensively reviewed over the last 10 years in [65-78]. The conversion of an epithelial cell to a mesenchymal cell is critical to metazoan embryogenesis and a defining structural feature of organ development, and follows a common and conserved program with hallmarks [69,77]. As Lamouille and coworkers suggest in [77] it also has some variation which depend on the cell type, tissue environment and signals that activate the EMT program. During EMT, epithelial cell–cell and cell–ECM interactions are weakened and epithelial cells become able to trans-differentiate into fibrogenic fibroblast-like cells [68]. Turning an epithelial cell into a mesenchymal cell requires alterations in morphology, cellular architecture, adhesion, and migration capacity [69]. Loss of epithelial apical-basal polarity, acquisition of a front-rear polarity and motility result from the disappearance of cell adhesion molecules, reorganization of cytoskeleton and changes in cell shape [70]. In many cases cells gain an increased ability to break ECM proteins, acquire resistance to senescence and apoptosis [73].

Research in this field revealed that cellular events of EMT occurs in three distinct biological settings with different functional consequences: (i) type 1 EMT acts during implantation, embryogenesis and organ development when can generate mesenchymal cells (primary mesenchyme) and then secondary epithelia after the mesenchyme undergoes a reverse MET; (ii) type 2 EMT as a source of fibroblasts and other related cells involved in tissue regeneration and organ fibrosis in response to persistent inflammation; (iii) type 3 EMT occurs in neoplastic cells that have undergone genetic and epigenetic changes, notably of oncogenes and tumor suppressor genes, and contributes to cancer progression and metastasis [72,76,79]. A main distinction between the first two types of EMT is that type 1 EMT produces mesenchymal cells, whereas type 2 EMT results in fibroblasts in mature tissues. But other than the fact that mesenchymal cells have a shape similar to fibroblasts and, like fibroblasts, express fibronectin and fibrilar collagens, there is no evidence that fibroblasts originate in primitive mesenchymal cells [78].

EMT induction. Successful EMT depends upon a combination of growth factors and cytokines associated with the proteolytic digestion of the epithelial basement membranes (BM) under the action of MMPs. Local expression of TGF-β, EGF, IGF-II or FGF-2 facilitates EMT by binding membrane receptors with kinase activity [65]. The effect of TGF-β on EMT induction depends on β1-integrin transduction, Smad-dependent transcription, Smad-independent p38MAP kinase activation and Rho-like GTPase-mediated signaling [80,81]. IGF-II also facilitates the intracellular degradation of E-cadherin [82], while FGF-2 and TGF-β are required for the expression of MMP-2 and MMP-9 to assist in BM breakdown [83]. Indeed, decrease of type IV collagen from the BM was associated with increased expression of MMP-2 and MMP-9 during human pathologies involving EMT [54,84]. Loss of BM integrity is essential for the increased interactions between epithelial and connective tissue layers that contribute to fibrosis. Several lines of evidence indicate that TGF-β signaling is causally linked with EMT, plays an important role in regulating epithelial plasticity and is one of the most significant lines of communication between stroma and epithelium in different organ fibrosis (renal, cardiac, pulmonary, and hepatic) [81].

Consequent cell and molecular events are engaged to initiate EMT and enable it to complete: i) activation of transcription factors; ii) expression of cell surface specific proteins; iii) reorganization and expression of cytoskeletal proteins; iv) synthesis of ECM-degrading enzymes. In many cases, these factors are used as biomarkers to prove cell passage from one phenotype to the other and EMT involvement in tissue remodeling (Figure 4).

Transcriptional regulation of EMT. EMT involves changes in gene expression that induce the loss of proteins associated with the epithelial phenotype and increased expression of proteins associated with a mesenchymal and migratory cell phenotype with concomitant alterations in cytoskeletal organization, cell adhesion and production of ECM [72]. Cellular plasticity that is the switch of epithelial to mesenchymal features is achieved through a well orchestrated program that involves the action of three families of transcription factors: Snail, ZEB (zinc-finger E-box-binding) and bHLH (basic helix-loop-helix). Expression of these factors is induced in response to TGF-β through different mechanisms and their function is finely regulated at transcriptional, translational and post-translational levels [77].

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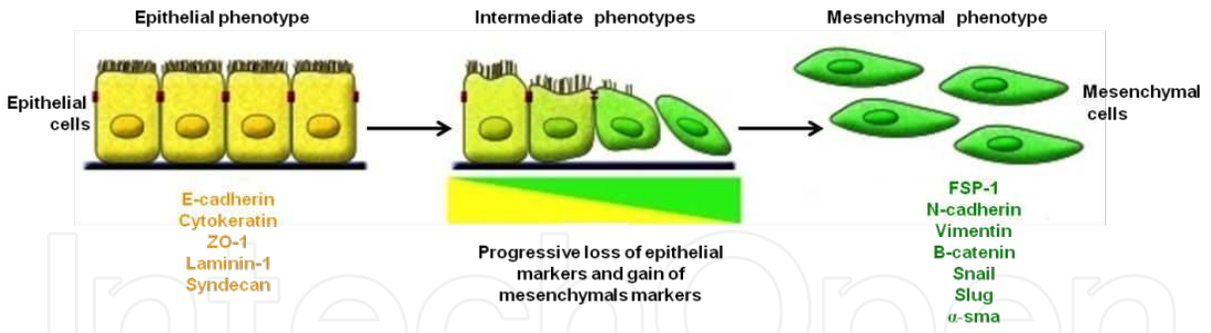

Figure 4. EMT is a functional transition from polarized epithelial cells into mobile cells able to secrete extracellular compounds (adapted after [72])

Snail family. Three Snail proteins have been identified in vertebrates: Snail 1 (Snail), Snail 2 (Slug) and Snail 3. They function as transcription repressors and their activity depend on the C-terminal zinc finger domain and the N-terminal SNAG domain [77]. Snail expression is induced in response to various growth factors. In cells that undergo TGF-β induced EMT Snail expression is mediated by Smad2/3 that form complexes with Smad4 and activates transcription by binding to Snail promoter [85]. Expression of Snails suppresses a spectrum of genes involved in maintaining the epithelial structure and function (Table 2) and enhances the expression of genes encoding vimentin and fibronectin leading to a full phenotype.

ZEB family. Two ZEB proteins have been identified in vertebrates, ZEB1 and ZEB2, which have two zinc-finger clusters at each end who mediates the interaction with DNA regulatory sequences. TGF-β induces the expression of ZEB proteins through an indirect mechanism mediated in part by Ets-1 and then ZEBs interact with Smad3 and repress the expression of epithelial marker genes (E-cadherin, claudins, ZO-3, plakophilin-2) and induce the expression of mesenchymal proteins (vimentin, N-cadherin, MMP-2) [86-88].

Helix-loop-helix family. HLH is a large family of transcription factors divided into seven classes based on their tissue distribution, dimerization ability and DNA-binding specificity [89]. The structure of HLH includes two parallel α-helices linked by a loop required for dimerization. E12, E47, Twist and Ids are involved in EMT. E12, E47 and Twist are able of DNA binding while Ids proteins are unable and act as dominant negative inhibitors [90]. Ectopic expression of E12 and E47 represses E-cadherin, plakoglobin or desmoplakin expression and induces mesenchymal markers, such as vimentin, fibronectin, N-cadherin or α5-integrin, and promotes migration and invasion [90]. Expression of Twist decreases E-cadherin, claudin-7 and occludin expression, increases that of N-cadherin and vimentin, and enhances migration and invasion [91]. Ids expression is repressed in response to TGF-β [92,93].

EMT proteome. Commonly used molecular markers of EMT could be grouped as follows: (i) decrease the amount of proteins associated with the epithelial phenotype; ii) abundance of some proteins; (iii) increased activity of selected proteins (Rho, GSK-3β); (iv) accumulation of proteins within the nucleus [69]. Table 2 listed common members of EMT proteome.

Delaminating of epithelia to facilitate movement is dependent on cell context and growth factor signaling and is accompanied by a decrease of apoptosis and mitosis [65].

In fibrotic diseases, TGF-β/Smad/Snail is a key signaling pathway [72,81]. Subsequently E-cadherin, cytokeratin, claudin and occludin are repressed, while FSP1 (fibroblast-specific protein-1), vimentin, fibronectin, Rho and MMP are increased [78].

According to [96] EMT represents the main source of fibroblasts in fibrotic pathology of connective tissues.

Generally, researches focused on few EMT markers and for this reason are not comprehensive. We specify that most information about the presence of EMT markers is indicated by the presence of proteins in epithelia and not only in fibroblasts, and as an example we'll discuss later the expression of FSP1.

	Name	EMT Type
Proteins that decrease in abundance	E-cadherin	1,2,3
	Cytokeratins	1,2,3
	Occludin	1,2,3
	Claudins	1,2,3
	ZO-1	1,2,3
	Collagen IV	1,2,3
	Laminin 1	1,2,3
Proteins that increase in abundance	N-cadherin	1,2
	Vimentin	1,2
	α-SMA	2,3
	Fibronectin	1,2
	FSP-1	1,2,3
	Snail, Slug	1,2,3
	ZEB	1,2,3
	Twist, E12/E47	1,2,3
	Ets-1	1,2,3
	MMP-2, MMP-9	2,3
	αvβ6 Integrin	1,3
Proteins that accumulate in the nucleus	β-catenin	1,2,3
	Smad2/3	1,2,3
	NF-κβ	2,3
	Snail, Slug	1,2,3
	Twist	1,2,3
	LEF-1	1,2,3
	Ets-1	1,2,3
	ZEB	1,2,3

Table 2. Epithelial-mesenchymal transition proteome (modified after [69,78])

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FSP1, also known as S100A4 is one of the most interesting proteins identified in the EMT proteome [94]. This is a fibroblast-specific protein member of the S100 superfamily of cytoplasmic, calcium-binding proteins. S100 members have been implicated in calcium signal transduction, cytoskeletal membrane interactions, microtubule dynamics, p53-mediated cell cycle regulation, cellular growth and differentiation. Because the precise function of FSP1 is not entirely clear, its interaction with cytoskeletal moieties suggest that FSP1 protein may be associated with mesenchymal cell shape to enable motility and its expression indicates the presence of a molecular program determining the fibroblast phenotype in many organ fibrosis (kidney, liver, heart, brain, lungs) [72,94,95]. FSP1 is a specific marker not only for fibroblasts but also for endothelial cells undergoing endothelial-mesenchymal transition (EndMT) [78,96].

5.2. Epithelial-mesenchymal transition in gingival overgrowth

The process of EMT is involved in the normal development and several pathologies of oral cavity. In oral tissues, type 1 EMT is associated to palate and root development, type 2 EMT could play a contributory role in GF and oral submucous fibrosis and type 3 EMT is responsible for progression, invasion and poor prognosis of oral squamous cell carcinoma [76,97].

Fibrosis which occurs in many epithelial organs (kidney, liver, lung, heart, intestine) begins as a part of a repair event, that normally generate fibroblasts by EMT mechanism in order to reconstruct tissues following inflammatory injury. It seems that in gingival fibrosis as in other organs, the main trigger for type 2 EMT is the cytokine bath released in response to persistent inflammation [78,98]. Inflammatory injury results in the recruitment of a diverse array of cells (mainly resident fibroblasts and macrophages) that release growth factors (TGF-β, PDGF, EGF, FGF-2) and MMPs, especially MMP2, MMP3 and MMP 9 [72]. Under the influence of these factors and others chemoatractants [83] the delaminated epithelial cells migrate towards the disruptions of the BM. In EMT a discontinuous BM accompanied by a decreased expression of collagen IV and laminin was reported [54]. First the epithelial cells loss the polarity, cell adhesion molecules are disrupted and cell-cell junctions disappears. The intermediate filament profile change from cytokeratins to vimentin, the F-actin rearranges to a mesenchymal shape and some cells begin to express α-smooth muscle actin (α-sma) [83]. Epithelial cells undergoing EMT are specifically labeled *in vitro* and also *in vivo* [65,99,100] by FSP1, collagen type 1 and α-sma [78,94].

Recently, was provided data about EMT origin of gingival fibroblasts in drug-induced GO. The authors reported that mesenchymal cells from the lamina propria raised after phenotypic changes of some cells from the basal and parabasal epithelial layers [54,60, 101]. These cells had a diminished expression of E-cadherin compared to others; meanwhile the majority of keratinocytes expressed FSP1. Besides phenytoin-induced GO, we reported similarly results in a case of syndromic GO (Figure 5.) [47].

As we mentioned before, the main growth factor involved in EMT is TGF-β1 that triggers activation of the transcription factors able to repress the expression of epithelial markers, for example E-cadherin.

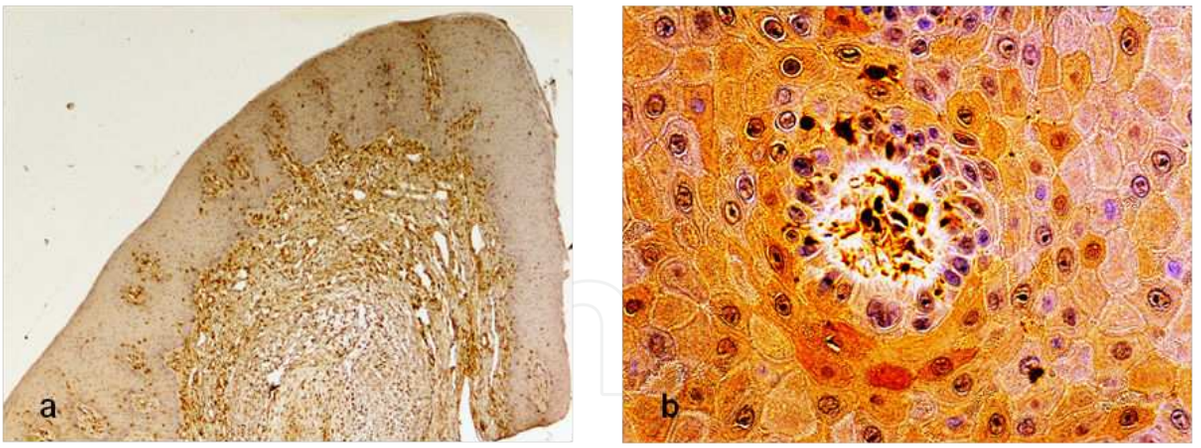

Figure 5. Syndromic GO. FSP1-positive reaction not only in fibroblasts but also in many keratinocytes, probably those that undergone EMT (IHC, a.x 100; b. x 400).

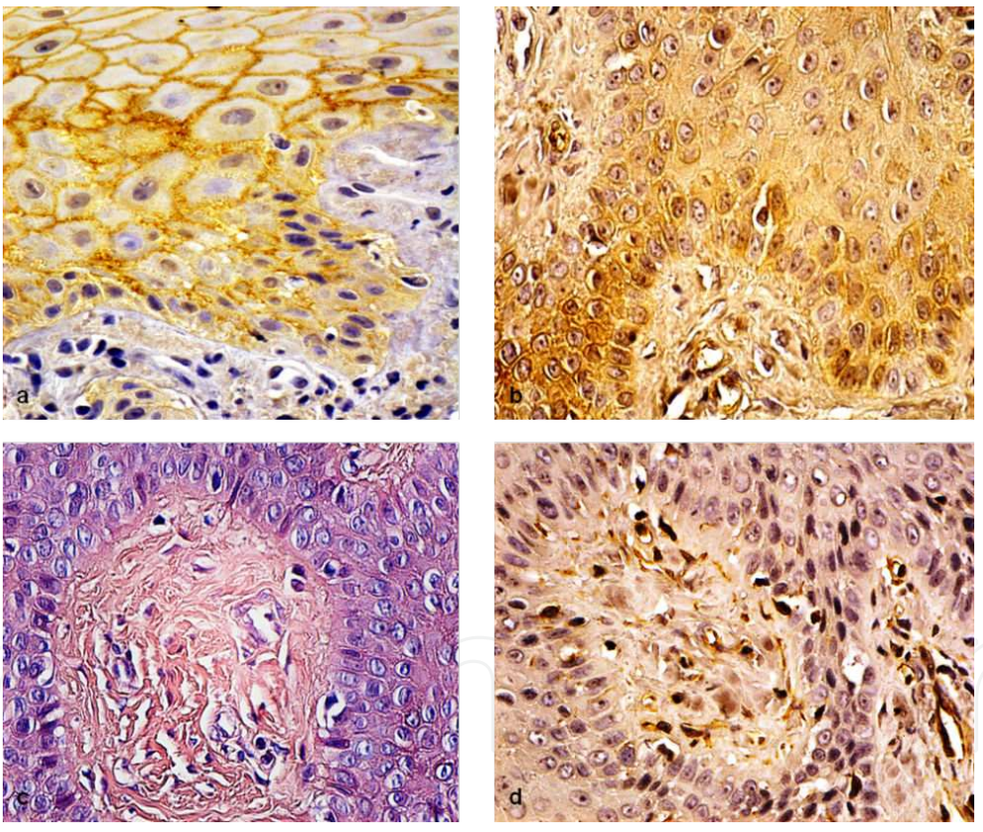

Figure 6. Idiopathic GO: a. Immunostaining for E-cadherin, x400; b. Immunostaining for TGF-β1, x200. c. Independent epithelial cells very close to the basal lamina, x400; d. FSP1 positive cells in the basal epithelium and the superficial connective tissue of the chorionic papilla, x 400.

Immunohistochemical studies that we performed on fibrotic GO samples revealed a diminished expression of E-cadherin in the basal epithelial layer in proximity of the BM suggesting that these cells undergo EMT while the immune reaction for TGF-β1 revealed many positive cells deep in the epithelium, mainly in the epithelial rete ridges [46,101]. A careful examination

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of these areas revealed independent epithelial cells detached from their neighbors, some of them extremely close to the basal lamina. In the lamina propria, adjacent to the BM we observed numerous intense FSP1 positive cells (Figure 6) (*unpublished data*).

The same results we reported in cases of phenytoin induced GO. Regarding the expression of FSP1 we observed an increased number of S100A4 positive cells both in the epithelium and lamina propria. At higher magnification we detected these FSP1 positive cells mainly in the basal epithelial layer nearby the disrupting BM and in the connective tissue close to the epithelium [101]. Tissues with phenytoin-induced GO showed significant reduction of E-cadherin expression in the epithelium compared with tissues from subjects without overgrowth where E-cadherin had a constant presence in the adherent junctions between keratinocytes. For the same samples we performed the assessment of the transcription factors Smad3 and Snail. We found an up-regulation in cells from profound epithelial layers. Often these positive cells were round or elongated, surrounded by a clear halo and we presumed that as a prove for lost of adhesion and the possibility to cross the disrupting BM to the connective tissue (Figure 7). Endothelial cells were also positive for these factors, especially for Smad3.

Overexpression of Snail, able to represses E-cadherin expression, and of MMP2 and MMP9 able to digest proteins from BM in the epithelium are downstream events of TGF-β1 biological effects [60, 102]. Experimental data proved that supplementation of epithelial cell cultures with TGF-β1 leads to loss of cell-cell adhesion through inhibition of E-cadherin gene expression and decrease of adherent junctions, tight junctions and desmosomes [72,102,103].

5.3. Connective tissue growth factor and the epithelial-mesechymal transition

As we mentioned before, besides TGF-β1 other growth factors could be involved in EMT such as IGF-II, EGF, FGF-2 and recently CTGF [104].

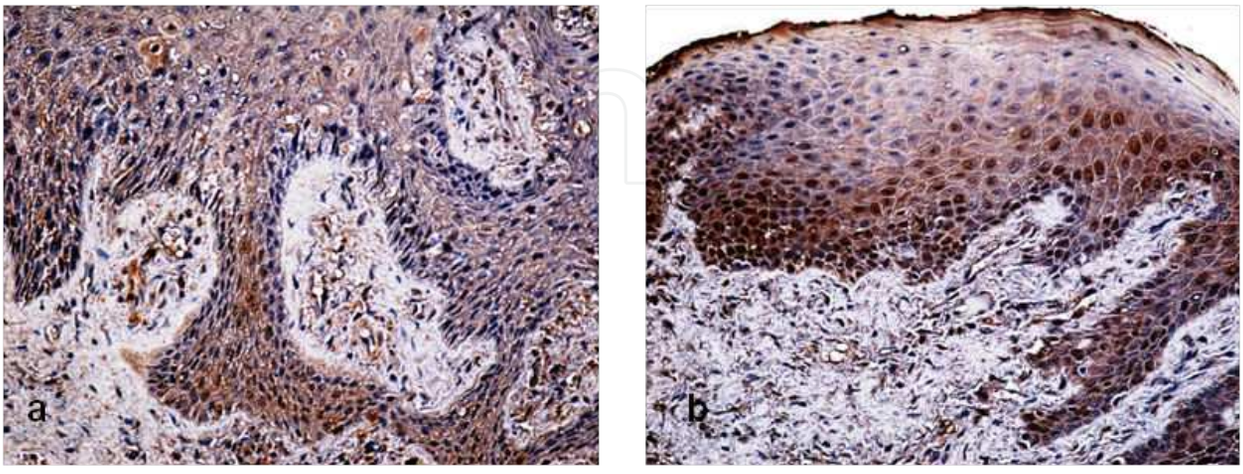

Figure 7. Phenytoin-induced GO. Expression of the transcription factors Smad 3 (a, x100) and Snail1 (b, x100).

TGF-β1 acts as a strong pro-fibrilogenetic factor through several mechanisms: (i) direct stimulation of collagen synthesis after the increase of number of highly collagen synthesizing fibroblasts through EMT; (ii) initiation of CTGF control on collagen synthesis [105]. Kantarci and coworkers in [104] showed a direct relation between the incidence of FSP1 positive cells and CTGF expression in drug-induced GO.

CTGF/CCN2 is a member of the CCN family whose members contain conserved cysteine-rich domains and have various biological activities, being important to stimulate proliferation of diverse cell types and to promote fibrosis [105]. 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 in CsA-induced overgrowth [44,106]. CTGF/CCN2 expression in connective tissue fibroblasts was positively related with the degree of fibrosis because CTGF is able to stimulate fibroblast proliferation and ECM synthesis.

We performed immunohistochemical studies to reveal the pattern of CTGF expression in various types of GO and we noted a constant intense CTGF positive reaction in all cases. We observed strong positivity in the epithelium and lamina propria not only in fibroblasts but also in endothelial and pro-inflammatory cells (Figure 8).

In phenytoin-induced GO and IGF, the most fibrotic types of GO, CTGF/CCN2 content was significantly higher compared to GO induced by other drugs (nifedipine, amlodipine) and controls. Similar results were reported by Kantarci and coworkers in [44] who revealed that CTGF/CCN levels were elevated in phenytoin-induced fibrotic lesions and HGF, the highest CTGF positive reaction being observed in the basal epithelial layer and the superficial connective tissue. Because the presence of CTGF in the epithelium is intriguing, they performed *in situ* hybridization to identify cells that express CTGF mRNA and confirmed the presence of a high amount of CTGF in the basal epithelial layer [44]. In the connective tissue, CTGF promotes local fibrosis but its presence in the epithelium could have a distinct significance as was mentioned for the uterine tissue where CTGF stimulates cell proliferation. The authors suggest that CTGF could also stimulate gingival cells proliferation, mesenchymal cells and also keratinocytes from the basal layer revealing an increased mitotic index in GO [44].

6. Role of mesenchymal cells in gingival overgrowth

The main cells of gingival connective tissue incriminated for increased collagen synthesis are logically the fibroblasts. Gingival fibroblasts are involved in ECM homeostasis through a dual effect. On the one hand, they are responsible for collagen synthesis and, on the other hand, by a process of phagocytosis, it performs ECM breakdown.

Under normal circumstances, but especially in organ fibrosis, fibroblasts show different origins. For example, in kidney fibrosis, the trans-differentiation of epithelial cells through EMT is responsible for 36% of fibroblasts, 14-15% originated in bone marrow stem cells-namely fibrocytes [99] and the rest from local fibroblast proliferation. [65]

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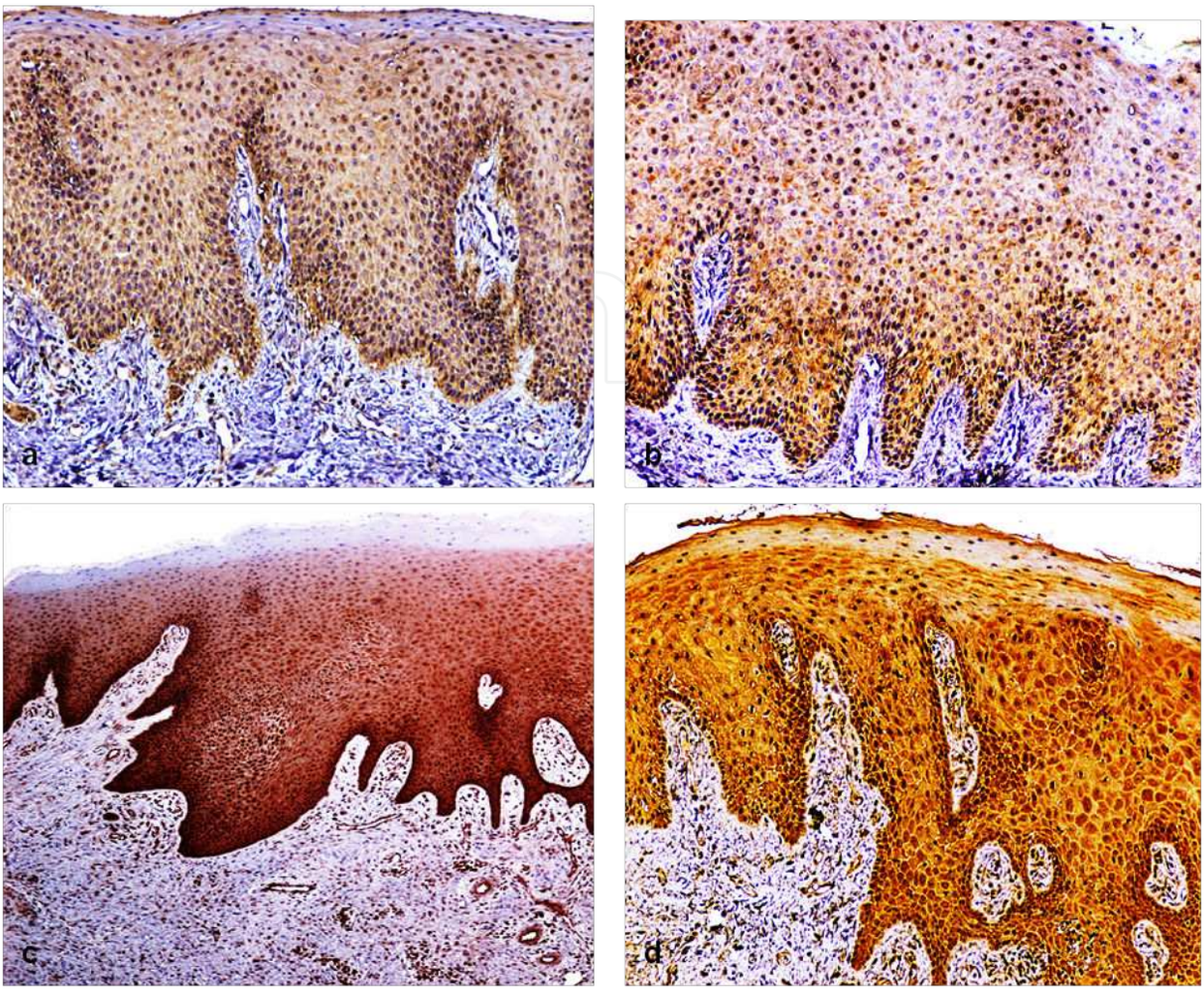

Figure 8. Immunostaining for CTGF in various samples of gingival fibromatosis: a, b. Idiopathic GF, x200, c. Phenytoin-induced GF, x100; d. Reactive GF, x200.

Kisseleva and Brenner showed in [99] that there are differences between the expression of several markers in fibrocytes and fibroblasts. The fibrocytes are cells involved in skin, kidney, liver and lung fibrosis. They have dual phenotypic features between fibroblasts and lymphocytes, and are defined as CD45+cells able to synthesize collagen with bone marrow origin, where they represents ≤1% of the cell population. Tissue injuries increase their number and after proliferation spread through blood into the damaged tissue where their proportion varies in relation to the tissue (5%-25%). [107,108]

In vitro the fibrocytes can differentiate into α-sma-positive myofibroblasts following stimulation by TGF-β1. The authors suggest that the role of these cells is not limited to tissue fibrilogenesis but to fulfill a role of intermediary in the biosignaling between the immune and fibrogenetic cells. This observation is based on the fact that fibrocytes express lymphoid markers (CD45, MHC II, MHC I), myeloid markers and adhesion molecules (CD54, ICAM-1) but also fibroblast markers (Thy-1, α-1 collagen). In addition, fibrocytes

secrete growth factors and cytokines, for example TGF-β1, that stimulate the local deposition of ECM constituents. [109]

The second type of fibroblast-like cells derived still from bone marrow is represented by fibroblasts which in contrast to fibrocytes do not express myelo-monocytic markers and hyperexpress α-sma *in vitro* [110]. These are the main cells responsible for lung fibrosis.

Morphologically in fibrotic GO were described two populations of fibroblasts: one with little cytoplasm, considered inactive, and the other, well represented, with abundant cytoplasm, endoplasmic reticulum and Golgi apparatus-the active form. [29,111]

Kantarci and coworkers reported a reduction of fibroblasts apoptosis and at the same time an increased fibroblasts proliferation regardless of the inflammatory infiltration in phenytoin-induced GO and HGF which may explain the increased fibrosis. [45]

Through an autocrine signaling TGF-β1 seems to be the stimulus for increased collagen synthesis in fibroblasts. Hakkinen and Csiszar in [112] advanced the hypothesis that in GF the onset of overgrowth along with dental eruption can be placed either on account of the differentiation of abnormal phenotype fibroblasts or following their activation by pro-inflammatory cells or by mechanical trauma of eruption.

Recently there have been proposed two ways of stimulating fibroblasts proliferation either the pathway induced by increased FAS (fatty acid synthase) expression [112,113] or by increased expression of c-myc (a nuclear proto-oncogene) which hyperexpression is associated with disturbances of cell proliferation. [114] Conflicting results regarding the proliferative activity of fibroblasts in GF can be explained either by the genetic heterogeneity of the pathology itself or by the small number of cases studied. [112]

Cells that undergo EMT reorganize their actin cytoskeleton in order to facilitate formation of membrane projections that include sheet-like membrane protrusions or lamellipodia and spike-like extensions or filopodia that enable cells to directional motility. [115] Finally, the result of both EMT and EndMT is the myofibroblast, a mobile cell rich in actin stress fibers that expresses α-sma. These processes have been named EMyt and EndMyt. [77,116,117]. The mesenchymal phenotype resulting after EndMT is characterized by the acquisition of mesenchymal markers, such as α-sma and N-cadherin and the complementary loss of endothelial markers, such as CD31/Pecam-1 and VE-cadherin. [117] The mechanism of EndMT was discovered in the process of heart-development but actually it had been implicated in a wide variety of pathological conditions like several organs fibrosis and as well as in cancer. [98] There is not a consensus regarding the fact that myofibroblasts of fibrotic tissue occur exclusively as a result of EMT, these cells having a very heterogeneous origin. In tissue injury the local fibroblasts become activated by local cytokines released from inflammatory and resident cells or by the change of the mechanical microenvironment. These cells become first proto-myofibroblasts – cells acquiring contractile stress fibers composed of cytoplasmic actin. [118,119] *In vivo* such a protomyofibroblast became a differentiated myofibroblast by *de novo* expression of α-sma, used for this reason as a molecular marker. [119]

Since only certain subpopulations of myofibroblasts, previously called activated fibroblasts, express α-sma [120,121] it has advanced the hypothesis that actin of smooth muscle

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could actually label the cells detached from the blood vessel walls as a response to local injury. [122,123] At least three local events are needed to generate α-sma–positive differentiated myofibroblasts: (i). The accumulation of biologically active TGF-β1, the main promoter of fibroblasts differentiation into myofibroblasts and trigger of EMT; (ii) The presence of specialized ECM proteins, like the ED-A splice variant of fibronectin. Some authors argue that in the presence of the granulation tissue fibroblasts gain progressively features of myofibroblasts including α-sma expression [118,124] and (iii) High extracellular stress raised from the mechanical properties of ECM and cell remodeling activity. In addition, in [119] have been suggested that bone marrow derived circulating cells known as fibrocytes represent an alternative source of myofibroblasts in skin wound healing or organ fibrosis. There are few reports in the literature referring to the evidence of myofibroblasts in reactive focal GO, HGF and drug induced gingival hyperplasia. [25,47,126,127] Schor and coworkers reported in [128] that the only tissues that do not develop post lesion scars are embryonic and gingival tissues. This special reactivity was due to the fact that gingival fibroblasts and skin activates TGFβ1 by different signaling pathways. [129] Following the experiments authors suggested that the lack of scars in gingival mucosa is due to the fact that mechanical stress, a normal condition for functional periodontal tissues and remodeling processes is translated into fibroblast proliferation, production of TGF-β1 and CTGF, but not in activating genes responsible for α-sma synthesis.

7. Conclusions and perspectives

EMT is a dynamic physio-pathological event that depends upon a fine crosstalk between signaling pathways. Understanding the molecular mechanisms involved in EMT may reveal new biological targets for an effective therapeutic control of fibrosis in syndromic and IGF.

Further studies are needed regarding the expression of genes that control the synthesis of ECM under the particularities of structure and function of oral mucosa which normally is constantly remodeled and, on the other hand, is in a continuous state of inflammation due to the contact with different external agents. In this respect, special attention should be paid to factors that govern the relationship between innate immunity and EMT.

List of abbreviations

GO-Gingival Overgrowth

EMT – Epithelial-Mesenchymal Transition

GF-Gingival Fibromatosis

HGF-Hereditary Gingival Fibromatosis

IGF-Idiopathic Gingival Fibromatosis

IL1A – Interleukin 1A

ECM-Extracellular matrix

CsA-cyclosporin

MMP – Matrix Metalloproteinases

TIMP – Tissue Inhibitors of Matrix Metalloproteinases

KGF-Keratinocyte Growth Factor

EGF-Epidermal Growth Factor

EGFr – Epidermal Growth Factor receptor

MET – Mesenchymal Epithelial Transition

BM – Basement Membrane

TGF – Transforming Growth Factor

IGF – Insulin-like Growth Factor

FGF – Fibroblast Growth Factor

ZEB – Zinc finger E-box binding

bHLH – basic Helix-Loop-Helix

FSP1 – Fibroblast Specific Protein-1

EndMT – Endothelial Mesenchymal Transition

PDGF – Platelet Derived Growth Factor

α-sma-α-smooth muscle actin

CTGF – Connective Tissue Growth Factor

EMyt – Epithelial Myofibroblast Transition

EndMyt-Endothelial Myofibroblast Transition

Author details

Ileana Monica Baniţă[1], Cristina Munteanu[1], Anca Berbecaru-Iovan[2],
Camelia Elena Stănciulescu[2], Ana Marina Andrei[2] and Cătălina Gabriela Pisoschi[2*]

*Address all correspondence to: c_pisoschi@yahoo.com

1 Department of Dentistry, University of Medicine and Pharmacy, Craiova, Romania

2 Department of Pharmacy, University of Medicine and Pharmacy, Craiova, Romania

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