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Growth Factors and Connective Tissue Homeostasis in Periodontal Disease

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1. Introduction

Periodontal disease is one of the major dental pathologies that affect human populations worldwide at high prevalence rates (Petersen, 2003). Periodontal diseases represents a family of heterogeneous chronic inflammatory lesions that involve the periodontium, a connective tissue protected by the epithelium, important to attach the teeth to the bone in the jaws and to support the teeth during function (Taylor, 2003). It is well known that periodontal diseases are caused by the interaction between periodontopathogens, almost gram-negative bacteria that grows on the teeth, and the host immune response to the chronic infection which results in tissue destruction (Ratcliff & Johnson, 1999; Reynolds & Meikle, 1997).

Gingivitis and periodontitis are the two main periodontal diseases and may be present concurrently. Gingivitis is a form of periodontal disease in which gingival tissues are inflamed but their destruction is mild and reversible while periodontitis is a chronic inflammatory response to the subgingival bacteria with irreversible changes (Armitage, 1999). Periodontium destruction is characterized by loss of connective tissue attachment and bone around the teeth in conjunction with the formation of periodontal pockets due to apical migration of the junctional epithelium (Champagne et al., 2003). Periodontal disease progression is episodic in nature on a tooth site level, but more recently, it has been realized that it is principally patient-based rather than site-based (Zia et al., 2011); the host related risk factors could be the key to better understand disease evolution. The available evidence shows that important risk factors for periodontal disease relate to poor oral hygiene, tobacco use, excessive alcohol consumption, stress, and diabetes mellitus (Laurina et al., 2009; Taylor & Borgnakke, 2008). Degrees of inflammation and fibrosis depend on these risk factors but recently the genetic basis of many aspects of the periodontal host response has been discussed in reference to disorders predisposing to periodontal disease (Bartold & Narayanan, 2006; Kinane & Hart, 2003).

Clinical hallmarks of periodontal disease are represented by the redness and swelling of the gingival margin around the neck of the teeth, recession of the gums, tooth looseness, changes in tooth alignment and halitosis (Taylor, 2003). Recently, it has been accepted that during chronic periodontal disease, morphological changes in the architecture of the extracellular matrix of the gingiva could occur and lead to gingival enlargement. This refers to the overgrowth of the gingiva characterized by the expansion and accumulation of the

connective tissue with occasional presence of increased cell number. These changes are unspecific, appearing in gingival enlargement associated to chronic inflammation but also when other risk factors exist (inheritance, systemic diseases, such as diabetes mellitus, and drugs administration). The most common form of gingival overgrowth is that drug-induced, by anti-seizure drugs, such as phenytoin, immunosuppressive agents, such as cyclosporine, and some calcium antagonists (verapamil, diltiazem, dihidropyridines, most notably nifedipine) (Dongari-Bagtzoglou et al., 2004; Seymour, 2006).

Histological assessment showed that independently of the etiological factor involved, changes of the mucosa refer both to the gingival epithelium and the lamina propria (Banita et al., 2008, 2011). There is now general agreement that all gingival overgrowth lesions contain fibrotic or expanded connective tissue with various levels of inflammation and an enlarged gingival epithelium. As soon as plaque accumulates adjacent to the gingival margin, inflammatory cells infiltrate in the subjacent connective tissue and initiate its destruction. Simultaneously with collagen destruction, wound repair occurs, which results in fibrosis and scarring coexisting at the foci of inflammation (Bartold & Narayanan, 2006). According to this sequence of events, the stages of inflammation, matrix destruction and repair succeed each other in the development of periodontal disease.

Important player for the regulation of gingival connective tissue homeostasis is the fibroblast, cell able to synthesize and breakdown the collagen fibers and other proteins from the ground substance. *In vitro* studies have shown that fibroblasts from human normal gingiva produce collagens type I and type III, while cells derived from gingiva of patients with chronic periodontitis failed to produce detectable amounts of type III collagen (Hammouda et al., 1980; Chavier et al., 1984, as cited in Bartold & Narayanan, 2006). Buduneli et al. (2001a) investigated total collagen content and collagen type I, III, IV, V and VI content in gingival connective tissue of chronic periodontitis as well as aggressive periodontitis patients and clinically healthy subjects. It was suggested that different collagen types present in various periodontitis categories may be related with diverse pathogenic mechanisms acting in these diseases. In our previous studies we observed the abundance of type I collagen in the extracellular matrix of the gingival tissue obtained from patients with chronic periodontitis. In accordance with reference data, we suggest two explanations for this: despite the degradation of the fibrilar collagen, cells are able to synthesize a new type of collagen, type I trimer which accumulates in the gingiva (Narayanan et al., 1985, as cited in Bartold & Narayanan, 2006) or that inflamed human gingiva contains fibroblasts with different phenotype than those from the normal tissue, the myofibroblasts, able to synthesize a large amount of collagen.

Pathogenic pathways involved in the imbalance of connective tissue homeostasis in periodontal inflammatory diseases are complex, and specific mediation is not completely understood. Activation of matrix metalloproteinases (MMPs) is one of the most important evolving under a rigorous control.

Growth factors and cytokines play an important role in regulation of the gingival extracellular matrix turnover. Tumor necrosis factor-α (TNF-α) and interleukins induce the expression of MMPs while transforming growth factor-β (TGF-β) down-regulates their synthesis and secretion and promotes the production of their natural tissue inhibitors, TIMPs (Bartold & Narayanan, 2006). Connective tissue growth factor (CTGF) is another

important mediator of tissue remodelling which stimulates fibroblasts to produce extracellular matrix constituents, so its expression correlates positively with the degree of gingival fibrosis (Heng et al., 2006; Trackman & Kantarci, 2004). Local conditions favour angiogenesis in periodontal tissues being characterized by an increased expression of the vascular endothelial growth factor (VEGF), this cytokine acting also to complete the greater ability of regeneration of the gingiva (Lucarini et al. 2009).

At the beginning, specialists tried to diagnose the stage of periodontal disease depending on the relation between the clinical appearances and the presence of some specific cell populations or specific matrix components (Havemose-Poulsen & Holmstrup, 1997; Romanos et al., 1993). Traditional clinical measurements (probing pocket depth, bleeding on probing, clinical attachment loss, plaque index) used for the diagnosis of periodontium health are often of limited usefulness because they are not sufficiently accurate to discern between previous periodontal disease and present disease activity. There is a need for development of new diagnostic tools to allow earlier detection of active disease, predict disease progression and evaluate the response to periodontal therapy, thereby improving the clinical management of patients with periodontal diseases. Advances in periodontal diseases diagnostic research are moving toward methods whereby periodontal risk can be identified and quantified by objective measures such as biomarkers. Gingival crevicular fluid (GCF) and salivary levels of several growth factors, cytokines and enzymes of host origin appear to hold the greatest promise as valuable biomarkers in assessing development of periodontal disease (Buduneli & Kinane, 2011; Giannobile et al., 2003; Gurkan et al., 2008; Goncalves et al., 2009; Kaufman & Lamster, 2000; Pisoschi et al., 2010; Wright et al., 2000).

In the last decade, scientists began to use signaling molecules such as growth factors in their quest to restore destroyed tooth support (Anusaksathien & Giannobile, 2002) and this reason request a very good knowledge of the biological actions of growth factors, both summative and redundant, in the specific "milieu" of periodontal diseases.

This paper highlights a brief review of the literature on growth factors involvement in periodontal disease and our contribution in this field, in order to sustain their use as biomarkers of active periodontal disease and future therapeutic tools.

2. Changes of the gingival tissues in periodontal disease

Periodontium includes four tissues located near the teeth: i) root cementum, ii) periodontal ligament; iii) alveolar bone, and iv)the part of the gingiva facing the tooth (dentogingival junction) (Nanci & Bosshardt, 2006). Gingiva or gums represent the mucosal tissue that covers the alveolar bone. Healthy gingiva is pale pink or pigmented and wrap tightly around the neck of the teeth (Taylor, 2003). Histologically, gingiva consists of two types of tissues - the epithelium that covers a connective tissue, chorion or lamina propria. A keratinized stratified squamous epithelium protects the lamina propria of the gingiva on its masticatory surfaces and a non-keratinized epithelium protects the lamina propria on its crevicular and junctional surfaces. During normal or pathological conditions such as inflammation, the periodontal connective tissues, including the gingiva, undergo many changes. Clinically detected gingival overgrowth – term used to substitute the former "gingival hypertrophy" or "gingival hyperplasia", because the changes of this clinical

condition are more complex (Dongari-Bagtzoglou et al., 2004) - is one of the alterations recently postulated to occur in chronic periodontitis. It is caused by a variety of etiological factors and is exacerbated by local bacterial biofilm accumulation, because the periodontopathogens products act on the gingival tissues activating cellular events that induce the alteration of connective tissue homeostasis and the destruction of the alveolar bone (Reynolds & Meikle, 1997). Clinical characteristics of different forms of gingival overgrowth have been previously reviewed (Marshall & Bartold, 1998, 1999; Seymour et al., 2000). Gingival overgrowth is characterized by enlarged and occasionally inflamed gums (the interdental papilla and the free gingival margins increase in size and thickness progressively covering the tooth crown). Besides the one associated with periodontal disease, gingival overgrowth can be inherited (hereditary gingival fibromatosis), sometimes associated with other systemic diseases (such as diabetes mellitus) or with idiopathic origin. The majority of cases, however, occur as a side-effect of systemic medications, including the anti-seizure drug phenytoin, the immunosuppressive agent cyclosporin A and certain anti-hypertensive calcium-channel-blockers (verapamil, diltiazem and dihidropyridines, most notably nifedipine) (Seymour, 2006; Trackman & Kantarci, 2004). There is now general agreement that all gingival overgrowth lesions contain fibrotic or expanded connective tissues with various levels of inflammation and an enlarged gingival epithelium. The degrees of inflammation, fibrosis, and cellular pattern depend on the risk factor if it is identified: the duration, dose, and identity of the drug, the quality of oral hygiene, and the individual susceptibility that stems from genetic factors and environmental influences. Histological assessment showed that independently of the etiological factor involved, changes of the mucosa are unspecific and refer both to the gingival epithelium and the lamina propria. Our previous studies of chronic periodontitis gingival samples revealed epithelial hypertrophy with a thick stratified squamous epithelium showing an alternation between keratinized and non-keratinized areas with the increase of the spinous layer (acanthosis) associated with acantholysis (Banita et al., 2008, 2011). Epithelium bended deeply into the lamina propria to form the so called "rete pegs" (Fig.1.).

Fig. 1. Gingival enlarged epithelium in chronic periodontitis (Masson staining x 10)

Gingival tissues assessed showed also the thickness of the lamina propria due to an excessive deposition of connective tissue. It was an important accumulation of thick collagen

bundles with a various number of fibroblasts and the constant presence of pro-inflammatory cells (lymphocytes and macrophages) and numerous *de novo* capillaries (Fig.2).
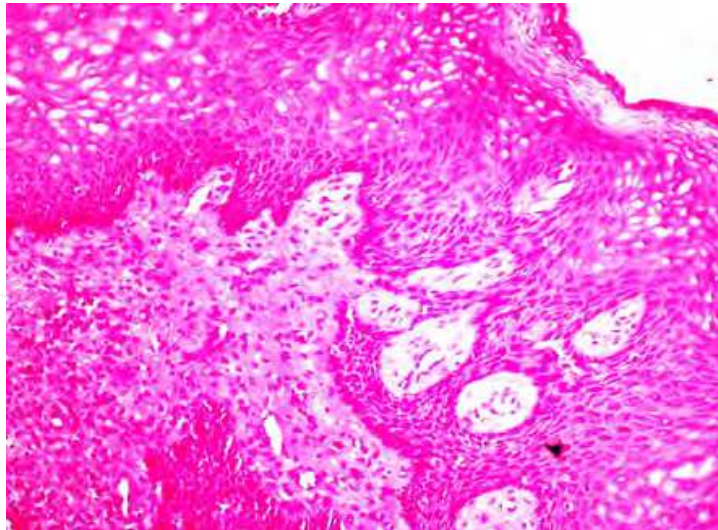

Fig. 2. Infiltration of connective tissue with inflammatory cells in periodontitis, de novo capillaries (Haematoxylin & Eosin staining x 10)

Using silver impregnation we noticed that the ratio between collagen types was changed, the extent of the yellowish coloured areas proving the presence of more type I collagen (Fig.3).

Fig. 3. Abundant collagen type I in chronic periodontitis (Gömöri silver impregnation x 4,5)

Our observations are in agreement with those of Lorencini and coworkers (2009) who, in their experimental model of ligature-induced periodontal disease noticed also this paradigm. They hypothesized that a kind of "frustrated repair" of the extracellular matrix can occur simultaneously with collagen breakdown and that the new configuration of the fibers can contribute to periodontal disease progression (Lorencini et al., 2009). Other authors postulated that with the developing of inflammatory lesion, the gingival collagens become more soluble, with increasing amount of collagen type V and the appearance of a

new collagen, type I trimer (Narayanan et al., 1985, as cited by Bartold & Narayanan, 2006). Taken together these results prove without doubt that there is a permanent link between inflammatory cell populations and extracellular matrix turnover in inflammatory gingival overgrowth. Gingival tissues are recognized for their remarkable ability of regeneration and healing after wounding. The ability of regeneration of the gingival epithelium is compulsory in order to maintain the homeostasis of the gingival mucosa. Lamina propria has also the ability to heal very quickly after wounding. Some areas of interest for researchers are the biological processes that control how the periodontal tissues respond to wounding, and how cells from the different tissues of the periodontium interact when more than one periodontal tissue is affected. It is generally agreed that gingival overgrowth results from an increase of extracellular matrix macromolecules infiltrated with various numbers of inflammatory cells.

3. Extracellular matrix homeostasis in periodontal disease

Connective tissue remodelling is essential for normal growth and development and many diseases have long been associated with the imbalance of the breakdown of the collagenous matrix of different tissues. Complex pathogenic pathways control the balance synthesis/degradation of the extracellular matrix. Macromolecules of interstitial connective tissues and basement membranes may be degraded by: *i)* matrix metalloproteinase (MMP)-dependent, *ii)* plasmin-dependent, *iii)* polymorphonuclear leukocyte serine proteinase-dependent reactions, and *iv)* a phagocytic pathway based on intracellular digestion of internalized material by lysosomal cathepsins (Birkedal-Hansen et al., 1993).

Since the early 1990s, interest has focused on works showing that connective tissue cells synthesize and secrete a family of proteinases, the MMPs, which can digest extracellular matrix macromolecules and play a major role in connective tissue breakdown.

3.1 Matrix metalloproteinases

Matrix metalloproteinases (MMPs), or matrixins, are enzymes derived from many types of mesenchymal cells, monocytes, macrophages and keratinocytes and can synergistically digest most of extracellular matrix (ECM) macromolecules (Reynolds et al., 1994; Whittaker & Ayscough, 2001). The MMP gene family encodes more than 20 human metal-dependent endopeptidases (Stamenkovic, 2003) divided into major five groups (see Tabel 1).

As we already mentioned, gingival tissue remodelling involve the balance between synthesis and accumulation of matrix components and their breakdown under the catalitic action of metalloproteases. Several MMPs have been identified in the inflamed gingival tissues: MMP-1, -2, -3, -8, -9, -13, produced by the keratinocytes, macrophages, polymorphonuclear leukocytes (Banita et al., 2011; Bartold & Narayanan, 2006; Bildt et al., 2008; Kubota et al., 2008; Kumar et al., 2006). Their activity could be different depending on the severity of disease and the needs for extracellular matrix digestion.

In a previous study regarding the expression of MMPs in gingival samples of chronic periodontitis we observed an enhanced expression of MMP-1 in keratinocytes from diseased epithelial layers (Fig.4). In some cases, MMP-1 expression extended to the lamina propria as inflammation progressed. MMP-1 increased activity could explain the change of collagen quality and quantity, since its preferred substrates are the type I and type III collagens.

Type of MMPs	Substrates	Members
Collagenases	Native fibrilar collagens (I, II, III, VII, VIII, X) Gelatins (limited) Other ECM molecules	MMP-1 (Collagenase 1) MMP-8 (Neutrophil collagenase) MMP-13 (Collagenase 3)
Gelatinases	Denaturated collagens (gelatins) Type IV collagen Other ECM molecules	MMP-2 (Gelatinase A) MMP-9 (Gelatinase B)
Stromelysins	Collagens III-V Gelatins (limited) Other ECM molecules MMPs	MMP-3 (Stromelysin 1) MMP-10 (Stromelysin 2)
Matrilysins	Collagen IV and X, gelatins Other ECM molecules MMPs	MMP-7 (Matrilysin 1) MMP-26 (Matrylisin 2)
Membrane type - MMPs	Collagen (I, II, III) Pro-MMP-2 Other ECM molecules	MT1-MMP - MT6-MMP (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, MMP-25)

Table 1. Major types of matrix metalloproteinases (MMPs)

Several other researchers reported an intense MMP-1 collagenolytic activity in fibroblasts and macrophages resident in the periodontal tissue (Beklen et. al., 2007; Kubota et al., 2008) and focused on the interrelation between MMP-1 and MMP-3 in order to amplify the proteolysis in chronic periodontitis (Beklen et. al., 2007).
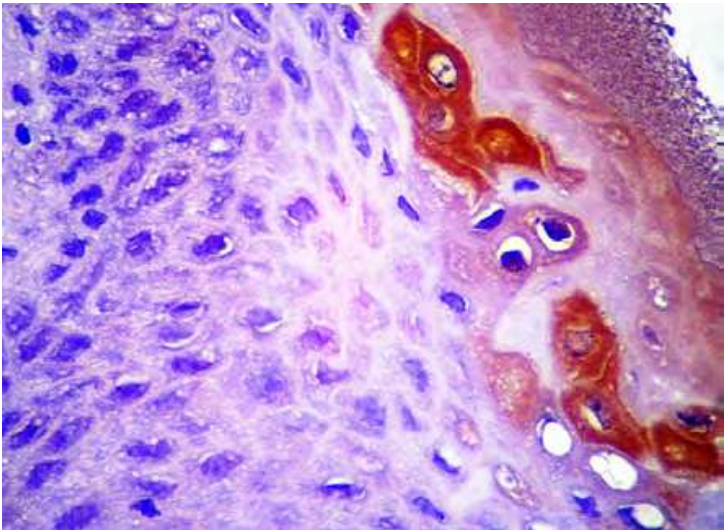

Fig. 4. Expression of MMP-1 in gingival epithelium in chronic periodontitis (IHCx40)

Regarding MMP-2, we observed an increased expression of this enzyme in many keratinocytes from the basal layer and in some cells from the basement membrane (Fig. 5); the active form of MMP-2 increased significantly as inflammation progressed.
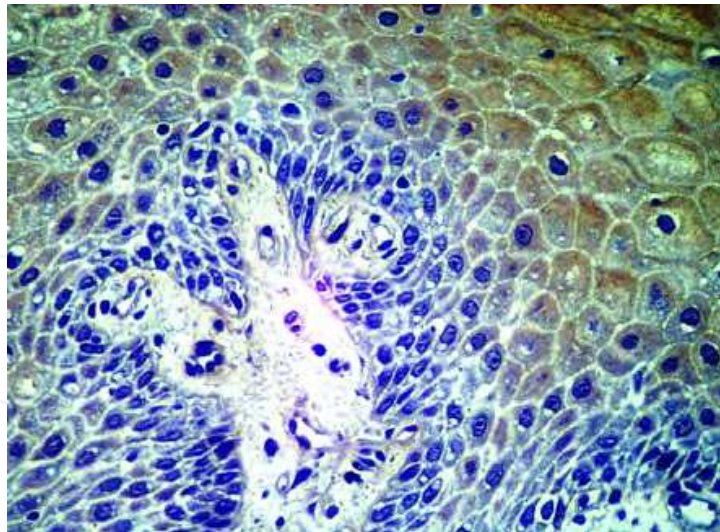
Fig. 5. Increased expression of MMP-2 in gingival epithelium and basement membrane in chronic periodontitis (IHCx20)

Its expression and distribution is justified by the fact that this gelatinase has an important role in the degradation of denatured collagen (predigested with MMP-1) and type IV collagen of the basement membrane, and these processes are increased in various stages of periodontal disease progression. MMP-2 is also able to increase matrix degradation by MMP-13 and neutrophil collagenase (De Souza & Line, 2002). The degenerative process causes the loss of attachment apparatus between tooth, epithelium, and connective tissue which accelerates inflammation and deepening of periodontal pocket. In the last decade, many research groups reported the putative role of other MMPs (MMP-7, MMP-25, MMP-26, MT1-MMP) as mediators of the alternating sequence inflammation-fibrosis during progression of periodontal disease (Emingil et al., 2006a, 2006b; Oyarzun et al., 2010).

3.2 Tissue inhibitors of matrix metalloproteinases

MMPs activity is controlled *in vivo* in three ways: *i)* these enzymes are synthesized and secreted as latent, inactive precursors and conversion to the active form require activation; *ii)* production of MMPs can be regulated by growth factors and cytokines; *iii)* their activity can be inhibited by endogenous serum and tissue inhibitors (Nagase, 1997, as cited by Bartold & Narayanan, 2006). Various tissues express one or more of the members of the most important group of inhibitors known as tissue inhibitors of MMP (TIMPs). These are specific inhibitors that bind MMPs in a 1:1 stoichiometry. All the four currently known TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) are very well conserved since they have been identified in vertebrates, including humans, insects and even in the nematode, *Caenoharbditis elegans* (Brew et al., 2000). Their expression is rigorous regulated during development and tissue remodelling under pathological conditions associated with unbalanced MMP activities, and changes of TIMP levels are considered to be important because they directly affect the level of MMP activity (Visse & Nagase, 2003). TIMP-1 and TIMP-3 expression is inducible, whereas TIMP-2 is largely constitutive (Verstappen & Van den Hoff, 2006). TIMPs are produced in many tissues, although not every tissue expresses all four inhibitors. Most mesenchymal and epidermal cells are able to produce TIMPs in various conditions (Rowe et al., 1997, as cited by Verstappen & Van den Hoff, 2006), but TIMP-4 expression is restricted to neural tissue, gonads, breast and skeletal muscle (Lambert et al., 2004).

Our studies regarding the modulation of MMP/TIMP balance in gingival overgrowth associated with periodontitis lead to the following observations related to TIMP activity. We noticed that the immune reaction for TIMP-1 was positive in few epithelial cells in periodontitis-affected gingival samples assessed (Banita et al., 2011). For TIMP-2 the immune response was quite different. We observed extended areas of TIMP-2 intense positive cells in the epithelium (Fig.6) and a lot of TIMP-2 positive pro-inflammatory cells and few fibroblasts in the lamina propria (Fig.7).
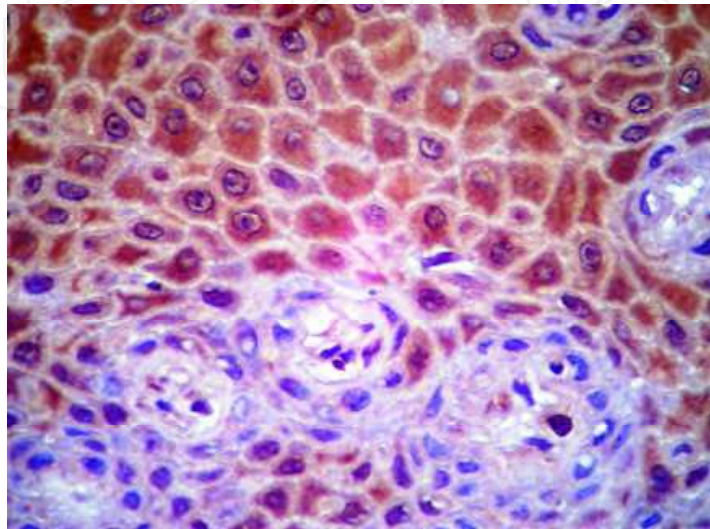

Fig. 6. TIMP-2 expression in gingival epithelium in periodontitis–affected gingiva (IHCx40)
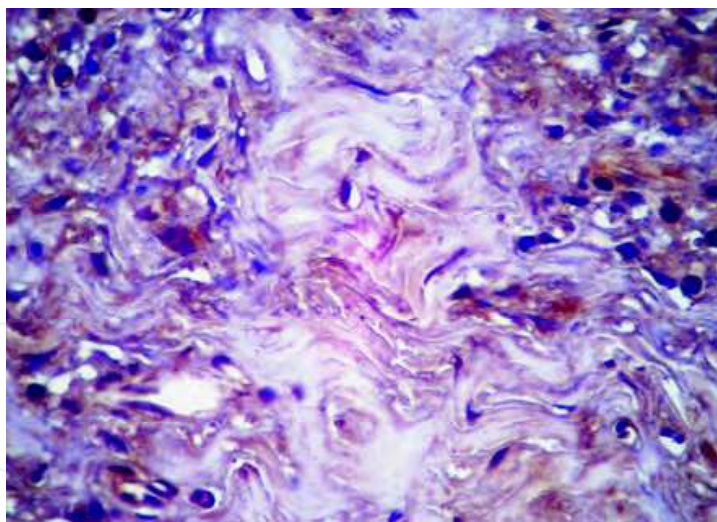

Fig. 7. TIMP-2 expression in periodontitis-affected gingival lamina propria (IHCx20)

Our results showed increased levels for both MMPs and TIMPs assessed in periodontitis-affected gingival tissue and are in accordance with other similar studies. Up-regulated MMPs and TIMPs expression with a modified MMPs/TIMPs ratio depending on the type of lesion (destruction or fibrosis) indicate that the imbalance between degradation and synthesis of the extracellular matrix components persists in periodontitis-affected gingiva and is responsible for an increased tissue breakdown in periodontitis. The reciprocal regulation of TIMPs and MMPs expression may depend on endogenous growth factors and cytokines.

4. Growth factors and cytokines in periodontal disease

The balance between MMPs and TIMPs influence on extracellular matrix homeostasis in different types of tissues is tightly controlled by growth factors, a class of polypeptide hormones. Other polypeptide mediators that affect matrix synthesis include several cytokines - interleukins (IL-1, IL-4, IL-6, IL-10) and tumour necrosis factor-α (TNF-α). By binding to specific cell-surface tyrosine kinase receptors, growth factors are able to regulate significant cellular events in tissue regeneration and repair, including cell growth, proliferation and differentiation, chemotaxis, angiogenesis, and extracellular matrix synthesis (Giannobile, 1996). Growth factors are classified as biological mediators that lack specificity sequestered in the extracellular matrix and molecules available from the circulation (Bartold & Narayanan, 2006). Growth factors demonstrate pleiotropic or multiple effects on wound repair in almost all tissues, including the periodontium.

Some of the most important growth factors exerting functions in healthy and diseased periodontium are listed below:

a.   Platelet-Derived Growth Factor (PDGF)
b.   Fibroblast Growth Factors (a-FGF and b-FGF)
c.   Transforming Growth Factors (TGF-α and -β)
d.   Connective Tissue Growth Factor (CTGF)
e.   Vascular Endothelial Growth Factor (VEGF)
f.   Insulin-like Growth Factors (IGF-I, IGF-II)
g.   Epidermal Growth Factor (EGF)
h.   Hepatocyte Growth Factor (HGF)

After an injury occurs, healing proceeds in a succession of "well orchestrated" cell-cell and cell-extracellular matrix interactions. In the process of normal wound healing, the growth factors act in conjunction to form a complex arrangement of molecules that regulate cellular activity and bordering the wound (Giannobile, 1996). Table 2 lists the sources and the main effects of some growth factors and cytokines important for extracellular matrix remodelling.

The role of growth factors is now recognized in connective tissue homeostasis during inflammation and fibrosis. Tissue repair studies conducted on animals provide evidence that soft tissue wound healing is enhanced by EGF, TGF-α and β, PDGF, acidic and basic FGF. Combinations of different growth factors yield greater repair than can be achieved by individual factors alone (Giannobile, 1996).

Growth factors mediate many events associated with turnover, repair and regeneration of periodontal tissues. Gingival epithelial cells, gingival fibroblasts, and periodontal ligament fibroblasts are the major cells involved in tissue repair. An appropriate response of these target cells to various growth factors depends on the expression of corresponding receptors.

a) Platelet-Derived Growth Factor. In vitro and *in vivo* studies suggest PDGF as the most thoroughly described growth factor associated with periodontal health. There are different isoforms of PDGF (PDGF-AA, -AB, -BB), and all have been shown to have a fibroblast proliferative activity *in vitro* (Giannobile, 1996). PDGF is present in increased levels in the human inflamed gingiva and is mainly localized to the pocket epithelium (Pinheiro et al., 2003).

Growth Factor	Source	Effect
PDGF isoforms	Platelets Macrophages Keratinocytes	Fibroblast and macrophage chemotaxis Fibroblast proliferation Extracellular matrix synthesis
FGF	Macrophages Endothelial cells	Fibroblast proliferation Angiogenesis
TGF-β1, 2	Platelets Macrophages	Fibroblast and macrophage chemotaxis Extracellular matrix synthesis Secretion of protease inhibitors
CTGF	Epithelial cells Vascular cells	Extracellular matrix synthesis
EGF TGF-α	Platelets Macrophages Keratinocytes	Reepithelization
IGF	Plasma Platelets	Endothelial and fibroblast proliferation Collagen synthesis
VEGF	Keratinocytes Macrophages	Angiogenesis
IL-1	Neutrophils	Activate growth factor expression in macrophages, keratinocytes and fibroblasts
TNF-α	Neutrophils	Activate growth factor expression in macrophages, keratinocytes and fibroblasts

Table 2. Sources and major functions of several growth factors and cytokines

It is possible that expression of PDGF contributes to the inflammatory changes that occur during periodontal diseases. PDGF supports the healing in the periodontal soft tissues in a variety of ways. Since PDGF is chemotactic for fibroblasts, it induces collagen synthesis, but also stimulates fibroblasts to synthesize the proteoglycans that supply the framework for extracellular matrix development. Lipopolysaccharide, the major constituent of the cell walls of gram-negative bacteria, inhibits the proliferation of gingival fibroblasts and PDGF decreases this inhibitory effect (Bartold & Narayanan, 1992). Reported data suggest that there may be cell specific differences in response to PDGF isoforms critical to periodontal healing that may be exploited in the development of efficient therapies (Mumford et al., 2001).

b) Transforming Growth Factor-β (TGF-β). TGF-β superfamily consists of several multifunctional structurally related growth and differentiation factors associated to the inflammatory response, known also to be involved in apoptosis, angiogenesis, wound healing and fibrosis (Frank et al., 1996; Lawrence, 1995). There are three TGF-β isoforms important for humans, TGF-β1, 2 and 3; their amino acid sequences are 70-80% homologous but they can be distinguished by the effects on cell growth, biological interactions and receptor binding abilities (Frank et al., 1996). TGF-β1 is expressed in epithelial, hematopoietic, and connective tissue cells (Massague, 1998). Because TGF-β1 exhibits both pro-inflammatory and anti-inflammatory properties besides its ability to stimulate synthesis of ECM molecules and to inhibit the breakdown of ECM, it has been intensively evaluated in relation to all types of gingival overgrowth.

Regarding TGF-β1 expression in chronic periodontitis, we noticed a positive reaction in some keratinocytes from the gingival basal epithelial layer (Fig.8), and pro-inflammatory cells infiltrating lamina propria (Fig.9).
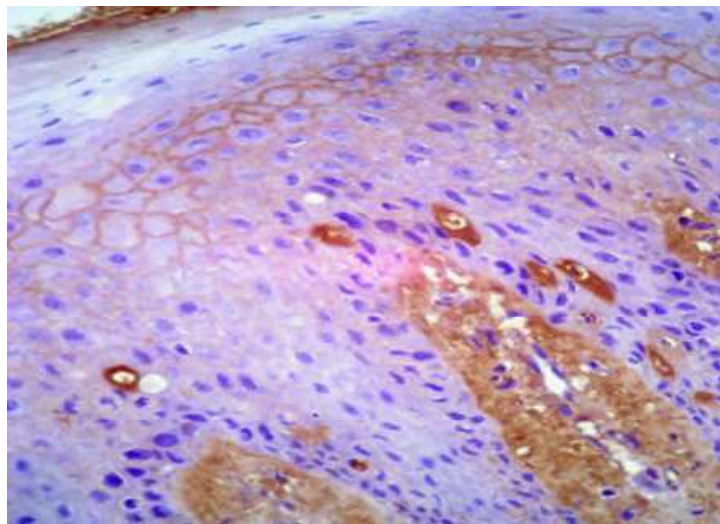

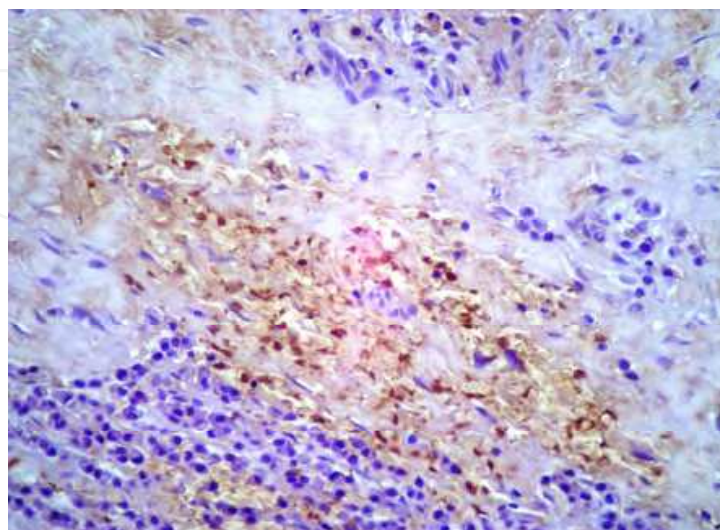Fig. 8. TGF-β1 expression in gingival epithelium in periodontitis (IHCx20)

Fig. 9. TGF-β1 expression in inflammatory cells infiltrating lamina propria of periodontitis-affected gingiva (IHCx20)

Our results confirm that increased gingival inflammation is associated with high levels of TGF-β isoforms. It is well known that TGF-β is one of several cytokines able to regulate inflammation and immune responses and the fact that TGF-β mediates leukocyte recruitment, adhesion and activation suggests that it play a key role in the host response to bacterial and immunological insults (Prime et al., 2004), even there is not a clear evidence for its role in the pathogenesis of the periodontal disease. But TGF-β has also a marked effect on ECM homeostasis, being an important mediator of fibroblast proliferation and ECM synthesis. The inverse relationship between TGF-β1 and MMP-1 expression in the gingival epithelium noticed in our studies could be in accordance with TGF- β1 action as inhibitor of MMP-1 activity and proves that TGF-β mediates a tight control of the MMP/TIMPs equilibrium and synthesis of matrix macromolecules. TGF-β1 pro-fibrotic role could be explained also by the stimulation of collagen synthesis in lamina propria. There are considerable data supporting the fact that under pathological conditions, TGF-β1 orchestrates a cross talk between parenchymal, inflammatory and collagen expressing cells and have a key role in control inflammation and fibrosis (Buduneli et al., 2001b; Ellis et al., 2004; Wright et al., 2001). The role of TGF-β1 in gingival overgrowth must be considered in line with natural development of the periodontal lesion, as inflammation preceedes fibrosis, and with its stadial activity. First, it acts as a pro-inflammatory cytokine that mediate the recrutation of monocytes-macrophages, their adhesion and action at site lesion, suggesting a key role in the host-response to the presence of the bacterial products. Finally, as the inflammation progresses, TGF-β1 overexpression in epithelial and fibroblasts could be a response to paracrine stimulation by other cytokines secreted by the pro-inflammatory cells. Despite the great interest for this growth factor, the precise role of TGF-β1 in the pathogenesis of periodontitis-induced gingival overgrowth is still under debate.

c) Connective Tissue Growth Factor (CTGF). The CCN family consists of six multifunctional members including CCN1 (Cyr61), CCN2 (connective tissue growth factor, CTGF), CCN3 (Nov), CCN4 (WISP1), CCN5 (WISP2), and CCN6 (WISP3) (Brigstock, 2003). The functions of this family include embryogenesis, wound healing, and regulation of ECM production. CTGF is a matricellular cysteine-rich peptide that plays a variety of important roles in cell development and differentiation and acts to promote fibrosis in many different tissues in cooperation with other growth factors and extracellular matrix proteins (Leask & Abraham, 2003). These findings rise the hypothesis that CTGF could play a role in gingival fibrosis.

In periodontitis, we observed a different pattern of CTGF distribution in gingival structures. Many samples showed an intense positive reaction in basal and parabasal epithelial layers but also in structures from the lamina propria (Fig.10). Higher CTGF staining in overgrown gingiva was accompanied by an increased number of fibroblasts and collagen fibers, in accordance with CTGF contribution to increase fibrosis. Fibrosis, as well as physiological wound repair and inflammation, involves the same molecules and cellular events (Bartold & Narayanan, 2006). As a consequence of inflammation, fibrosis can be the result of several events: abnormal release of mediators and persistence of changes in the abnormal growth factor/cytokine profile, and proliferation of cells with an abnormal phenotype responsible for the excessive extracellular matrix synthesis that characterize fibrosis. CTGF alone does not promote fibrosis. Recent studies indicate that CTGF binds to other factors, resulting in either inhibition or stimulation activity (Kantarci et al., 2006, Trackman & Kantarci, 2004). CTGF binding to VEGF results in inhibition of VEGF while CTGF binding to TGF-β1 is reported to be stimulatory (Trackman & Kantarci, 2004). Therefore simultaneous production of both TGF-β1 and CTGF is required to sustain fibrosis in gingival overgrowth.
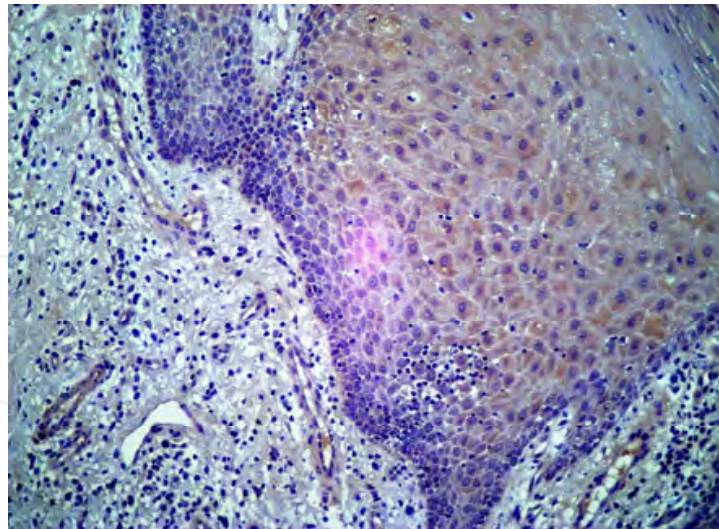
Fig. 10. Intense expression of CTGF in periodontitis-affected gingival epithelium (IHCx20)

d) Basic Fibroblast Growth Factor (bFGF or FGF-2). FGFs are a family of at least 23 structurally related polypeptides known to play a critical role in angiogenesis and mesenchymal cell mitogenesis. In periodontium, FGF-2 is present in the extracellular matrix, as well as in the cementum and can function as a local factor at the site (Gao et al., 1996). In periodontitis, the presence of bFGF was reported in the gingival epithelium, inflammatory cells and connective tissue (Laurina et al., 2009). They noted also a more increased expression of FGF receptor (FGFR) in hyperplasic gingival tissue compared to normal. One of their conclusions was that the expression of growth factors and their receptors in sulcular epithelium was lower than into the gingival epithelium and seems to be specific for periodontitis (Laurina et al., 2009).

e) Epidermal Growth Factor (EGF). EGF is a multifunctional cytokine with a variety of biological functions including epithelial growth and differentiation, and wound healing. In the periodontium, EGF seems to exert only a minor effect on the promotion of mitogenesis, chemotaxis, or matrix synthesis in periodontal ligament fibroblasts (Giannobile, 1996). He supposed that EGF receptors (EGF-R) localization in periodontal ligament fibroblasts may stabilize the periodontal ligament fibroblast phenotype or cellular physical characteristics.

Buduneli et al. (2001c) evaluated the expression of EGF-receptor (EGFR) in frozen sections of cyclosporine (CsA)-induced gingival overgrowth using immunohistochemical and semiquantitative techniques. Gingival biopsies were obtained from 12 renal transplant patients receiving CsA as well as from 9 systemically and periodontally healthy individuals. The authors suggested that CsA affects EGFR metabolism in gingival keratinocytes resulting in an increased number of cell surface receptors, which may eventually play a role in the pathogenesis of gingival tissue alterations. In chronic periodontitis, EGFR was regionally detected in gingival epithelium in some cases (Laurina et al., 2009). Other *in vivo* studies are needed to reveal the precise effects of EGF on soft periodontal tissue healing.

f) Insulin-Like Growth Factors (IGFs). IGFs are a family of mitogenic proteins that control growth, differentiation, and the maintenance of differentiated function in numerous tissues. The IGF family includes three ligands (insulin, IGF-I, and IGF-II), their corresponding cell surface receptors (IR, IGF-IR, and IGF-IIR), and at least six IGF-binding proteins (IGFBPs)

able to bind circulating IGFs and modulate their biological actions. Studies have suggested that IGF-I has an important involvement in periodontal wound healing and regeneration. IGF-I is chemotactic for cells that come from the periodontal ligament and demonstrates significant effects on the mitogenesis of periodontal ligament fibroblasts (Giannobile, 1996). IGF-I is able to prevent apoptosis in fibroblasts, to regulate DNA and protein synthesis in periodontal ligament fibroblasts *in vitro* and to enhance soft tissue wound healing in *vivo* (Werner & Katz, 2004). Regarding the IGF-IR expression in chronic periodontitis, Laurina et al. (2009) reported only a weak presence in the sulcular epithelium suggesting a potential role in regeneration of periodontal tissue. The effect of IGF-II on the metabolism of gingival fibroblasts is still uncertain.

h) Hepatocyte growth factor (HGF). HGF is a multifunctional cytokine involved in the repair and regeneration of various tissues and their protection from injury (Matsumoto & Nakamura, 1997) and recently, it has been linked also to the development of periodontal disease (Ohshima et al., 2001; Ohnishi & Daikuhara, 2003). HGF may be closely involved in the pathogenesis and progression of periodontal disease because it stimulates excessive proliferation and invasion of gingival epithelial cells and impair the regeneration of deep collagenous structures in the periodontium (Ohshima et al., 2001).

g) Vascular Endothelial Growth Factor (VEGF). Over the last two decades researchers have demonstrated that VEGF is a key regulator of physiological and pathological angiogenesis, because it induces endothelial cell proliferation, stimulates angiogenesis and increases vascular permeability (Ferrara, 2009). In the last decade, many groups focused their research on the angiogenic factors that contribute to periodontal healing. In periodontitis patients, VEGF was detected within vascular endothelial cells, neutrophils, plasma cells, and junctional, pocket and gingival epithelium (Booth et al, 1998). In a previous study on biopsies obtained from patients with type 2 diabetes associated gingival overgrowth, we detected VEGF expression in keratinocytes from the basal and spinous layers and in many *de novo* capillaries (Pisoschi et al., 2009). Other authors reported increased VEGF expression in epithelial cells and endothelial cells in periodontitis-affected gingiva (Guneri et al., 2004; Keles et al., 2010; Lucarini et al., 2009). Giannobile et al. (2003) suggested that VEGF could be an important growth factor for the onset of gingivitis and its progression to periodontitis. Taken together these observations conclude that VEGF expression is related to both maintenance of periodontal health and periodontal tissue destruction but the precise mechanism of neovascularization remains in debate.

5. Gingival crevicular fluid and salivary growth factors as potential biomarkers of periodontal disease

Traditional periodontal diagnostic methods include assessment of clinical parameters (probing pocket depth, bleeding on probing, clinical attachment loss, plaque index), and radiographs but these conventional techniques are of limited usefulness because they are not sufficiently accurate to discern between previous periodontal disorders and present disease activity. For this reason, development of non-invasive diagnostic tools has presented a significant challenge to periodontology. Recently, gingival crevicular fluid (GCF) and saliva are in the middle of high-throughput techniques aimed to validate tests for the objective diagnosis of disease, monitoring, and prognostic indicators. Oral fluids contain locally and systemically derived mediators of periodontal disease, including pathogens,

host-response, and bone-specific markers (Kinney et al., 2007). Most biomarkers assessed in oral fluids are inflammatory cytokines, but some collagen degradation products and bone turnover-related molecules have emerged as possible markers of periodontal disease activity, too. Most biomarkers in GCF and saliva are indicators of soft-tissue inflammatory events (pathogens infection and host inflammatory response) that precede the destruction of the alveolar bone (Buduneli & Kinane, 2011).

The detection of connective tissue-derived molecules may provide a more precise assessment of the breakdown of periodontal tissues, especially in light of the variability in the host response of different individuals (Giannobile et al. 2003).

Among those which are the potential candidates for oral fluid-based diagnostics of periodontal disease are included: alkaline phosphatase, MMP-8, MMP-9, MMP-13, osteocalcin, osteonectin, osteopontin, and pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (Arikan et al., 2008, 2011; Buduneli et al., 2008; Kinney et al., 2007; Miller et al., 2006; Ozçaka et al., 2011) but the list is still open.

Extracellular matrix molecules that are derived from the periodontium have been identified in GCF and saliva. Although growth factors function as molecular mediators of periodontal tissues repair their value as diagnostic biomarkers of periodontal tissue inflammation and/or destruction has yet elucidated.

Research in this area has reported the assessment of GCF and salivary levels of several growth factors for their potential to diagnose periodontal disease, including EGF, TGF-β, PDGF, HGF, and VEGF (Buduneli et al., 2001b; Chang et al., 1996; Gurkan et al., 2006; Hormia et al., 1993; Kamimoto et al., 2002; Sakallioglu et al., 2007; Wilczynska-Borawska et al., 2006).

We were also interested to seek a relationship between salivary levels of growth factors and other molecules (MMPs, TIMPs, TNFα) related to host response and morphological and clinical changes observed in periodontitis. We compared the salivary concentration of TGF-β1 and CTGF in patients with periodontal disease and healthy control subjects. We didn't obtain any significant difference for the salivary TGF-β1 levels between healthy subjects and those with gingivitis. Only the values obtained for salivary TGF-β1 in chronic periodontitis differ significantly from those found for control subjects but even in this case we observed a great difference between the smallest and the highest values (Pisoschi et al., 2010). This variation could be explained by the duration of the candidate risk factors on TGF-β1 levels, being well known that periodontal disease is characterized by periods of active tissue destruction and quiescence. Our findings are in accordance with the results reported by other researchers for the variation of TGF-β1 levels in patients with aggressive and chronic periodontitis (Gurkan et al., 2006). We also obtained a good correlation between salivary concentration and gingival expression of TGF-β1. So far, we didn't find any significant differences between salivary CTGF levels of patients with gingivitis, chronic periodontitis and controls (unpublished data). However, CTGF concentrations were higher in samples obtained from patients with type 2 diabetes associated gingival overgrowth (Pisoschi et al., 2011). Regarding the level of circulating and oral fluids TGF-β1 and CTGF, we agree with other researchers that variation in the analytical technique used in sample assessment might influence the values.

As we mentioned before, many reports sustained the importance of VEGF in the pathogenesis of periodontal disease. Therefore researchers attempted to find a correlation between GCF or salivary VEGF levels and its tissue expression in order to link this biomarker with the degree of periodontal damage. A recent study of Pradeep et al. (2011) that included patients with gingivitis and chronic periodontitis, reported an increase of VEGF concentration in GCF and a positive correlation between GCF and serum VEGF levels. Moreover a correlation between growth factor levels and clinical periodontal parameters before and after nonsurgical periodontal treatment was observed.

Even the results of much research have confirmed the presence of several growth factors in GCF and saliva, for many of them the relation between their variation and the severity of the periodontal disease is still unclear. In the future, high-throughput proteomic and metabolomic technologies will be critical in establishing whether a biomarker could be accurate to diagnose and predict the severity of the periodontal disease.

6. Growth factors as therapeutic tools in periodontal disease

Therapeutic approaches for treatment of periodontitis are divided into two categories: *i) anti-infective treatment*, and *ii) regenerative therapy*.

Several restoring techniques have been developed to regenerate periodontal tissues including guided tissue regeneration, bone grafting, and use of enamel matrix derivatives. Principles of guided tissue regeneration dictate that one of the goals of therapy is to modulate the repopulation of the wound with cells derived from the periodontal ligament rather than from the gingival tissues (Mumford et al., 2001). However, this technique is not associated with complete periodontal regeneration and a major complication and limiting factor in the achievement of periodontal regeneration is the presence of microbial periodontopathogens that contaminate wounds and reside on tooth surfaces as plaque-associated biofilms.

For more than a decade, periodontal researchers have been studying the potential of growth factors to achieve predictable periodontal regeneration. It is accepted that many growth factors are produced as inactive propeptides and stored in the cytoplasm (Anusaksathien & Giannobile, 2002). Cleavage of these propeptides and extracellular secretion of the mature forms provide the growth factors able to bind on their specific receptors.

Growth factor therapies are directed to stimulate the specific progenitor cells which are responsible for the regeneration of mineralized and nonmineralized tissues in the periodontium but also to limit periodontal degradation. Therapeutic application of growth factors to restore damaged tissue aims the regeneration through biomimetic processes, or mimicking the processes that occur during embryonic and post-natal development (Schilephake, 2002). The complexity of these events suggests that creating an optimal regenerative environment requires the combination of different growth factors as found in natural processes. But also the use of a single recombinant growth factor may induce several molecular, biochemical and morphological cascades that will result in tissue regeneration. Almost all the growth factors are believed to be potential targets for the periodontal regenerative therapy but few strategies are subjected to various phases of clinical trials. Growth factors tested for their contribution to periodontal regeneration include PDGF, IGF-I, TGF-β1, bFGF and some bone morphogenetic proteins (Giannobile et al., 1996, 2001;

Murakami et al., 1999; Taba et al., 2005). Results from the in vivo studies have shown that all of the above mentioned bioactive molecules, with the exception of TGF-β1, exhibited ability to promote periodontal tissue regeneration and suggest that there may be cell-specific differences critical to periodontal wound healing that may be exploited in the development of new therapies.

PDGF was the first growth factor to be evaluated in preclinical periodontal regenerative studies. In vitro studies had shown that exogenous application of PDGF at different concentrations (between 0,01 and 10 ng/ml) resulted in proliferation, migration and matrix synthesis in cultures of periodontal cells, including gingival and periodontal ligament fibroblasts, cementoblasts, preosteoblasts and osteoblasts in a time and dose dependence (Kaigler et al., 2006). From all its isoforms, PDGF-BB is the most effective on periodontal ligament cell mitogenesis and extracellular matrix macromolecules biosynthesis (Bartold, 1993; Ojima et al., 2003). Even periodontal ligament fibroblasts and gingival fibroblasts have been shown to proliferate rapidly, gingival fibroblasts have been shown to fill a wound space significantly faster than periodontal ligament cells and this is an unwanted effect (Mumford et al., 2001). Studies on combination growth factor therapy involving PDGF and IGF-I have consistently promoted the periodontal regeneration (greater osseous and new attachment response) in animal models and lead to the first study in humans using growth factors for periodontal regeneration. In their human phase I/II clinical trial, PDGF/IGF-I were considered safe when applied topically to periodontal osseous lesions, resulting in a significant improvement in bone growth and fill of periodontal defects, compared with standard therapy (Howell et al., 1997, as cited by Kaigler et al., 2006).

TGF-β plays a significant role in periodontal regeneration. It is pleiotropic, and can stimulate or inhibit cell growth, an action that can interfere with its therapeutic use (Clokie & Bell, 2003). TGF-β1 has been used for this application. The results of rhTGF-β1 for periodontal regeneration have not been consistent preclinically as shown in dogs and sheeps investigations (Mohamed et al., 1998; Tatakis et al, 2000, as cited by Kraigler et al., 2006). These studies showed little advantages in new bone formation and no improvement in cementum regeneration when treated with rhTGF-β1. Other research demonstrated that TGF-β1 increased the amount of bone healing adjacent to dental implants in minipigs (Clokie & Bell, 2003). TGF-β can also modulate other growth factors, such as PDGF, EGF, and FGF, by altering their cellular response or by inducing their expression. Combined therapies, which involved PDGF and TGF-β, have demonstrated synergistic effects and enhanced regeneration. Together, PDGF and TGF-β have stimulated gingival fibroblasts and periodontal ligament cells. Some authors reported that TGF-β, both alone and in combination with PDGF, led to a greater proliferation of periodontal ligament cells compared to the gingival fibroblasts. On the contrary, PDGF stimulated a significantly greater proliferation of gingival fibroblasts compared to periodontal ligament cells. Since periodontal proliferation at the diseased site is a desired feature in periodontal regeneration and because of the limited number of studies and inconsistent results, the use of TGF-β will be further emphasized and thoroughly investigated.

Several studies reported that FGFs can stimulate mitogenesis and chemotaxis in periodontal ligament cells (Takayama et al., 1997; Terranova et al., 1989). FGFs increased osteoblast proliferation, although they do not directly increase collagen production by differentiated osteoblasts. They have shown bFGF stimulates human endothelial and periodontal ligament

cell migration and proliferation on the dentin surfaces, and that the combination of bFGF with fibronectin can further enhanced periodontal ligament cell chemotaxis. Despite different concentrations of bFGF and different delivery systems used in the studies, all showed an improvement in the periodontal tissue regeneration. Studies that evaluated more than one concentration of bFGF suggested that its effects are dose dependent (Murakami et al., 2003).

The results from preclinical and initial clinical studies using growth factors are encouraging; however, some limitations exist with respect to bone volume and predictability. Trials utilizing topical growth factors have revealed difficulties in maintaining therapeutic levels of proteins and to obtain optimal outcomes *in vivo*; of great importance is to enhance the half-life of growth factors and their biological stability (Yun et al., 2010). Based on the results of studies that support *in vitro* biological functions of FGFs for tissue regeneration, the largest *in vivo* study in the field of periodontal regenerative therapy was initiated by Kitamura's team. This was a human clinical trial projected to determine the safety and efficiency of FGF-2 for clinical application. Their results support that topical application of three doses of FGF-2 during periodontal surgery could be efficient for the regeneration of periodontal tissue (Kitamura et al., 2008).

Future clinical application of growth factors in the regeneration of periodontal tissues will be achieved when their biological functions are maximized by the appropriate use of biomaterials and stem cells.

7. Conclusions

The biology of periodontal connective tissues is important to be understood in terms of development, pathology, regeneration and interrelationship between periodontitis interactions and various systemic diseases. Although growth factors function as molecular mediators of periodontal tissues, their value as diagnostic biomarkers for periodontal tissue inflammation and/or fibrosis is yet to be elucidated. High-throughput technologies applied for assessment of gingival crevicular fluid and saliva will give new promises for the use of growth factors as objective biomarkers in periodontal disease.

In earlier studies, the application of growth factors provided different degrees of success in stimulating wound healing in the periodontal areas. There is an imperious need to further evaluate the biologic mechanisms that may be responsible for the promotion of tissue regeneration by growth factors. Finally, studies on growth factors delivery and improved stability seek evidence to conclusively support the addition of growth factors strategy to the therapeutic protocol for regeneration of periodontal tissues.

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9. References

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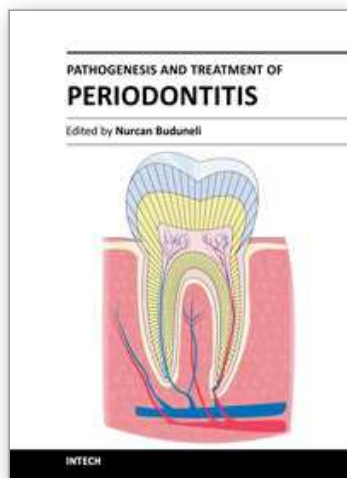
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