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Growth factors/cytokines/defensins and apoptosis in periodontal pathologies

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SUMMARY

In the recent past there has been an increased emphasis on morphogenetic tissue research of periodontal tissues. The aim of this study was to find qualitative and quantitative correlations in distribution and appearance of growth factors/cytokines/defensins and apoptosis in periodontal pathologies. Material and methods. Tissue was obtained from 5 controls and 6 chronical periodontitis patients 30-50 years of age referred to Latvian Institute of Stomatology. Histological investigations were performed at the Institute of Anatomy and Anthropology of Riga Stradins University. Results. Epithelial cells abundantly expressed IL10 in patients. The expression of β-defensins was very variable in both sulcular and gingival epithelium. TUNEL positive cells were observed in patients and control specimens with dominance in control group. Gingival epithelium showed moderate expression of bFGF whereas few to moderate cells were positive for bFGF in sulcular epithelium. Fibroblast growth factor receptor (FGF-1R) was abundant in gingival epithelium and in connective tissue cells, but almost not detectable in sulcular epithelium. Insulin-like growth factor receptor was not expressed in gingival epithelium and was weakly seen in basal layer of sulcular epithelium. Basic nerve growth factor expresion in both types of epithelium was numerous to abundant. Staining for the NGFR in the gingival epithelium was variable, with prevalence to be moderate whereas sulcular epithelium was free from any factor immunoreactivity. Conclusion. 1. Finding of apoptotic cells are variable and seems to correlate with the expression of defensins in oral epithelium in patients with periodontitis. 2. FGFR was expressed more than the bFGF, but in case with NGFR and bNGF situation was opposite. Although IGFRI was found in sulcular epithelium with no expression in gingival one suggesting about stimulation in regeneration/adaptation in periodontitis affected tissue. 3. 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.

Key words: growth factors, cytokines, defensins, apotosis, periodontal health.

INTRODUCTION

Morphogenetic tissue research in the craniofacial region has become very actual nowadays. This project is the first trial in Latvia that deals with examination of periodontal anomalies in molecular level.

Periodontal disease is one of the two major dental diseases that affect human populations worldwide at high prevalence rates (1, 2). An advanced periodontal disease with deep periodontal pockets (6 mm or more) affects 10% to 15% of adults worldwide (3). 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.

Periodontitis is a chronic disease characterized by the interaction between gram – negative bacteria and the host inflammatory response, which results in tissue destruction and tooth loss (4, 5, 6). Bacterial products act on the cellular constituents of the gingival tissues, activating cellular processes that induce the destruction of connective tissues and bone (7, 8). Pathogenic bacteria have virulence characteristics that can prevent their efficient detection and elimination by the

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host, disable host cells and humoral factors, and directly adversely affect the tissues (8). Activated tissue destructive cytokines is likely a major pathway for connective tissue attachment loss and bone loss.

In inflamed periodontal periodontal lesions, variety of cytokines is produced by lymphocytes, monocytes as well as by non-immune cells like fibroblasts, epithelial and endothelial cells (9). Cytokines are soluble, secreted glycoproteins which act as local signaling molecules to control and coordinate cellular behavior and function. Interleukin 10 (IL-10) is an anti-inflammatory cytokine that plays a role in periodontal disease by inhibiting synthesis of proinflammatory cytokines such as IL1, IL6, IL8, and tumor necrosis factor-α by monocytes and stimulating protective antibody production. It is found in inflamed periodontal tissues. Genetic polymorphisms in the IL10 gene might be useful as a marker to diagnose susceptibility to periodontitis and may influence the expression of the protein (10, 11).

Growth factors are generally considered to be a subset of cytokines. They are biological mediators which regulate connective tissue cell migration, proliferation and synthesis of proteins and other components of extracellular matrix. The response of target cells to growth factors depends on the expression their specific receptors. These receptors are transmembrane antigens which, on binding of the growth factors, produce a cascade of intracellular signals that stimulate chemotaxis, cell growth, differentiation and the production of extracellular matrix (12). The growth factor receptors therefore also show of fundamental importance in human periodontal growth and regeneration (13).

Epidermal growth factor (EGF) is a multifunctional cytokine with a variety of biological effects including stimulation of cell proliferation by binding to its specific EGF receptor. It is a small molecular weight polypeptide which is thought to have important functions in epithelial growth and differentiation and in wound healing. EGF exerts its action on cells through binding to a cell surface receptor. EGF receptors were expressed at high levels on the cell surface of basal cell layers of gingival epithelium (14).

Fibroblast growth factors (FGF) are a family of at least 23 structurally related polypeptides that are known to play a critical role in angiogenesis and mesenchymal cell mitogenesis. These growth factors bind to four high affinity tyrosine kinase receptors designated as fibroblast growth factor receptors (15). Basic fibroblast growth factor or fibroblast growth factor – 2 displays a wide variety of mesoderm and neuroectoderm derived cells and has a high efficiency of angiogenesis (16). 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 (17). The presence of high affinity receptors for FGF-2 has been reported for many cell types (18). The number of sites per cell varies from 2000 to 120,000 according to cell type studied.

The insulin like growth factors (IGF) is regulated by a series of at least six different binding proteins (insulin-like growth factor factor-binding proteins). As with other antagonists, insulin-like growth factor-binding proteins bind to the insulin-like growth factors with high affinity and typically may inactivate activity by preventing ligand binding. There are two insulin like growth factors – IGF1 and IGF2 both of which are found in high concentration in serum. IGF-I is capable of preventing apoptosis in fibroblasts by activation of multiple signal transduction pathways (19). IGF-1 has also been shown to regulate DNA and protein synthesis in periodontal ligament fibroblasts in vitro and to enhance soft tissue wound healing in *vivo* (20, 21). Studies have suggested variable responses of periodontal tissues to IGF-1 upon anatomical site, and a differential involvement of IGF-1 in periodontal wound healing and regeneration (12). IGF-IR (insulin growth factor 1) and PI3K (phosphoisositide 3-kinase) are two signaling molecules that could mediate IGF-I induced cell survival via common or independent pathways (22).

Nerve growth factor (NGF) appears to have multiple neuronal and non-neuronal functions in peripheral tissues. NGF is produced by target tissues and is required for development and maintenance of sympathetic sensory neurons. NGF regulates responsive cells by binding to high affinity (Type 1) and low affinity (Type 2) surface receptors. Only Type 1 receptor has been shown to internalize bound NGF, thus triggering biological responses (23, 24). Nerve growth factor (NGF) is a representative of neurotrophins responsible for the differentiation and survival of neurons (25, 26). NGF activates 2 different receptor classes: the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases (TrkA, TrkB, and TrkC) and the p75 receptor, a member of the tumor necrosis factor receptor superfamily. Neurotrophins regulate both cell death and cell survival through activations of Trk receptors and/or p75 neurotrophin receptor. It has been reported that neurotrophins are also produced from non-neuronal cells, such as leukocytes, osteoblasts, or fibroblasts, and act in many other ways on non-neuronal cells. It has been found that sulcular epithelium have intense p75-NGFR staining.

Antimicrobial peptides belong to first line of host defense mechanisms against infections in epithelial tissue. As part of innate immunity, defensins playing

an important role in maintaining tissue integrity in oral health are small cationic cysteine-rich antimicrobial peptides (27) and have a broad spectrum antimicrobial activity against gram-positive and gram-negative bacteria, fungi and viruses (28). They function by association with the anionic microbial surface, and then aggregate to form pores or disrupt microbial membranes, although new evidence suggests possible additional cytoplasmic targets. Defensins are localized in different sites in gingiva, suggesting that they are likely to serve different roles in several ecological niches of the periodontium (29). Human defensins are classified into two subgroups: α defensins and β defensins. Polimorphonuclear leucocytes express α defensins and β defensins are expressed by mucosal epithelial cells (30). It has been reported that α and β defensins are localized in different parts of gingival epithelium. β defensins are expressed in the oral and sulcular stratified epithelium of gingival, but α defensins are expressed in the junctional epithelium (31, 32).

Apoptosis or programmed cell death is an important determinant of the life span of cells in regenerating tissues; defined by distinct morphological and biochemical features (33, 34). It can be observed under both physiological and pathological conditions and plays an important role in the process of morphogenesis and homeostasis. The apoptotic cells shrinking and loss of cell-cell junctions results in detachment from the adjacent cells. Cells undergoing apoptosis are recognized by condensation of chromatin, the degradation of DNA into oligonucleosome-sized fragments, and the formation of plasma and nuclear membrane blebs (35). Eventually the cell breaks apart to form so-called apoptotic bodies. Apoptosis is one of the mechanisms involved in the pathogenesis of periodontitis thus regulates a cell turnover in gingival tissue.

The aim was to find qualitative and quantitative correlations in distribution and appearance of interleukin-10 (IL-10), antimicrobial peptides – β-defensins, growth factors:epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and its receptor one (FGF-R1), insulin-like growth factor receptor one (IGF-R1) and basic nerve growth factor (bNGF) and its receptor (NGFR), and also apoptosis in healthy and diseased periodontal tissues.

MATERIALS AND METHODS

The tissue samples obtained were identified as minimally inflamed gingival tissues or chronical periodontitis on the basis of clinical record, radiographs and macroscopical examination. Minimally inflamed gingiva (clinically healthy gingiva) was defined by absence on bleeding on gentle probing, no clinical signs of inflammation and a probing depth of 3 mm or less. Chronical periodontitis specimens were obtained from patients with a probing depth 5 mm and more and with an evidence of resorbtion of the alveolar bone. Tissue was obtained from 5 controls and 6 chronical periodontitis patients referred to Latvian Institute of Stomatology, Department of Periodontology for periodontal treatment. 30-50 year olds age group was chosen. Samples were taken during a routine periodontal flap surgery with an internal bevel incision and immediately fixed in 10% phosphate buffered formalin. Histological investigations were performed at the Institute of Anatomy and Anthropology of Riga Stradins University. Fixed periodontal tissue was embedded in paraffin and cut in 5 μm thin sections. After they were proceeded for interleukin-10 (Rabbit Policlonal to IL-10 / ab34843, w.d.1:400, *Abcam*, England), β-defensins (Anti-human β-Defensin 2 Antibody, AF 2758, w.d. 1:100, *R&D systems*, Deutchland), epidermal growth factor (Mouse anti-human to EGFR / M3563, w. D. 1:150, *DakoCytomation*, Denmark), basic fibroblast growth factor (Rabbit polyclonal to bFGF, w.d. 1:200, *Abcam*, England) and its receptor one (Rabbit polyclonal to FGFR1 / ab 10646, w.d. 1:100, *Abcam*, England), insulin- like growth factor receptor one (Anti-human IGF-I R Antibody, AF-305-NA, w.d. 1:100, *R&D systems*, Deutchland) and basic nerve growth factor (Rabbit polyclonal to NGF / ab 6199, w.d. 1:500, *Abcam*, England) and its receptor (Anti-human NGFR p75 / M3507, w.d.1:150, *DakoCytomation*, Denmark) by using of biotin-streptavidin immunohistochemistry (IMH) (36). Also apoptosis were detected by using of TUNEL kit (37).

Routine staining for hematoxylin and eosin was performed. Distribution of factors and substances was determined semi quantitatively by counting of positive structures in visual fields (0/+ – occasional, + – few, ++ – moderate, +++ – numerous positive structures in visual fields, ++++ – abundance) (38).

Permission of Ethical Committee of Riga Stradins University was received for the work with human material.

RESULTS

From controls only two responded to the common healthy tissue picture of periodont. In other cases morphological changes of controls were similar to those in periodontitis. In some cases hyperplasia of gingival epithelium and vacuolization of epithelial cells in sulcular epithelium was observed (Figure 1). Tissue demonstrated also infiltration with inflammatory cells – macrophages and lymphocytes (Figure 2).

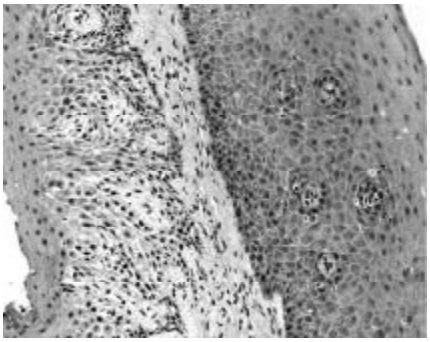

Fig. 1. Sulcular and gingival epithelium with hyperplasia of basal cells in patients with periodontitis. Heamatoxylin and eosin, x400.

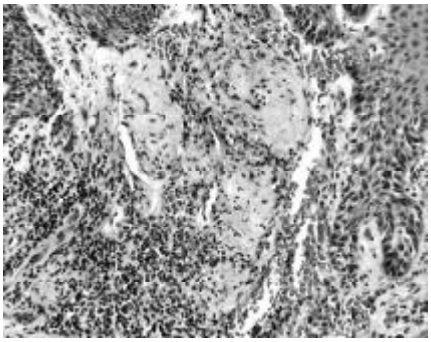

Fig. 2. Infiltration of connective tissues with inflammatory cells in patients with periodontitis. Heamatoxylin and eosin, x400.

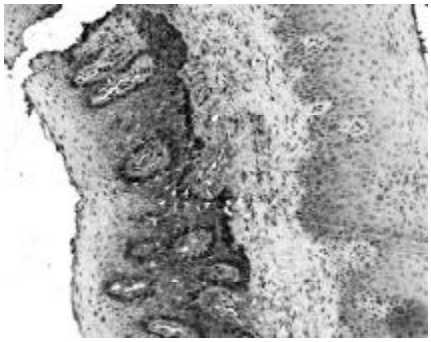

Fig. 3. Epithelial cells showing IL10 immunoactivity in patients. Immunohystochemistry, x200.

Epithelial cells abundantly expressed IL10 in patients (Figure 3) whereas in control group numerous positive cells were found. Also variable expression of IL-10 in connective tissues was seen both in patients and in controls. The expression of IL-10 was abundant in sulcular epithelium (pocket epithelium) and moderate in oral gingival epithelium (Table 1) (Table 2).

The expression of β-defensins was very variable in both sulcular (pocket) and gingival epithelium. There were even cases when no staining in sulcular epithelium was found, but averagely few positive cells were detected. Generally β-defensin in gingival epithelium was of occasional expression till numerous positive structures found (Figure 4).

TUNEL positive cells were observed in patients and control specimens with dominance in control group. Number of apoptotic cells in gingival epithelium often was higher than in sulcular, but common number of apoptotic cells was moderate (Figure 5).

Epidermal growth factor was regionally detected in basal layer of gingival epithelium only in one chronical periodontitis case (Figure 6).

Gingival epithelium showed moderate expression of bFGF (Figure 7) and weaker staining of the inflammatory cells and connective tissues. Few to moderate cells were positive for bFGF also in sulcular epithelium. Fibroblast growth factor receptor (FGF-1R) was abundant in gingival epithelium (Figure 8) and in connective tissue cells, but almost not detectable in sulcular epithelium.

Insulin-like growth factor receptor was not expressed in gingival epithelium and was weakly seen in basal layer of sulcular epithelium (Figure 9).

Basic nerve growth factor expresion in both types of epithelium was numerous to abundant. It also was found moderately expressed by inflammatory cells. Neuroendocrine cells marked by bNGF were detected in the gingival epithelium of two controls.

Gingival epithelium basal layer was strongly stained for nerve growth factor receptor while only moderate nerve fibers showed staining around blood vessels (Figure 10). Staining for the NGFR in the gingival epithelium was variable, with prevalence to be moderate whereas sulcular epithelium was free from any factor immunoreactivity**.**

DISCUSSION

Growth factors initiate many of the events associated with turnover, repair and regeneration of periodontal tissues. The corresponding receptors are thus of fundamental importance in maintaining periodontal

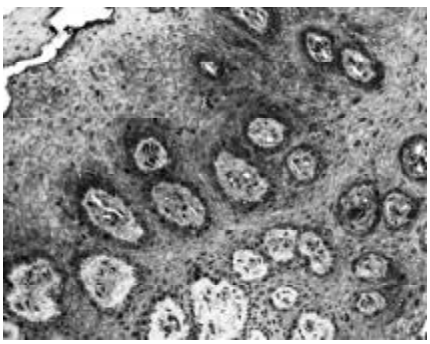

Fig. 4. Epithelial cells showing IL10 immunoreactivity in patients. Immunohystochemistry, x250

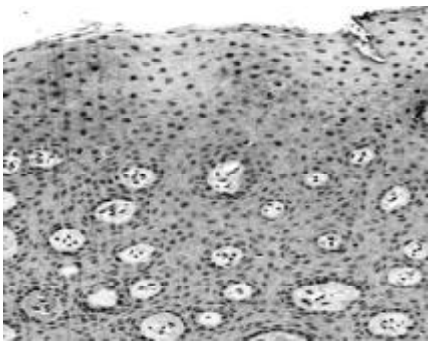

Fig. 5. Numerous apoptotic cellsin gingival epithelium in control. TUNEL, x250.

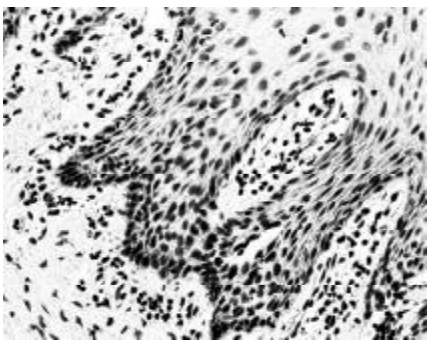

Fig. 6. Expression of EGFR in the basal layer of gingival epithelium in patient. Immunohystochemistry, x400.

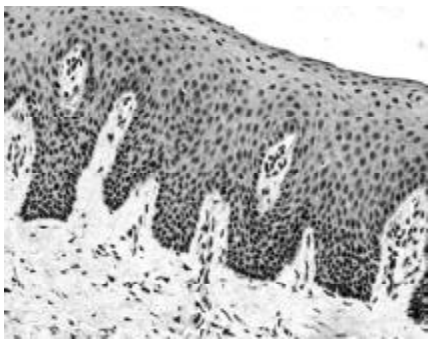

Fig. 7. bFGF expression from gingival epithelial cells in control. Immunohystochemistry, x250.

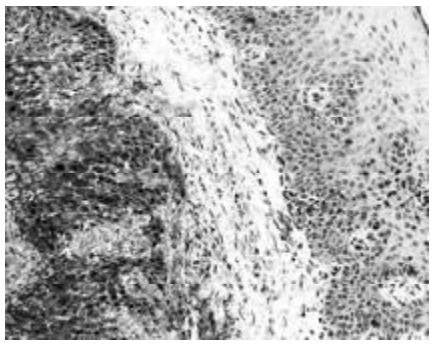

Fig. 8. Abundant expression of FGFR1 from sulcular epithelium and moderate from gingival epithelial cells in patients. Immunohystochemistry, x250.

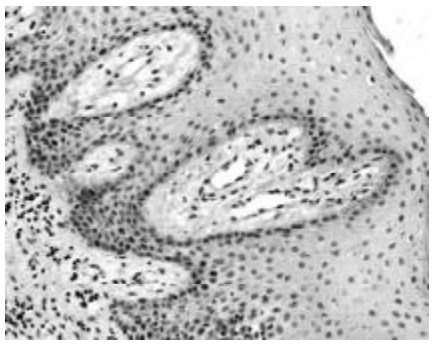

Fig. 9. Expression of IGFR1 from the the basal layer of sulcular epithelium in patients. Immunohystochemistry, x250.

integrity and mediating periodontal wound healing, but their expression in human gingival is not well defined. The most dominant expression of growth factors and their receptors in our study was seen in gingival epithelium in the specimens of periodontitis group. Normal gingival tissues expresses relatively lower amounts of the growth-factor receptors in contrast with inflamed or regenerating tissues where increased growth factor and their receptor prevalence are found (14). The biological properties of bFGF acting on variety of mesenchymal cell types may suggest that bFGF application is of great advantage for active induction of periodontal regeneration consisting of not only connective tissue regeneration, but also osteogenesis and cementogenesis. In such a way we suggest about inflammation that stimulates expression of the same growth factors and their receptors. However, interesting is the observed correlations between appearance of factor and receptors. So, FGFR were expressed more than the same bFGF, but in case with NGFR and bNGF situation was opposite**.**

The expression of FGFRI was reported to be higher in hyperplasic gingival tissue than in normal gingiva. An additional mechanism regulating FGF activity involves heparin or heparin sulfate proteoglicans, molecules which facilitate ligand receptor activation. Binding of ligand to FGFRI induces receptor dimerisation and activation of the receptor tyrosine kinase domain by autophosphorilation, followed by activation of downstream effector (39). Higher expression of FGFRI compairing to bFGF also can be due to possible appearance of acidic FGF (aFGF) in periodontal tissues. The FGFRI binds also to aFGF (40).

Beside of NGF role in maintenance and survival of peripheral and central nervous system and development of neuronal cells, it also has important functions on nonneuronal cells. It is also an autocrine survival factor for memory of B lymphocytes (41). The effect of NGF is mediated by activation of two different classes of plasma membrane receptor, high-affinity Trk tyrosine kinase receptors (TrkA, TrkB, TrkC) and low affinity p75 neutrophin receptors that form complexes. P75 receptors have a much wider distribution than any of Trk receptors in various cell types. Due to the relatively higher levels of p75, the majority of NGF binding is to p75 receptors, with the Trk receptors becoming low affinity. It is approved that p75 receptor can serve as putative cell marker in oral epithelium as it is expressed in the basal cell layer of both tips of the papillae and deep rete ridges in oral epithelium. Activation of receptor p75 without co-expression of Trk receptors, initiates apoptosis

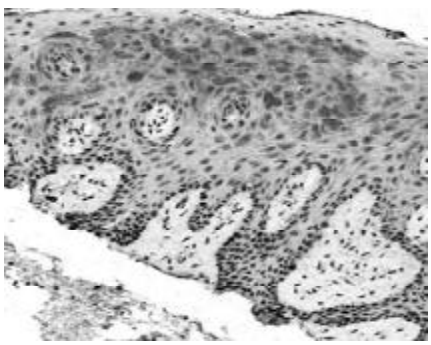

Fig. 10. Abundant expression of NGF from epitheliocytes of gingival epithelium in patients. Immunohystochemistry, x250.

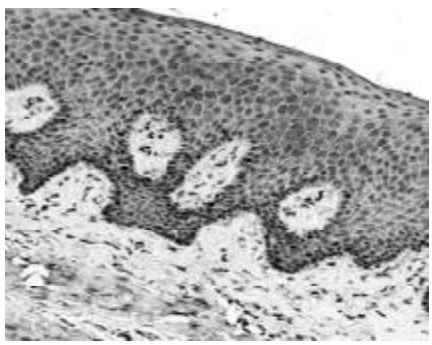

Fig. 11. Abundant expression of NGF from epitheliocytes of gingival epithelium in patients. Immunohystochemistry, x250.

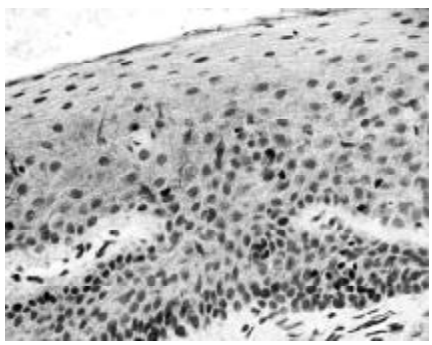

Fig. 12. Neuroendocrine cells marked by bNGF in the gingival epithelium of controls. Immunohystochemistry, x400.

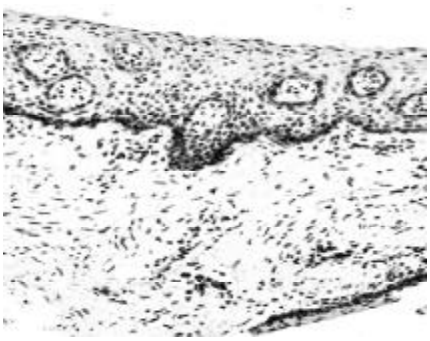

Fig. 13. Abundant expression of NGFR in the basal layer of gingival epithelium, Immunohystochemistry, x250.

(42). Thus, the cell-inducing or cell-survival actions of NGF are strongly dependent on the relative expression of p75 and Trk receptors on the target-cell populations. Absence of NGFR expression in sulcular epithelium correlates also with indistinct apoptosis in our patients and together with rich appearance of bNGF let us suggest about the common stimulating role of this factor in adaptation processes during periodontitis.

In general, IGFRI is the most important mediator of effects of IGFs on mitogenesis, apoptosis, cell motility, tumorogenesis and metastasis. IGFRI is a receptor that plays critical role in signaling cell survival and proliferation. Overexpression of IGFRI induces transformation, and activation blocks programmed cell death (apoptosis). Transcription of the IGFRI gene is upregulated by PDGF and bFGF (43). IGFRI is expressed in most body tissues and levels of IGFRI mRNA are modulated in a number of physiological and pathological states, including changes in the levels of various circulating and locally acting growth factors (44).

IGFRI may have only a limited effect on periodontal regeneration *in vivo* and as data shows play a more prominent part in the formation of new bone at later stages of periodontal wound healing process, as IGF enhances DNA syntesis in osteoclasts and has a significant role in osteogenesis (12). There may be a factors (inflammation, etc.) present that suppress the expression of the receptor as it seems in our patients with periodontitis.

The variable amount of antimicrobial peptides and inflammatory markers probably do not directly depend on inflammation, as different expression of IL10 and defensins was seen in both types of epithelium. Rather defensins appearance correlates with level of apoptosis and perhaps some other not cleared yet factor. Our observations are also not consistent with other findings reporting that apoptosis was a prevalent phenomenon in human clinically healthy gingival tissues that might be changed in diseased conditions and in accordance to the role of defensins that defend the tissues because inflammation was seen in almost all patients and control tissues.

Table 1. Expresion of Interleukin-10, β-defensins; apoptotic cells staining for TUNEL in gingival and sulcular epithelium.

	Gingival epithelium	Sulcular epithelium
IL-10	++	++++
β-defensins	0/+ / + / ++ / +++	0/+ / +
TUNEL	0/+ / + / ++ / +++	+

0/+ – occasional positive structures in visual field
+ – few positive structures in visual field
++ – moderate positive structures in visual field
+++ – number of positive structures in visual field
++++ – abundance of positive structures in visual field
Underlined is the most often seen appearance of positive structures.

Table 2. Growth factor and their receptors expression in gingival and sulcular epithelium

	Gingival epithelium	Sulcular epithelium
Epidermal growth factor **EGF**	+	-
Fibroblast growth factor **bFGF**	+ / ++	+ / ++
Fibroblast growth factor receptor **FGFR**	++++	-
Insulin like growth factor receptor **IGF-1R**	-	0/+ / +
Nerve growth factor **bNGF**	+++ / ++++	+++
Nerve growth factor receptor **NGFR**	0/+ / + / ++ / +++	-

0/+ – occasional positive structures in visual field
+ – few positive structures in visual field
++ – moderate positive structures in visual field
+++ – number of positive structures in visual field
++++ – abundance of positive structures in visual field
Underlined is the most often seen appearance of positive structures.

CONCLUSIONS

1. Finding of apoptotic cells are variable and seems to correlate with the expression of defensins in oral epithelium in patients with periodontitis.

2. FGFR was expressed more than the bFGF, but in case with NGFR and bNGF situation was opposite**.**

Although IGFRI was found in sulcular epithelium with no expression in gingival one suggesting about stimulation in regeneration/adaptation in periodontitis affected tissue.

3. The expression of growth factors and their receptors in sulcular epithelium was lower than into the with gingival epithelium and seems to be specific for periodontitis.

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