The involvement of TGF-β1 and CTGF in regional gingival overgrowth

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Abstract
Gingival overgrowth is a multifactorial and invalidating condition. Our research is about gingival overgrowth caused by gingival plaque, its purpose being the evaluation of the presence of gingivitis and/or parodontitis in patients with gingival growth and the extent in which there is a connection between gingival overgrowth and the inflammatory process that can contribute to an exceedingly stimulation of the overgrowth. Immunohistological study was conducted on human material – gingival mucosa that came from patients with ages between 20–65 years, divided into three groups: group I – control group, group II – patients with gingivitis, group III – patients with local or general periodontitis. The intensity of immunohistochemical staining of TGF-β1 and CTGF varies from one group to another, and also depends on the area of gingival mucosa that was observed. TGF-β1 has a crucial role in periodontal disease fibrogenesis by intensifying the action of CTGF.

Keywords: gingival overgrowth, gingivitis, periodontitis.

Introduction
Gingival overgrowth is the term used to describe the swelling of the gum, combining two major histological changes: the increase in the number of cells – hyperplasia and the swelling of tissue – hypertrophy, feature of gingival pathology. There are many risk factors involved in gingival overgrowth: hereditary factors (gingival fibromatosis), factors associated with a metabolic imbalance (diabetes, endocrinopathies) or it can be caused by drugs administration (antiepileptic – phenytoin, calcium pomp inhibitor – nifedipine or immunosuppressive drugs – cyclosporine A) \cite{1}.

Local induced gingival overgrowth, conditioned by acute or chronic inflammation of gingival mucosa – gingivitis or periodontitis is defined as a pathology induced by the lack of oral hygiene that causes bacterial plaque and the colonization of oral tissue by microbial pathogens \cite{2, 3}. Recently, a new classification system was imposed, for parodontitis – the most frequent histoclinical entities being chronic and aggressive periodontitis.

The main distinctions between the two aspects were the clinical occurrences, starting age, evolution, both being present locally or generally \cite{2, 4}. Most authors agree that histological changes are not specific, being, regardless of the etiological aspect, made up of various degrees of fibrosis or extracellular matrix hypertrophy, adding the growth of inflammation tissue. The level of inflammation, that of fibrosis or population of cells from the chorion may vary depending on the presence of bacterial plaque, and, recently proven, in aspect of individual sensibility induced by genetic factors \cite{5, 6}.

In the pathogenesis of periodontal disease, it is widely accepted the activation of an immune response because of parodontopathogens in bacterial plaque, inducing the alteration of conjunctive tissue and epithelial changes. Gingivitis is the initial stage of periodontal inflammatory disease and it can be reversible after treatment.

Periodontitis is characterized by chronic activation of the immune response, with the synthesis of some cytokines and proteases that induce a deep alteration of metabolism of the conjunctive tissue and in the end the destruction of periodontal tissue and the loss of the tooth \cite{7–10}.

Although the presence of bacterial infection is a paradigm of periodontal disease, the development of inflammation from gingivitis to parodontitis has, as recent studies show, multifactorial causes, one of them being gene polymorphism that induces the receptivity and evolution of parodontal disease, but there are also numerous question marks regarding the start and evolution of pathogen paths started by the manifestation of some genes.

The response of the host organism in the presence of some antigens is shown as the gathering of some pro-inflammatory cells (polymorphonuclears, lymphocytes, macrophages), whose response is followed by the release in the gingival tissue of some cytokines and chemokines \cite{6, 11}. If the inflammatory response is
high, it will determine, in 3–4 days from the start, a 70% destruction of the gingival collagen that will be replaced by granulated tissue [12].

Because of autocrine and paracrine stimulation of cells by TGF-β1, the release and synthesis of CTGF (connective tissue growth factor) is activated, whose role in fibrogenesis was recently showed [13–16].

There is a numerous number of studies regarding the relation between proinflammatory cytokines and fibrosis in organ fibrosis (liver, kidney, lung) but they are absent in studied literature regarding gingival overgrowth in periodontal disease.

The subject of this study is the research in the relationship between inflammation and the accumulation of connective tissue in gingival overgrowth from gingivitis, chronic and aggressive periodontitis. The relation inflammation–collagen deposition is seen through the study of immunohistochemical expression of TGF-β1 and evaluation of its impact at the activation of the main profibrillogenetic factor – CTGF.

Materials and Methods

The immunohistochemical study material was composed of fragments of human gingival mucosa, which came from patients of the Oral and Maxillofacial Surgery Clinic of the Emergency County Hospital of Craiova, and from some dental clinics in Craiova, ages ranging from 20 to 65 years.

The patients have been divided into the following categories:

• Group I: control group – eight cases, patients without gingival lesion;
• Group II: eight cases, patients that have been diagnosed with gingivitis, but without destructive lesions of support tissue;
• Group III: 15 cases, patients with local or generalized periodontitis;
  – IIIa: chronic periodontitis, eight patients;
  – IIIb: aggressive periodontitis, seven patients.

The inclusion in study groups was made according to actual rating (Armitage GC, 1999 [2]): the diagnose for generalized chronic periodontitis was made based on clinical attachment loss – periodontal pocket – with a depth >5 mm at over 30% of the measured sites, and the diagnose of aggressive generalized periodontitis was made on patients with ages between 18 and 40 years that had the loss of clinical attachment – parodontal pocket with a depth >6 mm at least three permanent teeth besides the first molar and incisors. All the patients signed an informed consent regarding the participation in this study.

We have excluded patients with gingival overgrowth caused by systemic disease, diabetes, endocrinopathies, or whose initial exam showed a treatment with corticosteroids, immunosuppressive drugs, seizure drugs or calcium blockers; we also excluded patients that had an antibiotic treatment in the last six months.

The tissue we extracted from each patient was of cubic shape, with a side of about 3 mm, containing epithelium and connective tissue.

The immunohistochemical reaction used, as a working technique, ABC/HRP (Avidin complexed with peroxidase biotinylated). In the immunohistochemical study, we used two antibodies, with characteristics and working conditions explained in Table 1.

Table 1 – Antibodies used in immunohistochemical study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Producer</th>
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<tbody>
<tr>
<td>Monoclonal mouse anti-human</td>
<td>1:300</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>TGF-β1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoclonal mouse anti-human</td>
<td>1:200</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>CTGF (6B13)</td>
<td></td>
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In the interpretation and report of immunohistochemical cases, we used the interpretation criteria in literature according to whom the intensity of staining is:

• (+++), when staining is intense positive or distributed specifically diffused, clearly visible in low magnification;
• (++), when staining is focal or relatively moderate, clearly visible only in moderate magnification;
• (+), when staining is soft or very focal, clearly visible only in high magnification;
• (±), when staining is reduced, at the limit;
• (−), when staining is negative.

Results

Immunohistochemical expression of TGF-β1

In normal gingiva, immunohistochemical expression of TGF-β1 was highly reduced, with a few cells with a recognized reaction, present in epithelium in the basal layer, at the edge with lamina propria, or in the lax connective tissue of chorions’s papillae (Figure 1).

In gingivitis, we have seen a few keratinocytes with a relative intense immunoreactivity shown in the deep layers of the epithelium; in lamina propria the positive response was seen in the chorion’s papillae, in some superficial situated fibroblasts, under the epithelial basal membrane, and a few in some endothelial cells, and also in the amorphous cellular matrix belonging to the superficial chorion (Figure 2).

In chronic parodontitis, the immunoreactivity for TGF-β1 was more intense in lamina propria, present both in fibroblasts and endothelial cells but mainly in proinflammatory cells – macrophages, lymphocytes, plasmocytes (Figures 3–5).

In aggressive parodontitis, the reaction of the gingival tissue for TGF-β1 was greatly increased, being positive in epithelium and in lamina propria. In the epithelium level, we have seen positive keratinocytes mainly in the basal layer and in the spinous one, but in some areas the staining was seen also in the space between this spinous layer keratinocytes, in the are occupied by intercellular thorns (Figure 6). In lamina propria, TGF-β1 was seen intracellular, in proinflammatory cells’ cytoplasm (plasmocytes, macrophages) but also in the extracellular matrix, where we have seen a variable positivity at collagenosis fibers level from fibrotic tissue areas (Figures 7 and 8).
Immunohistochemical expression of CTGF

In the gingival mucosa that came from patients in the control group, the reaction for CTGF was negative. In gingivitis, we have seen an extremely low immunoreaction, present in rare cells from epithelia or in lamina propria, in subpapillary chorion (Figure 9). Chronic parodontitis was best seen because of epithelial positive response, the groups of positive cells being distinguished from other groups by negative cellular clusters or those with diffused positive response (Figure 10).
Figure 7 – Aggressive periodontitis. Positivity of immunohistochemical reaction TGF-β1 at lamina propria level, in grain tissue and in areas of fibrous accumulation (ob. ×10).

Figure 8 – Aggressive periodontitis. Inflammatory infiltrate with cells positive for TGF-β1 (ob. ×40).

Figure 9 – Acute periodontitis. We can see rare positive cellular elements for CTGF in epithelium and in lamina propria (ob. ×10).

Figure 10 – Chronic periodontitis. Positive reaction for CTGF in large areas in the epithelium and in the dense, subpapillary chorion (ob. ×20).

Stronger magnification revealed that most reactive cells were found at the edge between epithelium and connective tissue (Figure 11). In aggressive periodontitis, the positive response was revealed in the epithelium, mainly in deep layers, basal and parabasal (Figure 12) and in lamina propria we have seen the presence of diffused immunostains, in the extracellular matrix and a few in some plasmocytary cells from inflammatory infiltrate spread through collagen accumulation (Figure 13).

Figure 11 – Chronic periodontitis. Detailed aspect of the immunoreaction for CTGF in epithelium (ob. ×40).

Figure 12 – Aggressive periodontitis. Positive reaction for CTGF on extended areas of the gingival epithelium (ob. ×10).
Discussion

The etiology of periodontal disease is the presence of bacterial biofilm attached to the surface of the tooth, being characterized from a histological point of view through accumulation of inflammatory tissue and through the changes in the structure of extracellular matrix from lamina propria.

Studies have shown that, as inflammation grows bigger, gingivitis evolving towards periodontitis with its two forms, chronic periodontitis, the most frequent, and aggressive periodontitis, inflammatory tissue initially dominated by lymphocytes and polymorphonuclears is replaced by granulated tissue rich in plasmocytic infiltrate, which represents the main contributor of inflammatory gingival overgrowth [4]. After this modification in the cellular pattern, there is also a massive degradation, around 60–70% of the collagen in gingival chorion [12, 17].

At the same time, the initial collagen type I, found mostly in gingival fibromucosa [18], is replaced still by fibrous conjunctive tissue so after examining the microscopic fields it shows the existence of both areas with inflammatory tissue and areas with fibrous accumulation, interpreted as scaring reactions of lesional repair [19].

Studies show that with scarring evolution and the development of the inflammation, collagen from the matrix becomes more soluble, the amount of type V collagen increases but there is also an initialization of the matrix becomes more soluble, the amount of type V collagen increases but there is also an initialization of the synthesis and production of numerous biomarkers (cytokines, chemokines, and growth factors) by epithelial cells or by mesenchymal ones, but the influence of these biomolecules on the gingival fibromucosa response is somehow modulated, as recent studies show, by individual sensibility [26]. At prone individuals, chronic presence of parodontopathogens alters the protective role of proinflammatory cells, tissular response being determined towards parodontal destruction [27].

Many studies show the abnormal presence or an increase in the level of cytokines pro or anti-inflammatory and pro or anti-fibrotic cytokines in gingival tissue, in the crevicular liquid or in the saliva of patients suffering from gingivitis or periodontitis [5, 16, 28, 29]. These molecules would determine immune response and would influence tissue destruction by affecting the production of matrix components and the activity of metalloproteinases, regularly by stimulating the synthesis of other cytokines or growth factors [30].

As the inflammatory process advances, we can see a specific pattern in cytokines production, different with inflammatory, diabetes or drug induced overgrowth [6]. In inflammatory cells, but also in numerous fibroblasts and in epithelial cells from gingival inflammations, there was an increase in the level of numerous interleukins IL-1α, IL-1β, IL-6, IL-8 and growth factors TGF-β, TNF-α, keratinocyte growth factor, VEGF (vascular endothelial growth factor) [4, 31].

In our work, we followed the dynamic expression of periodontal inflammation – TGF-β1. TGF-β and other 30 related proteins are members of a family of soluble mediators that contain three isoforms TGF-β (β1, β2, and β3), three activin isotypes, and about 20 types of bone morphogenetic proteins present with different subtypes in gingival tissue. Results obtained from immunohistochemical reactions have shown a steady increase in TGF-β1 expression from normal gingiva to aggressive and chronic periodontitis. In the epithelium, cytokine remained very least represented in all the studied cases; the positive reaction is present only in the keratinocytes of the deep layer of several cells, at the lamina propria interface. Two possible explanations could be considered for this positivity, both involving one of the physiological roles of TGF-β1: role in immunity and in multiplication and cell differentiation [32–34]. The first explanation would imply close proximity relationship that exists between deep layer keratinocytes and non-keratinocytary cells, “clear” cells, residing in the deep epithelial layer, most represented by a role in immune cells (lymphocytes and Langerhans’ cells); in which case TGF-β1 positive keratinocytes could issue paracrine stimulation to immune cells, with the role of a first barrier to parodontopathogens. A second explanation would be the stimulation of basal layer cell mitosis, resulting in an increase in the number of keratinocytes and increased epithelial thickness that we found in gingival overgrowth.

TGF-β1 for positivity was discreet in lamina propria of gingivitis reduced to a few cells located in the chorionic surface, instead, at the interface with the epithelium, in chronic periodontitis has spread to most of the fibroblasts and endothelial cells or inflammatory cells in gran tissue. In aggressive periodontitis, positivity had the same pattern but with an increased incidence

Figure 13 – Aggressive periodontitis. In lamina propria, positivity for CTGF is reduced to a few plasmocytes in areas of inflammatory infiltrate (ob. ×40).
and intensity. TGF-β1 has an important profibrogenetic role, not only by inhibiting the synthesis of metalloproteinases, but also by stimulating synthesis of collagen in lamina propria [35]. The cytokine TGF-β1 action derives from its key role as intermediary between parenchymatous, inflammatory and fibroblast cells [36]. TGF-β1 role of gingival overgrowth can be interpreted as the natural evolution of the periodontal lesion, inflammation preceding fibrosis [35], and TGF-β1 activity phase of this evolution: the initial pro-inflammatory cytokine that mediates inflammatory cell recruitment monocyte-macrophages, adhesion and activation at lesion level [37], suggesting a key role in host response to immune challenge initiated by the presence of bacteria. Subsequently, TGF-β1 expression that we noticed after the reactions causes excessive deposit of collagen by exceeding its physiological effect on healing (increasing the number of fibroblasts and increase their capacity for synthesis of collagen). There are studies in literature [38] who reported increased TGF-β1 expression intensity in epithelial cells and fibroblasts of chronic periodontitis as the inflammation progresses, suggesting that the cells could release proinflammatory cytokines that several isoforms of TGF stimulates the synthesis of this. Despite numerous studies, TGF-β1 role in the pathogenesis of periodontal disease in gingival overgrowth is still unclear, due to multifactorial nature of the disease and complex interaction of various cytokines involved.

Porodontopathogens responsible for gingival inflammation determine not only activation of non-specific immune response, but, just as shown in numerous studies and as we have noted on samples, immune cells are activated with the appearance of a considerable number of active plasma cells in immunoglobulin synthesis. Plasmocytary infiltrate was much better organized and aggressive chronic periodontitis than in gingivitis. An individual lesion found without exception in all cases of gingival inflammation was present in excess of dense connective tissue, represented mainly by collagen fibers, as we found in usual examined histological stains and special stains. If inflammation is a much-studied aspect in periodontal disease, accumulation of fibrotic mechanisms are less present in studied literature.

Accumulation of abnormal fibrotic tissue is an activation of repair and healing processes [39], physiological healing processes, inflammation and fibrosis involving the action of the same molecules and cellular events [6]. This is met in edentulous ridge mucosa, connective tissue of lamina propria showing a process of zonal collagen fibrosis associated with a perivascular inflammatory infiltrate in particular [40]. Among the molecules involved in deposition of connective tissue by stimulating the synthesis there are included connective tissue growth factor (CTGF). CTGF protein known as CCN2 is cysteine-rich, consisting of 349 amino acids, one of the six family members of profibrogenetic CCN protein, and can act as a consecutive effector activation by TGF-β in various tissues that develop fibrotic lesions [14]. CTGF has a mitotic role, promoting the division of some cell categories, including fibroblasts [41, 42]. Fibrosis by stimulating CTGF is supported by its various pathologies fibrotic overexpression: skin [43, 44] atherosclerotic [45] or hereditary gingival fibromatosis of gingival overgrowth [15, 46], or induced by phenytoin [47]. Our study showed immunohistochemical expression of CTGF in the gingival mucosa chorion, at fibroblasts level and sporadically in proinflammatory cells, particularly plasma cells in aggressive periodontitis. The most constant and striking positivity was studied in the extracellular matrix, however, especially in areas devoid of inflammatory infiltrate in chronic and aggressive periodontitis. In gingivitis, the connective immunoreactivity was negative. Sensitive similar results similar to ours are reported by other authors [15], following a comparative study of gingival fibromatosis in gingival overgrowth and the administration of phenytoin compared to normal gum. The authors also determine, as we do, intense extracellular CTGF expression, especially given that the accumulation of fibroblasts and the degree of fibrosis were more pronounced. They noted following immunohistochemical reactions performed a similar positivity evidenced by our responses to CTGF in gingival epithelium cells of keratinocytary overgrowth determined by gingival fibromatosis or phenytoin. These results seem to be surprising because growth factor CTGF positive cells appears not to occur but results were also observed by other authors and, for example in blood platelets, which accumulate CTGF after its endocytosis from bone marrow hematopoiesis [48]. In situ hybridization confirmed the increased levels of messenger RNA for CTGF in fibrotic areas in keratinocytes caused by phenytoin gingival overgrowth and gingival fibromatosis and compared to that of gingival overgrowth induced by cyclosporin A, where histological lesions are predominantly inflammatory and less fibrotic [47]. In gingival mucosa, CTGF is rapidly growing because of its stimulation by TGF-β1 and in turn induces the accumulation of insoluble collagen in culture fibroblasts [16]. We believe that the biological role of CTGF in gingival overgrowth may vary in gingival epithelium, which stimulates cell proliferation in lamina propria, which stimulates collagen synthesis in the fibroblasts.

**Conclusions**

The particular histological structure of fibromucosa gum consists of cells of epithelial origin and mesenchymal cells with specific phenotypes and pro-inflammatory cells print a particular evolution of the inflammatory process. In gingival overgrowth, periodontal disease caused by the presence of particular conditions like porodontopathogens induce synthesis of biomolecules and the interaction between cytokines and extracellular matrix between them and extracellular matrix homeostasis that alter the appearance of a clear imbalance of regulation’s turnover of its components. Biological mechanisms that induce gingival overgrowth are correlated with specific interactions between pro-inflammatory cytokines, including TGF-β1 is involved in both inflammation and the remodeling of extracellular matrix. TGF-β has a pivot role in periodontal
disease by potentiating fibrogenesis of CTGF action. During the progression of periodontal lesions, there is a constant interrelation between epithelial and mesenchymal elements; keratinocytes had a dual role, being the source of growth factors and the target of their autocrine action. Tissue damage in periodontal disease is represented not only by tissue destruction but also by accumulation of fibrous tissue.

References


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