

EFFECT OF ANTIBIOTIC TREATMENT ON TGF- β 1 EXPRESSION IN GINGIVAL TISSUES OF PATIENTS WITH CHRONIC PERIODONTAL DISEASE

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Abstract

Chronic periodontitis is initiated by subgingival biofilm depots that induce a release of a cascade of inflammatory molecules in periodontal structures which finally lead to the destruction of the supporting tissues and alveolar bone. One of these cytokines is the transforming growth factor- β 1 (TGF- β 1) associated with inflammatory host response, but also involved in healing and fibrosis. Correlations between periodontal tissues degradation and TGF- β 1 expression have been intensively investigated, but the studies regarding modulation of TGF- β 1 expression under systemic antibiotic therapy are scarce. In this study we assessed the effect of two combinations of antibiotics used to treat chronic periodontitis on the relation between TGF- β 1 expression and the indexes used for clinical evaluation of *periodontium* destruction. Gingival samples collected from patients with untreated chronic periodontitis and those receiving either the combination amoxicillin-metronidazole or spiramycin-metronidazole were processed for paraffin embedding and then stained for routine histological examination and immunohistochemistry in order to assess the morphological changes and to detect the localization of TGF- β 1. TGF- β 1 displayed different patterns of expression between the groups included in the study, being weaker and scarce in samples from patients treated with antibiotics in contrast with untreated subjects. The clinical index was in accordance with immunohistochemical results. As a conclusion, TGF- β 1 may have a contribution not only to the pathogenesis of periodontal disease but also in the healing mechanism by means of antibiotic treatment.

Rezumat

Parodontita cronică este inițiată de depozitele de biofilm subgingivale care induc eliberarea unei cascade de molecule inflamatorii în structurile parodontale și conduc, în final, la distrugerea țesuturilor de susținere și a osului alveolar. Una dintre aceste citokine este TGF- β 1 (*transforming growth factor β 1*) asociat cu răspunsul inflamator al gazdei, dar implicat și în procesele de vindecare și fibroză. Corelațiile între degradarea parodontiului și exprimarea TGF- β 1 au fost intens investigate, dar studiile referitoare la modularea expresiei TGF- β 1 după administrarea sistemică a unor antibiotice sunt rare. În acest studiu am evaluat efectul a două combinații de antibiotice folosite pentru tratamentul parodontitei cronice asupra relației între exprimarea TGF- β 1 și indicii folosiți pentru evaluarea clinică a alterării parodontiului. Probele de țesut gingival prelevate de la pacienți cu parodontită cronică netratată și cei care fac tratament cu una dintre combinațiile amoxicilină-metronidazol sau spiramicină-metronidazol au fost prelucrate pentru includere în parafină și apoi colorate pentru analiza histologică de rutină și imunohistochimie pentru a evalua modificările morfologice și a detecta localizarea TGF- β 1. TGF- β 1 a prezentat o exprimare diferită la grupurile incluse în studiu, pozitivitatea fiind mai slabă ca intensitate în probele prelevate de la pacienții care au urmat tratamentul cu antibiotice, comparativ cu cei netratați. Indicele clinic a fost în concordanță cu rezultatele studiului imunohistochimic. În concluzie, TGF- β 1 poate contribui nu doar la patogenia bolii parodontale, dar și la mecanismul vindecării sub acțiunea tratamentului cu antibiotice.

Keywords: chronic periodontitis, TGF- β 1, antibiotic treatment, metronidazole, amoxicillin, spiramycin

Introduction

Chronic periodontitis is a multifactorial polymicrobial infection that has high prevalence worldwide. The subgingival bacterial biofilm plays

a crucial role in the pathogenesis of chronic periodontitis, inducing a chronic inflammation that extends deep into the periodontal tissues and finally determines the destruction of supporting connective tissue and loss of alveolar bone [1, 2]. Recent

studies emphasize that the microorganisms resident in the dental plaque are only the triggers, but the periodontal disease progression is the result of host-bacterial interactions which induce a release of a cascade of active pro-inflammatory biomolecules [3]. Therefore, the clinical expression of chronic periodontitis depends on how the host responds to the virulence of anaerobic and microaerophilic parodontopathogens able to damage the periodontal tissues [4].

In the presence of bacterial infection, pro-inflammatory cytokines released induce the synthesis and activation of matrix metalloproteinases (MMPs) – the central mediators which promote periodontal disease progression [5]. The overproduction of MMPs is accompanied by an augmented breakdown of collagen, the dominant structural framework of the *periodontium* [6]. One of the cytokines involved in the inflammatory and immune responses in the periodontal tissues is the transforming growth factor- β isoform 1 (TGF- β 1) [7]. TGF- β 1 is secreted by various cells including neutrophils, monocytes and lymphocytes. This cytokine is involved in immune suppression, apoptosis and cell growth inhibition, but also in wound healing, tissue remodelling and regeneration [8]. It modulates the inflammatory response and inhibits the destruction of extracellular matrix proteins by promoting the synthesis of natural tissue inhibitors of MMPs (TIMPs) [9].

Many previous studies have focused on the correlation between increased TGF- β 1 levels in gingival tissues and periodontal disease [10, 11]. So, TGF- β 1 seems to be a multifunctional cytokine that has both pro-inflammatory and anti-inflammatory roles in controlling the inflammatory infiltration of gingival tissues during the initiation and progression of periodontitis. The pro-inflammatory feature of TGF- β 1 is represented by the release of pro-inflammatory cytokines, such as interleukine-1 (IL-1) and tumour necrosis factor- α (TNF- α). Its anti-inflammatory role includes the suppression of humoral immune responses. This cytokine also increases the synthesis of extracellular matrix by stimulating many cells [12]. Moreover, TGF- β 1 plays an important role in the regulation of collagen metabolism in pathologic conditions such as chronic periodontitis [13].

However, the expression of TGF- β 1 following periodontal therapy, especially after systemic antibiotic treatment, has not been studied in detail yet, the data been rare and contradictory. The effects of metronidazole administration on pro-inflammatory cytokines production were the most investigated in different studies [14, 15]. As a conclusion of these studies, metronidazole seems to inhibit the pro-inflammatory activity of TGF- β 1. In a previous study, we reported the effects of two

combinations of antibiotics recommended for the systemic treatment of periodontitis (amoxicillin and metronidazole, respectively spiramycin and metronidazole) on some mediators of the changes of gingival extracellular matrix [16].

In this study we assessed the effects of these combinations of antibiotics on the relation between the expression of TGF- β 1 in gingival tissues and the clinical bleeding on probing (BOP) index for a better understanding of the healing progression after systemic antibiotic therapy.

Materials and Methods

Patients. A total number of 30 subjects, females and males, aged from 31 to 64 years, were included in the study, diagnosed with chronic periodontitis in the Department of Oral Surgery from the Faculty of Dentistry and in a private medical office from Craiova, Romania. Medical and dental histories were recorded. None of the patients had current manifestations of systemic diseases which may have affected gums health. The study was performed with the approval of the Ethics Committee of the University of Medicine and Pharmacy of Craiova and a written informed consent was obtained from each participant in the study. Subjects' selection was made according to the clinical and radiographic criteria proposed by the 1999 International World Workshop for a Classification of Periodontal Diseases and Conditions. All the patients had at the first visit a probing pocket depth of ≥ 6 mm in multiple sites of all four quadrants of the dental arches. They also presented a severe alveolar bone and clinical attachment loss of ≥ 5 mm. Three different groups of patients were enrolled:

- control group (n = 6) - patients treated by scaling and root planning without treatment with antibiotics or other antimicrobial agents;
- Ist group (n = 14) - patients treated by scaling and root planning followed immediately by adjunctive oral administration of an association of amoxicillin (500 mg $\times$ 3/day) and metronidazole (250 mg $\times$ 3/day) over a period of 7 days;
- IInd group (n = 10) - included patients treated by scaling and root planning followed immediately by adjunctive oral administration of an association of spiramycin (500 mg $\times$ 3/day) with metronidazole (250 mg $\times$ 3/day) over a period of 7 days.

All subjects were evaluated clinically and radiographically at the first visit and bleeding on probing index (BOP) was assessed at six sites per tooth for whole mouth.

Histology. Gingival samples collected during extraction and surgery included pocket epithelium and adjacent connective tissue. Gingival tissues were fixed in 4% buffered paraformaldehyde and

then processed for paraffin embedding. Sections of 3 μ m were cut and stained with haematoxylin-eosin (H&E) for usual examination in light microscopy.

Immunohistochemistry. Serial sections of 3 μ m from all groups were dewaxed in xylene and rehydrated *via* graded alcohols. Antigen retrieval was performed after microwave incubation of sections in citrate buffer, pH 6. Endogenous peroxidase activity was blocked with methanol and 0.3% hydrogen peroxide. Sections were treated with normal serum in order to block unspecific binding and then incubated over night with monoclonal mouse anti-human TGF- β 1 (clone Sc-21735, Santa Cruz Biotechnology Inc., SUA), dilution 1:100 in phosphate buffer saline (PBS), pH 7.4-7.6. Detection of TGF- β 1 was performed using avidin-biotin-peroxidase method (Vectastain, Vector Laboratories, USA), 3,3'-diaminobenzidine tetrahydrochloride (Sigma Aldrich Co.) and hydrogen peroxide were used for colour development and Mayer haematoxylin for nuclear counterstaining. A negative control replacing the primary antibody with PBS was performed each time.

Evaluation. Slides were evaluated with a Nikon Eclipse microscope coupled to a digital camera.

The response of the immunohistochemical reaction was verified by two different observers according to the following: - negative reaction – absence of the brown deposits in analysed structures; - moderate or strong positive reaction according to the intensity of brown deposits in all microscopic fields observed for each slide.

The statistical analysis was performed using non-parametrical techniques. Kruskal-Wallis was used for intergroup comparisons before or after therapy. Wilcoxon test was used for comparison of all study groups before and after treatment with a significance level $p < 0.001$. All data analysis was performed using a statistical package (SPSS 10.0, Abacus Concepts).

Results and Discussion

We compared the baseline values of BOP index with the results obtained after one week of treatment with the combinations of antibiotics. As the results had not a Gaussian distribution, in order to compare the data we used the Wilcoxon test (Table I). Comparing the values at baseline and after treatment, we obtained highly significant differences ($p < 0.001$).

Table I

Bleeding on probing (BOP) index values before and after treatment

	BOP index before treatment (mean $\pm$ SD)	BOP index after treatment (mean $\pm$ SD)	P value (Wilcoxon test)
Control	56.329 $\pm$ 15.060	24.076 $\pm$ 6.439	0.006
Ist Group (A+M)	61.469 $\pm$ 15.223	19.589 $\pm$ 5.826	0.00001
IInd Group (S+M)	59.287 $\pm$ 13.629	17.741 $\pm$ 6.990	0.00001

Histological aspects were different in case of periodontal therapy. As one can observe in Figure 1, an important inflammatory response in all gingival tissues before treatment was noted on H&E staining samples. Chronic pro-inflammatory

infiltrate, represented mainly by lymphocytes and plasma cells, was localized especially nearby the basal lamina, in the deep epithelial layers and in the superficial lamina propria, the so-called chorionic papillae (Figure 1 a, b).

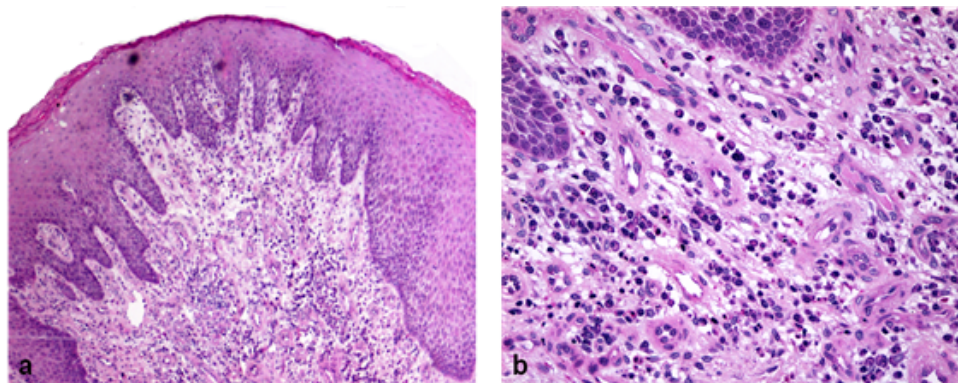


Figure 1.

Gingival tissue from a patient with untreated chronic periodontitis.

- Inflammatory infiltrate in deep epithelial layers and in superficial loose connective tissue (H&E, x100);
- In the granulation tissue, inflammatory cells are mainly lymphocytes and plasma cells (Detail, H&E, x 400).

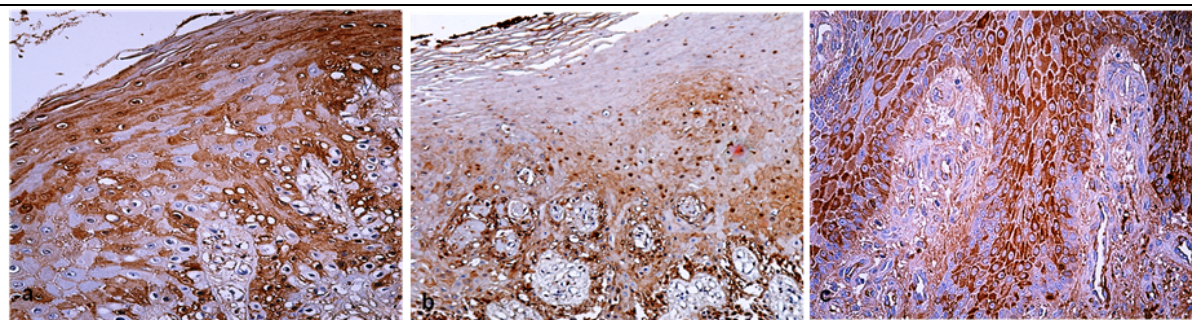


Figure 2.

Immunohistochemical reaction for TGF- β 1 in cases of untreated chronic periodontitis.

- a. TGF- β 1 positive reaction in epithelial layers, especially in keratinocytes from the deep layer and inflammatory cells infiltrated between keratinocytes (IHC, x200);
- b. "Puzzle shape" positive reaction for TGF- β 1 in the epithelium, more intense in cells surrounding the chorionic papillae (IHC, x400);
- c. Positive reaction in epithelial layers of ridges and in the superficial connective tissue (IHC, x 200).

The TGF- β 1 positive reaction displayed different patterns between the groups included in this study. In the samples obtained from patients with untreated chronic periodontitis, the immunohistochemical reaction for TGF- β 1 was positive in the epithelial layers, sometimes mainly in keratinocytes from the deep layer, in the so called rete-pegs, while in the superficial layer the reaction was negative (Figure 2a). Other cases of chronic periodontitis display a patchy positive reaction in all epithelial layers, distributed like a "puzzle", but constantly increased in the cells from the top of the epithelial ridges (Figure 2b).

We also found an intense positive TGF- β 1 reaction in the loose connective tissue underlying the epithelial ridges, especially in the new formed vessels from the papillary chorion and in the pro-inflammatory cells from *lamina propria* (Figure 2c).

Immunohistochemical reactions for TGF- β 1 in the samples obtained from the patients treated with antibiotic combinations were generally more limited and discreet as intensity. In samples treated with the combination amoxicillin-metronidazole, the reaction for TGF- β 1 was positive in the epithelial layers displaying the same "puzzle" pattern, but also in the desmosomes between keratinocytes (Figure 3a). In the chorion, positive reaction for TGF- β 1 was noted in macrophages, fibroblasts and very rarely in the endothelial cells (Figure 3b.). In gingival samples obtained from patients treated with spiramycin and metronidazole, the TGF- β 1 expression was very similar to that observed for the Ist group, a positive reaction being observed especially in keratinocytes from the basal and spinous epithelial layers. In the chorion, the TGF- β 1 positive reaction was limited to some pro-inflammatory and endothelial cells (Figure 4).

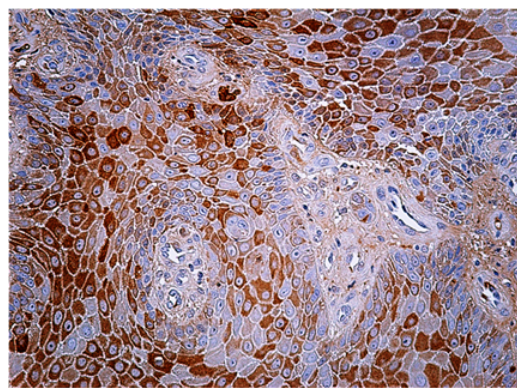


Figure 4.

Chronic periodontitis treated with spiramycin-metronidazole association - transversal section through the papillae. Less intense positive reaction for TGF- β 1 in keratinocytes from the basal layer and in few pro-inflammatory cells (IHC, x 400)

Contrary to healthy *periodontium*, chronic periodontitis is characterized by high levels of inflammatory mediators in periodontal tissues or in the gingival crevicular fluid as a result to the presence of periodontal pathogens [17]. Pro-inflammatory cytokines are the most important mediators involved in host defence, but an increased or prolonged release of these can lead to pathological periodontal conditions [18]. TGF- β 1 is one of these cytokines that has both pro-inflammatory and anti-inflammatory effects on periodontal tissues during the initiation and progression of chronic periodontitis. It might switch from pro- to anti-inflammatory role and so it acts against excessive immune and inflammatory host response and limits periodontal tissues degradation [19].

It is generally accepted that TGF- β 1 has a crucial role in regulation of collagen metabolism both in physiologic and pathologic conditions [20].

TGF- β 1 modulates the inflammatory response by reducing inflammation. It also inhibits cell proliferation and differentiation, destruction of extracellular matrix proteins by suppressing MMPs and inducing their inhibitors [21]. This cytokine can be used to assess the pathogenesis and healing process of chronic periodontitis because previous studies reported an increase of TGF- β 1 expression both in gingival crevicular fluid and gingival tissues of patients with different clinical forms of periodontal disease [22, 23]. Our results are in agreement with those reported by the authors cited since we noted a decrease of TGF- β 1 expression in cases of periodontitis treated with antibiotics.

As we showed, the therapy of chronic periodontitis with systemic antibacterial agents, especially the association of two antibiotics, leads to a different expression of this pro-inflammatory cytokine. We observed a reduced TGF- β 1 expression in case of the treatment with the association amoxicillin-metronidazole or spiramycin-metronidazole. The therapeutic effect of these two combinations of antibiotics must be considered a positive result due to a reduced inflammatory effect. For this reason, the values of clinical parameters were reduced in the groups treated with antibiotics compared to the control group ($p < 0.001$).

Gurkan et al. has shown an increased TGF- β 1 level in gingival crevicular fluid of patients with gingivitis, chronic periodontitis and generalized aggressive periodontitis [24]. In this study, TGF- β 1 levels showed the same trend of increasing as the severity of periodontitis progress. An increased TGF- β 1 expression in gingival tissues or in gingival crevicular fluid was also observed following various periodontal treatment procedures, especially after administration of adjunctive subantimicrobial dose of doxycycline therapy [25].

In the present study we observed a TGF- β 1 positive reaction with a different pattern between the groups included. In samples of the control group, the positive reaction was increased in keratinocytes from the deep epithelial layers but also in fibroblasts, inflammatory cells and some endothelial cells from the *lamina propria*. In gingival tissues of the two groups treated with both associations of antibiotics, TGF- β 1 expression was decreased mainly in the connective tissue. This decrease can be explained by the reduced number of pro-inflammatory cells after antimicrobial treatment. At the same time we noted a decrease of expression in fibroblasts and endothelial cells, which can determine the inhibition of metalloproteinases which promote tissue turnover.

The variation of clinical index was in accordance with the immunohistochemical results. BOP index was significantly lower in both groups treated with

antibiotic associations than before treatment ($p < 0.001$).

For the group treated with the association spiramycin-metronidazole, BOP index was lower compared to the Ist group, but the difference between these two groups was not significant ($p > 0.05$).

In some instances, an adequate oral hygiene associated with conventional mechanical therapy such as scaling and root planning stop the progression of periodontal tissue lesions. But, in many cases, systemic antibiotics and chemotherapeutics need to be prescribed as adjuvant to non-surgical or surgical therapy for patients with chronic periodontitis.

Many studies have demonstrated the effectiveness of systemic antibiotic administration on periodontal pathogen flora [26, 27]. However, the immunomodulatory effects of antibiotic therapy have not been investigated extensively. Nevertheless, antibacterial agents could have adjunctive potential therapeutic effects by interaction with host response to bacterial infection [28].

The therapeutic modalities that modulate the host response by systemic antibiotic administration have been researched, especially using isolated immunocytes *ex vivo* [29], but the investigations regarding the possible immunomodulatory effects of antimicrobial agents are scarce.

Until now many researchers studied the effects of metronidazole widely used in periodontal therapy by systemic or local administration, alone or in combination with other antibiotics, especially amoxicillin and ciprofloxacin, but rarely spiramycin [30, 31]. Metronidazole was prescribed to the patients with chronic periodontitis, like an adjunctive local or systemic antibiotic administration to non-surgical mechanical treatment or surgical therapy [32].

In 2010, Rizzo et al. have investigated the effects of metronidazole administration on the production of pro-inflammatory cytokines [33]. They have demonstrated that metronidazole inhibited significantly *Porphyromonas gingivalis* development and also the release of some pro-inflammatory cytokines (IL-1 β , IL-6 and TNF- α). These cytokines are expressed in higher concentrations in inflamed periodontal tissues and caused alveolar bone resorption. As a result of this study, metronidazole seems to inhibit the pro-inflammatory activity of TGF- β 1 on the release of these cytokines. The conclusion of this research was that metronidazole could significantly reduce the inflammatory response and contributes to limit the destructive effects on the periodontal tissues. The therapy with metronidazole had the ability to diminish the alveolar bone resorption and also to

stop host destructive progress in chronic periodontitis.

Our results are in accordance with those obtained by Rizzo et al. sustaining the ability of metronidazole in combination with amoxicillin or spiramycin to control the inhibition of pro-inflammatory cytokines release. This finding suggests a possible therapeutic application of these two associations of antibiotics.

Conclusions

The research showed that TGF- β 1 might be involved both in the pathogenesis of periodontal disease and the healing of *periodontium* after treatment. In association with other mediators, an increase of TGF- β 1 expression in gingival tissues has an important role in progression of chronic periodontitis. Elsewhere, after adjunctive periodontal therapy with systemic antibiotic associations, TGF- β 1 might reduce the inflammation and remodelling of periodontal tissues.

For the future, we consider that is necessary to investigate the molecular mechanisms by which these antibiotic associations exert immunomodulatory effects in periodontal disease.

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