

Assessment of transforming growth factor beta one (TGF- β 1) immunohistochemical (IHC) expression profile in the gingival tissue of patients with different forms of periodontal diseases

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ABSTRACT

Background: This study evaluate the immunohistochemical expression profile of transforming growth factor beta-1 in inflamed gingival tissue of patients with gingivitis and chronic periodontitis compared to healthy subjects and, determine the correlation between this cytokine and the clinical periodontal parameters, intensity of inflammation and chronic periodontitis severity.

Materials and methods: Gingival tissue specimens were taken from 23 chronic periodontitis patients, 20 gingivitis patients and 20 periodontally healthy subjects. The periodontal status was evaluated by dichotomous measurements of the clinical periodontal parameters (PLI, GI, BOP, PPD, CAL). The gingival specimens were fixed immediately in 10% formalin and processed routinely into paraffin blocks for further immunohistochemical analysis.

Results: A highly significant statistical difference was observed between the study groups regarding the PLI, GI, the percentage of bleeding on probing sites and the intensity of inflammation. TGF- β 1 expression profile statistical analysis showed a high statistical significant difference among the study groups, A highly significant statistical difference was found between the PPD Scores while a significant statistical difference was revealed among the CAL Scores when the expression profile of TGF- β 1 was compared. TGF- β 1 was positively correlated with the clinical periodontal parameters (PLI, GI, and BOP) as well as with the intensity of inflammation in the three study groups. In chronic periodontitis group a highly significant positive linear correlation between the PPD and CAL with the TGF- β 1 expression profile.

Conclusions: TGF- β 1 expression profile showed variations as the destructive character of the periodontal disease changed. Therefore, it could be proposed that TGF- β 1 might contribute both to inflammatory regulation and remodeling events during periodontal disease.

Key words: Transforming growth factor beta-1, periodontal diseases, gingival tissues. (J Bagh Coll Dentistry 2013; 25(Special Issue 1):96-101).

الخلاصة: الهدف من هذه الدراسة هو تقييم نمط التعبير لعامل النمو المحول بيتا في الانسجة اللثوية الملتهبة للمرضى الذين يعانون من التهاب اللثة، مرض النساغ المزمن ومقارنتهم بالاصحاء عن طريق التحليل المناعي واختبار العلاقة بين هذا السيتوكين مع معلمات اللثة السريرية. شدة التهاب اللثة وشدة مرض النساغ المزمن. تم اخذ 23 عينة من مرضى النساغ المزمن و 20 عينة من مرضى التهاب اللثة و 20 عينة من الأشخاص ذوي الانسجة اللثوية السليمة وجرى تقييم حالة اللثة عن طريق قياس المعلمات اللثوية (معامل الصفيحة الجرثومية، معامل التهاب اللثة، النزف عند التسمير، عمق الجيب اللثوي ومستوى الانسجة الرابطة). تم تثبيت العينات اللثوية على الفور في 10% فورمالين تحضيراً لمعالجتها بشكل روتيني وطمرها بشمع البارافين لأغراض التحليل المناعي. في نتائج البحث لوحظ وجود فروقات احصائية عالية بين المجموعات الداخلة في الدراسة فيما يخص معامل الصفيحة الجرثومية، معامل التهاب اللثة، النزف عند التسمير وشدة الالتهاب. اظهرت النتائج الاحصائية لنمط تعبير عامل النمو المحول بيتا وجود فروقات عالية ضمن المجموعات الداخلة في الدراسة. تم ايجاد فروقات احصائية عالية بين المستويات المختلفة لعمق الجيب اللثوي والانسجة الرابطة عندما تمت مقارنة نمط تعبير عامل النمو المحول بيتا. ارتبط نمط تعبير عامل النمو المحول ايجابيا مع جميع المعلمات اللثوية السريرية ومع شدة الالتهاب وتم ايجاد علاقة خطية ايجابية بين كل من عمق الجيب اللثوي ومستوى الانسجة الرابطة مع تعبير عامل النمو المحول بيتا. استنتجت هذه الدراسة تقترح ان التغييرات في عامل النمو المحول بيتا يمكن ان يساهم في كل من تنظيم الالتهابات وعملية اعادة بناء الانسجة اللثوية.

INTRODUCTION

Transforming Growth Factor Beta-1 is one of the key cytokines with pleiotrophic properties that has both pro-inflammatory and anti-inflammatory features in regulation of the inflammatory infiltrate. This cytokine is a multifunctional cytokine that is involved in angiogenesis, immune suppression, extra cellular matrix synthesis, apoptosis and cell growth inhibition and it is pro-inflammatory, since it is chemoattractant for neutrophils, monocytes, mast cells and lymphocytes and also causes the release of pro-inflammatory cytokines, such as interleukine-1 (IL-1), interleukine-6 (IL-6) and tumor necrosis factor- α (TNF- α), by these cells, anti-inflammato-

ry properties of this cytokine include suppression of cell-mediated as well as humoral immune response ^(1, 2).

The above features of TGF- β 1 make it an interesting protein to monitor in the pathogenesis of periodontal disease. Limited knowledge on the role of TGF- β 1 in various periodontal disease types and particularly in advanced periodontitis forms warranted the conduction of this study in order to investigate TGF- β 1 expression profile in tissue sections of subjects with healthy periodontium as a control group and patients with gingivitis and chronic periodontitis as study groups and test whether TGF- β 1 expression profile is correlated with the clinical periodontal parameters, intensity of microscopic inflammation and the severity of the periodontal diseases.

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MATERIALS AND METHODS

Patients and Biopsies

A total of 63 subjects (38 male, 25 female) attending the outpatient clinic at the Department of Periodontics in the Teaching Hospital of Dentistry College- Baghdad / Iraq, Al khadhimya specialized dental centre as well as patients from private practices were recruited for the study. Exclusion criteria included the presence of less than 20 natural teeth, pregnancy, non smoker, any systemic condition that could affect the host's periodontal status or that would require antibiotics for monitoring or treatment procedures (e.g. heart conditions and joint replacements); use of antibiotics and/or anti-inflammatory drugs within the last 3 months; and professional cleaning or periodontal treatment within the last 6 months. The periodontal status was evaluated by dichotomous measurements of the following clinical periodontal parameters (PLI, GI, BOP, PPD, CAL), the first measurements were at the initial periodontal examination and the second measurements were recorded before tissue sampling at the time of periodontal surgery.

Measurements were performed at four sites per tooth for whole mouth excluding the 3rd molar. Chronic periodontitis group was firstly subdivided into two scores according to the PPD which are:

Score (1): includes the examined sites with PPD range of 5-6 mm.

Score (2): includes the examined sites with PPD range of ≥ 7 mm.

Then secondly into 3 scores according to measured CAL for better estimation of the disease severity:

Score (1): includes the sites with CAL range of 3-4 mm.

Score (2): includes the sites with CAL range of 5-6 mm.

Score (3): includes the sites with CAL ≥ 7 mm.

All the patients underwent the 1st phase of periodontal therapy. Based on the recorded gingival index (GI), probing pocket depth (PPD), clinical attachment level (CAL) and radiographic evidence of bone loss; subjects were categorized into three groups:

- Group I (healthy) consisted of 20 subjects /age range (25-40) years with clinically healthy periodontium, PPD ≤ 3 mm and CAL = 0.
- Group II (gingivitis) consisted of 20 subjects/ age range (25-40) years who showed clinical signs of gingival inflammation, gingival enlargement and false pocket formation, PPD ≤ 3 mm and had no attachment loss (CAL = 0) or radiographic bone loss.

- Group III (chronic periodontitis) consisted of 23 subjects/ age range (30-50) years with at least four sites with a PPD ≥ 5 mm and CAL > 3 mm with radiographic evidence of bone loss.

Tissue sampling

Each patient underwent periodontal surgery, independently of this study, as a part of their routine periodontal treatment. The chronic periodontitis patients were arranged for full-thickness mucoperiosteal flap/debridement, the gingivitis patients with gingival enlargement were arranged for gingivectomy procedure while the healthy subjects underwent crown lengthening procedure or tooth extraction for orthodontic indication or extraction of impacted 3rd molar according to the designated treatment plan. Informed consent was obtained from the patients to collect, preserve and analyze the gingival tissues for this study. Biopsies were obtained from the suitable sites at the time of surgeries.

Tissue preparation and staining

All samples and positive controls were presumably fixed in 10% formalin, and processed routinely into paraffin blocks. From each paraffin embedded tissue block (samples and controls); serial sections were cut as follows: Sections of 4 μ m thickness were mounted on normal glass slides, stained with hematoxylin and eosin (Hand E), and histopathologically re-evaluated, the intensity of inflammation assessed according to Farhad et al⁽³⁾ and then photographed. Two other 4 μ m thick sections for each case were cut and mounted on positively charged slides (Fisher scientific and Eschsuperfrost plus, USA) for immunohistochemical staining with monoclonal antibodies. Negative and positive tissue controls were included into each immunohistochemical run (according to the manufacturer). For Transforming Growth Factor beta (TGF- β) monoclonal antibody, two tissue blocks of Human breast cancer were used.

Immunohistochemical staining

Five micrometer thick sections were cut and mounted on (Biocare, USA) positively charged slides, then de-paraffinized and rehydrated. For immunohistochemical staining by TGF- β (US biological) monoclonal antibodies; then the sections were immersed in 0.3% hydrogen peroxide (H₂O₂) to block the endogenous peroxidase activity, washed in phosphate-buffered saline (PBS), and then incubated in 10% normal serum to block any non-specific binding of antibodies. The tissue sections were incubated with monoclonal mouse anti-human TGF- β (diluted 1:30) antibodies over night at 37 °C. The bounded antibodies were detected by the

streptavidin-biotin complex method, after an immunoreaction, the sections were counterstained with Hematoxylin.

Quantitative assessments

Evaluation of inflammation on H and E stained specimens

According to the method proposed by Farhad et al⁽³⁾, quantitative assessment of the inflammatory cells was performed in 5 separate fields of each H and E stained specimen at 400x magnification of light microscope. The mean count of inflammatory cells for the 5 fields was determined, and severity of tissue inflammatory response was classified as follows figure (1):

- Grade 0: absence of inflammatory cells or presence of fewer than 5 cells.
- Grade 1 (mild reaction): presence of 5 to 25 cells.
- Grade 2 (moderate reaction): presence of 25 to 125 cells.
- Grade 3(severe reaction): presence of more than 125 cells.

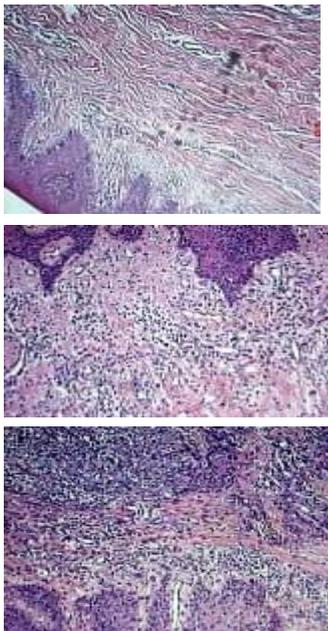


Figure 1: Top; photomicrograph showing healthy gingiva. Middle; photomicrograph showing chronic gingivitis (moderate), Bottom; photomicrograph showing Chronic Periodontitis (severe), 100 X (H and E).

Evaluation of immunohistochemistry results

Immunohistochemical signal specificity was demonstrated by the absence of immunostaining in the negative control slides and its presence in recommended positive controls. Membranous / or membranous and cytoplasmic staining pattern was considered positive for TGF- β 1 immunostaining, according to the manufacturer's

data sheets and were studied by light microscope under 10X objective. In each tissue section five representative fields were selected for TGF- β 1 Maps immune-staining evaluation. Positively stained cells were counted in fields (0.03 mm^2) determined by an ocular grid at 400x magnification. Cells edging the upper and left grid lines were included, whereas cells edging the bottom and right lines were excluded from the counts. Cells were counted within 5 randomly chosen areas in the CT. Cell density was calculated as cells/ mm^2 ⁽⁴⁾. In the context of this research (TGF- β expression) term would be used as a synonym for the (TGF- β 1 expressing inflammatory cell density), figures (2 and 3).

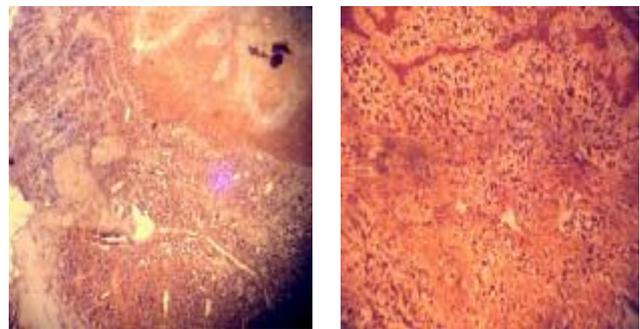


Figure 2 and 3: Chronic gingivitis with positive brown -staining of TGF- β 1 in the inflammatory cells TGF- β 1 ECD = 160 cells/ mm^2 (200 X) (left photo). Right photo represents chronic periodontitis with positive brown staining of TGF-B1 in the inflammatory cells TGF-B1 ECD=130cells/ mm^2

Statistical analysis

Data are calculated and entered into a computerized data base structure. Statistical analysis was done using SPSS software. Mean and SD, t-test, Chi square, ANOVA test, Mann-Whitney test, Kruskal-Wallis test and Pearson correlation coefficient (r) were used. Level of significance was 0.05.

RESULTS

A highly significant statistical difference was observed between the study groups regarding the PLI, GI, the percentage of bleeding on probing sites, table (1) and the intensity of inflammation, table (2). TGF- β 1 expression profile's statistical analysis showed a high statistical significant difference among the study groups, table (3).

A highly significant statistical difference was found between the PPD Scores with a significant statistical difference among the CAL Scores when

the expression profile of TGF- β 1 was compared Table (4). TGF- β 1 was positively correlated with the clinical periodontal parameters (PLI, GI, and BOP) as well as with the intensity of inflammation in the three study groups, table (5). In chronic periodontitis group a highly significant positive linear correlation was revealed between the PPD and CAL with TGF- β 1 expression profile, table (6), figures (4 and 5).

DISCUSSION

In the present study, TGF- β 1 ECD was evaluated in patients with different forms of periodontal disease and in healthy subjects. The results demonstrated significantly elevated TGF- β 1 ECD in patients with chronic periodontitis and gingivitis compared to healthy subjects ($p < 0.001$). Elevated levels of TGF- β 1 ECD in advanced periodontitis and gingivitis could suggest that this cytokine is one of the components that contribute to the extent of inflammatory response. TGF- β 1 exerts both anti-inflammatory and pro-inflammatory effects on host cells during the onset and progression of periodontal disease. It is a critical mediator in resolution of inflammation and indicates ongoing wound healing and chronic inflammation during host response^(5,2). Any or all of these TGF- β 1 dependent mechanisms could contribute both to the initiation and regulation of inflammation and connective tissue destruction in periodontal diseases. The gingivitis group showed a higher significant TGF- β 1 ECD as compared to the control group this result is in line with other studies⁽⁶⁻⁸⁾.

The presence of TGF- β 1 in the gingival overgrowth is attributed to (besides to its role as pro-inflammatory cytokine) its important pro-fibrogenetic role, not only by inhibiting the synthesis of metalloproteinases, but also by stimulating synthesis of collagen in lamina propria⁽⁹⁾. TGF- β 1 role in gingival overgrowth can be interpreted as the natural evolution of the periodontal lesion, inflammation preceding fibrosis, and TGF- β 1 activity phase of this evolution: the initial pro-inflammatory cytokine⁽¹⁰⁾, suggesting a key role in host Response to immune challenge initiated by the presence of bacteria. Subsequently, TGF- β 1 over expression that we noticed could be a response to paracrine stimulation by other cytokines secreted by the pro-inflammatory cells causing excessive deposit of collagen by exceeding its physiological effect on healing (increasing the number of fibroblasts and increase their capacity for synthesis of collagen).

The elevated TGF- β 1 ECD in periodontitis groups compared to gingivitis might indicate the

counterbalancing role of TGF- β 1 in periodontal tissue breakdown by acting against exaggerated immune and inflammatory host response. TGF- β 1 might switch from pro- to anti-inflammatory role in order to regulate immune-inflammatory responses and limit tissue degradation with the progression of periodontal disease to a more destructive state as in periodontitis⁽⁴⁾.

Results obtained from immunohistochemical reactions of the present study have shown a steady increase in TGF- β 1 expression from normal gingiva to chronic periodontitis this is in agreement with other studies^(4,11,12). A highly significant strong positive correlation was revealed between the PLI and TGF- β 1 expression, this is in accordance with others^(11,13).

Plaque with its components of bacteria and their products, in addition to directly recruiting and activating leukocytes at sites of infection, indirectly influence the inflammatory events through the induction of cytokines such as TGF- β 1⁽¹⁴⁾. Transforming Growth Factor Beta 1 expression correlated to the GI in highly significant strong positive correlation and this is in agreement with Gürkan et al.⁽¹¹⁾. In keeping with its dichotomous nature, recent evidence suggests that overproduction and/or activation of TGF- β 1 contribute to persistent inflammation and that antagonists of TGF-beta delivered locally can break the cycle of leukocyte recruitment and fibrotic sequelae. On the other hand, systemic routing of TGF-beta can also inhibit inflammatory pathogenesis by multiple mechanisms as exemplified by systemic injections⁽¹⁵⁾. The results showed the presence of positive significant correlation between the TGF- β 1 ECD and BOP. The findings of the study are in consistence with the results of Gurkan⁽¹¹⁾.

BOP represents the earliest sign of the gingival inflammation⁽¹⁶⁾ hence such an objective index is expected to be sensitive to the molecular changes that take place during the progression of the periodontal disease and correlates with quantitative and qualitative expression of the pro/anti-inflammatory cytokines. A significant positive correlation was established between the intensity of inflammation and TGF- β 1 expression profile, this is consistent with⁽⁶⁾. Our results confirm that increased gingival inflammation accompanying the progression of periodontal disease is associated with high levels of TGF- β 1. A direct relation exists between the magnitudes of the inflammation process with the level of this cytokine⁽¹⁷⁾.

TGF- β 1 has the ability to induct and potentiate the action of other inflammatory cytokine resulting in multiplying the magnitude of the

inflammatory condition, TGF- β in the presence of IL-6 drives the differentiation of T helper 17 (Th17) cells, which can promote further inflammation⁽¹⁸⁾, TGF- β orchestrates the differentiation of both Treg and Th17 cells in a concentration-dependent manner⁽¹⁹⁾, In addition, TGF- β in combination with IL-4, promotes the differentiation of IL-9- and IL-10-producing T cells, which lack suppressive function and also promote further tissue inflammation^(20,21).

Regarding the severity of chronic periodontitis, Statistical analysis showed a highly significant positive linear correlation between PPD and CAL with the expression profile of TGF- β 1 this in agreement with the findings of other studies^(6,7,11,22). Also a highly significant difference was found between PPD scores while CAL scores showed a significant difference this is in agreement with Skaleric et al⁽²²⁾. These results represent that in human periodontal disease TGF- β 1 shows a concomitant elevation trend as the disease severity progresses. In other words, TGF β 1 expression profiles were higher in patient groups as there is little doubt that excessive and/or continuous production of this cytokine in inflamed periodontal tissues is responsible for the progress of periodontitis and periodontal tissue destruction⁽²³⁾.

However, it remains unknown whether advanced periodontal destruction at sites of severe chronic periodontitis is due to insufficiently elevated anti-inflammatory cytokine levels. Advances in periodontal treatment strategies showed that by antagonizing the activity of TGF-beta with neutralizing antibodies, a causal relationship between this cytokine, inflammation, and pathogenesis has been demonstrated. Administration of anti-TGF-beta to sites of chronic destructive inflammation not only blocked leukocyte recruitment and activation, but also inhibited the subsequent destruction of bone and cartilage characteristics of such lesions^(14,24,25).

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Table 1: The mean values of PLI, GI and the percentages of sites with BOP among the study groups

	C	G	CP	P	SIG	
PLI	0.514±0.271	1.699±0.330	1.467±0.570	<0.001	HS	
GI	0.435±0.338	1.536±0.370	1.343±0.451	<0.001	HS	
BOP	SCORE 0	95.40%	75.40%	84.30%	<0.001	HS
	SCORE1	4.50%	24.50%	15.60%		

Table 2: Distribution of study groups according to different inflammation grades

	C	G	CP	P	Sig.	
Intensity of inflammation	Mild	45%	0	0	<0.001	HS
	Moderate	55%	40%	17%		
	Severe	0	60%	83%		

Table 3: The mean ±SD of TGF-β1 expressing cell density (TGF-β1 ECD) in the study groups

	Control	Gingivitis	Chronic Periodontitis	*P-Value	Sig	
TGF-β1 Expressing Inflammatory Cells Density(Cell\mm²)	Mean	44	147.50	156.87	<0.001	HS
	±SD	26.852	29.470	24.242		

Table 4: TGF-β1 ECD means of each PPD and CAL score

	Mean Cell Density	P- Value	Sig.	
PPD	Score1	127.69	0.003	HS
	Score2	162.33		
CAL	Score1	137.5	0.020	S
	Score2	150.9		
	Score3	191		

Table 5: The correlation between PLI, GI and BOP of the study groups with TGF-β1 ECD

TGF-β1 ECD	Control		Gingivitis		Chronic periodontitis	
	r	p-value	r	p-value	r	p-value
PLI	0.695	0.01	0.819	<0.001	0.801	<0.001
GI	0.601	0.005	0.819	<0.001	0.619	<0.001
BOP	0.69	<0.001	0.71	<0.001	0.65	<0.001
Intensity of Inflammation	0.876	<0.001	0.785	<0.001	0.478	0.021

Table 6: The correlation between PPD and CAL means with the TGF-β1 expression

		Chronic periodontitis	
		PPD	CAL
TGF-β1 ECD	r	0.586	0.558
	P-value	0.003	0.006

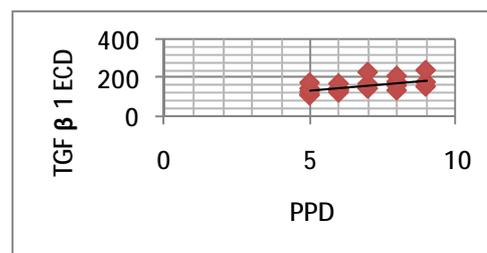
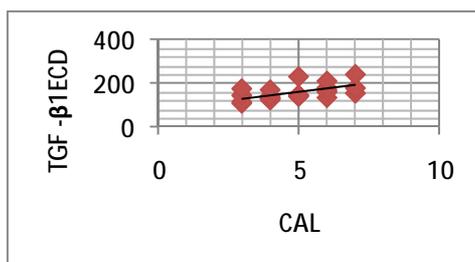


Figure 4 and 5: Scatter plots showing the linear correlation between PPD and CAL with TGF-β1 expression.