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Original Article

In vivo association of immunophenotyped macrophages expressing CD163 with PDGF-B in gingival overgrowth-induced by three different categories of medications

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ABSTRACT

Aims: This study was carried out to identify and outline the degree of relationship between immunophenotyped macrophages expressing CD163 and PDGF-B in cyclosporine-A, phenytoin, and nifedipine-induced gingival overgrowth.

Methods: Eighty adult male albino rats were selected and divided into four equal groups. Group I received no treatment. Rats of groups II, III, and IV were administered cyclosporine-A, phenytoin, and nifedipine, respectively. Routine tissue processing was carried out for staining with CD163 and PDGF-B. The results of this study were analyzed statistically.

Results: Group I exhibited score 0 gingival overgrowth while group II yielded score 3 with blunt and bulbous gingival crests. Rats of group III showed score 2 with knife edge and group IV revealed less pronounced gingival overgrowth and mostly the gingival crest was knife edge. Group II had the highest mean value for CD163 while group I showed the lowest value. In addition, group II had the highest mean value for PDGF-B while group I showed the lowest value. Statistically, there was an overall significant difference between the studied groups as well as between each two groups.

Conclusion: Strong association exists between immunophenotyped macrophages expressing CD163 and PDGF-B in GO induced by these medications. In addition, CD163 and PDGF-B upregulated in cyclosporine-A-induced GO compared to phenytoin and nifedipine medications.

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1. Introduction

Drug-induced gingival overgrowth (DIGO) is a side-effect and unwanted outcome with three systemic medications.¹ These medications include the immune suppressor cyclosporine-A (CsA), the anti-seizure drug phenytoin (PNT), and calcium channel blockers (CCBs).^{2,3} The incidence of this side effect can be as high as 70% in transplant patients, 65% in epileptics, and 30% in hypertensive patients.^{4,5}

In 1997, Iacopino et al.⁶ reported that the clinical presentation of inflamed and hyperplastic gingival tissues are associated with specific macrophage phenotypes which express the essential platelet derived growth factor-beta (PDGF-B) in PNT-induced hyperplastic tissues. In contrary, Wright et al. concluded that macrophage-derived PDGF B plays a negligible pivotal role in the pathogenesis of DIGO.⁷

It is well known that macrophages respond to environmental cues by adopting either “classically” activated (M1) proinflammatory or “alternatively” activated (M2a, M2b, M2c, and M2d) wound healing phenotypes.^{8,9} M2a macrophages secrete the highest levels of PDGF-B, a chemoattractant stabilizing pericytes, and also promote anastomosis of sprouting endothelial cells in vitro.^{10,11} Although PDGF is synthesized, stored, and released by platelets upon activation, it is also produced by a plethora of other cells, including smooth muscle cells, activated macrophages such as alveolar macrophages, and vascular endothelial cells.¹²

Macrophages of M2c type express high levels of cluster of differentiation 163 (CD163), which has been shown to be associated with tissue repair and remodeling of the wound and promoting cell proliferation in mice.¹³ In 2004, Lau et al.¹⁴ reported that CD163 immunoreactivity was observed in resident macrophages of all normal tissue samples including lymph node and tonsil, red pulp macrophages of spleen, bone marrow, alveolar macrophages of the lung, and Kupffer cells of the liver. CD163 is not expressed in splenic white pulp macrophages and germinal center tingible body macrophages.

To date, several studies have tried to determine the pathogenesis of DIGO but the molecular mechanisms that trigger such condition are not fully elucidated. In this context, this study was carried out to assess the possible relationship between immunophenotyped macrophages expressing CD163 and PDGF-B in gingival overgrowth (GO)-induced by three different categories of medications. The research null hypothesis was that no associations between immunophenotyped macrophages expressing CD163 and PDGF-B in GO-induced by these medications.

2. Materials and methods

2.1. Animal subjects

Eighty adult male albino rats, weighing 150–200 g were selected. These animals were housed and cared according to the guidelines of the Medical Research Center, Faculty of Medicine, Mansoura University. They were kept in a light controlled room with (12:12-h) light dark cycle and temperature ($\pm 22^{\circ}\text{C}$).

Relative humidity (65–70%) was kept constant. They received commercial soft diet and water. The rats were classified randomly into four equal groups.

Group I: Rats of this group were considered as negative control and received no treatment.

Group II: The rats of this group were administered CsA in mineral oil via gastric feeding once daily for 7 weeks. The dissolved drug was given by gastric syringe, each rat received 30 mg/kg daily.¹⁵

Group III: The rats of this group were administered PNT after trituration with Tween 80 solution. They received PNT via subcutaneous injection twice daily for 6 weeks. Rats were subcutaneously injected with 100 mg/kg per day.¹⁶

Group IV: The rats of this group were administered nifedipine dissolved in dimethyl sulfoxide via gastric feeding once daily for 6 weeks. The dissolved drug was given by gastric syringe, and each rat received 30 mg/kg daily.¹⁷

2.2. Medicine supplementation

CsA: Soft gelatinous capsule of CsA (100 mg, Sandimmune, Novartis Pharmaceuticals Corporation, Hanover, Germany) was dissolved in 16.5 cm³ mineral oil.

PNT: Soft gelatinous capsule of PNT sodium (100 mg, El-Nasr Pharmaceuticals Chemical Co. “ADWIC” A.R.E.) was diluted in 0.5% Tween 80 (0.5 ml Tween 80 dissolved in 99.5 ml distilled water) (El-Nasr Pharmaceuticals Chemical Co. “ADWIC” A.R.E.).

Nifedipine: Sustained release tablet of nifedipine (Adalat LA 30 mg, Bayer Schering Pharma AG, Germany) was dissolved in 16.5 cm³ dimethyl sulfoxide.

At the end of the experiment, all the rats were euthanized with an overdose of halothane. The lower jaw was dislocated by pulling it down, and biopsies were collected by the following two methods. First, a spoon excavator was used to reflect the marginal gingiva, then it was continually resected from the right side of the mandible opposite molar region using a scalpel. Second, mandibular specimens from the left side of the mandible, including molar teeth with surrounding bone and gingival tissue, were cut in a buccolingual direction using a scalpel.

2.3. Histological examination

The obtained specimens were prepared at 4 μm and stained with:

- 1 Hematoxylin and eosin stain (H&E).
- 2 Expression of CD163 and PDGF-B was identified immunohistochemically. The product information about the antibodies used in the present study is illustrated in [Table 1](#).

2.4. Scoring and evaluation of GO using H&E stained slides

Microscopic analysis was performed on the buccal gingiva of decalcified specimens by two-blinded calibrated examiner using a light microscope (Olympus, New York, USA). Each examiner had approximately 15 years of experience in

Table 1 – Immunohistochemical markers used in the present study.

Antigen	Expression	Clone	Dilution	Supplier
CD163	Cytoplasmic	Mouse anti rat monoclonal antibody	1:200	AbD Serotec, Kidlington, UK
PDGF-B	Cytoplasmic	Rabbit anti rat polyclonal antibody	1:200	Bioss Inc., Massachusetts, USA

Table 2 – Statistical results for intraexaminer and interexaminer variability attributed to scored samples.

Features	Intraexaminer variability			Interexaminer variability		
	First reading, mean $\pm$ SD	Second reading, mean $\pm$ SD	P value	First reading, mean $\pm$ SD	Second reading, mean $\pm$ SD	P value
Degree of gingival overgrowth	2.6 $\pm$ 0.6	2.4 $\pm$ 0.7	0.600	0.3 $\pm$ 0.6	0.3 $\pm$ 0.4	0.726
Shape of gingival crest	0.3 $\pm$ 0.2	0.3 $\pm$ 0.1	0.153	0.5 $\pm$ 0.2	0.5 $\pm$ 0.1	0.661

investigating the biopsy specimens under the light microscope in their practical laboratories. One expert examiner read specimens and two days later, the same examiner read all the specimens again to evaluate the intraexaminer variability. Then, the same specimens were read by the second examiner to assess the interexaminer variability (Table 2). A modified scoring method was done according to the index described by Banthia et al. in 2014.¹⁸ The crown length was divided into three equal parts (cervical, middle and occlusal). The hyperplastic index measured the degree of GO in an apico-coronal direction by means of 4-point scale:

- (0) No GO (gingiva still in the cervical one-third),
- (1) Mild (gingiva overgrown to the middle one-third),
- (2) Moderate (gingiva overgrown to the lower half of the occlusal one-third) and
- (3) Severe (gingiva overgrown to the upper half or above the occlusal one-third).

2.5. Evaluation of immunostain

Slides were photographed using Olympus® digital camera installed on Olympus® microscope with 1/2 X photo adaptor, using X objective. The resulted images were analyzed on Intel® Core I3® based computer using VideoTest Morphology® software (Saint-Petersburg, Russia) with a specific built-in automated object counting and stain quantification routines. Immunoreactivity for CD163 and PDGF-B was assessed and scored as follows:

- (0) =100% of cells were negative,
- (1) Mild = (>0% to <10%) of cells were positive,
- (2) Moderate = (>10% to <50%) of cells were positive and
- (3) Intense = (>50%) of the cells were positive.

2.6. Statistical analysis

The data were tabulated, coded, and then analyzed using statistical package for social science (SPSS version 17.0). For the analytical statistics, significant differences were tested using multivariate analyses of variance (ANOVA) to compare between more than two groups for the numerical parametric

data, followed by post-hoc least significant difference (LSD) for multiple comparisons. Moreover, the Kruskal-Wallis test was used to compare between more than two groups for categorical data, followed by the Mann-Whitney U test to compare two groups. Partial correlation analysis was done between CD163 positive macrophages and PDGF-B in both epithelial and mesenchymal tissues. The statistical tests were based on a type 1 error value of 5% ($\alpha = 0.05$) and on a power of 0.85 sample size. P value <0.05 represents level of significance.

3. Results

3.1. 1-Heamatoxylin and eosin stain findings

3.1.1. Degree of GO

Group II, III, and IV rats showed GO with variable extents over the crown of teeth. While group I showed score 0 GO with knife edge gingival crest (100%), group II yielded score 3 GO with blunt gingival crest (60%) and score 3 with bulbous gingival crest (40%). Group III showed score 2 GO with knife edge (90%) and score 2 with blunt gingival crest (10%). Group IV revealed less pronounced GO with knife edge (100% score 1).

The Kruskal-Wallis statistical test revealed an overall significance between the different groups within examination period. The highest mean rank was for group II (66.23), while the lowest one was with group I (19.43). The mean ranks for groups III and IV were 45.60 and 30.75, respectively. The Mann-Whitney U revealed significant differences between groups I & II, I & III, I & IV, II & III, II & IV, and between III & IV (Tables 3 and 4).

3.1.2. Histological findings

Group I: The epithelium of this group showed a keratinized stratified squamous epithelium covering a core of connective tissue. The epithelium revealed normal arrangement of its layers. The basement membrane was flat with no extended rete processes. The underlying connective tissue showed normal arrangement of its fibers, blood vessels with few randomly distributed chronic inflammatory cells (Fig. 1A).

Group II: The epithelium of this group showed increased epithelial thickness with hyperorthokeratosis. The length and broadness of the rete processes were increased within the underlying connective tissue. The basal cells showed

Table 3 – Kruskal-Wallis statistical test for the degree of GO for the different groups and its statistical significance.

	Ranks			Kruskal Wallis Test	
	Group	Mean rank	Chi-Square	df	Sig
GO	I	19.43	53.62	3	0.000
	II	66.23			
	III	45.60			
	IV	30.75			

Table 4 – Mann-Whitney U statistical test for multiple comparisons for the degree of GO for the different groups and its statistical significance.

Groups	I*II	I*III	I*IV	II*III	II*IV	III*IV
Mann-Whitney U	0.000	51.000	127.50	63.000	22.500	110.00
Wilcoxon W	210.00	261.00	337.50	273.00	232.50	320.00
Z	-5.73	-4.60	-2.81	-4.17	-5.10	-2.81
Asymp. Sig. (2-tailed)	0.000	0.000	0.005	0.000	0.000	0.005
Exact Sig. [2*(1-tailed Sig.)]	0.000	0.000	0.049	0.000	0.000	0.014

disturbance and crowding. The suprabasal layer showed mitotic activity. The spinous cell layer showed acanthosis and hyperchromatism. The connective tissue showed increased in the amount of collagen fiber and inflammatory cells (Fig. 1B).

Group III: The epithelium of this group revealed slight hyperplasia as compared to that of group I and it appeared lesser than that of group II. Rete processes were slightly elongated as compared to group I. Also, they were narrower than those of group II. The hyperkeratosis was detected, but it was lesser than that of group II. The epithelial layers appeared more or less as group II but with limited suprabasal mitotic activity and mild acanthosis. The inflammatory cells were distributed throughout the connective tissue. The fibrous tissue is highly collagenized and hypovascularized (Fig. 1C).

Group IV: The epithelium in this group revealed acanthosis and slightly elongated rete processes. The cells of basal layer were crowded. The keratin layer thickness was less than that of groups II and III. The underlying connective tissue revealed coarse and dense collagen bundles. Fibroblasts appear spindle in shape (Fig. 1D).

3.2. Immunohistochemical findings

3.2.1. CD163

CD163 immunoreactivity was characterized by a granular cytoplasmic or cytoplasmic and membrane staining patterns. Few cells were positively stained in the subepithelial connective tissue for CD163 in group I (Fig. 2A). Group II had shown an

Fig. 1 – Group I showing keratinized stratified squamous epithelium covering connective tissue core (A). Group II shows elongated rete process (B). Group III reveals mild acanthosis and the fibrous tissue is highly collagenized and hypovascularized (C). Group IV shows mild acanthosis with slightly elongated rete process (D). Epithelium (Epi), basement membrane (Bm), connective tissue (Ct), spinous cells (Sp), basal cells (B), collagen (C), fibroblast (F), rete processes (Rp), abnormal mitosis (M), blood vessels (Bv), inflammatory cells (Ic), and keratin (Ke) (H&E: 200×).

Fig. 2 – Group I showing few cells positively stained in the subepithelial connective tissue (A). Group II shows CD163 intense reaction throughout the whole thickness of the subepithelial connective tissue with few scattered cells in the basal layer of the epithelium (B). Group III shows moderate reaction to CD163 throughout connective tissue with few scattered cells in the epithelium (C). Group IV shows mild reaction to CD163 in the connective tissue with no reaction in the epithelium (D) (ABC: 400×).

intense reaction to CD163 throughout the whole thickness of the subepithelial connective tissue with a few scattered cells in the basal layer of the epithelium (Fig. 2B). Group III exhibited moderate reaction to CD163 throughout connective tissue with few scattered cells in the epithelium (Fig. 2C). Group IV exhibited mild to moderate reaction for CD163 in

the connective tissue with no reaction in the epithelium (Fig. 2D).

3.2.2. PDGF

Group I showed very mild reaction to PDGF-B within macrophages, endothelial cell and fibroblast of the connective tissue

Fig. 3 – Group I shows very mild reaction to PDGF-B within the connective tissue (A). Group II shows intense expression to PDGF-B within epithelium and connective tissue and most of the reaction was at basal cell layer (B). Group III shows moderate reaction to PDGF-B while epithelium showed mild expression (C). Group IV shows mild reaction within the connective tissue to PDGF-B (D) (ABC: 400×).

Table 5 – Two way-MANOVA and LSD post-hoc test for CD163 and PDGF-B in the epithelial and mesenchymal tissues for all groups.

Pillai's Trace (F ratio and P value) ^a				LSD post hoc (Mean $\pm$ SD) ^b			
Groups	Tissues	Groups*Tissues		Groups	CD163	PDGF-B	
(1508.024, 0.001)	(1057.497, 0.001)	(209.552, 0.001)			Epithelial	Epithelial	Mesenchymal
Two way-MANOVA (F ratio and P value)*	Groups	CD163	(1037.68, 0.001)	I	0.01 $\pm$ 0.00 [†]	0.02 $\pm$ 0.00	0.08 $\pm$ 0.01 [≠]
		PDGF-B	(3564.36, 0.001)	II	0.06 $\pm$ 0.00 [†]	0.23 $\pm$ 0.01	0.75 $\pm$ 0.03 [≠]
	Tissues	CD163	(1993.61, 0.001)	III	0.05 $\pm$ 0.01 [†]	0.18 $\pm$ 0.01	0.54 $\pm$ 0.02 [≠]
		PDGF-B	(185.87, 0.001)	IV	0.04 $\pm$ 0.00 [†]	0.15 $\pm$ 0.02	0.43 $\pm$ 0.01 [≠]
	Groups*	CD163	(618.06, 0.001)				
	Tissues	PDGF-B	(111.84, 0.001)				

^a Two way-MANOVA.
^b LSD.
Superscripted symbols in the same column mean significant difference at $P < 0.05$.

while epithelium was negative (Fig. 3A). Group II exhibited intense expression to PDGF-B within epithelium and connective tissue and most of the reaction was at basal cell layer (Fig. 3B). Group III revealed moderate reaction to PDGF-B while epithelium showed mild expression (Fig. 3C). Group IV exhibited connective tissue mild reaction to PDGF-B (Fig. 3D).

3.2.3. Statistical results

The mean values and standard deviation (SD) for CD163 and PDGF-B are illustrated in Table 5. Regarding CD163, the highest mean values were in group II in both epithelial (0.06 ± 0.00) and mesenchymal (0.32 ± 0.03) tissues, while the lowest mean values were in group I in both epithelial (0.01 ± 0.00) and mesenchymal (0.05 ± 0.01) tissues. The mean values for groups III and IV for the epithelial tissue were 0.05 ± 0.01 and 0.04 ± 0.00 , respectively. The mean values for groups III and IV for the mesenchymal tissue were 0.11 ± 0.00 and 0.08 ± 0.01 , respectively. Regarding PDGF-B, the highest mean values were in group II in both epithelial (0.23 ± 0.01) and mesenchymal (0.75 ± 0.03) tissues while the lowest mean values were in group I in both epithelial (0.02 ± 0.00) and mesenchymal (0.08 ± 0.01) tissues. The mean values for groups III and IV for the epithelial tissue were 0.18 ± 0.01 and 0.15 ± 0.02 , respectively. The mean values for groups III and IV for the mesenchymal tissue were 0.54 ± 0.02 and 0.43 ± 0.01 , respectively.

Two way-MANOVA test for CD163 and PDGF-B revealed an overall significant difference between the different groups ($P = 0.001$), epithelial or mesenchymal expression ($P = 0.001$) and there was a significant interaction between different groups and the epithelial or mesenchymal expression ($P = 0.001$) (Table 5). LSD post-hoc test for multiple comparisons of CD163 and PDGF-B revealed significant differences between groups I & II, I & III, I & IV, II & III, II & IV, and III & IV (Table 5).

Two-tailed Pearson correlation revealed significant differences between CD163 and PDGF-B for epithelial expression in group I ($P = 0.032$), group II ($P = 0.020$), group III ($P = 0.024$), and group IV ($P = 0.001$). In addition, significant differences were found between CD163 and PDGF-B for mesenchymal expression in group I ($P = 0.011$), group II ($P = 0.018$), group III ($P = 0.033$), and group IV ($P = 0.012$). Very weak positive correlation was found between CD163 and PDGF-B for epithelial expression in group I (0.067) and strong positive

correlations were found in group II (0.850), group III (0.795), and group IV (0.786). Very weak positive correlation was found between CD163 and PDGF-B for mesenchymal expression in group I (0.079) and strong positive correlations were found in group II (0.912), group III (0.867), and group IV (0.811) (Table 6).

4. Discussion

In this study, group I showed normal epithelium and connective tissue of the gingival mucosa. This finding is in accordance with the normal histology of gingiva of albino rats as described by Garant in 2003.¹⁹ Group II showed acanthotic epithelium with hyperorthokeratosis and elongated rete processes within the underlying collagenous and vascular connective tissue. This is in agreement with Rahman et al.,²⁰ Gonçalves et al.,²¹ Seibel et al.,²² and Piloni et al.²³ They indicated that thickening of the epithelium and elongated rete processes appeared to be a characteristic feature of CsA-induced GO. Spolidorio et al.²⁴ and Nurmenniemi²⁵ attributed increased epithelial thickness in CsA-induced GO to the influence of connective tissue on epithelial architecture causing an increase in the mitotic activity in the oral epithelium. In addition, Sume et al.²⁶ attributed collagenous fibrous stroma found in group II of present study to the possible association between GO and epithelial mesenchymal transition which is a process of trans-differentiation of epithelial cells into fibroblast like cells.

Microscopic analysis of group III biopsies of the present study revealed a redundant fibrous tissue stroma with excessive amount of collagen and increased number of fibroblasts. Frequently, the surface epithelium has elongated rete processes. These findings are in agreement with Gurgel et al.,²⁷ Kanno et al.,²⁸ McKeivitt and Irwni,²⁹ and Fujii et al.³⁰ as they reported that CsA, PNT, and nifedipine led to phased and drug-related gene expression patterns, resulting in impaired collagen metabolism. Leask et al.³¹ and Chujo et al.³² found an interaction between certain fibroblasts subpopulation and collagen with PNT and its metabolite. In this study, group IV epithelium revealed acanthosis and slightly elongated rete processes. This is accordance with Shimizu et al.³³ who suggested that nifedipine induces epithelial overgrowth not by increase in keratinocyte proliferation but by prolongation of cell life through reduction of apoptosis. Regarding amount of

Table 6 – Partial correlations for CD163 and PDGF-B in the epithelial and mesenchymal tissues for all groups.

	Epithelial								Mesenchymal							
	I		II		III		IV		I		II		III		IV	
	CD163	PDGF-B	CD163	PDGF-B	CD163	PDGF-B	CD163	PDGF-B	CD163	PDGF-B	CD163	PDGF-B	CD163	PDGF-B	CD163	PDGF-B
CD163																
Pearson correlation	1	0.067	1	0.850	1	0.795	1	0.786	1	0.079	1	0.912	1	0.867	1	0.811
Sig. (2-tailed)		0.032		0.020		0.024		0.001		0.011		0.018		0.033		0.012
PDGF-B																
Pearson correlation	0.067	1	0.850	1	0.795	1	0.786	1	0.079	1	0.912	1	0.867	1	0.811	1
Sig. (2-tailed)	0.032		0.020		0.024		0.001		0.011		0.018		0.033		0.012	

collagen fiber in group IV, it was less than that of groups II and III; this is in agreement with Kataoka et al.³⁴ as they noted reduction in collagen fiber phagocytosis by fibroblast after 30 days of CsA use before macroscopic detection of GO.

CD163 antibody aids in identifying cells of monocyte/macrophage lineage in normal and neoplastic conditions. This antibody reacts with human scavenger receptor cysteine-rich protein CD163 (p155, M130) found on mononuclear phagocytes including human monocytes and macrophages.³⁵ The present study showed elevated expression of CD163 in group II compared to groups III and IV. This is in accordance with Dill et al.³⁶ and Iacopino et al.⁶ as they concluded that PNT, CsA, and nifedipine GO tissues contain subpopulations of macrophages and other inflammatory cells. These cells differ from those in healthy control gingival tissues. These altered macrophage phenotype results in an increased production of cytokines and growth factors.

PDGF-BB is most effective on PDL cell mitogenesis and matrix biosynthesis.³⁷ The present study showed elevated expression PDGF-B in groups II compared to groups III and IV. This observation is convenient with Plemons et al.³⁸ who found that healthy gingiva is in a continuous state of wound repair and support the hypothesis that CSA-induced GO is associated with enhanced macrophage PDGF-B gene expression rather than an increase in the number of PDGF-B producing macrophages. In addition, Dill et al.³⁶ analyzed RNA isolated from GO associated with CsA and PNT and reported that expression of PDGF gene was 47-fold greater than control. This causes persistent proliferation of connective tissue, matrix formation, and fibrosis. PDGF also increases lysyl oxidase activity which is utilized in the final enzymatic step of collagen cross linking and thereby causes ECM accumulation.³⁹

5. Conclusion

- (1) CsA-induced GO likely more extensive than PNT and nifedipine-induced GO.
- (2) CD163 expressed by M2c immunophenotyped macrophages upregulated in CsA-induced GO compared to PNT and nifedipine.
- (3) PDGF-B expressed by M2a macrophages, endothelial cells, and platelets upregulated in CsA-induced GO compared to PNT and nifedipine.
- (4) Strong association exists between CD163 and PDGF-B in GO induced by these medications.
- (5) CD163 and PDGF-B play an essential biological role in GO.

Conflicts of interest

The authors have none to declare.

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