Clinical Study

Expression Profile of IL-35 mRNA in Gingiva of Chronic Periodontitis and Aggressive Periodontitis Patients: A Semiquantitative RT-PCR Study

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Background. Proinflammatory and anti-inflammatory cytokines play a key role in the pathogenesis of periodontal diseases. Secretion of bioactive IL-35 has been described by T regulatory cells (T_{regs}) and is required for their maximal suppressive activity. T_{regs} are involved in the modulation of local immune response in chronic periodontitis patients. *Objective*. Hence, the present study was aimed to investigate the expression of IL-35 mRNA in chronic periodontitis and aggressive periodontitis patients. *Materials and Methods*. The present study was carried out in 60 subjects, which included 20 chronic periodontitis patients, 20 aggressive periodontitis patients, and 20 periodontally healthy controls. IL-35 mRNA expression in gingival tissue samples of all subjects was semiquantitatively analyzed using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). *Results*. The present study demonstrated the expression of IL-35 mRNA in gingival tissues of all the three groups. IL-35 mRNA expression was highest in chronic periodontitis subjects (6.87 ± 2.32) as compared to the aggressive periodontitis group (4.71 ± 1.43) and least seen in healthy patients (3.03 ± 1.91). *Conclusion*. The increased expression of IL-35 in chronic and aggressive periodontitis suggests its possible role in pathogenesis of periodontitis. Future studies done on large samples with intervention will strengthen our result.

1. Introduction

Periodontal diseases are considered to be multifactorial diseases, where putative periodontopathogens trigger inflammatory and immune responses. It is characterized by irreversible loss of alveolar bone and connective tissue attachment in the periodontium which ultimately results in the loss of teeth. These pathogens are known to produce proteolytic enzymes as well as elicit signals in resident gingival cells or in immune cells infiltrating the gingival tissues that result in immune responses; these responses lead to either the successful removal of the pathogens or to host mediated destruction of the periodontal tissues [1].

Gingival inflammation is regulated by reciprocal interactions between various cell types including, leukocytes, epithelial cells, fibroblasts, and endothelial cells. Among the many immune and inflammatory mediators known cytokines have attracted special attention. Proinflammatory and anti-inflammatory cytokines play a key role in the pathogenesis of periodontal disease thereby influencing destruction, remodeling, and repair of periodontal tissues [2].

Interleukin-12 (IL-12) is one such cytokine which appears to contribute to inflammatory response in numerous physiological and pathological processes. The IL-12 family, which is evolutionarily linked to the IL-6 cytokine superfamily, is composed of IL-12, IL-23, IL-27, and IL-35 [3]. The IL-12 related cytokines are heterodimeric proteins comprised of an α chain (p19, p28, or p35) and a β chain (p40 or Ebi3). IL-35, the newest member of IL-12 family, is composed of the IL-27 β chain Ebi3 and the IL-12 α chain p35.

Secretion of bioactive IL-35 has been described in forkhead box protein 3 (Foxp3)[+] regulatory T cells (T_{regs}),

peripheral $\gamma\delta$ T cells, CD8$^+$ T cells, and placental trophoblasts [3]. IL-35 has been shown to be an inhibitory cytokine that may be specifically produced by T$_{reg}$ cells and is required for maximal suppressive activity [4]. Loss of IL-35 expression results in reduced *in vivo* suppressive capacity of T$_{regs}$. Also IL-35-expanded CD4+CD25+ T-cell population expressed Foxp3 and produced elevated levels of IL-10 [5].

A previous studies had shown that patients with chronic periodontitis presented increased frequency of T lymphocytes and CD4$^+$CD25$^+$ T cells in the inflammatory infiltrate of gingival tissues and exhibited the phenotypic markers of T$_{regs}$ such as Foxp3. These results indicate that T$_{regs}$ are found in the chronic lesions and must be involved in the modulation of local immune response in chronic periodontitis patients [6].

The search for potential markers associated with the severity, as well as the susceptibility of periodontal disease, has recently been receiving considerable attention [7]. Although roles of many other cytokines have been evaluated in the pathogenesis of periodontal diseases, the exact role of IL-35 has not been studied yet. So the current analytical cross-sectional descriptive study was carried out to evaluate and compare the expression of IL-35 mRNA in gingival tissues of healthy controls, chronic periodontitis, and aggressive periodontitis patients by RT-PCR. We also intended to correlate the expression levels of IL-35 with the severity of disease process among the 3 groups so as to obtain an insight into the probable role of IL-35 in immunopathogenesis of periodontal diseases.

2. Materials and Methods

2.1. Study Population. The present study included 60 subjects in the age range of 21–59 years (32.4 ± 10.7) who visited the outpatient Department of Periodontics, PMNM Dental College and Hospital at Bagalkot and was divided as follows: group 1 : 20 chronic periodontitis patients, group 2 : 20 aggressive periodontitis patients, and group 3 : 20 periodontally healthy controls. The exclusion criteria used were smoker, patients with systemic diseases like diabetes mellitus, hepatitis, and HIV infection which are known to influence the periodontal disease; patients who received periodontal treatment for at least 6 months prior to sampling and recording were excluded from the study. Patients with diseases of oral hard and soft tissue except caries and periodontitis, use of antibiotics and analgesics within three months prior to study, and pregnant women and lactating mothers were also excluded. After explaining the nature of the study and the method of sample collection, the patients signed an informed consent form. Verbal consent was obtained from all the participants before obtaining the gingival tissue samples. The study protocol was approved by the Ethical Committee of PMNM Dental College & Hospital, Bagalkot, Karnataka, India.

2.2. Oral Examination. Probing Pocket Depth (PPD) and Clinical Attachment Loss (CAL) were recorded with a graduated Williams's periodontal probe at six sites on each tooth for all the groups. Gingival inflammation was scored using Gingival Index (GI). Debris and calculus was scored using the simplified oral hygiene index (OHI-S) [1].

Study population was divided as healthy subjects showing absence of clinical manifestations of periodontal disease with at least 20 teeth present. Chronic periodontitis patients comprised of at least 20 natural teeth and a minimum of six teeth with periodontal pockets ≥5 mm and clinical attachment loss ≥3-4 mm and aggressive periodontitis patients with proximal attachment loss of ≥5 mm affecting at least three teeth other than first molars and incisors and bone loss as assessed by radiographs [8].

2.3. Gingival Tissue Specimen Collection. Sixty gingival tissue samples were biopsied from 20 chronic periodontitis, 20 aggressive periodontitis, and 20 healthy sites. Patients received local anaesthesia and biopsies were obtained by surgical excision from labial/buccal surface of the gingival margin/papilla, of multirooted teeth. Two parallel vertical incisions, 2 to 3 mm apart from each other were made with a scalpel equipped with a number 15 Bard Parker blade followed by an incision perpendicular to the two vertical ones'. The surgical blade point was directed to the portion of the alveolar bone crest in order to obtain a well-oriented and representative biopsy. The gingival tissue sample was stored and transported in RNA SAVE SOLUTION (Life technologies India Pvt Ltd.) for RNA stabilization [8].

2.4. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). RNA was isolated from these tissues using the total RNA isolation kit (Chromous Biotech Pvt. Ltd.) followed by reverse transcription (Chromous Biotech Pvt. Ltd.) according to the manufacturer's instructions. The kit contained MMuLV Reverse Transcriptase to synthesize first strand cDNA and high fidelity polymerase for PCR (ChromTaq polymerase). All components of the kit were stored at −20°C.

The following primers were used for RT-PCR (Bioserve Pvt Ltd. USA): IL-12 p35 CTGCATCAGCTCATCGAT-GG (forward) and CAGAAGCTAACCATCTCCTGGTTT (reverse); EBi3 TGT TTC CCT GAC TTT CCA GG (forward) and GGG GCA GCT TCT TTT CTT CT (reverse). The PCR cycle consisted of 94°C for 15 s, 55°C for 30 s, and 68°C for 60 s. Samples were amplified for 23 to 33 cycles. The reaction was terminated by heating at 72°C for 5 min. The PCR product (5 μL) were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. The bands of the PCR product were visualized on a UV transilluminator. The results were expressed on a scale of 1 to 10 using TOTAL LAB Software. (Nonlinear Dynamics Ltd., UK).

2.5. Statistical Analysis. Data were expressed as means and standard deviations. The statistical difference between the groups was tested using Kruskal Wallis ANOVA test, Mann-Whitney U test, and Tukeys multiple post hoc test. Simple pairwise correlations were calculated according to the Karl Pearson's correlation coefficient. (SPSS 15.0 IBM Corporation Ltd., USA).
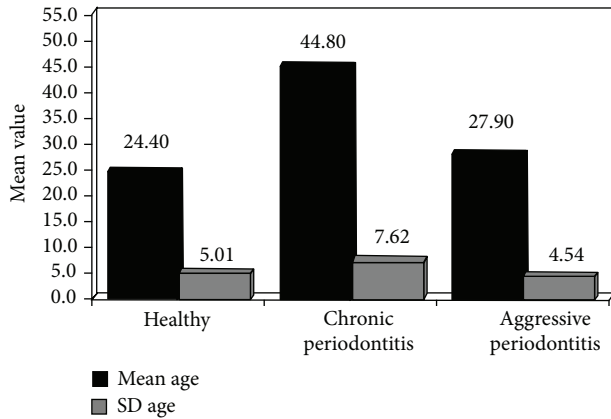
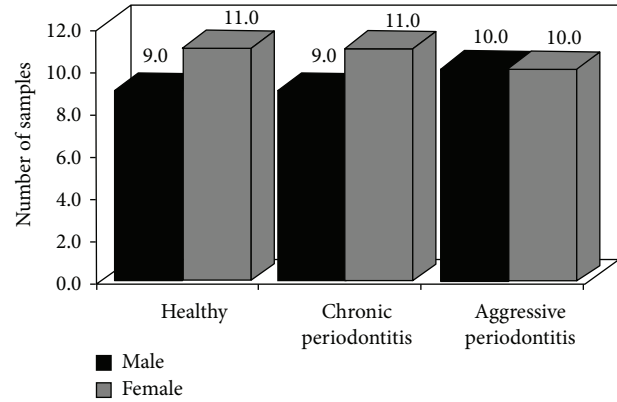FIGURE 1: Mean and SD of age according to study groups.

FIGURE 2: Distribution of study samples according to sex.

3. Results

The various clinical parameters as well as IL-35m RNA levels were analyzed in gingival tissue samples for all the 3 groups. Mean age of subjects were 24.40 ± 5.01 years in healthy group, 44.80 ± 7.62 years in chronic periodontitis group, and 27.90 ± 4.54 years in aggressive periodontitis group. All the three groups were gender matched (Figures 1 and 2).

Comparison for mean clinical attachment level and mean probing depth for healthy controls, chronic periodontitis, and aggressive periodontitis groups was statistically significant (Table 1). This was also true when pairwise comparison among healthy versus CP, healthy versus AgP, and CP versus AgP, was carried out. Correlation between IL-35 mRNA expression with Gingival index, OHI-S, PD, and CAL by Karl Pearson's correlation coefficient method in three groups (Table 3).

IL-35 mRNA expression for healthy controls, chronic periodontitis, aggressive periodontitis were 3.03 ± 1.91, 6.87 ± 2.32, and 4.71 ± 1.43, respectively. The difference in the IL-35 mRNA expression in healthy, CP, and AgP was found to be statistically significant. Statistically significant difference was found after pairwise comparison amongst the groups relating to IL-35 mRNA expression (Table 2).

4. Discussion

Periodontitis is a chronic inflammatory condition of the periodontium which develops in response to local infectious challenge. Periodontopathogenic antigen-driven host cell responses serve as initiators of chronic inflammatory disease; secondary endogenous degenerative pathways may act as auxiliary magnification loops to propagate inflammation and local tissue destruction. Since these pathways may be exemplified by the release of inflammatory cytokines, researchers have of late attempted to link various clinical and pathological entities to the specific immune responses elicited [9].

Recently IL-35, a T_{reg} cell specific cytokine that is required for the maximum regulatory activity of T_{reg} cells *in vitro* and *in vivo* has drawn considerable attention. IL-35 has been shown to be expressed by resting and activated T_{regs} but not

by T effector (T_{eff}) cells [10]. Subsequently, previous studies have described that T_{reg} cell subset is involved in the attenuation of the inflammatory reaction and bone loss after periodontal infection [11, 12]. The current case control study was carried out to evaluate, compare, and correlate the expression of IL-35 mRNA in gingival tissues of healthy controls, chronic periodontitis, and aggressive periodontitis patients by RT-PCR so as to obtain an insight into the probable role of IL-35 in immunopathogenesis of periodontal diseases.

In the present study, we demonstrated the expression of IL-35 mRNA in gingival tissues of healthy, CP, and AgP subjects. The levels of IL-35 mRNA were more in the chronic periodontitis subjects (6.87 ± 2.32) as compared to the aggressive periodontitis group (4.71 ± 1.43) and was least expressed among the healthy patients (3.03 ± 1.91). Comparisons among all the three groups as well as pairwise comparisons showed statistically significant differences.

In a recent literature by Wei et al. IL-35 has been shown to be secreted by T_{reg} cells and is required for the immunological capacity of T_{reg} cells. Consequently some reports have demonstrated an increased frequency of T_{reg} cells in periodontal diseased tissues suggesting that T_{reg} infiltration could reflect an attempt to control tissue destruction but could also be indicative to have a destructive role in periodontitis [11, 12]. T_{reg} cells have also been shown to impair the immune response against infectious agents which could be potentially deleterious in periodontal environment [13].

Previously, it has been established that T_{reg} expression is elevated by stimulation with *Porphyromonas gingivalis* antigens in periodontitis patients [14]. *P. gingivalis* is associated with disease progression associated alveolar bone loss in established periodontitis patients [15]. IL-35 has been known to expand T_{reg} cells; thus, it can be hypothesized that this increase in T_{reg} cells may be attributed to the increased levels of IL-35 in periodontitis cases. To the best of our knowledge and the available literature there is no direct published evidence supporting the expression of IL-35 in gingival tissues and this is the first study where IL-35 mRNA expression has been evaluated.

In experimental models, T_{regs} have been predicted to play a role in the maintenance of chronic infections such

TABLE 1: Comparison of three groups with respect to GI and OHI-S Scores by Kruskal Wallis ANOVA and Mann-Whitney U test.

| | Mean | SD | Kruskal Wallis ANOVA Test | | Mann Whitney U Test | | |
			H value	P value	Healthy versus CP	Healthy versus AgP	CP versus AgP
GI							
Healthy	0.91	0.14					
CP	2.06	0.21	40.2052	0.0000[*]	0.0000[*]	0.0000[*]	0.1046
AgP	1.85	0.45					
OHI-S							
Healthy	0.80	0.43					
CP	3.83	0.88	40.5283	0.0000[*]	0.0000[*]	0.0000[*]	0.1105
AgP	3.38	0.86					
PD							
Healthy	2.45	0.48					
CP	6.38	0.76	199.33	0.0000[*]	0.0001[*]	0.0001[*]	0.0012[*]
AgP	7.36	1.11					
CAL							
Healthy	1.41	0.48					
CP	5.10	0.93	214.86	0.0000[*]	0.0001[*]	0.0001[*]	0.0001[*]
AgP	6.66	0.97					

[*]$P < 0.05$.

TABLE 2: Comparison of three groups with respect to PD, CAL and IL-35 mRNA by one-way ANOVA and tukeys post hoc test.

| | Mean | SD | One-way ANOVA Test | | Tukeys multiple post hoc test | | |
			F value	P value	Healthy versus CP	Healthy versus AgP	CP versus AgP
IL-35 mRNA							
Healthy	3.03	1.91					
CP	6.87	2.32	17.5338	0.0000[*]	0.0001[*]	0.0358[*]	0.0024[*]
AgP	4.71	1.43					

[*]$P < 0.05$.

TABLE 3: Correlation between IL-35 mRNA expression with Gingival index, OHI-S, PD, and CAL by Karl Pearson's correlation coefficient method in three groups.

Group	Gingival index	OHI-S	PD (mm)	CAL (mm)
Healthy controls	0.1780	0.2510	0.1470	−0.1117
Chronic periodontitis	−0.3724	−0.0883	−0.3824	−0.0956
Aggressive periodontitis	0.0738	0.2874	−0.1079	−0.1447

$r = 1$.

as infection by Leishmania, HSV, and *Schistosoma mansoni*, with persistence of pathogens, consequently enabling the disease reactivation [16]. Similar results were reported by Collison et al., where they showed that T_{reg} cells induced *in vivo* generation of iTR35cells and IL-35 under inflammatory conditions in intestines infected with *Trichura muris*. Thus, elevated levels of IL-35 could also be involved in unresolved chronic infection/inflammation in periodontitis.

In periodontitis, the effector immune response has to be regulated to control the bacterial growth, dissemination, and to prevent tissue damage. Cardoso et al. indicated that T_{regs} accumulate within the gingiva of periodontitis patients

limiting the effector immune responses, promoting pathogen survival, and maintaining the chronicity of the disease [6]. This is consistent with the higher levels of expression of IL-35 mRNA in chronically inflamed gingival tissues of chronic periodontitis patients.

Also, previous studies have mentioned that expression of IL-35 following activation with anti-CD3/anti-CD28-coated beads remained low for 3 days, suggesting that human T_{reg} cells do not express IL-35 upto 2 days after stimulation. However, it showed a steep increase after 3-day-period. This may be hypothesized as one of the reasons for higher levels in chronic periodontitis patients as compared to the aggressive periodontitis patients [17].

Further in the present study, IL-35 mRNA expression was also demonstrated in healthy gingival tissue sample, even though at lower frequencies than in periodontal lesions. Previous studies have shown IL-35 expression in extracts of the trophoblast component of a human full-term normal placenta, thus, suggesting that it may be important in immune regulation [5]. A previous study by Ernst et al. has demonstrated an increased frequency of T_{regs} in healthy tissues suggesting a role for T_{regs} in the maintenance of periodontal health [18]. This could explain the lower levels of expression of IL-35 in healthy gingival tissues when compared to that of aggressive and chronic periodontitis patients. Contrastingly,

a recent literature has mentioned the failure of human T_{reg} cells to express IL-35 constitutively but how be may able to express it when induced [19]. However, presence of IL-35 even at low levels in healthy tissues may be attributed to variation in the inflammatory tissues considering the presence of the vast number of commensal bacteria colonizing the gingival sulcus and the absence of absolute pristine gingiva even in clinically healthy sites. Also previously existence of T_{reg} cells have been shown to be present constitutively in gingival tissues which may explain the possible mechanism by which immune responses to these microbes are controlled and periodontal destruction is thus prevented [12].

As no previous studies have addressed the role of IL-35 in periodontal disease, we could not compare our results with any other study. However, based on the differential roles of T_{reg} cells in healthy as well periodontitis sites, the mechanism of involvement of IL-35 needs to be addressed. Although we may hypothesize that in healthy sites IL-35 mediated T_{reg} cells control the immune pathology so as to avoid periodontal tissue destruction, while in established periodontitis lesions IL-35 mediated T_{reg} cells may be recruited into the lesion in an attempt to suppress tissue destruction possibly through a negative feedback mechanism. Moreover, the upregulation of T_{reg} and regulatory cytokines at the same time suggests a need for further studies regarding the reciprocal regulation of these IL-35 and T_{reg} in the pathophysiology in periodontal disease. However within the limitation of the study, the present findings indicate that IL-35 is associated with the pathogenesis of periodontitis.

From the present study, it can be concluded that the expression of IL-35 mRNA may be associated with the immunopathogenesis of chronic and aggressive periodontitis as well as it may play a significant role in maintaining the immune balance in healthy state. Further studies with larger sample size and precise detection of protein levels and IL-35 variation during progression of disease are required to elaborate the respective roles of IL-35 in the pathogenesis and progression of periodontal disease as well as in periodontal tissue homeostasis.

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