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IMMUNOHISTOCHEMICAL EXPRESSION OF TOLL-LIKE RECEPTOR PROTEIN RP105 IN CHRONIC AND AGGRESSIVE PERIODONTITIS.

Dental Science

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ABSTRACT

Introduction: TLR4 act as the principal sensors of innate immune responses, especially for the Lipopolysaccharide leading to the unregulated inflammatory response, which would otherwise cause tissue destruction. This is regulated by Radio protective 105 (RP105) a TLR4 protein homologue.

Objective: To investigate the expression of RP105 in chronic and aggressive periodontitis patients.

Material and methods: 60 subjects, 20 periodontally healthy controls, 20 chronic periodontitis and 20 aggressive periodontitis were included. Gingival index, oral hygiene index-simplified, probing depth and clinical attachment loss were recorded followed by immunohistochemical evaluation of RP105 expression in the gingival tissue samples and the results were statistically evaluated through 1-way ANOVA and Mann-Whitney U test.

Results: Significant expression of RP105 in gingival connective tissue of three groups (P=0.0000*). The expression of RP105 in gingival connective tissue of healthy group was higher in comparison to chronic periodontitis subjects and was statistically significant (P = 0.0082*).

Conclusion: The present study demonstrated weak RP105 expression in the gingival epithelium owing to the fact that RP105 is an anti-inflammatory marker.

KEYWORDS

Introduction

Periodontitis being a chronic microbial infection affects the gingiva and bone supporting the teeth. Bacterial plaque stimulates the host inflammatory response leading to tissue damage. Innate immunity provides multicellular organisms with immediately available defense mechanisms against a wide variety of pathogens without requiring prior exposure.[1,2] Recognition of microbial determinants sets in motion early humoral and cellular mechanisms for defense. Invading bacteria releases its products thereby recruiting the adhesion molecules over vascular endothelial cells, further contributing to the recruitment of leucocytes to the focus of infection; later induce the synthesis and release of pro-inflammatory cytokines, viz: TNF and IL-1 that amplify the response to infection. [3] Thus the innate response to microbial challenge controls and instructs the adaptive immune response.

In contrast to adaptive immune response, innate immune response are antigen receptor-independent and induced by invariant molecular structure in pathogen i.e. pathogen associated molecular patterns (PAMP) via, pattern-recognition receptors (PRR's).[4] However, these immune responses are energy-intensive, and have the potential for damaging the host.

Responses to this type were found recently to be mediated by a human homologue of the Drosophila toll protein.[5] TLR's, which recognize and distinguish PAMP viz: bacterial lipopolysaccharide (LPS) [6] Lipoproteins and LPS were identified as principal compounds of the immune-stimulatory activity of microbial membranes. There components have turned out to be ligands for TLR's. [7,8]

Expression of TLR2, TLR4 and CD14 was appreciated in the human gingival fibroblasts through - DNA micro-array analysis among patients with periodontitis and periodontally healthy subjects.[9] However, the LPS responsiveness was not always well documented by the presence of TLR4. It has the ability to signal in response to a variety of endogenous `danger signals'. [4] Thus any other additional molecule which could assess and locate the LPS was required to be investigated.

Although Myeloid differentiation 2 (MD-2) was initially thought to be the molecule similar to MD-1, recently studies have shown that MD-2 is expressed as an extracellular domain of TLR4 thereby imparting LPS responsiveness. Similarly MD-1 is associated with the extracellular domain of Radioprotective 105 (Rp105), which appears to be a biologically important modulator of TLR4 signaling. It is also been assigned as Cluster of Differentiation (CD180). [10]

TLR4 acts as a mediator between a LPS of the invading Gram-negative bacteria and the immune cells like the neutrophils, macrophages and B cells for their activation. Yoshinori Nagai et al (2000) demonstrate that RP105 which is a homologue of TLR4 had a similar response of B cells to LPS. In an animal study done in mice, the gene mutation of RP105 was analyzed to assess the expression of B cells and its correlation to LPS. The data suggest the existence of the TLR4–RP105 signaling module in the LPS-induced B cell activation.[11]

For activation of a host defense, presence, expression and initiation of a proinflammatory response is critical. But, intense and inappropriate inflammation can itself be maladaptive. Several direct, negative regulators of TLR signaling have been found, including MyD88s, IRAK-M, Etc. Among these, RP105 stands out for its apparent specificity for the inhibition of TLR4 signaling and control over the inflammatory over expression.[7]

Further, studies also suggest that RP015 and TLR-4 expression on dendritic cells and macrophages is quiet similar, suggesting its role as TLR4 inhibitor. [12] However the distribution and quantification of RP105 in the chronic and aggressive periodontitis gingival tissue samples is not been previously attempted.

Hence, considering the role of RP105 as an anti-inflammatory marker thereby controlling the TLR4 levels the present study is based on the hypothesis that if considered so, what will be the amount of distribution and expression of the biomarker in the gingiva and connective tissue in health and disease. The aim of study is to quantify and compare the RP105 expression in Healthy, Chronic periodontitis (CP) and Aggressive periodontitis (AgP) groups and correlating its expression with the severity of periodontal disease.

Material and methods:

Source of data: The present study was carried out in the Department of Periodontics, PMNM Dental College and Hospital, which included 60 subjects in the age range of 18-60 years (mean age =32.4±10.6 years) who were divided as: Group 1: 20 CP patients, Group 2: 20 AP patients and Group 3: 20 periodontally healthy patients. Exclusion criterias were 1) the patients with diseases influencing periodontal disease like diabetes mellitus, hepatitis, and HIV infection. 2) smokers 3) patients who received periodontal treatment for at least 6 months prior to sampling 4) with diseases of oral hard and soft tissue 5) use of antibiotics and analgesics within three months of study 6) pregnant women and lactating mothers.

After explaining the nature of the study and the method of sample collection, the patients signed informed consent form was obtained.

The study protocol was approved by the college Ethical Committee board.

Clinical recordings:
An appropriate proforma to obtain precise recording of patient's details like the chief complaint, medical, medicational and dental history was prepared and recorded. Each patient underwent a comprehensive periodontal examination as part of his/ her routine assessment. The GI [Loe and Silness 1963], [13] OHI-S [Green and Vermillion, 1964], [14] Probing Depth and CAL using graduated periodontal probe were recorded. [15,16]

Periodontally healthy subjects were those showing absence of clinical manifestations of periodontal disease with presence of at least 20 teeth. Similarly, subjects with at least 20 remaining teeth and presence of periodontal pocket of ≥5mm and CAL of ≥3-4mm involving at least 6 teeth were categorized as chronic periodontitis subjects and those with similar findings with CAL of ≥5mm affecting at least three teeth other than first molars and incisors and bone loss as verified by radiographs are considered as aggressive periodontitis subjects. [17]

Gingival tissue specimen collection: [18]
Gingival tissue samples for group 1 were obtained during extraction of tooth for the purpose of orthodontic treatment and during third molar extraction. Gingival specimens for group 2 and group 3 were collected from the periodontitis subjects undergoing extraction of the tooth with hopeless prognosis, after completion of scaling.

Gingival biopsies were harvested from the buccal or labial surfaces. While obtaining gingival tissue from group II and group III it was made sure that biopsies should include connective tissue and junctional epithelium. The vertical incisions were connected by a horizontal incision at the approximate level of the alveolar crest. The selection was made on the basis of maximum area of inflammatory cells in the connective tissue with proper technical orientation of the section. Around 2mm wide tissue was excised and the biopsies were fixed in 10% buffered formalin and were transported to the laboratory.

Immunohistochemistry (IHC) procedure: [19]
Specimens obtained were processed through series of steps to retrieve antigen out by immersing the slides in citrate buffer and incubating in enzyme retriever (BioGenex) for 95°C for 10 minutes. The endogenous peroxidase activity was blocked with peroxide block for 10 minutes, rinsed and blotted gently around the section. Non specific binding of IgG was blocked with buffered casein + 15mM in phosphated buffer solution (PBS).

Then the primary antibody LEAFTM anti-human RP105 a Polyclonal antibody purified by affinity chromatography (1µg/ml) procured from Biolegend; (San Diego, CA, USA and distributed by Labmate Asia, India Pvt Ltd) was added and incubated at 20°C for 1hour. The Antibody was diluted in 1 ml of sterile Phosphated Buffer Solution. The secondary antibody staining was done as per the instructions of the kit manufacturer (BioGenex). The sections were counter stained with Meyer's Hematoxylin for 5 to 10 minutes and rinsed with tap water. The sections were immersed in ammonia water for 10sec and mounted for further analysis.

Quantification of cells
Stained slides were first screened and the area for examination was determined by the observers, using a light microscope (Leica DMRB) equipped with a digital video camera. Each slide was analyzed for the intensity of RP105 positive stained cells in the epithelium and connective tissue. The level of intensity of RP105 expression in the epithelium and connective tissue was quantified in semiquantitative method. Grading was done in following manner using a 0 to +3 scales.[20]

No expression (0); negative -to-weak specific immunohistochemical staining in <5% of cells, weak expression (+1); weak specific immunohistochemical staining in 5-30% of cells or strong specific staining in <5% of cells, moderate expression (+2); weak specific immunohistochemical staining in 30-100% of the cells or strong specific staining in 5-75% of cells. Strong expression (+3); strong specific immunohistochemical staining in 75-100% of cells.

Statistical analysis:
Power analysis for a one-way ANOVA with 3 groups was conducted in G*Power to determine a sufficient sample size using an alpha of 0.05, a power of 0.80, and a large effect size (f = 0.40) (Faul et al., 2013). Based on the aforementioned assumptions, the desired sample size was 63. But as the study progressed the samples for aggressive and chronic periodontitis fell short and in all the three groups the rounded figure of 20 in each was considered. To test the difference between the groups statistically, Mann Whitney U test, Kruskal Wallis ANOVA test and Tukeys multiple post hoc test were used. Further according to Spearman's rank co-relation co-efficient method pair wise correlations were calculated. The P value was set to <0.05. (SPSS 15.0 IBM Corporations Ltd., USA).

Results:
Assessment of levels of RP105 and the clinical parameters among the groups and there variations were analyzed. Mean age of the subjects were 24.4±5.01 years in healthy group, 44.80±7.62 in chronic periodontitis group, and 27.90±4.54 in aggressive periodontitis group. All the three groups were gender matched. (Table1).

Table 1: Comparison of gender distribution in three groups of subjects studied

Group	Male	%	Female	%	Total
Healthy (Group 1)	9	45.00	11	55.00	20
Chronic periodontitis (Group 2)	9	45.00	11	55.00	20
Aggressive periodontitis (Group 3)	10	50.00	10	50.00	20
Total	28		32		60

On comparing the means of GI, OHI-S, PD and CAL for three groups, the results were statistically significant. (Table2)

Table 2: Comparison of three groups with respect to GI, OHI-S, PD and CAL by Kruskal Wallis ANOVA and Mann Whitney U test

	Mean	SD	Kruskal Wallis ANOVA Test		Mann Whitney U Test		
			H value	P value	Healthy vs CP	Healthy vs AgP	CP vs AgP
GI							
Healthy	0.91	0.14	40.2052	0.0000*	0.0001*	0.0822	0.0001*
CP	2.06.	0.21					
AgP	1.85	0.45					
OHI-S							
Healthy	0.80	0.43	94.4803	0.0000*	0.0001*	0.1570	0.0001*
CP	3.83	0.88					
AgP	3.38	0.86					
PD							
Healthy	1.95	0.76	136.2020	0.0001*	0.0001*	0.0205*	0.0001*
CP	5.85	0.93					
AgP	6.07	1.17					
CAL							
Healthy	1.80	0.62	150.5465	0.0000*	0.0001*	0.0002*	0.0001*
CP	5.20	1.01					
AgP	6.55	1.00					

* p<0.05

similarly the pairwise comparison among Healthy verses CP, Healthy verses AgP and CP verses AgP when performed, the results were found to be significant. Through Spermans rank correlation coefficient method the significant correlation between RP015 expression with GI, OHI-S, CAL and PD was appreciated. (Table 3)

Table 3: Correlation between RP105 expression and OHI-S, GI, PD and CAL by Spearmans rank correlation coefficient method in three groups .

Groups	Parameters	Rank correlation coefficient	t-value	p-value
Healthy	OHI-S	0.0120	0.0510	0.9599
	GI	0.0184	0.0781	0.9386
	PD	0.0742	0.3156	0.7559
	CAL	0.0569	0.2417	0.8118
Chronic periodontitis	OHI-S	0.2111	0.9163	0.3716
	GI	-0.4162	-1.9420	0.0680
	PD	0.5267	2.6286	0.0170*
	CAL	0.0381	0.1618	0.8733
Aggressive periodontitis	OHI-S	0.1715	0.7384	0.4698
	GI	0.2376	1.0377	0.3131
	PD	-0.0903	-0.3849	0.7048
	CAL	-0.1762	-0.7593	0.4575

*P<0.05 and r =1

Rp105 expression was demonstrated in gingival epithelium and underlying connective tissue among the three study groups as represented by the four-grade (0; +1; +2; +3) intensity of immunohistochemical staining. Negative or weak RP105 expression in the gingival epithelium was seen owing to the fact that the RP105 is an anti-inflammatory marker. (Table 4)

Table 4: Comparison of three groups with respect to PD, CAL and RP105 by one-way ANOVA and Tukeys multiple post hoc test

	Mean	SD	One-way ANOVA Test		Tukeys multiple post hoc test		
			F value	P value	Healthy vs CP	Healthy vs Agp	CP vs AgP
Rp105 Healthy	**3.02**	**1.90**	**17.5217**	**0.0000***	**0.0082***	**0.0000***	**0.259***
CP	**6.88**	**2.31**					
AgP	**4.70**	**1.44**					

***p<0.05**

Expression of RP105in Healthy controls, chronic periodontitis and aggressive periodontitis were 3.02 ± 1.90, 6.88 ± 2.31 and 4.70 ± 1.44, respectively. Also this difference of RP105 expression in three groups was found to be statistically significant. (Table 5)

Table 5: Distribution of RP105 expression in the gingival connective tissue in the three groups of subjects studied

Grades	Healthy	%	Chronic periodontitis	%	Aggressive periodontitis	%	Total
Grade 0	2	10.00	2	10.00	1	5.00	5
Grade 1	2	10.00	6	30.00	11	55.00	19
Grade 2	4	20.00	10	50.00	8	40.00	22
Grade 3	12	60.00	2	10.00	0	0.00	14
Total	20	100.00	20	100.0	20	100.00	60

Discussion

In the present study, the in-vivo expression of RP105 in periodontally healthy, chronic periodontitis and aggressive periodontitis patients was carried out to specify the role of RP105 in triggering periodontal infection. This is the first study, showing the immunohistochemical mapping of RP105 in periodontal tissues in health and disease. In the present study an attempt has been made to localize and quantify the expression of RP105 in epithelium and connective tissue among these groups using human monoclonal RP105 anti-body.

Studies showed that the RP105-MD1 complex acting through the extracellular domain of RP105 regulates TLR4 signaling in conventional antigen presenting cells, likely via direct inhibition of LPS-TLR4 binding. [21] The activation of lymphocytes during innate immune responses play an important role in the efficient recruitment of activated B-cells into antigen-driven adaptive responses. [22] Further more anti-RP105 antibody has an ability to induce polyclonal activation of B-cells in-vitro performing an anti-inflammatory role.

In this study, the expression of RP105 were detected in both gingival epithelia and underlying connective tissue in all gingival samples and the varied expressions of RP105 were observed in all the groups. With the earlier evidences regarding TLR4 and RP105, [23] it was shown that in chronic periodontitis patients the levels of RP105 may be increased if it's in homologue with TLR4 or may decrease if it's expressing a negative variation. [24] Which, in accordance with the present study have shown a down-regulation in RP105 levels in chronic and aggressive periodontitis patients and in turn an increased levels are appreciated in the healthy connective tissue. (Figure 1)

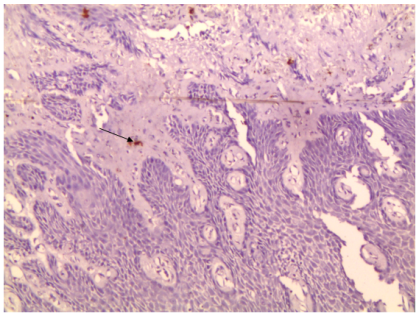

Figure 1: Brown color immunohisto localization of RP105 on the dendritic cells in the connective tissue of Gingival tissue sample of Chronic periodontitis patient

Previous studies (Y Kikuchi et al 2001 and Y Sun et al 2010) have suggested that RP105 is solely expressed on B lymphocytes, dendritic cells, macrophages and peripheral blood cells and not on the normal epithelial cells. [25, 26] similarly, in present study in addition to its expression in inflamed oral epithelia, cell surface localization of RP105 shows a negative expression of RP105 in different layers. Being an anti-inflammatory marker, expression in the epithelium is not appreciated. Also the decreased levels of RP105 in the chronic and aggressive periodontitis suggest the susceptibility to infection.

Role of RP105 in regulating the cell mediated immune response by the suppression of antigen presenting cells (APC) function and regulating T-cell development was shown by Yoshinori Tada et al (2005) in his study, which suggests or draw us to a conclusion that the levels of RP105 may override TLR4 levels in few cases where in the levels of dendritic cells preventing RP105 adhesion on their surface are increased as part of inflammatory regulators. Further studies should be conducted to ascertain whether inflammation was the only reason for the decrease of RP105 expression in CP/AgP patients.

The present investigations suggest that RP105 regulates TLR4 expression while providing one point of control in inflammatory process in chronic and aggressive periodontitis subjects and on other aspect, plays a key role in maintaining the immune balance in healthy state. RP105 is a critical receptor for regulation of TLR4 function in B cells and could also be an important target for immunoregulation & future research will help clarify the role of RP105 as a master regulator of TLR2 and TLR4 and will help to determine the potential for RP105 as a target for antibody-based therapy.

The clinical parameters were assessed to determine the periodontal status of the subject but not to compare with the biomolecule. Overall weak expression of RP105 in case of chronic and aggressive periodontitis is suggestive of a strong interaction between RP105 combating the increased levels of TLR4 during inflammation wherein the RP105 MD-1 complex acts as a receptor molecule to TLR4. However further research to probe in the reason behind increased levels of RP105 in case of healthy subjects is acceptable.

Limitations: Analyzing coexpression of MD1 with RP105 and its association with TLR4 would definitely prove to be more specific in assessing the immune reaction, which was not possible to carry out because of the financial restrain. As the laboratory procedures are technically sensitive, some errors are suspected. Hence, necessity to carry out further prospective studies with large sample size to confirm the role of RP105 in pathogenesis of periodontitis and to affirm the observations of our study is required.

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