Possible Involvement of IL-21 and IL-10 on Salivary IgA Levels in Chronic Periodontitis Subjects


*Laboratory of Immunology and Molecular Biology, São Leopoldo Mandic Institute and Research Center, Campinas/SP, Brazil; †School of Dentistry, Federal University of Maranhão, São Luiz/MA, Brazil; and ‡Department of Periodontics, Dental Research Division, Guarulhos University, Guarulhos/SP, Brazil

Received 12 May 2011; Accepted in revised form 16 July 2011

Correspondence to: Dr M. H. Napimoga, Laboratory of Immunology and Molecular Biology, São Leopoldo Mandic Institute and Research Center R. José Rocha Junqueira, 13 – Campinas/SP, Brazil, E-mails: marcelo.napimoga@gmail.com or napimogamh@yahoo.com

Abstract

Specific cytokines and the costimulatory protein CD40 play role in inducing immunoglobulin (Ig)A production by B cells in the humoral immune response. However, to date, the role of these mediators was not investigated in chronic periodontitis. Therefore, the aim of this study was to assess the local levels of interleukin (IL)-21, IL-21 receptor (IL-21R), IL-4, IL-10 and CD40 ligand (CD40L) on chronic periodontitis subjects and their relationship with the salivary levels of IgA. Gingival biopsies and un-stimulated saliva were collected from chronic periodontitis (n = 15) and periodontally healthy (n = 15) subjects. The mRNA levels of IL-4, IL-10, IL-21, IL-21R, CD40L in the gingival biopsies were evaluated by quantitative real-time polymerase chain reaction. The salivary levels of IgA and the levels of IL-4 and IL-10 in the gingival biopsies were analyzed by ELISA. The mean levels of IgA were significantly higher in the chronic periodontitis compared to the healthy group. However, the expression of IL-21R and CD40L did not differ between groups. The IL-10 was significantly elevated at mRNA and protein levels in chronic periodontitis when compared to periodontally healthy group (P < 0.05). Conversely, the mRNA levels as well as the protein amount of IL-4 were significantly lower (P < 0.05) in chronic periodontitis than healthy ones. In conclusion, the upregulation of IL-21 and IL-10 and downregulation of IL-4 in periodontitis tissues may be collectively involved in the increased levels of salivary IgA in chronic periodontitis subjects.

Introduction

The mucosal immune system generates frontline immune protection at the interface between the host and the environment by forming a highly integrated system of lymphoid organs collectively known as mucosa-associated lymphoid tissue, which play a crucial role in antibody formation [1]. The antibody immunoglobulin (IgA) is the predominant immunoglobulin secreted by oral mucosal sites, considered one of the most important protein contributing to microbial defence from toxins, viruses, and bacteria by means of direct neutralization or prevention of microbial binding to the mucosal surface [2].

Previous studies have long demonstrated that the humoral immune response, especially mediated by secreted IgG and IgA, plays protective role in the pathogenesis of periodontal diseases, including gingivitis, chronic and aggressive periodontitis. It was previously showed that the levels of salivary IgA directed to Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans were significantly higher in subjects with deeper periodontal probing depth (PD) compared to healthy subjects [3]. Moreover, the serum IgA- and IgG-class antibody levels against A. actinomycetemcomitans and P. gingivalis were higher in the pathogen carriers compared with the non-carriers, and clearly higher in the carriers with periodontal pockets compared with the carriers without pockets [4]. Also, it was reported that the level of specific salivary IgA antibodies against mycobacterial heat shock protein (HSP) 65 was significantly increased in patients with gingivitis compared to healthy and periodontitis subjects [5]. Besides, a positive correlation between the levels of salivary IgA directed to GroEL (a chaperonin) and periodontal disease severity and,
suggested that salivary IgA to GroEL may have a protective role by reducing the inflammatory response induced by GroEL derived from periodontal pathogens [6]. Interestingly, IgA levels positively correlated with serum C-reactive protein suggesting the involvement of oral infection on systemic inflammation and coronary artery disease prevalence [7].

The primary function of B cells is to produce antigen-specific Ig. Naïve B cells present the amazing ability to alter the effector function of Ig molecule by isotype switching, which is a critical component of B cell differentiation and generation of protective humoral immune responses [8]. Recently, it has been demonstrated that some Th-secreted cytokines is essential to stimulate naïve B cells to produce Ig. Interleukin (IL)-21 induces naïve B cells to switch expression of IgA, especially IgA1. In addition, IL-10 amplifies secretion of IgA induced by IL-21 [9], consistent with the role of IL-10 in regulating IgA responses [10]. In contrast, IL-4 dramatically attenuates IL-21-induced switching to IgA secretion while the neutralization of endogenous IL-4 increases the levels of IgM and IgA [9]. In addition to B cell antigen receptor and receptors for cytokines such as IL-4, IL-10, IL-21, the CD40, an integral membrane protein found on the surface of several cells, upregulates the expression of DNA-editing enzyme called activation-induced cytidine deaminase (AID) and triggers the induction of somatic hypermutation (SHM) and class-switch recombination (CSR) from IgM to IgG or IgA [11–13]. It has been proposed that IL-21 in combination with CD40 costimulation is even more effective in inducing IgA production by B cells [14]. Therefore, the presence of IL-21/IL-10/CD40L has been proposed to be critical for isotype switching to IgA by naïve B cells.

To date, the possible relationship between the mediators related to Ig production and the levels of IgA was not evaluated in chronic periodontitis subjects. Therefore, the aim of this study was to assess the gingival levels of IL-21, IL-21 receptor (IL-21R), IL-4, IL-10 and CD40 ligand (CD40L) and the salivary levels of IgA in chronic periodontitis subjects, when compared to periodontally healthy ones.

Materials and methods

Subjects. Thirty systemically healthy individuals, 15 with chronic periodontitis and 15 periodontally healthy subjects (aged 34–60 years) were selected from the population referred to the Periodontal Clinic of Guarulhos University, from January 2009 until July 2010. Subjects who fulfilled the following described inclusion/exclusion criteria were invited to participate in the study. All eligible subjects were informed of the nature, potential risks, and benefits of their participation in the study and signed their informed consent. This study protocol was previously approved by the Guarulhos University’s Ethics Committee in Clinical Research (protocol # 100/2007).

Inclusion and exclusion criteria. All subjects should be >30 years old and present at least 15 teeth (excluding third molars). Periodontitis subjects should be diagnosed with generalized chronic periodontitis [15] and should have at least one tooth indicated to extraction due to advanced periodontitis (probing depth (PD) and clinical attachment level (CAL) >7 mm with bleeding on probing (BoP)). Periodontally healthy subjects should require gingival removal during periodontal aesthetic surgery for the correction of gingival discrepancies and asymmetries.

Exclusion criteria were pregnancy, lactation, current smoking, and smoking within the past five years, periodontal or/and antibiotic therapies in the previous six months, use of mouthrinses containing antimicrobials in the preceding two months, systemic condition that could affect the progression of periodontal disease (e.g. diabetes, immunological disorders) and long-term administration of anti-inflammatory and immunosuppressive medications.

Clinical examination. All clinical examinations were performed by one examiner (VRS) who was calibrated, as previously described [16]. The intra-examiner variability was 0.21 mm for PD and 0.22 mm for CAL. The clinical parameters, registered dichotomously [i.e. BoP], were calculated by the Kappa-Light test and the intra-examiner agreement was >0.85. The following parameters were assessed at six sites of all teeth, excluding third molars, using a manual periodontal probe (UNC15, Hu-Friedy, Chicago, IL, USA): plaque index (PI), BoP (presence/absence), suppression (SUP, presence/absence), marginal bleeding (MB, presence/absence), PD (mm) and CAL (mm).

Experimental groups. Based on their periodontal status, the subjects were divided into one of the following groups:

1. Periodontally healthy (n = 15; control): Subjects with no sites with CAL >3 mm and <20% of sites presenting BoP and/or MB.

2. Chronic periodontitis (n = 15): Subjects with generalized chronic periodontitis, presenting more than 30% of the sites with PD and CAL >4 mm and at least one tooth with indication to exodontia due to advanced periodontitis.

Saliva sampling. The saliva samples were obtained around 8:00 a.m. Volunteers were instructed not to brush their teeth during the preceding 12 h and not to drink or eat anything for 1 h before sampling to avoid contamination with non-salivary components. Approximately 500 µl of saliva was transferred to 1.5 ml tubes in which 10 µl of 250 mM EDTA had been added. Samples were placed on ice and processed within 1 h after collection. Saliva samples were clarified by centrifugation at 13,000 g at 4 °C for 10 min, and the supernatants were
collected and frozen at −70 °C until laboratory analysis. Total concentration of protein in saliva was determined by the method of Bradford to check for variations in salivary flow (Sigma-Aldrich, St Louis, MO, USA).

**Gingival biopsies sampling.** For the chronic periodontitis group, the gingival biopsies were collected from teeth indicated for exodontia due to advanced periodontitis in order to obtain representative areas of the periodontal inflammation. If the patient had two or more teeth with these characteristics, biopsy only one tooth with the worst diagnosis was included. For the periodontally healthy group, gingival biopsies were collected from teeth without signs of clinical inflammation, indicated for gingivoplasty due to esthetical reasons. All samples included junctional and supracrestal epitheliums and connective gingival tissue. The gingival biopsies were divided into two portions. One portion was immediately placed in microcentrifuge tubes containing 250 μl phosphate-buffered saline and protease inhibitor cocktail (Sigma-Aldrich), and homogenized (Kinematica Polytron PT3100, Littau-Luzern, Switzerland), and then centrifuged at 13,000 g for 5 min at 4 °C. The resulting supernatants, devoid of debris, were stored at −70 °C until subjected to cytokine measurements by ELISA. The additional portion was stored in a tube containing RNA later (Ambion Inc., Austin, TX, USA) and stored at −20 °C for subsequent assays.

**Enzyme linked immunosorbent assay (ELISA).** Total levels of IgA were determined by ELISA using microtiter plates (Costar 3590, Corning, NY, USA) coated for 24 h at 4 °C with 2 μg/ml of goat IgG anti-human IgA (Southern Biotech, Birmingham, AL, USA) in carbonate-bicarbonate buffer, pH 9.6. After being coated, plates were washed and blocked for 1 h at room temperature with bovine serum albumin (0.1%) in phosphate-buffered saline (PBS), pH 7.5. Diluted saliva samples (1:200 in PBS, pH 7.5) were applied in triplicate, and plates were incubated for 2 h at room temperature. All experiments included serial dilutions (1.0, 0.5, 0.25, and 0.125 μg/ml) of a standard sample of human IgA antibody purified from serum (Southern Biotech). The secondary antibody was biotin-conjugated goat IgG anti-human IgA (Southern Biotech) at a dilution of 1:14,500. After incubation with a solution of streptavidin, conjugated with alkaline phosphatase (Southern Biotech) (1:500 in PBS, pH 7.5), antibody reactions were revealed by incubation with the substrate p-nitrophenyl phosphate disodium. In order to obtain the A405 units, plates were read in an ELISA plate reader (Epoch, Biotek, Winooski, VT, USA). Negative controls included the uncoated wells without saliva and primary antibody. For determination of IgA concentrations, absorbance values were plotted against the standard curve obtained for the serial dilutions of the purified human IgA within a linear range. IgA levels were expressed as pg/ml of saliva.

The gingival biopsies were analyzed by ELISA for IL-4 and IL-10 using commercially available ELISA kits (Quantikine; R&D Systems Inc., MN, USA). Assays were carried out according to the manufacturer’s recommendations using human recombinant standards. The optical density was measured at 450 nm according to recommendation. Results are reported as total amount (pg/mg) of each cytokine. Sites with cytokine levels below the detection limit of assay were scored as 0 pg.

**RNA extraction.** The gingival biopsies stored in RNA later (Ambion) were evaluated for mRNA levels of IL-4, IL-10, IL-21, IL-21R, CD40L and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Total RNA from the biopsies was isolated by the Trizol method (Gibco BRL; Life Technologies, Rockville, MD, USA) according to the manufacturer’s recommendations using human recombinant standards. The optical density was measured at 70°C and stored at −70°C. The RNA concentration was determined from the optical density using a micro-volume

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene</th>
<th>Gene Bank (NM)</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40 Ligand</td>
<td>CD40L</td>
<td>NM_000747.2</td>
<td>F 5’-TGTTAACAACAGGAGGAGGGAGA-3’</td>
</tr>
<tr>
<td>Interleukin 21</td>
<td>IL-21</td>
<td>NM_021803.2</td>
<td>F 5’-TCAAGATGGCACATGTGAAAA-3’</td>
</tr>
<tr>
<td>Interleukin 21R receptor</td>
<td>IL-21R</td>
<td>NM_021798.3</td>
<td>F 5’-TCAAGATGGCACATGTGAAAA-3’</td>
</tr>
<tr>
<td>Interleukin 4</td>
<td>IL-4</td>
<td>NM_000389.2</td>
<td>F 5’-AACGGCTGACAGGGACCT-3’</td>
</tr>
<tr>
<td>Interleukin 10</td>
<td>IL-10</td>
<td>NM_000572.2</td>
<td>F 5’-GAGCTCATGACATGGAAGTACGA-3’</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>GAPDH</td>
<td>NM_002046.3</td>
<td>F 5’-GCACCGCAAAGGCTGAGAAC-3’</td>
</tr>
</tbody>
</table>

F: forward; R: reverse.

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spectrophotometer (Nanodrop 1000, Nanodrop Technologies LLC, Wilmington, NC, USA).

Real-time PCR reactions. Reverse transcription total RNA was DNase treated (Turbo DNA-frees, Ambion Inc., Austin, TX, USA), and 1 μg was used for cDNA synthesis. The reaction was carried out using the First-Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD, USA), following the manufacturer’s recommendations.

Primer design. Primers were designed using the Primer Express 3.0 probe design software (Applied Biosystem, Foster City, CA, USA). The primer sequences are presented in Table 1.

PCR Reactions. Quantitative real-time polymerase chain reaction (qPCR) was performed in the 7300 Real Time PCR (Applied Biosystem) using the SYBR Green PCR Master Mix (Fermentas). The reaction product was quantified with the Relative Quantification tool, using GAPDH as the reference gene. Negative controls with SYBR Green PCR Master Mix and water were performed for all reactions.

Statistical analysis. The statistical analysis was performed using a software program (GraphPad Prism 4.0, La Jolla, CA, USA). Data were first examined for normality by the Kolmogorov-Smirnov test and, since the data achieved normality, parametric method was employed. The percentages of sites with visible plaque accumulation, BoP, SUP, the means PD, CAL were computed for all teeth. Clinical parameters, mRNA data, the levels of cytokines and IgA were averaged into both groups. The differences in clinical parameters, age, mRNA levels, IgA, and cytokines levels between groups were compared using Student’s t-test. The level of significance was set at 5%.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Healthy (n = 15)</th>
<th>Periodontitis (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>42.2 ± 6.3</td>
<td>49.0 ± 5.8</td>
</tr>
<tr>
<td>M/T</td>
<td>6.09</td>
<td>7.98</td>
</tr>
<tr>
<td>PI (%)</td>
<td>12.2 ± 8.2</td>
<td>60.0 ± 12.3</td>
</tr>
<tr>
<td>Sampled teeth</td>
<td>5.2 ± 2.2</td>
<td>63.0 ± 24.0</td>
</tr>
<tr>
<td>BoP (%)</td>
<td>3.4 ± 4.1</td>
<td>48.3 ± 19.1</td>
</tr>
<tr>
<td>SUP (%)</td>
<td>0</td>
<td>66.7 ± 4.0</td>
</tr>
<tr>
<td>Sampled teeth</td>
<td>0</td>
<td>3.5 ± 7.2</td>
</tr>
<tr>
<td>PD (mm)</td>
<td>2.1 ± 0.5</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>Sampled teeth</td>
<td>2.3 ± 0.4</td>
<td>4.2 ± 0.8</td>
</tr>
<tr>
<td>CAL (mm)</td>
<td>2.3 ± 0.2</td>
<td>8.0 ± 1.6</td>
</tr>
</tbody>
</table>

M. male; F. female; PI: plaque index; BoP: bleeding on probing; SUP: suppuration; PD: probing depth; CAL: clinical attachment level. Different letters indicate statistically significant differences between healthy and periodontitis subjects (Student t-test; P < 0.05).

Results

Table 2 summarizes the demographic characteristics and the clinical parameters of the study population. There were no differences in the mean age and gender distribution between groups (p > 0.05). As expected, the levels of all periodontal parameters were lower in the control group when compared to chronic periodontitis group considering full-mouth and the teeth selected for gingival biopsies levels (p < 0.05).

Salivary levels of antibody were normalized by comparing the IgA antibody in ELISA to the total protein (Bradford method) found in the saliva. The mean level of total protein found in the saliva of the periodontal disease individuals was 1471.60 ± 438.09 μg/ml, and from healthy individuals was 1056.79 ± 381.13 μg/ml. The normalized mean levels of IgA (pg/ml) in total saliva are presented in Figure 1. The total IgA antibody levels were significantly higher in the chronic periodontitis group compared to periodontally healthy ones (P < 0.05).

As observed in Fig. 2A, the gingival mRNA levels for IL-21 was significantly higher (P < 0.05) in the chronic periodontitis group when compared to the healthy group. On the other hand, the expression of IL-21R (Figure 2B) and CD40L (Fig. 2C) did not differ between groups (p > 0.05).

IL-10 was significantly elevated at mRNA and protein levels in chronic periodontitis group when compared to periodontally healthy group (P < 0.05) (Fig. 3A and B) and CD40L (Fig. 2C) did not differ between groups (p > 0.05). As well as the protein amount of IL-4 (Fig. 4A) as well as the protein amount of IL-4 (Fig. 4B) were significantly lower (P < 0.05) in chronic periodontitis group than healthy ones.

Figure 1 Amount of IgA in saliva normalized with the total protein. Box plot of the levels of salivary IgA observed between healthy and periodontitis groups. * Significant difference between groups (P < 0.05; Student t-test).
Discussion

Cytokines influence B cell development and homeostasis by regulating their proliferation, survival and function, including the production of Ig. It has been demonstrated that Ig secretion is affected by Th-secreted cytokines such as IL-21, IL-10 and IL-4 and by CD40L [9, 10]. However, the role of these specific mediators of Ig isotype switching in the B cell response on periodontal diseases remains unclear. Therefore, this study evaluated for the first time the gingival levels of some mediators related to Ig isotype switching (IL-21, IL-21R, IL-4, IL-10 and CD40L) and the salivary levels of IgA in chronic periodontitis subjects. Overall, the results demonstrated that the salivary levels of IgA were upregulated in periodontitis subjects at the same time that the gingival levels of IL-21 and IL-10 were increased and the levels of IL-4 were decreased in periodontitis tissues. Together, these results suggested that some Th-secreted cytokines are probably involved in the generation of IgA by B cells in periodontitis tissues that, in turn, may be one of the most important sources of IgA in the saliva of chronic periodontitis.

Figure 2 Distribution of the total mRNA of IL-21 (A), IL-21R (B), and CD40L (C) in the gingival tissue of healthy and chronic periodontitis subjects. The relative quantification of each gene, expressed as fold-change, was obtained using the \(2^{-\Delta\Delta Ct}\) method and by normalizing the mRNA expression to GAPDH. The results are expressed as mean and standard deviation of mRNA fold change. The symbol * indicates significant difference between groups (\(P < 0.05\); Student t-test).

Figure 3 (A) Distribution of the total mRNA of IL-10 in the gingival tissue of healthy and chronic periodontitis subjects. The relative quantification of this gene, expressed as fold-change, was obtained using the \(2^{-\Delta\Delta Ct}\) method and by normalizing the mRNA expression to GAPDH; (B) Distribution of the concentration (pg/mg) of IL-10 in the gingival tissue from healthy and chronic periodontitis subjects. The symbol * indicates significant difference between groups (\(P < 0.05\); Student t-test).
chronic periodontitis tissues when compared to healthy ones. In addition, the levels of IL-4 were lower in periodontitis tissues than healthy biopsies. Concomitant with the increased expression of IL-21 and IL-10 and decreased in IL-4 levels in periodontitis tissues; the amounts of salivary IgA were significantly higher in periodontitis subjects. Together, these data suggest that the abovementioned role of IL-21, IL-10, and IL-4 in Ig isotype switching might also take place in chronic periodontitis and indicate an immunomodulation of the oral mucosal tissues in subjects under periodontal pathogens challenge.

The role of these cytokines has been already investigated in periodontitis; however, the majority of the studies have focused on the functions of cytokines on the Th1/Th2 or Th17/Treg responses. In accordance to the present results, previous studies showed that IL-21 was highly expressed in gingival biopsies of chronic periodontitis [24] and the levels of IL-21 in gingival crevicular fluid decreased after treatment of chronic periodontitis [19]. Furthermore, our findings confirm previous observations in which lower levels of IL-4 [25, 26] and higher levels of IL-10 [27, 28] were associated with periodontitis. In addition, in agreement with present study, the levels of IgA against different pathogens have been found to be higher in subjects with periodontal disease [3, 4, 6]. Therefore, salivary IgA, the most abundant immunoglobulin isotype in saliva seems to be potentially protective against periodontal pathogens and their virulence factors [6, 29]. Accordingly, the selective IgA primary immunodeficiency (IgAD) predisposes to oral mucosal infections, supporting the role of IgA in inhibiting mucosal colonization and invasion of pathogens [30], although the loss of IgA did not result in an increase in periodontitis levels in IgAD individuals [30, 31]. In this study, we suggested that the higher amount of the IgA found in the saliva of the chronic periodontitis subjects may have a direct relationship with the higher expression of IL-21 and IL-10 and lower expression of IL-4 in periodontitis tissues. Since IL-21 and IL-10 are involved in the isotype switching, their synergistic actions on B

Figure 4  (A) Distribution of the total mRNA of IL-4 in the gingival tissue of healthy and chronic periodontitis subjects. The relative quantification of this gene, expressed as fold-change, was obtained using the 2^ΔΔCt method and by normalizing the mRNA expression to GAPDH; (B) Distribution of the concentration (pg/mg) of IL-4 in the gingival tissue from healthy and chronic periodontitis subjects. The symbol * indicates significant difference between groups (P < 0.05; Student t-test).
lymphocyte subpopulation in the diseased periodontal tissues may explain the elevated IgA amounts in the saliva in chronic periodontitis subjects.

In conclusion, the results of the present study suggested that upregulation of IL-21 and IL-10 and downregulation of IL-4 in periodontitis tissues may be collectively involved in the increased levels of salivary IgA in chronic periodontitis subjects. Since only cytokine profiles and salivary IgA level were evaluated and, no characterization of naïve B cell switch in the periodontal lesions was performed, these preliminary findings are still not enough to definitely define the mechanisms of Ig iso-type switching on chronic periodontitis. However, our results may provide new insights into the possible role of Th-secreted cytokines in driving humoral immune response on periodontal tissue breakdown.

Acknowledgment

The authors thank Ms Jeruza P. Bossonaro for technical assistance and São Paulo State Research Foundation (São Paulo, São Paulo, Brazil) for its financial support (#2008/09687-0; #2008/04280-0).

References