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Short communication

Relationship between salivary leukotriene B_4 levels and salivary mucin or alveolar bone resorption, in subjects with periodontal health and disease

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Objective: Here we determine the salivary levels of leukotriene B_4 (LTB_4) and its relation with salivary mucin and alveolar bone level.

Background: LTB_4 is a membrane-derived lipid mediator formed from arachidonic acid. It is among the most potent stimulants of polymorphonuclear leukocytes providing the first host defense against infections. Leukotrienes also induce bone resorption. Because LTB_4 is present in the oral cavity the aim of the present study was to explore the role of LTB_4 in patients with periodontal disease.

Methods: Eighty-one subjects were clinically examined and distributed into four groups, namely, clinically healthy, mild, moderate and severe periodontitis, according to periodontal status, classified into values of clinical attachment level and probing pocket depth. Unstimulated saliva was collected for 5 min. Salivary LTB_4 was determined by an immune assay method, mucin was determined by a colorimetric method and radiographic assessment used to determine alveolar bone level.

Results: Patients with mild periodontitis showed a decrease in salivary LTB_4 levels while patients with severe periodontitis showed increased LTB_4 levels. A significant positive correlation was observed between salivary LTB_4 and clinical attachment level, salivary mucin concentration or alveolar bone level.

Conclusion: The close relation between salivary LTB_4 and mucin levels suggested that LTB_4 might be involved in the defense mechanism of the oral cavity. The correlation of LTB_4 with the alveolar bone level indicates that they are one of the mediators responsible for bone resorption.

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Leukotriene B_4 (LTB_4) is a potent lipid mediator of inflammation produced through the sequential actions of 5-lipoxygenase, 5-lipoxygenase activating protein and LTA_4 hydrolase (1). Although LTB_4 is known to exert broad proinflammatory effects, evidence is accumulating regarding the antimicrobial functions of LTB_4 (2,3). Furthermore, the LTB_4–BLT_1 pathway was found to be important for linking early immune responses and the multiple classes of effector cells associated with acquired immunity (4).

LTB_4 is found in saliva from healthy subjects. It is produced by cells taken from the oral cavity or secreted by salivary glands (5). In this regard, we observed that in submandibular glands from rats with experimental periodontitis there was an increase in leukotriene production associated with mucin production (6). Because mucin plays a major role in the oral defense mechanism and leukotrienes participate in the fine-tuning of the immune system (7), their presence in the oral cavity may be associated to the non-immunological defense mechanism.

LTB_4 also plays a significant role in bone metabolism. Local administration of LTB_4 to mouse calvarial bone increases osteoclastic bone resorption *in vivo*, likely due to an increase in osteoclast formation and activation of mature osteoclasts (8). LTB_4 production is enhanced in inflammatory bone-resorptive diseases such as rheumatoid arthritis (9), osteoarthritis (10) and periodontitis (11).

Periodontitis is a multifactorial poly-microbial infection initiated by the presence of gram-negative bacteria, which accumulates in the gingival crevice region (12). The presence of periodontal pathogenic bacteria leads to the establishment of an inflammatory process in periodontal tissues, which may lead to the destruction of periodontal ligament and adjacent supporting bone, causing tooth loss (13,14). Levels of LTB_4 are elevated in gingival crevicular fluid of patients with periodontitis (15) and, bearing in mind the LTB_4 effect on osteoclasts, its presence may be associated to bone resorption.

LTB_4 may have two different roles in the oral cavity, as a defense mechanism or as an inflammatory mediator in periodontal disease. The present study, therefore, aimed to examine: (i) LTB_4 levels in saliva from subjects with periodontal health and different status of periodontal disease; (ii) relationship between salivary LTB_4 levels and salivary mucin levels; and (iii) relationship between salivary LTB_4 levels and alveolar bone levels (ABL).

Material and methods

Subjects

A total of 81 adult subjects, distributed in four groups namely, clinically healthy, mild, moderate and severe chronic periodontitis, participated in this study. They were enrolled at a private dental clinic and agreed to participate. Before the study, the health of the subjects was ascertained by using detailed medical histories and clinical examinations. Exclusion criteria included smokers, diabetes, immune suppression and medicine use.

The protocol was approved by the Ethics Committee of the School of Dentistry, University of Buenos Aires, and the study was conducted in accordance with the Declaration of Helsinki (version 2008).

Periodontal diagnosis

The periodontal status was determined according to the classification of Page and Eke (16), and according to this, subjects were distributed in four different groups namely: healthy (absence of periodontal disease); mild (< 2 interproximal sites with clinical attachment level (CAL) $\geq$ 4 mm and < 2 interproximal sites with probing pocket depth (PPD) $\geq$ 5); moderate (≥ 2 interproximal sites with CAL $\geq$ 4 mm and ≥ 2 interproximal sites with PPD $\geq$ 5); and severe (≥ 2 interproximal sites with CAL $\geq$ 6 mm and ≥ 2 interproximal sites with PPD $\geq$ 5). Data of the number, age and gender of subjects of each group are shown in Table 1. At the first visit the assessment of clinical parameters was carried out by one trained and calibrated examiner (G.S.; calibrated by the Argentine Dental Association and the University of Buenos Aires). All periodontal disease measurements were performed in four quadrants using a first-generation probe (Hu-Friedy Mfg. Co., Chicago, IL, USA). PPD (measurements were rounded off to the nearest millimeter marking) and CAL (measuring the distance from the cement–enamel junction to the bottom of the probable pocket) were assessed at six sites per tooth and bleeding on probing (scored as: −, no bleeding or +, bleeding within 30 s after probing) at four sites per tooth and expressed as the percentage of positive sites.

Collection of saliva

Whole saliva was collected by spiting into an ice-cooled graduated vessel. Subjects spat out every 30 s for 5 min. The volume of saliva was recorded and expressed as mL/min. The resulting saliva was stored in aliquots at −20°C until determinations were performed. Saliva was collected at 10.00 h by one calibrated examiner the day after periodontal diagnosis.

Determination of leukotriene B_4 concentration

The concentration of LTB_4 in saliva was measured by the Enzyme Immuno Assay kit from Cayman Chemicals Co. (Ann Arbor, MI, USA). The concentration (pg/mL) in the samples was calculated according to the reference calibration curves of standards supplied with the kit.

Determination of mucin concentration

Mucin concentration was determined using the Alcian blue method described by Hall *et al.* (17) and modified by Sarosiek *et al.* (18). Briefly, aliquots of diluted saliva (1 : 10) were incubated for 30 min in a 1% solution of Alcian blue in 50 mM sodium acetate buffer 25 mM $MgCl_2$, pH 5.8 under constant agitation at room temperature. Following incubation, the samples were centrifuged for 20 min at 705 *g*, pellets washed in 95% ethanol, vortexed

Table 1. Number, gender and age of the subjects that participate in this study classified according to Page and Eke (16)

Data/group	Clinically healthy	Mild periodontitis	Moderate periodontitis	Severe periodontitis
Number	20	20	21	20
Age	34.7 (27–41)	38.5 (30–46)	41.6 (33–57)	46.8 (32–61)
Gender	14 M, 6 F	14 M, 6 F	17 M, 4 F	14 M, 6 F

gently for 10 s and after 5 min, centrifuged for 20 min at 705 *g*. Mucin–dye complexes were dissociated by the addition of a 1 : 2 dilution of Aerosol OT (Sigma Chemical Co., St Louis, MO, USA) in distilled water, brief mixing and sonication. Subsequently, samples were extracted with equal volumes of ethyl ether under vigorous shaking. The resulting solution was centrifuged for 15 min at 705 *g* and the dye concentration determined spectrophotometrically at 605 nm in the aqueous layer.

Determination of alveolar bone level

Serial dental radiographs were taken of the incisor/canine/pre-molar/molar regions using a standardized periapical projection technique. On the radiographs, the ABL was assessed by measuring the distance in mm from the cement–enamel junction to the alveolar bone crest, i.e. the point at which the periodontal ligament space was considered to have a normal width (19). The measurements were made by the use of a magnifying lens (7 ×) to the nearest 0.5 mm at all mesial/distal tooth surfaces reproduced in the radiographs. The intra-examiner reproducibility of ABL measurements was determined by repeated assessments of 10 randomly selected subjects. Of the measurements, 96% were reproduced within a difference of ± 0.5 mm. The error of the method corresponded to 6% of the variance for the mean ABL in the population sample.

Statistical analysis

Statistical significance of differences was determined by analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison test. Pearson's analysis were done using GRAPHPAD Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA). The level of statistical significance is given when $p < 0.05$.

Results

Leukotriene B_4 levels

The salivary levels of LTB_4 are shown in Fig. 1A. As can be seen, patients with mild periodontitis showed a significant ($p < 0.001$) decrease in LTB_4 concentration with respect to all other groups, while patients with severe periodontitis had increased LTB_4 levels ($p < 0.001$). A significant positive correlation ($p < 0.0001$) was observed between age and LTB_4 salivary concentration (Fig. 1B). Sex did not influence the salivary LTB_4 level (male, 273.1 ± 20.2 pg/mL; female, 271.6 ± 34.6 pg/mL).

Relationship between salivary Leukotriene B_4 and clinical parameters

When it was analyzed the correlation between salivary LTB_4 concentration and the clinical parameters, PPD and CAL, only a good and positive correlation ($p < 0.0001$) was observed between LTB_4 and CAL (Fig. 2).

Clinical data and salivary mucin

Table 2 shows the data for bleeding on probing, PPD, CAL, ABL and salivary mucin concentration from all subjects studied, classified in to clinically healthy and with mild, moderate or severe periodontitis. According to the classification, all clinical parameters increased as the periodontal disease progressed. Salivary mucin, like LTB_4, was decreased ($p < 0.01$) in patients with mild periodontitis and increased in patients with moderate and severe disease ($p < 0.001$).

Relationship between leukotriene B_4 and mucin and alveolar bone level

With the aim of evaluating the relationship between salivary LTB_4 concentration with salivary mucin and ABL, Pearson's correlation analysis was carried out. As can be seen in Fig. 3, a significant ($p < 0.0001$) positive correlation was observed among the three parameters.

Discussion

In the present study, the levels of LTB_4 in saliva of patients with chronic periodontal disease were determined. Whole saliva was chosen because

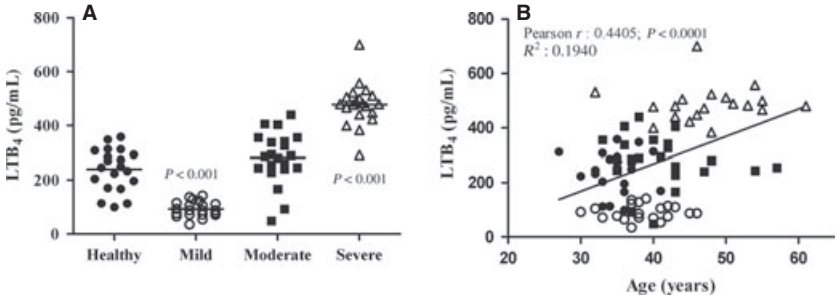

Fig. 1. (A) LTB_4 levels in saliva of subjects without periodontitis (healthy) and with mild, moderate and severe periodontal disease, *p* values are vs healthy group. (B) Univariate Pearson's analysis between salivary LTB_4 and age. LTB_4, leukotriene B_4; healthy, •; mild, ○; moderate, ■; severe, △.

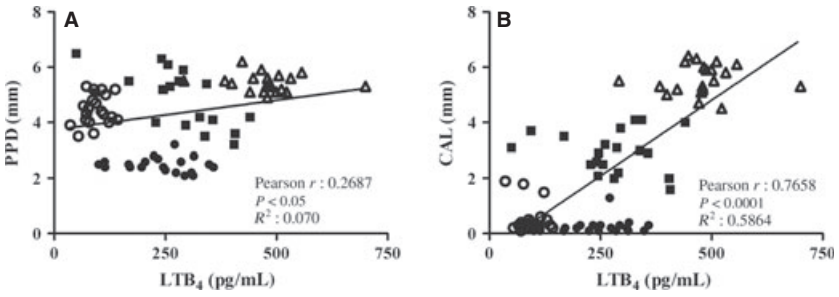

Fig. 2. Univariate Pearson's analysis between salivary LTB_4 and PPD in (A) and CAL in (B). CAL, clinical attachment level; LTB_4, leukotriene B_4; PPD, probing pocket depth; healthy, ●; mild, ○; moderate, ■; severe, △.

LTB_4 are involved in the defense mechanism of the oral cavity and thus, they could be produced not only in periodontal pockets but also may have another origin. The relationship between salivary LTB_4 concentration and salivary mucin and ABL was also examined in healthy subjects and in patients with mild, moderate and severe chronic periodontitis. The study included both male and female patients. There were fewer female participants because of their reluctance to participate. However, the statistical analysis showed no influence of sex on LTB_4 levels.

The results showed that patients with mild periodontitis had decreased levels of LTB_4. LTB_4 is involved in leukocyte chemotaxis, lysosomal enzyme secretion, neutrophil degranulation, adhesion molecule expression, defensins and nitric oxide production, phagocytosis, and other functions (20). Leukotrienes are produced during the interaction of phagocytes and microorganisms *in vitro* and in experimental infections *in vivo* (20). Pharmacologic or genetic approaches to reduce or block the leukotriene biosynthesis pathways decrease the phagocytic and antimicrobial activities against bacteria (21), fungi (22) and parasites (23,24). In addition, immunodeficient individuals, such as HIV patients, are characterized by low leukotriene production (25), which has been associated with impaired immune responses and infection control. Thus, it is possible that the low levels of LTB_4, in patients with mild periodontitis, may be related to a decrease in defense mechanisms that facilitate the beginning of the disease. The fact that mucin, known to be involved in the non-immunological defense mechanism of the oral cavity, was also decreased in these patients, support this hypothesis. In line with the results reported here, low LTB_4 levels in gingival crevicular fluid (15) or a depressed LTB_4 chemotactic response (26) have been found in patients with aggressive periodontitis. For ethical reasons patients with mild periodontitis received periodontal treatment, thus, the evolution of the disease could not be evaluated. The increase in LTB_4 levels in patients with moderate and severe periodontitis could suggest that, although the levels of LTB_4 were low at the beginning, they responded to the inflammatory stimulus and progress to the chronic form of the disease. On the other hand, a significant positive correlation was observed between LTB_4 level and age. Bearing in mind that participants in the mild group were younger than those in the moderate and severe groups, an influence of age on LTB_4 level could not be rejected. Correspondingly, aggressive periodontitis, which shows an altered production of LTB_4, is observed during teenage years (27).

The Pearson's analysis between LTB_4 and CAL showed significant positive correlation, as previously reported for mucin (28). This degree of correlation implies a relation between mucin secretion and the inflammatory process. As mucin is involved in the defensive response, it is possible to speculate that the increase in salivary LTB_4 concentration is also related to the defense mechanism. In this regard, a significant positive correlation between LTB_4 and mucin supports the above hypothesis.

A significant positive correlation between salivary LTB_4 and salivary mucin with ABL was observed. Because the ability of leukotrienes to induce bone resorption has been described (29), these results could imply a relation between LTB_4 and alveolar bone loss. The fact that salivary mucin also correlated with ABL, but mucin does not induce

Table 2. Data of BOP, PPD, CAL, ABL and salivary mucin concentration obtained from healthy subjects and in patients with mild, moderate and severe periodontitis

Sign/group	Clinically healthy	Mild periodontitis	Moderate periodontitis	Severe periodontitis
BOP (%)	0	8 ± 0.7***	18 ± 2***	22 ± 2***
PPD (mm)	2.49 ± 0.06	4.44 ± 0.12***	4.87 ± 0.2***	5.43 ± 0.07***
CAL (mm)	0.25 ± 0.06	0.53 ± 0.12	2.94 ± 0.16***	5.57 ± 0.12***
ABL (mm)	0.96 ± 0.04	1.28 ± 0.05**	1.50 ± 0.07***	2.28 ± 0.10***
Mucin (μg/mL)	1919 ± 93.3	1438 ± 145**	2575 ± 81.9***	3098 ± 47.6***

ABL, alveolar bone level; BOP, bleeding on probing; CAL, clinical attachment level; PPD, probing pocket depth.
The data are expressed as the mean ± SEM.
BOP is expressed as the percentage of positive sites.
**Significantly different from healthy subjects ($p < 0.01$).
***Significantly different from healthy subjects ($p < 0.001$).

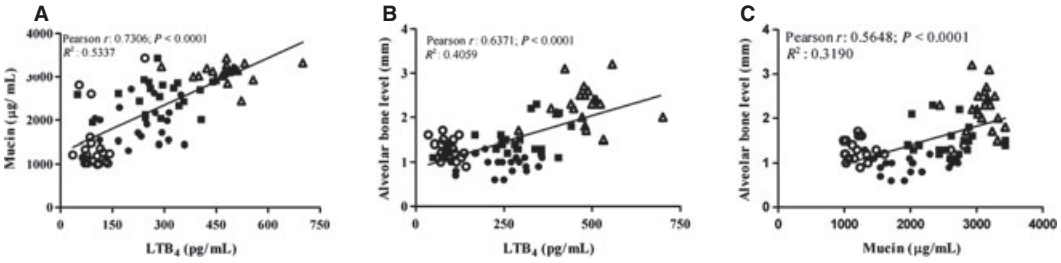

Fig. 3. Univariate Pearson's analysis between salivary LTB_4 and salivary mucin in (A), LTB_4 and alveolar bone level in (B) and salivary mucin and alveolar bone level in (C). LTB_4, leukotriene B_4; healthy, ●; mild, ○; moderate, ■; severe, △.

alveolar bone resorption, allows speculation that both mucin and LTB_4 increase in saliva as periodontitis progresses.

In conclusion, although further research is needed, these results give a new insight into the role of LTB_4 in periodontal disease.

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