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Expression of toll-like receptors 2, 4 and 9 is increased in gingival tissue from patients with type 2 diabetes and chronic periodontitis

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Background and Objective: Broad evidence indicates that diabetes both increases the risk and hastens the progression of periodontal disease. Likewise, chronic inflammation or infections seem to provoke insulin resistance and thereby contribute to the development of diabetes and its complications. Innate immune responses, which appear to be altered in individuals with diabetes, are usually mediated by the recognition of pathogens through toll-like receptors (TLRs). The constitutive expression of some TLRs has been reported in healthy human gingival tissue. Interestingly, the expression of TLRs 2 and 4 is increased with the severity of periodontal disease. Considering that the inflammatory reaction is exacerbated in individuals with diabetes and periodontitis, we suspected that the expression of some TLRs might be increased in gingival tissue in these patients.

Material and Methods: In this study, we analyzed, by immunofluorescence, the expression of TLRs 2, 3, 4 and 9 in gingival tissues from healthy individuals and from periodontal patients with or without type 2 diabetes.

Results: We found that the expression levels of TLRs 2, 3, 4 and 9 were higher in all periodontal patients than in healthy individuals. The expression of some TLRs was increased in subjects with periodontitis and diabetes relative to subjects with periodontitis but without diabetes; this increase in expression was found particularly in TLR2 and TLR9 in the connective tissue and in TLR4 at the epithelial region.

Conclusion: These data suggest that the expression of these TLRs 2, 3, 4 and 9 in gingival tissue is higher in individuals with diabetes because its inflammatory reaction is exacerbated. Additionally, the expression of these TLRS is positively regulated with the severity of periodontal disease.

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Diabetes is a chronic metabolic disease characterized by hyperglycemia that results in defects in insulin secretion, insulin properties, or both. The two main types of diabetes are classified primarily on the basis of their underlying pathophysiology. Type 1 diabetes, which constitutes approximately 5–10% of all cases, results from

autoimmune destruction of insulin-producing β-cells in the pancreas, leading to a total loss of insulin secretion, whereas type 2 diabetes, which accounts for approximately 90% of all cases, results from insulin resistance rather than from the total absence of insulin production (1).

Type 2 diabetes is highly prevalent, currently affecting over 217 million people worldwide; the prevalence is expected to increase rapidly in the coming decades and to reach at least 366 million by 2030 (2). The growing incidence of type 2 diabetes represents a significant burden to human health because of its numerous and often frequent complications. These include nephropathy, retinopathy, neuropathy, cardiovascular disease, peripheral vascular disease, stroke and periodontitis (3). Many factors are known to contribute to the development of diabetes and its complications. These include genetics, diet, sedentary lifestyle, perinatal factors, age and obesity. Nevertheless, the recent proposal that chronic inflammation or infection can provoke insulin resistance, thereby contributing to the development of diabetes and its complications, has gained interest (4).

Gingivitis and periodontitis are among the most common chronic infections worldwide (5). Periodontal disease is a microbe-induced chronic inflammatory condition that leads to destruction of the tooth-supporting tissues because it provokes gingival inflammation, periodontal tissue destruction and alveolar bone loss (6). A large evidence base suggests that diabetes is associated with an increased prevalence, extent and severity of periodontal disease (7). It has been argued that individuals with diabetes are more susceptible to infections, such as periodontitis, because diabetes alters the function of cells involved in the first line of host defense, including neutrophils, monocytes and macrophages. The adherence, chemotaxis and phagocytosis of neutrophils are often impaired (8–10). Additionally, monocytes from subjects with diabetes are pre-activated by hyperglycemia (11–14). However, diabetes-enhanced inflammation may affect the oral environment and increase the severity of periodontitis because when diabetic individuals become infected, they might present a more severe inflammatory reaction that provokes wider tissue destruction (15) and delays wound healing (16). In agreement with this proposal, an inflammatory state has been associated with diabetes because some markers of inflammation are elevated (17). High levels of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6, are present in subjects with diabetes (18). The concentrations of leptin, adiponectin, resistin (17) and the acute-phase proteins (19) are also elevated. The advanced-glycation end products (AGEs), which are augmented in diabetes (20), may also contribute to inflammation and infection. In addition, it has been suggested that inflammation may promote insulin resistance (21). Indeed, several of the molecules that are elevated in diabetes have been demonstrated to be responsible for inducing insulin resistance, such as TNF-α (22), resistin (23) and free fatty acids (FFAs) (24).

In general, this evidence indicates that diabetes may exacerbate periodontal disease through the upregulation of inflammatory mediators, indicating that the innate immune mechanisms are altered in diabetes. The innate immune response is usually mediated by the recognition of pathogens through a set of germline-encoded receptors that have evolved to recognize conserved pathogen-associated molecular patterns shared by large groups of organisms. It has been shown that this recognition is mediated mainly through a family of receptors, known as toll-like receptors (TLRs) (25), which are stimulated by different microbial stimuli (26). On binding the appropriate ligand, TLR signaling leads to the production of proinflammatory cytokines and to the up-regulation of costimulatory molecules (27). Therefore, TLRs also play a role in linking the innate and adaptive immune responses (28). These receptors are known to be expressed in cells of the immune system, in particular antigen-presenting cells, but they are also increasingly recognized as being expressed in other cells (29).

Certainly, several types of cells from the oral cavity express some TLRs along with other molecular pattern innate immune receptors. Indeed, oral epithelial cells constitutively express TLR2, TLR4, nod-like receptors (NOD)1 and NOD2 (30). The level of expression of *TLR2* and *TLR4* mRNAs in gingival tissues has been found to be increased with severity of the periodontal disease, as it was higher in gingival tissues from patients with chronic periodontitis than in gingival tissues from patients with gingivitis or from healthy controls (31). Studies have also shown that human gingival fibroblasts express TLR2 and TLR4, which are expressed at significantly elevated levels in inflammation (25). Immunohistochemical studies confirmed that the expression of some TLR is increased with the severity of periodontal disease. The localization of TLR2 and TLR4 has been demonstrated in healthy human gingival tissue; however, it has been observed that in the connective tissue zone subjacent to the epithelial pocket, the proportion of TLR-positive cells found in patients with severe chronic periodontitis is higher than found in patients with moderate or slight periodontitis (32).

Also, in a report where the immunohistochemical localization of TLRs 1–10 was studied, consistently higher TLR expression was found (particularly in the connective tissue) within the periodontitis group compared with the healthy group (33).

The above-mentioned studies indicate that the expression of some TLRs is elevated when the inflammatory reaction is higher, and that their expression seems to be positively correlated with the severity of periodontal disease. Various markers of inflammation are elevated in diabetes, and the inflammatory reaction is exacerbated in individuals with diabetes and periodontitis; therefore, we considered that in the gingival tissue of these patients the expression of some TLRs might be increased. Hence, in this study, we analyzed the expression of TLRs 2, 3, 4 and 9 in gingival tissues from healthy individuals and from periodontal patients, with or without type 2 diabetes, using immunofluorescence.

Material and methods

Patients and samples

Thirty subjects were selected from the population referred to the Dental Specialty Hospital 'Dr. Honorato Villa Acosta', in Mexico City, Mexico. Individuals recruited included 10 healthy patients without periodontal disease and type 2 diabetes, 10 patients with periodontal disease only and 10 patients with both periodontal disease and type 2 diabetes. In patients with chronic periodontitis, gingival samples were collected during routine periodontal flap operations after phase I (initial phase) conventional periodontal therapy, which includes scaling and root planing. Samples from the 10 healthy controls were obtained during tooth-extraction operations performed for fully impacted, retained wisdom teeth. The diagnosis of chronic inflammatory periodontal disease (CIPD) was based on the criteria of the American Academy of Periodontology classification of pathological values reported. In this study, inclusion criteria of chronic periodontitis comprised patients with at least 20 natural teeth; a minimum of six periodontal pockets > 4 mm that bled on probing; and clinical attachment loss of > 1 mm in combination with periapical radiographs revealing crestal bone loss, which was detected at a distance > 3 mm between the alveolar crest and the cemento–enamel junction around the affected teeth. The age range of patients with periodontitis was 37–72 years.

The inclusion criteria for healthy controls comprised the absence of clinical manifestations of periodontal disease, at least 20 natural teeth present, a normal oral glucose tolerance test result, fasting plasma glucose within the normal healthy range (60–110 mg/dL), no history of periodontal treatment and no history of systemic diseases. Age, gender and weight of healthy controls matched those of the subjects with periodontitis.

The inclusion criteria for patients with chronic periodontitis and type 2 diabetes mellitus were the following: male or female patients (37–72 years of age) with periodontitis and diagnosed with type 2 diabetes mellitus at least 6 months prior to the flap operation (diagnostic range 6 months to 12 years); and a fasting plasma glucose level of at least 7.8 mM. The mean fasting plasma glucose level in the type 2 diabetes mellitus group, recorded before phase I periodontal therapy, was 180 mg/dL. All subjects with type 2 diabetes mellitus were not well controlled (as they presented very high blood glucose levels) when they were recruited; however, at the time of the surgery, their periodontal clinical parameters had significantly improved as a result of the phase I therapy. Nevertheless, they still required surgery. With the exception of the inclusion criterion of a diagnosis of diabetes mellitus type 2 in the group with CIPD and type 2 diabetes mellitus, the exclusion criteria for all control and CIPD groups were identical; they comprised patients with systemic diseases, patients with evidence of modifiers of periodontal disease (type 1 and type 2 diabetes mellitus, osteoporosis and medications known to influence periodontal tissues), pregnant or lactating women and subjects who had taken systemic antibiotic, anti-inflammatory, hormonal or other assisted drug therapy in the 3 months prior to the start of the study.

The study was conducted in accordance with the World Medical Association's Declaration of Helsinki, and the research protocol was approved by the Local Ethical Committee at the UNAM FES Iztacala. Written informed consent was obtained from all study subjects before the sample tissues were acquired.

Periodontal evaluation

The periodontal evaluation was performed by a single-blinded, calibrated and well-trained examiner (34). The intraclass correlation coefficient at the site level ranged between 0.69 and 0.84 mm. All teeth present, with the exception of the third molars, were examined. Probing was made on six sites per tooth to examine periodontal clinical parameters (probing depth, bleeding on probing and clinical attachment level). To estimate the severity and extension of periodontal disease between patients with periodontitis, with and without diabetes, the mean values of clinical attachment level and the percentage of affected sites with attachment loss were recorded in patients with periodontitis at baseline and 4 weeks following phase I therapy.

Tissue processing

Gingival samples were washed with saline solution and fixed in 50 mL of a freshly prepared zinc salt-based fixative (ZSF) solution [0.1 M Tris base buffer containing 0.05% calcium acetate (pH 7–7.4), 0.5% zinc acetate and 0.5% zinc chloride], as previously described in the literature (35). The ZSF solution was replaced with fresh solution twice after 48 h. Then, tissue samples were processed using a routine histological technique, dehydrated in a graded alcohol series (70, 80, 90 and 100%), for 30 min each time, transferred to butanol for 24 h, immersed twice in paraffin for 24 h and finally mounted using Paraplast (Leica Microsystems AG, Wetzlar, Germany). Tissue slices, of 5–6 μm thickness, were cut on a microtome and mounted on poly-L-lysine-coated slides (Sigma Chemicals, St Louis, MO, USA).

Immunofluorescence

A direct immunofluorescence technique was used to detect TLRs on mounted sections of 5 μm thickness. Tissue sections were briefly deparaffinized in xylol and rehydrated in graded alcohols before antigenic recovery was performed with 5% urea in water for 30 min. Nonspecific binding was blocked with 7% bovine serum/0.05% Triton X-100 in 0.01 M phosphate-buffered saline (PBS), pH 7.0, for 1 h. Then, slides were incubated in 0.05% Triton X-100 (Bio-RadLaboratories Inc., Hercules, CA, USA) in PBS for 5 min. The tissue sections were incubated for 24 h at 4 °C with one of the two experimental antibody combinations – either (i) TLR2 and TLR4 or (ii) TLR3 and TLR9 – and at least two slides per sample were tested. We used

the following fluorochrome-labeled mouse (ms) or rat (rt) monoclonal antibodies (mAbs) against human TLRs: 1:50 dilution in PBS/0.07% Triton X-100 (v/v): (ms) mAb TLR2 (TLR2.3) phycoerythrin (PE)-labelled (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), (ms) mAb TLR4 (HTA125) fluorescein isothiocyanate (FITC)-labelled (Santa Cruz Biotechnology), (ms) mAb TLR3 (TLR3.7) FITC-labelled (Santa Cruz Biotechnology) and (rt) mAb TLR9 PE-labelled (eBioscience, San Diego, CA, USA). At the end of the incubation, the slides were washed and dehydrated in a graded alcohol series (70, 80, 90, 100%, for 3 min each) and mounted in Entellan (Merck, Darmstadt, Germany). Immunofluorescence images were acquired using a laser confocal scanning microscope (TCS/SP-22; Leica Microsystems AG). At least three representative images were captured and analyzed per slide.

We performed negative controls in each case. To set the capture parameters to levels at which the autofloresecence was eliminated from gingival tissue samples, the samples were processed using the same procedure described above; however, the antibody-incubation protocols were omitted. Thereafter, the experimental samples were examined using the set parameters. In addition, we performed control experiments for staining specificity; these included incubation with appropriate isotype-matched nonspecific antibodies or with nonimmune serum. No specific staining was seen in either case.

The images shown in the figures are representative for each case. They are maximum projections of five sequential optical sections (scanned 20 times each), collected at 0.5-μm intervals, and comprise the full thickness of the tissue section, captured using a 63×, 1.4-numerical aperture (NA) oil-immersion objective on the *Z*-axis direction.

We measured, using the aid of CONFOCAL LEICA software, the fluorescence intensity in five different gingival epithelial and connective tissue regions, from each optical section captured, per image. This was performed for all gingival tissue samples obtained from control and periodontal patients, with or without diabetes. The data obtained were normalized by area and analyzed using one-way analysis of variance followed by Bonferroni post-hoc tests using the GRAPHPAD PRISM software (GraphPad Software Inc., La Jolla, CA, USA). $p < 0.05$ was considered to be statistically significant.

Statistical methods

As it has been established that diabetes increases the risk of developing destructive periodontal disease and that the expression levels of TLR2 and TLR4 seem to correlate with the severity of the disease (31–33), we expected to find a higher expression of some TLRs in patients with diabetes. The sample size selected was calculated using the basic formula (36) set forth below; the required values were inferred from our own pilot study and from typical statistical tables:

$$n = \frac{2[z(1-\alpha/2) + z(1-\beta)]^2}{\Delta^2},$$

where $z(1-\alpha/2)$ and $z(1-\beta)$ represent percentage points of the normal distribution for the statistical significance level of 5%, $z(1-\alpha/2) = 1.96$ and a power of 80%, and $z(1-\beta) = 0.8416$; and $\Delta = \delta/s$ represents the standardized difference (i.e. the difference in fluorescence intensity of a TLR between healthy and periodontal patients divided by its standard deviation). The standardized difference (Δ) was estimated from our own preliminary study in which the fluorescence intensity of TLR2 was measured in the connective regions of five gingival tissue samples per group. We established that the mean fluorescence intensity of TLR2 presented by controls was 10.5 units, while the mean values found in patients with periodontitis with and without diabetes were 22.6 and 15.7 units, respectively. We considered that a difference of 5 units vs. the control may be considered a relevant effect δ, this delta value was estimated from the difference between the mean fluorescence intensity values: $\delta = \bar{X}_1 - \bar{X}_2$). On the other hand, we used a common standard deviation 's' of 4.03 between groups. Therefore, the standardized difference used in our study, $\Delta = \delta/s$, was $1.24 = 5/4.03$. Based on these calculations, it was decided that 10 subjects per group were necessary to provide 80% power with a significant level α-value set to 0.05.

Results

The expression of TLRs 2, 3, 4 and 9 was detected by immunofluorescence in gingival tissues from all healthy individuals or in gingival tissues from periodontal patients with or without diabetes. However, among the three groups, differences were found regarding the expression level, distribution and localization of the different TLRs analyzed. In general, the highest expression of TLRs was detected in samples from patients with periodontitis and diabetes, while the lowest expression was found in tissues from healthy individuals.

Histopathological characteristics

The top row of panels in Fig. 1 show representative gingival tissues from the three groups studied, which were stained with hematoxylin and eosin to determine the histological characteristics. In the healthy gingival tissue (left panel), a typical keratinized stratified squamous epithelium with minimal inflammatory infiltrate was observed. In the central and right panels, gingival tissues of patients with chronic periodontitis, without or with diabetes, respectively, are shown. In patients with periodontitis, with or without diabetes, keratinized stratified squamous epithelium showed changes such as edema and exocytosis. These observed changes seemed to be an effect of the chronic inflammatory infiltrate found predominantly in lymphocytes and plasma cells, which agrees with the high degree of inflammatory reaction reported in periodontal disease. The fibroblastic reaction was very similar in periodontal tissues of patients with or without diabetes; however, there was a more pronounced inflammatory response with increased vascular proliferation in tissues of patients with diabetes. As expected, we found that phase I periodontal

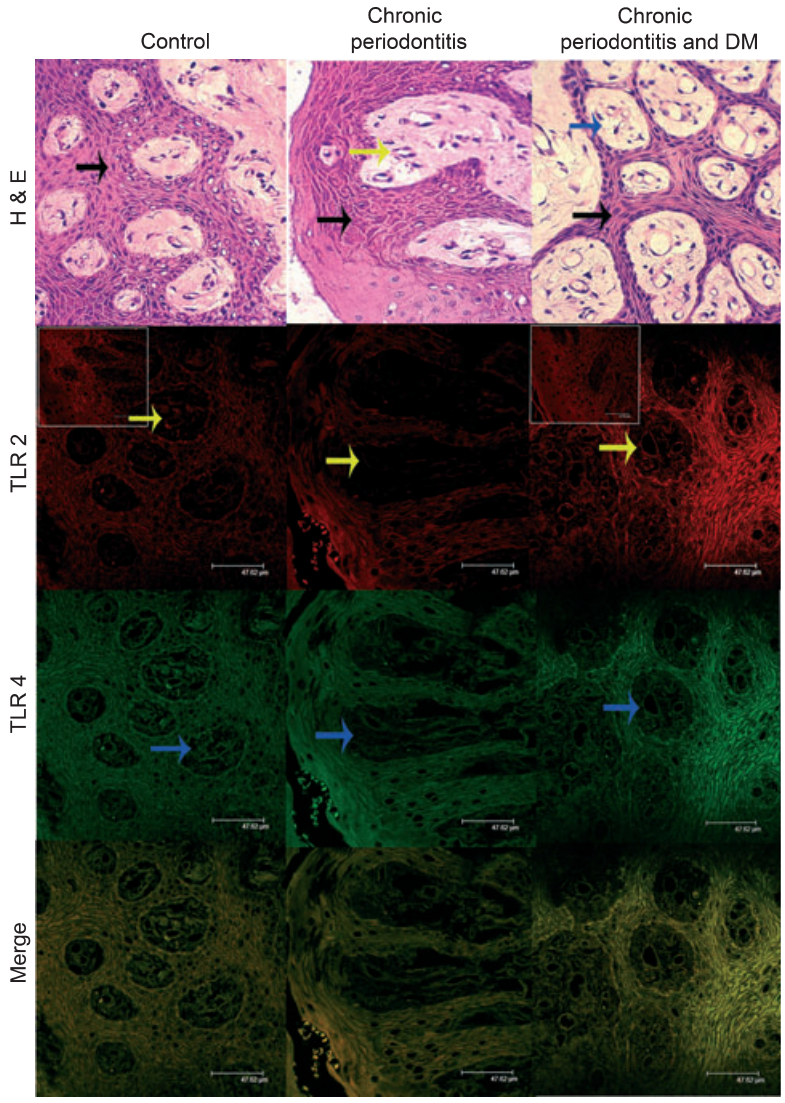

Fig. 1. Immunolocalization of toll-like receptors (TLRs) 2 and 4 in gingival tissue from healthy controls and from patients with chronic periodontitis, with or without type 2 diabetes mellitus (T2DM). From left to right, the images shown in the figure include the following: healthy controls, patients with chronic periodontitis and patients with chronic periodontitis and T2DM. Representative images of hematoxylin and eosin (H&E)-stained tissue sections are shown in the upper panels. Direct immunofluorescence staining for detection of TLR2 (red) and TLR4 (green) was performed in gingival samples as described in the Materials and methods, and representative images captured using a Leica confocal microscope are shown. Merged images in the bottom panels show colocalization (yellow) of TLR2 and TLR4 in gingival tissues. The black arrows indicate sulcular epithelial zones, yellow arrows indicate vascular endothelium and blue arrows indicate connective tissue. The images shown are maximum projections, captured with a 63×, 1.4-numerical aperture (NA) oil-immersion objective.

therapy, given to all patients with periodontitis, led to a significant reduction in periodontal clinical parameters (probing depth, bleeding on probing and a gain in clinical attachment level) ($p < 0.001$). Before and after phase I therapy, the severity and extension (percentage of diseased sites) of periodontal disease parameters (37) were still higher in patients with diabetes. Indeed, patients with diabetes presented significantly higher mean clinical attachment level values, along with higher percentages of sites with loss of attachment, before and after phase I therapy (Table 1).

Expression and localization of TLRs 2 and 4 in gingival tissue

The panels of the second and third rows in Fig. 1 show tissue sections, processed as described in Material and methods, to detect TLR2 (red) and TLR4 (green). The panels of the bottom row of Fig. 1 show the images merged (yellow) to determine the colocalization of TLR2 and TLR4. TLR2 expression in healthy gingival tissues was lower than in the tissues of patients with periodontal disease, with or without diabetes. In patients with periodontitis, TLR2 expression

Table 1. Severity and extension of periodontal disease between patients with and without type 2 diabetes mellitus (T2DM)

	Patients with CIPD ($n = 10$)	Patients with CIPD and T2DM ($n = 10$)	
	CAL[a] (mm), %SLA[b]	CAL[a] (mm), %SLA	p-value[c]
Before phase I	2.41, 62.91	3.42, 79.31	< 0.001
After phase I	2.02, 49.01	3.00, 69.85	< 0.001
p-value[d]	< 0.0001	< 0.002	

[a]CAL, clinical attachment level, mean values recorded 'before' (at baseline) and 'after' (4 weeks following phase I).
[b]%SLA, percentage of sites with loss of attachment.
[c]CIPD vs CIPD + T2DM.
[d]Before vs after phase 1, $N = 10$.

was higher in the epithelium, as shown in the inset panel (i.e. the upper left square), where expression of TLR2 is evident in the stratum granulosum and corneum. In tissues of patients with periodontal disease associated with diabetes, TLR2 expression was increased in the epithelium, connective tissue and vascular endothelium compared with the other two groups (healthy controls and periodontitis without diabetes). TLR4 expression in healthy gingival tissues was lower than in the tissues of patients with periodontal disease. TLR4 was expressed strongly in the epithelium and connective regions of gingival tissues from patients with periodontitis, specially in patients with diabetes. In the merged images (bottom panels), all demonstrate the colocalization of TLR2 and TLR4 in the epithelium, the connective tissue, as well as in the vascular endothelium of the gingival tissues. Additionally, the expression of TLR2 predominated in the connective tissue and vascular endothelium of the gingival tissues with greater intensity in patients with periodontitis and diabetes compared to healthy tissues and patients with periodontitis without diabetes mellitus (Fig. 1).

Expression and localization of TLRs 3 and 9 in gingival tissue

In the first and second rows of panels in Fig. 2, are shown tissue sections prepared, as described in Materials and methods, for the detection of TLR3 (red) and TLR9 (green). In the third row of panels in Fig. 2, the merged images demonstrate the colocalization of TLR3 and TLR9 (yellow).

In healthy gingival tissue, TLR3 is expressed more weakly in the epithelium and in the connective tissue than in gingival tissue from patients with chronic periodontitis. A strong expression of TLR3 was observed in patients with periodontitis with and without and diabetes; which was detected in the epithelium, connective tissue and vascular endothelium. TLR9 was expressed in the healthy gingival tissue, but at a lower level than in tissues from periodontal patients. TLR9 was expressed strongly in the epithelium of gingival tissues from patients with periodontitis, but weakly in the connective tissue. An important difference in the intensity of the expression of TLR9 in the epithelium, connective tissue and vascular endothelium was observed in the gingival tissues from patients with periodontitis and diabetes when compared with healthy tissue (Fig. 2).

When the expression of TLR3 and TLR9 was analyzed in the merged figures, we detected colocalization of both receptors in the epithelium, connective tissue and vascular endothelium in both healthy and periodontal gingival tissues. However, a higher intensity of TLR9 expression was found in the epithelium and connective tissue of patients with periodontitis, while a higher intensity of expression of TLR9 was found in the connective tissue and vascular endothelium of patients with periodontitis and diabetes (Fig. 2).

Analysis of TLR expression in gingival tissues

To estimate the expression level of TLRs, fluorescence intensity was measured using CONFOCAL LEICA software (arbitrary units) in the gingival epithelial (Fig. 3A) and gingival connective (Fig. 3B) tissue regions; this was performed for all gingival tissue samples, obtained either from control or periodontal patients with or without diabetes. The data obtained were analyzed with GRAPHPAD PRISM software, using one-way analysis of variance followed by the Bonferroni post-hoc test, to compare TLR expression among healthy controls and patients with periodontitis and diabetes. A p-value of < 0.05 was taken to be statistically significant. Marked differences were observed in the localization pattern and reaction intensity of the different TLRs analyzed between healthy and periodontal patients, but in general, the expression of every TLR analyzed was higher at the epithelial region than at the connective region in gingival tissues from all patients ($p < 0.0001$). However, as shown in Fig. 3, the gingival epithelial and gingival connective tissues of patients with periodontal disease (either with or without diabetes) displayed significantly higher levels of expression of TLRs 2, 3, 4 and 9 ($p < 0.0001$) with respect to tissues from healthy individuals. In gingival epithelial tissue (Fig. 3A), the expression level of the different TLRs was variable. The highest expression of TLR4 was found in the group with periodontitis and diabetes ($p < 0.05$). In contrast, the expression level of TLR2 was elevated in the group with diabetes relative to the healthy group ($p < 0.01$); however, its expression was similar to that found in the periodontitis group without diabetes ($p > 0.05$). The expression of TLR3 and TLR9 at the epithelium tended to be higher in the periodontitis group with diabetes than in the periodontitis group without diabetes; however, this difference was not statistically significant ($p > 0.05$). In the gingival connective tissue of patients with periodontal disease associated with diabetes, TLR2 and TLR9 were

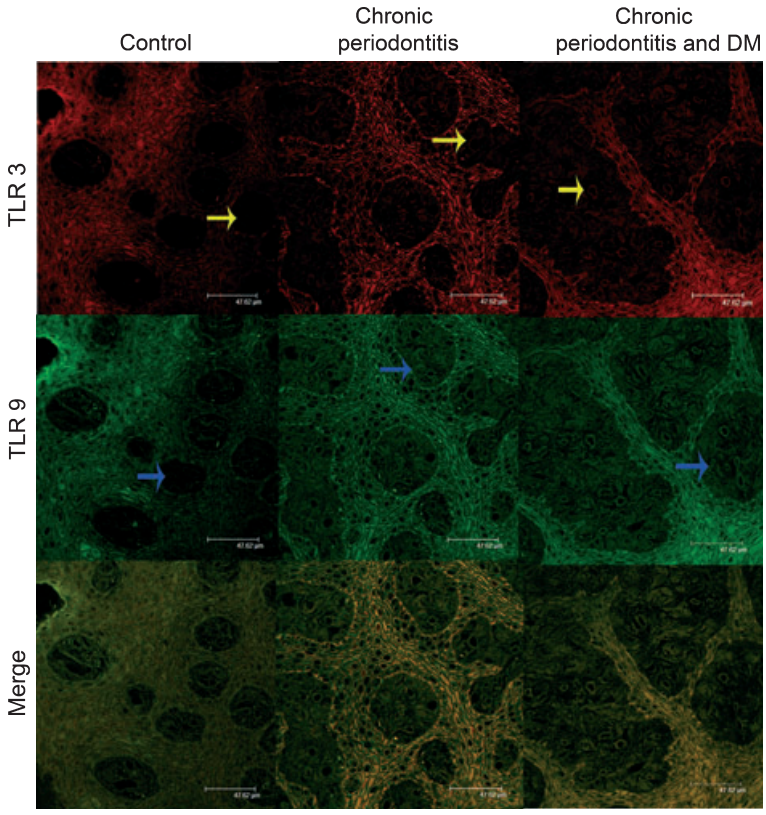

Fig. 2. Immunolocalization of toll-like receptors (TLRs) 3 and 9 in gingival tissue from healthy controls and from patients with chronic periodontitis with or without type 2 diabetes mellitus (T2DM). From left to right, the images shown in the figure include the following: healthy controls, chronic periodontitis and chronic periodontitis with T2DM. Direct immunofluorescence staining for detection of TLR3 (red) and TLR9 (green) was performed in gingival samples as described in the Material and methods, and representative images captured with a Leica confocal microscope are shown. Merged images in the bottom panels show colocalization (yellow) of TLR3 and TLR9 in gingival tissues. The black arrows indicate sulcular epithelial zones, yellow arrows indicate vascular endothelium and blue arrows indicate connective tissue. The images shown are maximum projections, captured with a 63×, 1.4-numerical aperture (NA) oil-immersion objective.

expressed at significantly higher levels than in gingival connective tissue of subjects with periodontitis but without diabetes (Fig. 3B). In contrast, the expression levels of TLR3 and TLR4 did not vary significantly between the periodontitis groups with and without diabetes ($p > 0.05$).

Discussion

The results of the present study showed, using confocal microscopy, that TLRs 2, 3, 4 and 9 are expressed in human gingival tissue. The results also confirmed and extended previous evidence showing the expression of diverse TLRs in the various types of cells in healthy human gingival tissue. This expression is significantly exacerbated in patients with periodontal disease. Furthermore, the present work shows, for the first time, that the expression of some TLRs is significantly increased in gingival tissue from patients with periodontal disease associated with type 2 diabetes, particularly TLR2 and TLR9 in the connective tissue and TLR4 at the epithelial region; this is with respect to tissues from subjects without diabetes and with or without periodontitis. These data suggest that the expression of these TLRs in the gingival tissue is higher in individuals with diabetes because an inflammatory reaction is exacerbated. It also demonstrates that the expression of these receptors is positively regulated with the severity of periodontal disease. Therefore, this study supports the possible involvement of the TLR system in the pathogenesis of periodontal disease.

Our data coincide with previous reports showing that the inflamed periodontium is infiltrated by TLR-expressing inflammatory cells, whereas healthy gingival tissue displays significantly lower levels of TLR expression (32,38,39). Likewise, our immunohistochemical data are in agreement with data showing that gingival epithelial cells and fibroblasts also express TLRs (39–42). Those previous reports have suggested that the expression levels of TLR2 and TLR4 in particular, may be related to the severity of periodontal disease. Accordingly, we also observed that the expression levels of these same receptors were increased in patients with periodontitis, but in addition, we noticed that TLR3 and TLR9 expression was also augmented in periodontitis. Moreover, we found that the expression levels of TLRs 2, 4 and 9 were even higher in individuals with diabetes than in those without diabetes. However, differences were found in the gingival region at which

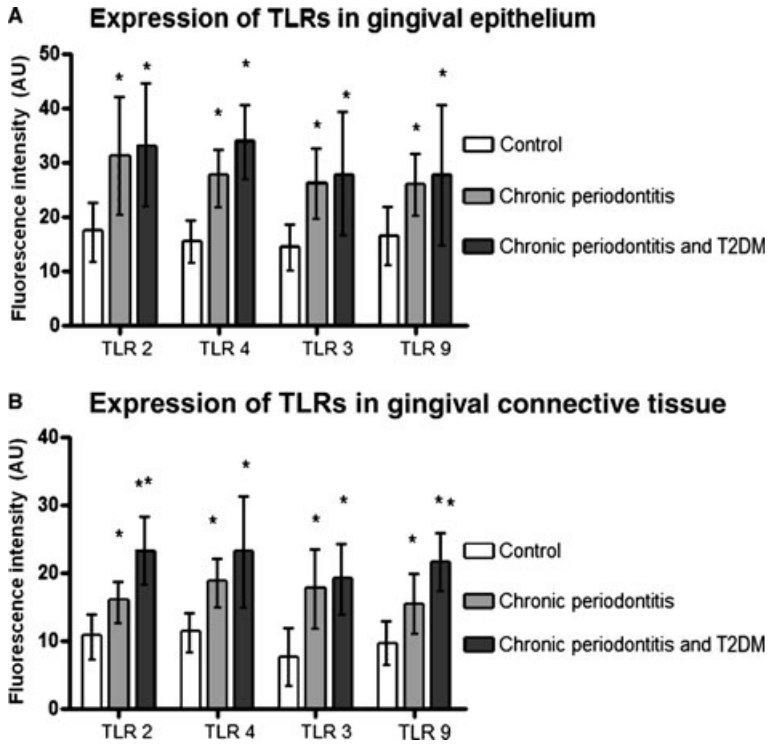

Fig. 3. Analysis of toll-like receptor (TLR) expression in gingival epithelium (A) and gingival connective tissue (B). Direct immunofluorescence staining for the detection of TLRs was performed in gingival samples as described in the Material and methods, and three images from each slide were captured using a confocal microscope. Fluorescence intensity (expressed in arbitrary units) was measured with CONFOCAL LEICA software in five different gingival epithelial tissue (upper panel) and gingival connective tissue (lower panel) regions per image. For all gingival tissue samples, the data obtained were normalized by area and analyzed using one-way analysis of variance followed by the Bonferroni *post hoc* test using the GRAPHPAD PRISM software. $p < 0.05$ was taken to be statistically significant. Data represent the mean fluorescence intensity ± standard deviation of epithelial and connective zones for each TLR analyzed. Significant differences are indicated as follows: *vs. healthy tissue; **vs. periodontitis without diabetes. AU, arbitrary units; T2DM, type 2 diabetes mellitus.

they were overexpressed; TLR2 and TLR9 were augmented only in the connective tissue, whereas TLR4 was increased particularly in the epithelium.

Therefore, our findings are consistent with the notion sustaining a correlation between TLR expression with disease severity activity or with the degree of inflammation because, as expected, we observed that the degree of inflammation and tissue damage was more severe in the gingival tissue of the group with diabetes than in individuals without diabetes. In accordance, TLR expression is also increased in a plethora of inflammatory disorders, including atherosclerosis and type 1 diabetes (12,43–45). However, this is the first study showing a higher expression of TLRs in type 2 diabetes.

Several explanations can be proposed to explain the differences in degree of inflammation and TLR expression seen between individuals with and without diabetes. In terms of function, TLRs (particularly TLR2, and to a much lesser extent TLR4) have been shown to regulate important immune and inflammatory responses to periodontal bacteria *in vivo* and *in vitro* (46–55). In fact, TLR2, rather than TLR4, is the predominant TLR involved in the recognition of *Porphyromonas gingivalis*, one of the species of bacteria most frequently associated with periodontitis. *P. gingivalis* is a gram-negative organism that expresses a lipopolysaccharide. Additionally, current evidence suggests that *P. gingivalis* may have also evolved ways to evade or subvert the TLR system, which is sensed primarily through TLR2, as shown *in vitro* and *in vivo* (48,51). In spite of those data, the role of TLR4 in periodontal disease cannot be ruled out because additional evidence, including the results of the present study, sustain that the expression level of this receptor is positively related to the severity of this disease (25,31,55). Although it is well known that TLRs are activated by both endogenous and exogenous agonists of microbial and nonmicrobial origin, the differences in expression of TLR2 and TLR4 between periodontal patients with and without diabetes, may more likely be related to differences in the host immunoinflammatory response, instead of being explained on the basis of differences of the microbial stimulus. This is thought to be true given that a similar composition of subgingival microbiota has been detected in subjects with periodontitis, with or without diabetes (3,56).

Although bacteria constitute an essential etiologic factor of periodontitis, alterations in the host inflammatory reaction to bacterial challenge seem to play a predominant role in

mediating periodontal tissue damage. An alternative explanation is that other, nonmicrobial, ligands may be activating TLRs. In fact, it has been proposed that the inflammation in individuals with diabetes is being exacerbated by nonmicrobial mechanisms. Additionally, it has been suggested that TLR4 may be a molecular link among nutrition, lipids and inflammation because TLR4 is the receptor for lipopolysaccharides and nutritional fatty acids, which demonstrate circulating levels that are often increased in obesity. This is recognized as the most important factor contributing to insulin resistance. Lipopolysaccharides and nutritional fatty acids can also activate TLR4 signaling in adipocytes and macrophages (57). Based on the evidence that TLR4 may link innate immunity and fatty acid-induced insulin resistance, we speculate that elevated levels of FFAs in individuals with diabetes may explain, in part, why the expression of TLR4 was higher in the epithelial gingival tissue of this group of patients than in the groups without diabetes. However, the presence of periodontopathogens is abundant and has been reported to be similar in both periodontal groups (56,58); thus, perhaps FFAs may not be participating as significantly as TLR ligands at the epithelial layer, where pathogen-associated molecular patterns may be the main TLR ligands. FFAs may instead contribute as ligands in the connective tissue in individuals with diabetes. However, although we observed that in the connective tissue the expression level of TLR4 tended to be higher in patients with diabetes, than in periodontal patients without diabetes, this difference was not significative. Interestingly, the expression and activity of TLRs can be differentially regulated by saturated and unsaturated fatty acids (59), suggesting the need for additional studies to determine whether fatty acids could serve as alternative therapies for inflammatory diseases, such as diabetes, in which the TLRs seem to be involved.

Even so, to clearly establish whether or not diabetes alters the expression of TLRs in periodontal tissues, and is dependent (or not) on the composition of the bacterial periodontal flora, further studies are still needed. Preferably these studies should include: (i) a group with diabetes without periodontal disease; (ii) the examination of additional parameters, such as analysis of the composition of periodontal biofilm; (iii) a higher number of patients and measurements of glycosylated hemoglobin (HbA1c) levels before and after phase I treatment; and (iv) the analysis of cytokine and *TLR* mRNA expression in frozen, not fixed, samples. We recommend particularly this last point, because we were unable to isolate RNA from the fixed samples in the current study to complement our immunohistochemical results (data not shown), even when following some recommendations indicated to improve the efficiency of RNA extraction from long-term preserved, buffered formalin-fixed, paraffin-embedded tissues (60). For example, tissues were digested with proteinase K at 55°C overnight, followed by treatment with DNase I and by incubation in Tris–EDTA buffer (pH 7.0) at 70°C for 1 h. Therefore, although fixation with standard zinc salts solution seems to be a good strategy for immunohistochemical applications, as our results indicate, it does not allow further RNA analysis, as the RNA is significantly degraded. However, recently it has been shown that a novel zinc-based fixative containing zinc trifluoroacetate, instead of zinc acetate, appears to be appropriate for RNA and protein preservation, but this has not yet been tested in long-term fixed tissues (61).

Connective tissue is composed primarily of fibroblasts that are also endowed with innate immune receptors, such as TLRs and NODs, and are capable of producing inflammatory cytokines (25,41); thus, this tissue can participate actively with recruited inflammatory cells in the inflammatory process associated with periodontal disease. In contrast, epithelial cells are in constant contact with commensal bacteria, and we found that they express significantly higher levels of TLRs than the connective tissue both in healthy and periodontal patients; it has been reported that upon recognition of their ligands, they produce antimicrobial peptides but do not produce significant levels of inflammatory cytokines (30). Therefore, the role of TLRs in gingival epithelial cells could be to sense and to differentiate pathogenic and commensal organisms, but they do not appear to contribute significantly to the inflammatory process. A possible biological significance of the difference found in epithelial and connective tissue regions in TLR expression may be related to periodontal bacteria and endogenous ligands triggering different responses, such as the various products released during the inflammatory process. Moreover, in patients with diabetes, additional TLR ligands, such as receptor for advanced glycation end products (AGEs), FFAs and high glucose concentrations (discussed below), may also contribute to the inflammatory response.

It is possible that the activation of oxidative stress provoked by hyperglycemia in the diabetic patients with periodontal disease in the present study, may also have induced the augmented expression of TLRs 2, 4 and 9 found in patients with diabetes. Several pieces of evidence suggest that oxidative stress plays a causative role in the pathogenesis of diabetes and its complications; it has been shown to increase insulin resistance both in animal models and in patients with diabetes. Additionally, it has been well demonstrated that chronic elevations of glucose and possibly of other metabolites, including FFAs (6), are the major factors responsible for the activation of oxidative stress. Even though the precise cellular mechanisms responsible for the generation of reactive oxygen species (ROS) in diabetic tissues are not entirely known, studies have shown a potential mechanism. In the absence of an appropriate compensatory response from the endogenous antioxidant network, the system becomes overwhelmed (creating a redox imbalance), leading to the activation of stress-sensitive signaling pathways, such as nuclear factor-κB (NF-κB), p38 MAPK, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/c-Jun),

protein kinase C (PKC), AGE/RAGE, sorbitol, and others (62). Interestingly, it has been recently shown that high glucose induces the expression of TLR2 and TLR4, via PKC-α and PKC-δ, respectively, by stimulating NADPH oxidase in human monocytes. Moreover, high glucose has been shown to induce inflammatory cytokines, chemokines, p38 MAPK, ROS, PKC and nuclear factor-κB (NF-κB), in both clinical and experimental systems (11,63–68).

However, in individuals with sustained hyperglycemia, proteins become glycolated to form AGEs (53), which serve as a major link between the various diabetic complications. Formation of AGEs also occurs in the periodontium, and their harmful effects on other organ systems may be reflected in periodontal tissues as well. Likewise, the receptor for AGEs is increased in gingival tissues of subjects with type 2 diabetes compared to controls without diabetes. (3) In addition to exogenous microbial products, TLRs can recognize endogenous ligands such as AGEs; therefore, it is also possible that these ligands might participate in the initial activation of TLRs before the onset of diabetes.

Although numerous mechanisms have been proposed to explain the impact of type 2 diabetes on the periodontium, inflammation seems to be a major component in the pathogenesis of diabetes and diabetic complications (4,7,69). A large body of evidence demonstrates that diabetes both increases the risk and hastens the progression of periodontal disease; interestingly, this high incidence has been seen to be reduced by the effective control of hyperglycemia (70,71). It has been shown that patients with diabetes and periodontal infection have a greater risk of worsening glycemic control in comparison to patients with diabetes, but without periodontitis (3,58). This has led to the proposal that the emergence of periodontitis may signal the onset of diabetes. Furthermore, a higher prevalence and severity of gingival inflammation and periodontal destruction is seen in patients with poor glycemic control. Conversely, periodontal intervention trials suggest that treatment of periodontal infection may have a positive effect on the diabetic condition, not only for maintenance of oral health but also for its possible effect on improving glycemic control and breaking this vicious cycle that worsen both diseases (3,7).

However, recent studies suggest that the relationship between periodontal disease and diabetes is complex. In fact, striking relationships between periodontal disease and the development of the vascular complications of diabetes, in particular cardiovascular and kidney disease (72,73), have been reported. Whether these relationships are caused primarily by the hyperglycemia that typically accompanies periodontal disease, or by other mechanisms, remains to be determined. Some proposed mechanisms for the link between periodontitis and the complications of diabetes include chronic systemic inflammation associated with increased concentrations of circulating cytokines and inflammatory mediators, direct infection of the vasculature extending beyond the oral cavity, an autoimmune response to the chronic periodontal infection that leads to endothelial dysfunction, or common susceptibility factors that lead to increased susceptibility to periodontal disease and to vascular diseases simultaneously (7,74). Perhaps several or all of these mechanisms are involved.

Various studies have demonstrated that elevated levels of inflammatory products in patients with diabetes, insulin resistance or obesity are associated with other risk factors. For example, these patients present high serum levels of the acute-phase reactants fibrinogen and C-reactive protein; proinflammatory cytokines, such as TNF-α, interleukin-1 and interleukin-6; vascular adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and E-Selectin; and chemokines, such as monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1 (MIP-1). Within these factors, TNF-α is the major cytokine responsible for inducing insulin resistance at the receptor level. TNF-α prevents autophosphorylation of the insulin receptor and inhibits second messenger signaling via inhibition of the enzyme tyrosine kinase (3). Furthermore, signaling through TLRs results in the activation of the transcription factor NF-κB which may lead to the transcription of proinflammatory cytokines, such as TNF-α (27). Therefore, the elevated expression of TLRs in the periodontal tissue of subjects with diabetes suggests that TLRs may participate in the induction and maintenance of a low-grade chronic systemic inflammation. Thus, they may play a critical role in the pathogenesis of diabetes and periodontal disease.

Additionally, in individuals with diabetes, gingival TLR expression and tissue damage may be exacerbated as a result of functional alterations of the infiltrating inflammatory cells. Indeed, the function of cells involved in the innate immune response, including neutrophils, monocytes and macrophages, is altered in many people with diabetes. It has been proposed that diabetes may result in the impairment of neutrophil adherence, chemotaxis and phagocytosis; this may facilitate bacterial persistence in periodontal pockets and significantly increase periodontal destruction (9,10). While neutrophils are often hypofunctional in diabetes, other immunoinflammatory responses are up-regulated in these patients. For example, in subjects with diabetes, macrophages and monocytes often exhibit an elevated production of proinflammatory cytokines and mediators, such as TNF-α, in response to periodontal pathogens; in the periodontal environment, this may increase host tissue destruction (11–14). Likewise, elevated TNF-α levels are found in the blood and gingival crevicular fluid of periodontal patients with diabetes. Moreover, the level of proinflammatory cytokines in the gingival crevice fluid has been related to the level of glycemic control in patients with diabetes (3).

Alternatively, periodontal bacteria may exacerbate diabetes through enhanced proinflammatory effects because they cause chronic activation of the innate immune system and play a major role in altering glucose tolerance. If the expression of TLRs is increased during prolonged inflammation or infection, as our results support, this increased expression may

increase the levels of inflammatory cytokines thought to promote hyperglycemia. We propose that the level of TLR expression, and thus the severity of periodontal disease, may be regulated through the ability of the TLR ligands to selectively modulate the TLR-mediated biological activity. However, further studies are needed to elucidate the precise role of TLRs on the pathogenesis of diabetes and periodontal disease.

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