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DNA methylation status of the *IL8* gene promoter in oral cells of smokers and non-smokers with chronic periodontitis

Naila F. P. Oliveira[1], Gilcy R. Damm[1], Denise C. Andia[1], Cristiane Salmon[2], Francisco H. Nociti Jr.[2], Sérgio R. P. Line[1] and Ana Paula de Souza[1]

[1]Laboratory of Molecular Biology, Department of Morphology, Division of Histology, School of Dentistry of Piracicaba, University of Campinas-UNICAMP, Piracicaba, SP, Brazil ; [2]Department of Prosthodontics/Periodontics, Piracicaba Dental School, University of Campinas-UNICAMP, Piracicaba, SP, Brazil

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Abstract

Aim: This study analysed the status of DNA methylation in the promoter region of the *IL8* gene in oral mucosa cells from healthy, smoker and non-smoker subjects with chronic periodontitis and compared these findings among groups with mRNA levels.

Material and Methods: Genomic DNA from epithelial oral cells of 41 healthy subjects, 30 smokers with chronic periodontitis and 40 non-smokers with chronic periodontitis were purified and modified by sodium bisulphite. Genomic DNA from blood leucocytes and gingival cells from biopsies of 13 subjects of each group were also purified and modified by sodium bisulphite. Modified DNA was submitted by methylation-specific polymerase chain reaction (PCR) (MSP), electrophoresed on 10% polyacrylamide gels and stained with SYBR Gold. Total RNA from gingival cells was also isolated using the TRIzol reagent, and real-time PCR performance was used to detect the levels of interleukin-8 mRNA.

Results: Our results indicate that individuals with chronic periodontitis, independent of smoking habit, have a higher percentage of hipomethylation of the *IL8* gene than those controls in epithelial oral cells ($p < 0.0001$), and expression of higher levels of interleukin-8 (IL-8) mRNA than controls in gingival cells ($p = 0.007$). No significant differences among groups were observed in gingival cells and blood cells.

Conclusion: We conclude that inflammation in the oral mucosa might lead to changes in the DNA methylation status of the *IL8* gene in epithelial oral cells.

Key words: epigenetic; IL-8; inflammation; methylation; periodontitis; smokers

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Chronic periodontitis is an infectious disease characterized by a destructive inflammatory process that affects the supporting tissues of the tooth, and promotes loss of the soft connective tissues and alveolar bone (Williams 1990). These events are dependent on a combination of associated factors, including the individual's genetic and epigenetic characteristics, which creates the clinical phenotype.

The early stage of periodontal disease is characterized by an intense accumulation of inflammatory cells in the gingival tissue (Williams 1990, Seymour et al. 1996), which contribute to later degenerative events. It is recognized that interleukin-8 (IL-8) is one of the most potent factors responsible for inducing neutrophil chemotaxis, directing the migration of these cells to the inflammation site (Remick 2005). IL-8 is constitutively produced and secreted by a wide range of cells such as macrophages, monocytes, fibroblasts and epithelial cells. The production of IL-8 can be induced by multiple stimuli including lipopolysaccharide (LPS), live bacteria and early proinflammatory cytokines (Remick 2005). IL-8 seems to play a relevant role in tissue destruction

Conflict of interest and source of funding statement

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in periodontal disease. High levels of IL-8 mRNA were observed in a culture of human gingival fibroblasts after treatment with IL-1β and epidermal growth factor, showing that these cells are able to respond after stimuli, releasing IL-8 (Yucel-Lindberg & Brunius 2006). The production of this cytokine is stimulated by periodontopathogens (Gursoy et al. 2008), and the levels of IL-8 are increased in the crevicular fluid and tissues in patients with peridontitis (Tsai et al. 1995, Gamonal et al. 2000, 2001).

DNA methylation is a crucial mechanism in the control of gene activity and nuclear architecture. Dinucleotides CpG ''methylable'' are not randomly distributed in the human genome; they appear in CpG-rich regions denominated as CpG islands, which are present in the promoters of many genes. Some transcriptional factors (i.e., Sp1) can interact only with non-methylated DNA sequences, whereas methylation of cytosine abolishes interactions (Clark et al. 1997). This in turn leads to less effective transcription of certain genes or even silences the gene. Thus, methylation defects include genome hypomethylation (resulting in epigenetic activation of proto-oncogenes and retroelements) and localized aberrant hypermethylation of CpG islands, resulting in transcriptional repression of many important genes as tumour suppressor genes (Ting et al. 2006).

Several authors have reported an aberrant methylation status in genes associated with oral cancer (Kulkarni & Saranath 2004, Kato et al. 2006), lung cancer (Blanco et al. 2007), oesophagi cancer (Eads et al. 2001, Guo et al. 2006, Ishii et al. 2007) colon cancer (Lee et al. 2004, Takahashi et al. 2006) and gastric cancer (Esteller et al. 2001a; Kang et al. 2003a; Chang et al. 2006).

Although alterations in the methylation pattern have been extensively implicated in cancer processes, recent studies have shown that changes in the methylation status of ILs and other inflammatory factors can modulate the inflammatory process (Abolhassani et al. 2008, Nile et al. 2008). In fact, aberrant methylation has been detected in some inflammatory diseases such as gastritis (Kang et al. 2003b), cystic fibrosis (Shuto et al. 2006, Furuta et al. 2008) and chronic kidney disease (Stenvinkel et al. 2007). It is also known that the expression of IL-8 can be modulated by the methylation status of its promoter (De Larco et al. 2003).

It is well known that smoking is a risk factor for periodontal disease (Brothwell 2001). Likewise, recent experimental studies have shown that tobacco smoking can alter the methylation status of gene promoters. Nicotine can directly induce methylation in DNA (Marsit et al. 2006, Soma et al. 2006). Additionally, changes in the promoter methylation of the TSLC1/IGSF4 tumour suppressor genes have been associated with a poor prognosis (Kikuchi et al. 2006). Therefore, the aim of the present study was to investigate the methylation status in the gene promoter of IL-8 in cells of the oral epithelium, gingival tissue and blood leucocytes of healthy subjects and to compare it with the findings in cells of smokers and non-smokers with chronic periodontitis. We also analysed the levels of IL-8 mRNA in these groups.

Material and Methods

Subject population

This study was approved by the Institutional Review Board of the Piracicaba Dental School, University of Campinas. All patients were informed about the nature of the proposed treatment, and informed consent forms were signed. A convenience sample of unrelated subjects >25 years old, male and female, was recruited for the study. The patients were from the Southeastern region of Brazil. All subjects were in good general health and had at least 20 teeth in the mouth. All subjects were subjected to anamnesis and to clinical and periodontal examination. Exclusion criteria included any systemic disorder that would affect the periodontal condition (with the exception of smoking), current pregnancy or lactation and patients who had used systemic antibiotics or anti-inflammatory medication within 6 mo before baseline. The smoking habit was recorded, and patients who had consumed five cigarettes/day for at least 5 years were classified as smokers. Only patients who had never smoked were included in the nonsmoker group. The diagnosis and classification of disease were made on the basis of clinical parameters and consisted of a physical examination, medical and dental history, probing depth, assessment of attachment loss [clinical attachment loss (CAL)], tooth mobility and observation of gingival bleeding on probing. Measurements of probing depth and attachment level were recorded at six points around each tooth. Subjects were included in clinical categories according to periodontal disease and smoking habit:

(1) *Healthy group (never smoked)*: Subjects found to exhibit no signs of periodontal disease as determined by the absence of CAL and no sites with probing depth >3 mm.
(2) *Non-smoking chronic periodontitis (never smoked)*: Subjects with at least three teeth exhibiting sites $\geqslant 5$ mm CAL, in at least two different quadrants.
(3) *Smoking chronic periodontitis*: Subjects with at least three teeth exhibiting sites $\geqslant 5$ mm CAL, at least two different quadrants and had consumed five cigarettes /day for at least 5 years.

Table 1 shows the clinical and demographic characteristics of the participants of the study.

Biopsy collection

Gingival biopsies were obtained from single teeth from subjects undergoing periodontal surgery for periodontal disease-related (test groups) and non-disease-related reasons (control group). Following blocks anaesthesia, tissues on the tooth surface where periodontal pockets were evaluated were incised to include the entire soft tissue walls of the pockets to be investigated. Entire pocket,

Table 1. Mean values and standard deviation for the clinical parameters in the selected sites, and demographic and behavioural data in smokers and non-smokers

	Control ($n = 41$)	Smokers ($n = 30$)	Non-smokers ($n = 40$)
Age (years)	46.2 ± 14.1	47.03 ± 6.49	44.94 ± 9.17
% men	33.4	55.8	40.5
% women	66.6	44.2	59.5
Probing depth (mm)		7.8 ± 2.02	7.9 ± 2.18
Cigarettes/day		15.6 ± 7.32	
Cigarette consumption (in years)		22.3 ± 9.26	

junctional epithelia and connective tissues were removed. Then, the samples were divided into groups.

DNA extraction and bisulphite modification

Samples of epithelial oral cells were collected by mouthwash with dextrose 3% and the DNA was purified using 8 M ammonium acetate and 1 mM EDTA according to Aidar & Line (2007). DNA from gingival cell were obtained from biopsies and stored in a tube containing RNAholder (Bioagency, São Paulo, SP, Brazil) and frozen at -70°C until analyses. Total DNA was purified using the TRIZOL reagent (Invitrogen, Carlsband, CA, USA) following the manufacturer's recommendation. DNA from leucocytes was purified after red cell haemolysis according to Aidar & Line (2007) to be used as an endogenous control of methylation status. Then, 1 μg of purified DNA was modified by sodium bisulphite, whose principle exploits the different sensitivities of cytosine and 5-methylcytosine (5-MeC) to deamination by bisulphite under acidic conditions, in which cytosine undergoes conversion to uracil while 5-MeC remains unreactive (Herman et al. 1996). DNA already modified by sodium bisulphite was purified the using Wizard SV Gel and polymerase chain reaction (PCR) Clean-Up System kit (Promega Corporations, Madison, WI, USA) and stored at -70°C. One sample was methylated in vitro using 3 U of the recombinant *SssI* CpG methylase (New England Biolabs, Mississauga, ON, USA) in the presence of 160 μM S-adenosylmethionine at 37°C overnight to serve as a control of the methylated reaction.

Methylation-specific PCR (MSP)

Differences in DNA sequences after treatment with sodium bisulphite were detected by MSP. A fragment of 173 bp was amplified with specific primers for either methylated (foward 5′aaaattttcgttatatttcg3′/reverse 5′tccgataacttttttatatcat3′) or unmethylated (forward 5′aaaatttttgttatattttg3′/reverse 5′tccaataacttttttatatcat3′) targets (GenBank accession number M28130). Each MSP reaction incorporated 100ng of bisulphite-modified DNA, 1 μM of each primer and 1 × Go Taq Green Master Mix (Promega Corporations) in a final reaction of 25 μl. The methylated and unmethylated cycle conditions were as follows: 95°C × 5 min.; 30 cycles × (95°C × 45 s, 47°C × 45 s, 72°C × 45 s); and 72°C × 7 min. Amplified PCR samples were carried on 10% polyacrylamide gels and subjected to electrophoresis. DNA bands were detected after SYBR Gold stain (Invitrogen).

Bisulfite sequencing

Some samples were subjected to automatic sequencing to verify the specific methylated and unmethylated amplified sequences. After MSP, fragments were eluded from agarose gel, purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega Corporations) following the manufacturer's recommendation and subjected to automatic sequencing in 20 μl containing 1 μl of DNA, 3 μM of sense primer and 8 μl of Big Dye Terminator Ready v.2.0 (Applied Biosystems, Foster city, CA, USA).

RNA extraction

Gingival biopsies were collected and immediately stored in a tube containing RNAholder® (Bioagency) at room temperature for 24 h. Then samples were frozen at -70°C until analyses. Total RNA was purified using the TRIzol reagent (Invitrogen) following the manufacturer's recommendation.

Real-time PCR

Reverse transcription

One microgram of total RNA was treated with DNase (Invitrogen) and 500 ng was used for cDNA synthesis. The reaction was carried out using the First-strand cDNA synthesis kit (Invitrogen) following the manufacturer's recommendations.

Reverse transcription PCR

A fragment of 172 bp using specific primers (forward 5′gccaagagaatatccgaactttaat3′/reverse 5′ctggctagcagactaggg3′) was amplified by a quantitative PCR that was performed in the LightCycler® system (Roche Diagnostics GmbH, Indianapolis, IN, USA) using the Fast-Start DNA Master Plus SYBR Green kit (Roche Diagnostic GmbH.). The reaction product was quantified using the Relative Quantification tool (LIGHTCYCLER® Software 4; Roche Diagnostics GmbH), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene.

Statistical analysis

The difference in the methylation status in the groups was compared by the χ^2 test at a level of 5%. With respect to gene expression analysis, an inter-group analysis was performed using the non-parametric Kruskal–Wallis test at the level of 5%, followed by Dunn's test when differences were detected. The linear correlation between methylation status and IL-8 expression was tested by the Spearman coefficient.

Results

Automatic sequencing confirmed the methylated and unmethylated sequences amplified by MSP (Fig. 1). The results of DNA purified from epithelial oral cells showed that the majority of individuals in the control group are positive for both conditions, methylated and unmethylated, in the *IL8* gene. However, a high percentage of subjects of the periodontitis groups, smokers and non-smokers have epithelial cells positive only to the unmethylated condition. Practically all subjects in the healthy control group were methylated in contrast to only 23% and 20% of subjects of periodontitis smoking and periodontitis non-smoking groups, respectively (Fig. 2). This occurred independent of age, and the difference between control

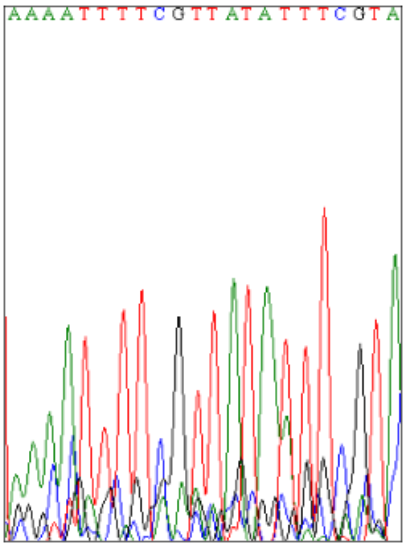

Fig. 1. CpG-methylated sites in the *IL8* promoter confirmed after automatic sequencing.

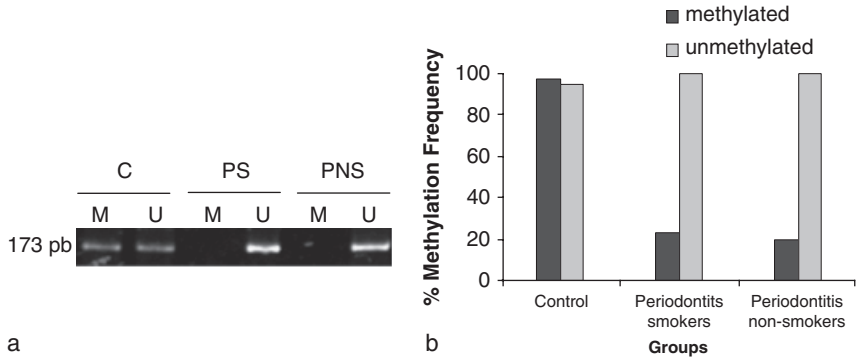

Fig. 2. (a) Bands of Interleukin-8 (IL-8) methylation-specific polymerase chain reaction. C, control; PS, periodontitis smokers; PNS, periodontitis non-smokers; M, methylated; U, unmethylated. (b) Methylation frequency of the *IL8* gene promoter in oral epithelial cells of healthy subjects and subjects with chronic periodontitis (control group, $N = 41$; periodontitis smokers $N = 30$; periodontitis non-smokers, $N = 40$).

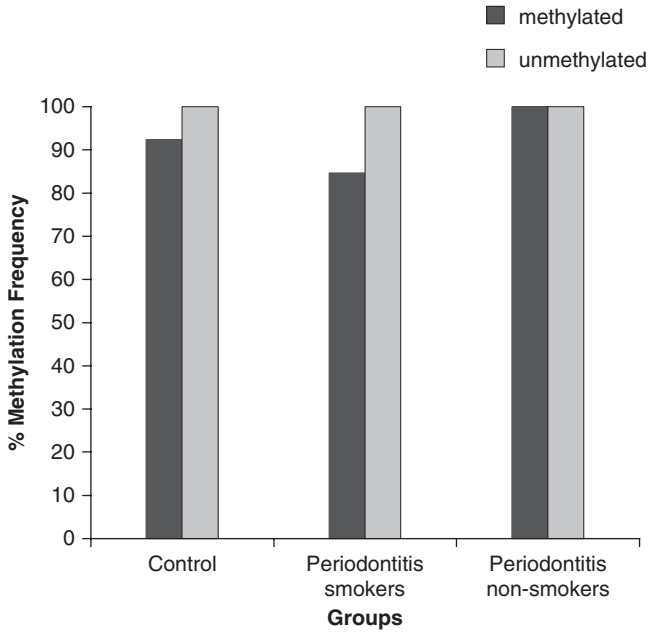

Fig. 3. Methylation frequency of the *IL8* gene promoter in gingival cells of healthy subjects and subjects with chronic periodontitis (control, $N = 13$; periodontitis smokers, $N = 13$; periodontitis non-smokers, $N = 13$).

and periodontitis groups was strongly significant ($p < 0.0001$; χ^2 test).

The same was not observed for DNA purified from gingival tissue cells and blood leucocytes. No difference was observed between the methylated and the unmethylated conditions for the *IL8* gene promoter. The majority of the subjects were positive for both conditions in the three different groups (Figs 3 and 4).

Data for gene expression analysis demonstrated that IL-8 mRNA levels were higher in the periodontitis than in the healthy group (Fig. 5). This difference was statistically significant ($p = 0.007$; Kruskal–Wallis test).

Discussion

Epigenetics highlights how habits such as smoking and alcohol drink, diet, environment and infections may after the tissue behaviour. Studies of DNA methylation patterns have emerged as an important field of research in several pathologies, if not all diseases including chronic periodontitis, because this event may modify the genetic transcription dramatically. The transcriptional repression caused by DNA methylation seems to be mediated by the methyl binding protein (MBP) family such as MeCP1 and MeCP2 (Wade 2001). While MeCP1 requires multiple methylated CpG sites to bind to DNA and promote chromatin condensation, the MeCP2 protein is able to bind to only one methylated CpG site. It is known that the interaction between only one CpG site and DNA enhancer elements can be enough to modify gene transcription (Bird 1986). The promoter of IL-8 is not a typical CpG island promoter as it does not contain repeat sequences of CpG dinucleotides. The CpG sites are sparse in the *IL8* gene promoter. It is worth mentioning that most studies on DNA methylation are focused on gene promoters containing multiple CpG sites and that the analysis of the methylation status in promoters containing sparse CpG sites has been largely neglected.

The CpG dinucleotides selected in the present study are located between nucleotides -136 and $+43$ of the *IL8* gene promoter. It contains a basal modulator transcription region, consisting of four *cis*-regulator elements, an NF-κB (-82 to -70), a CCAAT/enhancer-binding protein (-94 to -84) and AP-protein (-126 to -120)-binding sites. The fourth *cis*-regulator element is located between nucleotides -90 and -83, being activated by the Oct-1 factor, which down-regulates the *IL-8* transcription (de Larco et al. 2003). These sites are located near TATA and CCAAT boxes, which are important for the initiation of transcription.

Most individuals in the control group showed an *IL8* methylation status positive to both conditions, methylated and unmethylated, in epithelial oral cells, gingival tissue cells and blood cells, while a high frequency of periodontitis group individuals showed completely unmethylated epithelial oral cells. The differences between healthy and diseased individuals were probably due to the differential response of distinct cell types to products of bacterial infection or inflammation. While buccal mouthwashes contained mainly epithelial cells (Aidar & Line 2007), gingival tissues include not only epithelial cells but also fibroblasts, blood cells, macrophages and some other cell types. It is worth mentioning that basal cells that give rise to oral epithelium remain in the oral cavity during the individual's whole life, while connective tissue fibroblasts, inflammatory and blood cells present in periodontal tissues are continuously renewed. Thus, the long-term exposure of basal epithelial cells to bacterial products and inflammatory cytokines may be a possible cause of the specific

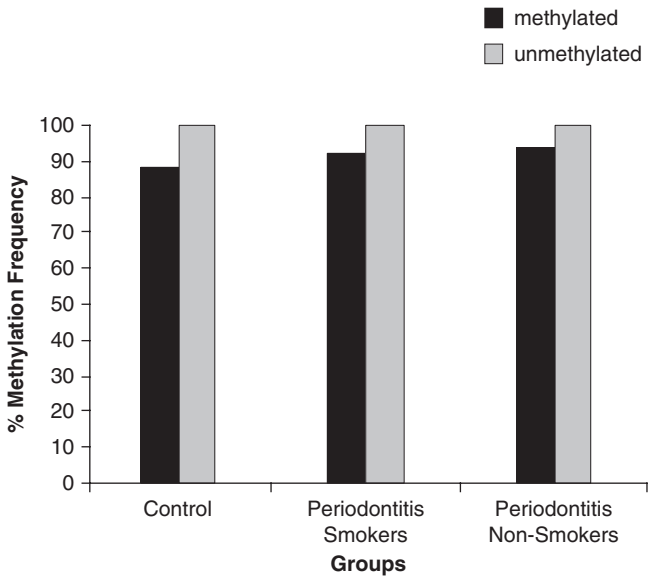

Fig. 4. Methylation frequency of the *IL8* gene promoter in leucocytes from the peripheral blood of healthy subjects and subjects with chronic periodontitis (control, $N = 13$; periodontitis smokers, $N = 13$; periodontitis non-smokers, $N = 13$).

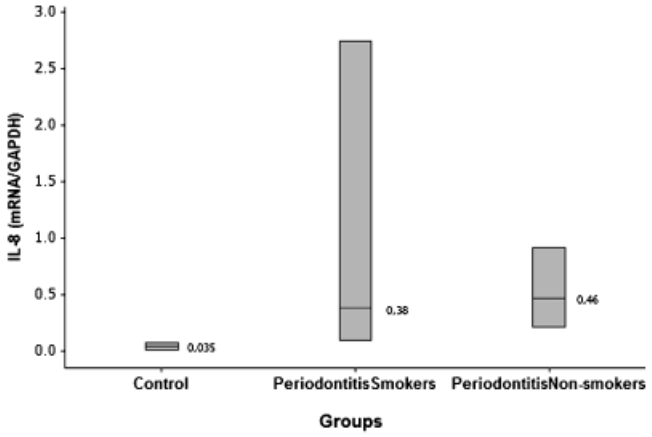

Fig. 5. Interleukin-8 mRNA levels from gingival cells of healthy subjects and subjects with chronic periodontitis (control, $N = 13$; periodontitis smokers, $N = 13$; periodontitis non-smokers, $N = 13$).

loss of methylation observed in this tissue. It is also important to mention that besides buccal epithelial cells, the salivary rinses could also contain some other cells such as neutrophils and monocytes, especially in patients with chronic peridontitis. It seems, however, that this is a minor contamination as it was not detected in the PCR reactions, where DNA was unmethylated.

The relationship between gene methylation and local pathogens has been reported. One of the examples is the association of *Helicobacter pylori* (*H. pylori*) presence and modified methylation status in gastric cancer. The *H. pylori* infection increases the risk of methylation in genes responsible for tumour development, including transcription erasing of the CDH1 gene (Ushijima 2007). In this context, oral infection may also lead to epigenetic alterations, locally within gingival tissue or more globally, with widespread effects (Offenbacher et al. 2008). The synthesis of IL8 can be regulated by virulence factors produced by bacterial cells. The synthesis of IL8 by human fibroblast cells is enhanced by the forsythia detaching factor, a cysteine protease produced by periodontopathogen *T. forsythia* (Tomi et al. 2008). Interestingly, incubation of oral epithelial cells with the *Porphyromonas gengivalis* virulence factors lysine-specific gingipain (Kgp) and high-molecular-mass arginine-specific gingipain (HRgpA) resulted in a decrease in the production of IL8, while arginine-specific gingipain 2 (RgpB), from the same source, increased IL-8 production (Uehara et al. 2008). Therefore, it is plausible to assume that the methylation of IL8 and other cytokines, in diverse cell types present in gingival tissues, may be influenced by bacterial virulence factors. In fact, bacterial lipopolysacharide, a known potent inflammatory agent, was shown to induce de novo methylation in the CpG sites of mouse embryonic cells (Tatemichi et al. 2008). We predict that future investigation on this subject may help to unravel the molecular mechanisms by which bacterial cells modulate periodontal inflammation.

Cigarette smoke is known to induce global hypomethylation (Smith et al. 2007). Interestingly, in the present study, smoking was not found to interfere with the loss of methylation in the cells collected in the mouthwash. Previous studies also shown inconclusive results on the risk of smoking on predisposed epigenetic modification in lung cancer (Guo et al. 2004). Other authors have suggested that a clear correlation between methylation status in certain genes and tobacco may only be observed in heavy smokers who have a tobacco history of more than 50 years and who started cigarette smoking at an early age.

Finally, we wonder whether tissue inflammation could be a consequence of the methylation status modifications or a cause of it. Fifty years ago, Slaughter et al. (1953) reported that ''field cancerization'' could appear in oral-stratified squamous epithelium cell sites before oral carcinoma development. Today, it is known that ''field cancerization'' represents sites that contain genetic and epigenetic alterations. ''Field cancerization'' has been described in gastric mucosa (Maekita et al. 2006), colorectal cancer developed from ulcerative colitis (Issa et al. 2001), Barret oesophagus (Eads et al. 2000), lung epithelium (Guo et al. 2004), chronic hepatitis (Kondo et al. 2000), kidney disease (Arai et al. 2006) and breast cancer (Yan et al. 2006). It is thought that at least some types of inflammation might lead to alterations in the epigenetic regulation (Ushijima 2007). Therefore, the role of periodontal inflammation in the development of epigenetic abnormalities in oral mucosa must not be discarded.

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Address:
Ana Paula de Souza
Laboratory of Molecular Biology
Department of Morphology
Division of Histology
School of Dentistry of Piracicaba
University of Campinas
Av. Limeira 901
CEP 13414-018
Piracicaba
SP
Brazil
E-mail: anapaulapardo@fop.unicamp.br

Clinical Relevance

Scientific rationale of for the study: The diagnosis and prognosis of periodontal inflammation are closely linked to a combination of associated factors that create the clinical phenotype. The biology architecture of this phenotype includes inflammatory and immunologic responses against a microbial composition of biofilms that are orchestrated by individual genetic and epigenetic compositions. Alterations in the DNA methylation status represent an epigenetic event that may modify the genetic transcription drastically. Several authors have reported an aberrant methylation status in genes associated with cancer and there is increasing evidence that DNA methylation is critical for regulating the inflammatory response in a dynamic manner. We have investigated the methylation status in several candidate genes implicated in the host response to oral microbial infection and/or involvement in the destruction of periodontal tissues during inflammation. In the present study, we present the results found in the *IL8* gene promoter in samples of non-smoker healthy subjects and smokers and non-smoker subjects with chronic periodontitis.

Principal findings: Our results showed that the *IL8* gene promoter shows a decrease in the methylation status in CpG sites in epithelial cells of the oral mucosa of individuals with chronic periodontitis, independent of smoking habit. The same was not observed in gingival cells and blood cells.

Practical implications: These results show for the first time epigenetic changes in oral cells affected by chronic periodontitis and this may provide new insights into the role of inflammation in oral biology. It may help to understand the molecular events that are occurring in the oral cells and may aid the development of new methods of diagnosis and treatment in the future.