The Profile of Inflammatory Cytokines in Gingival Crevicular Fluid around Healthy Osseointegrated Implants

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ABSTRACT

Objective: Regardless of gingival health and subgingival microbiology, production of cytokines within peri-implant tissues may be different from that of teeth. The objective of this study was to describe the peri-implant levels of pro-inflammatory cytokines and subgingival microbiology in clinically healthy sites.

Materials and Methods: Subgingival plaque and gingival crevicular fluid (GCF) were obtained from 28 clinically healthy implants and 26 teeth selected from 24 individuals. Microbial composition was determined by selective anaerobic culture techniques. Pro-inflammatory cytokines were quantified by flow cytometry analysis of GCF. The concentration of cytokines between implants and teeth were compared with the independent *t*-test.

Results: The concentration of cytokines was higher in GCF from healthy implants than in teeth. The profile of cytokines was characteristic of an innate immune response. A more frequent detection of periodontopathic bacteria was observed in teeth than implants. Cultivable levels of periodontopathic bacteria were similar between implants and teeth.

Conclusions: Despite gingival tissue health and scarce plaque accumulation, the profile of inflammatory cytokines in implant crevicular fluid was distinctive of an innate immune response and in higher concentration than in teeth. Other than bacterial stimulus, intrinsic factors related to implants may account for more cytokine production than teeth.

KEY WORDS: cytokines, gingival crevicular fluid, osseointegrated implants, peri-implant crevicular fluid

INTRODUCTION

Cytokines are important modulators of both normal and pathologic processes within the periodontium. In fact, the same cytokines are released during normal turnover and during inflammatory events in gingival connective tissue and in bone tissue. For example, Interleukin (IL)-1β, tumoral necrosis factor (TNFα), receptor activator for nuclear factor κB ligand (RANK-L), IL-6, IL-8 are all released by both events in these tissues.[1,2]

The main inflammatory event observed around teeth and implants is plaque-induced inflammation. The continuous balance between the host immune response and potential subgingival pathogens soon determines the clinical condition around osseointegrated dental implants. As early as 1 week after implant placement, a complex subgingival microbiota can be observed.[3,4] Peri-implant connective tissues respond to bacterial accumulation with an inflammatory reaction.[5,6] However, over-production of pro-inflammatory cytokines can alter connective and bone tissue metabolism[7,8] and release of tissue damage markers such as aspartate aminotransferase and alkaline phosphatase.[9,10] Actually, during plaque accumulation within a 21-day

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period, gingival inflammation developed around teeth and implants. The results showed that the levels of IL-1β increased over time.[11] Additionally, a recent study[12] showed that there is an increased gene expression of IL-12, TNFα and RANK-L as inflammation increases and that specific cytokine expression can affect the severity of peri-implant disease. Because IL-1β, TNFα and RANK-L are important modulators of bone and connective tissue metabolism, the study provides evidence supporting that these inflammatory mediators may lead to both periodontal and peri-implant tissue breakdown.

Overproduction of cytokines is clear when clinical inflammation is evident, but the profile and levels of cytokines released under normal conditions are still unknown. The study of inflammatory markers in peri-implant crevicular fluid (PICF) around healthy implants would help establish the baseline level for comparisons during different stages of peri-implant disease. In a previous investigation,[13] we analyzed the production of cytokines (IL-1β, TNFα, IL-6) around clinically healthy teeth and dental implants and examined their relationship to putative periodontal pathogens. Although no specific microbiologic profile was observed and no viral activity (HCMV, Human Cytomegalovirus) detected, teeth allowed for more colonization by *Porphyromonas gingivalis*, *Tanerella forsythia*, *Fusobacterium sp.* Microscopic structural differences between dental and implant surfaces could account for this finding. Although a tendency to greater cytokine production was observed around implants in contrast to teeth, a specific explanation for this finding is not available.

In conditions with minimal bacterial accumulation (clinical health), inflammatory cytokines may still be produced in substantial amounts[14,15] and the result of this reaction should be further investigated. Structural and biologic conditions around implants are different from that of natural teeth; therefore, the aim of the present study was to describe the levels of inflammatory cytokines and subgingival microbiology in clinically healthy implant sites as compared with sites in clinically healthy teeth.

MATERIALS AND METHODS

The study protocol was approved by the Institutional Review Board at the University of Southern California (USC). The study sample comprised patients from the USC Advanced Periodontics Clinics who had previously received dental implants. Informed consent and Health Insurance Portability and Accountability Act (HIPAA) information were given and signed.

Patients were partially edentulous and exhibited the presence of one or more clinically healthy dental implants in function. At least one tooth and one implant in each patient were included based on the following conditions: absence of clinical inflammation and bleeding on probing, probing depth ≤4 mm and no bone loss beyond the first thread of the implant or bone loss around teeth assessed by radiographs.

The instruments used to determine the clinical parameters for healthy implants and teeth included the CP12 (Hu-Friedy, Chicago, IL, USA) periodontal probe and conventional peri-apical radiographs taken in a parallel cone manner.

All patients were in good systemic health. Patients were excluded if they had any of the following criteria: periodontal and/or implant signs of disease (bleeding on probing, clinical attachment loss >3 mm, pocketing >4 mm, radiographic bone loss), were pregnant or lactating, taking antibiotics three or less months, taking nonsteroidal anti-inflammatory drugs 2 weeks prior to sample collection. The same clinician conducted all examinations and samplings of the patients.

Microbiologic Sampling and Analysis

Supragingival plaque was carefully removed using a gauze and the sites isolated with cotton rolls. Three sterile paper points were placed around implants and teeth until resistance was felt and kept in place for 30 seconds. Care was taken in order to avoid bleeding during sampling. Paper points were immediately transferred to a vial containing VMGA III and processed for bacterial culture at the Oral Microbiology Testing Laboratory at USC within 24–48 hours at 25°C.

Samples were analyzed for the presence of periodontopathic bacteria, according to methods by Slots.[16] Briefly, the samples were incubated in a CO_2 anaerobic culture and brucella blood agar medium was incubated at 35°C in an anaerobic jar for 7 days. The TSBV medium was incubated in 10% CO_2 at 37°C for 4 days. Presumptive identification of the following periodontopathogens were performed to previous methods described by Slots: *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia/nigrescens*, *Tannerella forsythia*, *Campylobacter sp.*, *Eubacterium sp.*, *Fusobacterium sp.*, *Micromonas micros*,

Eikenella corrodens, *Capnocytophaga sp.*, *Dialister pneumosintes*, Gram negative enteric rods, Staphylococci spp., and yeasts. The values are expressed as the frequency detection of each pathogen and levels as the mean percentage (%) ± standard deviation in positive sites.

Gingival Crevicular Fluid (GCF) Sample Collection and Analysis

GCF around implants (PICF) and teeth (GCF) were collected using periopaper strips (Oraflow Inc., NY, USA). Immediately after subgingival plaque sampling, isolation with sterile gauze was performed for all sites sampled to prevent saliva from contaminating the strips and paper points. Sterile periopaper strips were placed for 1 minute into the mesio-buccal and disto-buccal implant sites and at the mesio-buccal and disto-buccal sites of healthy appearing teeth. The strips were carefully placed into sterile 1.5-mL volume low protein-binding centrifuge tubes pre-labeled and previously filled with 100 µL sterile phosphate buffered saline. These were immediately placed on dry ice during transportation and stored at –70°C until flow cytometry processing.

A cytometric bead array (BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit, BD, San Jose, CA, USA) was used for the detection and quantification of the following cytokines: interleukin-8 (IL-8), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor α (TNFα), and interleukin-12p70 (IL-12p70). Data was acquired in a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Before processing for multiplex cytokine array assay, the periopaper strips were incubated at 4°C for 12 hours and then vortexed in a cold room (4°C) for 20 minutes. Samples were processed according to manufacture's instructions. Values were expressed as pg/mL.

Statistical Analysis

Clinical and demographic data are presented as the mean ± standard deviation. The concentration of cytokines is depicted as the mean ± standard error of the mean. Differences for the concentration of cytokines between implants and teeth were assessed with the independent *t*-test. The detection of each pathogen is presented as the frequency of detection (%) and the cultivable microbiota as the mean % ± standard deviation in positive sites. The frequency detection of microorganisms was assessed with the chi-square test and the cultivable levels with the Kruskall-Wallis test. Statistical significance was assumed when $p \leq .05$. Analysis of data was conducted using statistical package (SPSS v 15, Inc., Chicago, IL, USA).

RESULTS

Table 1 describes the demographic and clinical parameters of study sample. A total of 24 subjects, with a mean age of 57 years, participated in the study. In general, 28 implants of different types and 26 teeth were selected for sampling. Gingival tissues around implants and study teeth were free of clinical signs of disease. Radiographic images around implants and teeth showed no signs of bone loss.

The concentration of cytokines in peri-implant and GCF is depicted in Table 2. An overall trend for higher values was observed around implants. The highest value corresponded to IL-8 in implants as compared with teeth and this difference was statistically significant. The concentration of TNFα was two-fold higher around implants than around teeth ($p < .05$). Although IL-6 was four times higher around implants compared with when around teeth, the difference did not reach statistical significance.

TABLE 1 Demographic and Clinical Data of Study Sample

Parameter	Subjects	
Number	24	
Age, mean ± SD	57 ± 10	
Gender	Female 15/Male 9	
	Implants	Teeth
Number	28	26
Implant type (n)	Nobel Biocare SG (2)	
	3i Osseotite XP (10)	
	3i Osseotite NT (2)	
	3i Osseotite Certain (5)	
	3i Osseotite (2)	
	3i Osseotite Micromini (1)	
	3i Osseotite ex Hex (1)	
	Straumann Standard Plus RN (1)	
	Straumann Standard Plus WN (4)	

Nobel Biocare, Yorba Linda, CA, USA.
3i, Palm Beach Gardens, FL, USA.
Straumann, Andover, MA, USA.
SD: standard deviation.

TABLE 2 Quantification of Cytokine Concentration in Peri-Implant and Gingival Crevicular Fluid

Cytokines	Implants pg/mL ± SEM	Teeth pg/mL ± SEM	$p < .05$
IL-8	2,486.19 ± 481.61	1,470.55 ± 165.70	0.001
IL-1β	596.03 ± 106.62	509.44 ± 99.36	0.600
IL-6	33.104 ± 20.41	8.02 ± 2.08	0.061
IL-10	5.81 ± 1.24	4.37 ± 1.22	0.109
TNFα	4.36 ± 0.72	1.92 ± 0.23	<0.001
IL-12	1.22 ± 0.20	1.27 ± 0.23	0.533

Cytokine values are expressed as the mean (pg/mL) ± SEM.
SEM, standard error of the mean.

While the frequency detection of important periodontopathic bacteria (*P. gingivalis*, *T. forsythia*, *Fusobacterium sp.*) was higher in teeth than in implants, cultivable levels were similar (Table 3). Although mean percentages of the cultivable microbiota in positive sites were comparable between implants and teeth, a tendency for more cytokines was observed in implants (Table 2). No statistically significant differences for the subgingival microbiota were observed between groups.

DISCUSSION

This study determined the concentration of proinflammatory cytokines in PICF and GCF under clinically healthy conditions. The clinical appearance of gingival surrounding tissues in implants and teeth suggested a clinically healthy state. However, the subclinical inflammatory state that has been proposed can only be confirmed by histologic methods. It is accepted that the constant colonization of bacteria in the gingival sulcus induces the stimulation of epithelial, connective tissue and immune cells.[17] Although important periodontal pathogens such as *P. gingivalis*, *T. forsythia*, and *P. intermedia* were detected in subgingival biofilm samples, they are not correlated to periodontal/peri-implant status. Enteric rods were also detected but their role in periodontal disease is still unknown. Because of the cross-sectional limitations of this study, it is not possible to ascertain if transient colonization was a factor.

TABLE 3 Frequency Detection and Cultivable Subgingival Microbiota around Implants and Teeth

Microorganism	Implants		Teeth	
	Frequency (%)	% ± SD Microbiota Positive Sites	Frequency (%)	% ± SD Microbiota Positive Sites
Aggregatibacter actinomycetemcomitans	0	0	0	0
Porphyromonas gingivalis	7.1	4.2 ± 0.6	15.3	4.2 ± 1.2
Prevotella intermedia	7.1	5.4 ± 1.1	7.6	6.5 ± 2.1
Tannerella forsythia	14.2	4.6 ± 1.4	26.9	4.2 ± 1.7
Campylobacter sp.	10.7	5.4 ± 0	19.2	5.6 ± 1.6
Eubacterium sp.	3.6	3.1	7.6	6.2 ± 1.1
Fusobacterium sp.	10.7	5.1 ± 1.2	26.9	5.3 ± 1.9
Micromonas micros	3.6	4.6	11.5	3.5 ± 1.1
Enteric rods	14.3	9.2 ± 3.3	15.3	8.2 ± 4.2
Eikenella corrodens	0	0	0	0
Dialister pneumosintes	0	0	3.8	2.3

The cultivable microbiota is presented as the mean % ± SD in positive sites as explained in Materials and Methods.
SD, standard deviation.

The cytokines studied here correspond to the innate immune response and have a great impact in promoting inflammation.[18] It is not known what the threshold level of these cytokines is in a healthy periodontium. In contrast, it is known that the concentration increases with clinical signs of peri-implant and periodontal disease in comparison with less inflamed samples, indicating an association between clinical inflammation and cytokine production.[7,8,11,12]

Cytokines are polypeptides acting as molecular messengers that communicate information between cells and are categorized as interleukins, growth factors, chemokines, and interferons. In a cytokine dose–response curve, there may be no effect below a certain concentration; however, after this level, an exponential increase in response could occur, followed by a plateau or reduced response of cytokine concentration. The concentration of the cytokines studied here reflects a state of immune response to bacterial accumulation capable of establishing a balance between the host and bacteria. Innate response cytokines are necessary for the recruitment of polymorphonuclear leukocytes (PMNs) (IL-8) and subsequent stimulation of monocytes, macrophages, and PMNs (IL-1β, TNFα). Vascular changes are also related to these cytokines (IL-1β, TNFα), allowing for the transmigration of inflammatory cells within connective tissues and through the sulcus.[18] It is interesting to note a decreasing gradient in the production of cytokines, which would be biologically plausible for the response observed (Table 2, IL-8 > IL-12). First, IL-8 induces chemotaxis of PMNs while IL-1β and TNFα prepare blood vessels for the diapedesis of cells (PMNs, monocytes, T cells) to the connective tissue and sulcus.[17] Depending on the concentration, IL-6 may act as anti-inflammatory by inhibiting the production of IL-1β and TNFα in conjunction with IL-10. In addition, the detection of IL-12 suggests that a Th1 response may be in action. A Th1 response is necessary for the control of extracellular pathogens and for improving the innate cellular response (phagocytosis) and protective immunoglobulins.[19–21] The sequential events related to these cytokines, reveals a proper environment that would be observed when the immune system is appropriately controlling the bacteria and hence, periodontal/peri-implant health. However, a careful analysis of IL-1β and TNFα is necessary because they are important stimulants of osteoclastic activity in diseased sites.[22–24] Within the limits of the present study, it is not possible to know whether the values detected (cytokine production pg/mL) could indicate that osteoclastic activity is present because absolute zero (0) values were not observed and clinical and radiographic parameters suggested health. On the other hand, when plaque matures, the balance between the host/bacteria is broken. Higher values of pro-inflammatory cytokines and clinical signs of inflammation are evident. Nevertheless, the concentration of cytokines needed to indicate that a specific site is in jeopardy of losing attachment and bone is unknown.

Whether the placement of dental implants induces an immune reaction that may be responsible for some of the clinical failures is debatable.[25] For many years, titanium has been considered as the most biologically compatible material for the fabrication of implants of different kinds. This study found higher concentrations of cytokines around implants than around teeth. A reaction to titanium molecules, difference in peri-implant anatomy, and plaque accumulation may account for this phenomenon. Nonetheless, implants were as healthy as one can expect under good clinical circumstances. Therefore, it is likely that the results may be a reaction to the implant that has no significant implications for peri-implant health. Animal studies have found macrophage accumulation and fibrous collagenous tissue surrounding titanium subcutaneous discs.[26] Another study in rats showed that intravenously administered titanium dioxide (TiO_2, 5 mg/Kg) produced neither toxicity nor elevation on cytokines[27] suggesting its biocompatibility. But in the case of connecting dental implants to the oral environment under function and plaque accumulation, the scenario may be different. Because implants are not entirely inert, a slow liberation of titanium molecules is possible[28] and in conjunction with bacterial accumulation, this would give a conceivable explanation for the results observed here.[25]

The results from the present study showed that with almost undetectable biofilm accumulation and the surrounding tissues clinically healthy, there is a release of cytokines into the sulcus. Of interest was that higher cytokine levels were observed around implants as compared with around teeth, even though cultivable counts of bacteria were almost equal. This finding could be explained by the many unique properties of implants (eg, surface characteristics, chemical composition) and their distinct relationships with surrounding bone and a marginal implant–gingivae interface (junctional

epithelium) that is exposed to the oral environment. These exceptional differences between implants and teeth may create environmental changes (eg, deeper sulcus) that could modify the production of cytokines.

CONCLUSIONS

The subgingival microbiota around clinically healthy implants presented similar composition to teeth sites. In contrast, the frequency detection of periodontopathic bacteria was higher in teeth as compared with implants. Regardless of gingival tissue health and scarce plaque accumulation, the profile of inflammatory cytokines in implant crevicular fluid was distinctive of an innate immune response and in higher concentration than in teeth. Other than bacterial stimulus, intrinsic factors related to implants may account for more cytokine production.

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REFERENCES

1. Bartold PM. Turnover in periodontal connective tissues: dynamic homeostasis of cells, collagen and ground substances. Oral Dis 1995; 1:238–253.
2. Chau JF, Leong WF, Li B. Signaling pathways governing osteoblast proliferation, differentiation and function. Histol Histopathol 2009; 24:1593–1606.
3. Quirynen M, Vogels R, Pauwels M, et al. Initial subgingival colonization of "pristine" pockets. J Dent Res 2005; 84:340–344.
4. Quirynen M, Vogels R, Peeters W, van Steenberghe D, Naert I, Haffajee A. Dynamics of initial subgingival colonization of "pristine" peri-implant pockets. Clin Oral Implants Res 2006; 17:25–37.
5. Tillmanns HW, Hermann JS, Tiffee JC, Burgess AV, Meffert RM. Evaluation of three different dental implants in ligature-induced peri-implantitis in the beagle dog. Part II. Histology and microbiology. Int J Oral Maxillofac Implants 1998; 13:59–68.
6. Nociti FH Jr, Cesco De Toledo R, Machado MA, Stefani CM, Line SR, Gonçalves RB. Clinical and microbiological evaluation of ligature-induced peri-implantitis and periodontitis in dogs. Clin Oral Implants Res 2001; 12:295–300.
7. Cochran DL. Inflammation and bone loss in periodontal disease. J Periodontol 2008; 79(Suppl):1569–1576.
8. Graves D. Cytokines that promote periodontal tissue destruction. J Periodontol 2008; 79(Suppl):1585–1591.
9. Paolantonio M, Di Placido G, Tumini V, Di Stilio M, Contento A, Spoto G. Aspartate aminotransferase activity in crevicular fluid from dental implants. J Periodontol 2000; 71:1151–1157.
10. Paknejad M, Emtiaz S, Khoobyari MM, Gharb MT, Yazdi MT. Analysis of aspartate aminotransferase and alkaline phosphatase in crevicular fluid from implants with and without peri-implantitis. Implant Dent 2006; 15:62–69.
11. Schierano G, Pejrone G, Brusco P, et al. TNF-alpha TGF-beta2 and IL-1beta levels in gingival and peri-implant crevicular fluid before and after de novo plaque accumulation. J Clin Periodontol 2008; 35:532–538.
12. Duarte PM, de Mendonça AC, Máximo MB, Santos VR, Bastos MF, Nociti FH Jr. Differential cytokine expressions affect the severity of peri-implant disease. Clin Oral Implants Res 2009; 20:514–520.
13. Nowzari H, Botero JE, DeGiacomo M, Villacres MC, Rich SK. Microbiology and cytokine levels around healthy dental implants and teeth. Clin Implant Dent Relat Res 2008; 10:166–173.
14. Sakai A, Ohshima M, Sugano N, Otsuka K, Ito K. Profiling the cytokines in gingival crevicular fluid using a cytokine antibody array. J Periodontol 2006; 77:856–864.
15. Teles R, Sakellari D, Teles F, et al. Relationships among gingival crevicular fluid biomarkers, clinical parameters of periodontal disease, and the subgingival microbiota. J Periodontol 2010; 81:89–98.
16. Slots J. Rapid identification of important periodontal microorganisms by cultivation. Oral Microbiol Immunol 1986; 1:48–57.
17. Delima AJ, Van Dyke TE. Origin and function of the cellular components in gingival crevice fluid. Periodontology 2000 2003; 31:55–76.
18. Dixon DR, Bainbridge BW, Darveau RP. Modulation of the innate immune response within the periodontium. Periodontology 2000 2004; 35:53–74.
19. Ebersole J. Humoral immune responses in gingival crevicular fluid: local and systemic implications. Periodontology 2000 2003; 31:135–166.
20. Gemmel E, Seymour GJ. Immunoregulatory control of Th1/Th2 cytokine profiles in periodontal disease. Periodontology 2000 2004; 35:21–41.
21. Gemmel E, Yamazaki K, Seymour GJ. The role of T cells in periodontal disease: homeostasis and autoimmunity. Periodontology 2000 2007; 43:14–40.
22. Hultin M, Gustafsson A, Hallström H, Johansson LA, Ekfeldt A, Klinge B. Microbiological findings and host response in patients with peri-implantitis. Clin Oral Implants Res 2002; 13:349–358.
23. Konttinen YT, Lappalainen R, Laine P, Kitti U, Santavirta S, Teronen O. Immunohistochemical evaluation

of inflammatory mediators in failing implants. Int J Periodontics Restorative Dent 2006; 26:135–141.

24. Silva N, Dutzan N, Hernandez M, et al. Characterization of progressive periodontal lesions in chronic periodontitis patients: levels of chemokines, cytokines, matrix metalloproteinase-13, periodontal pathogens and inflammatory cells. J Clin Periodontol 2008; 35:206–214.
25. Cadosch D, Chan E, Gautschi OP, Filgueira L. Metal is not inert: role of metal ions released by biocorrosion in aseptic loosening – current concepts. J Biomed Mater Res A 2009; 91:1252–1262.
26. Chehroudi B, Ghrebi S, Murakami H, Waterfield JD, Owen G, Brunette DM. Bone formation on rough, but not polished, subcutaneously implanted Ti surfaces is preceded by macrophage accumulation. J Biomed Mater Res A 2010; 93:724–737.
27. Fabian E, Landsiedel R, Ma-Hock L, Wiench K, Wohlleben W, van Ravenzwaay B. Tissue distribution and toxicity of intravenously administered titanium dioxide nanoparticles in rats. Arch Toxicol 2008; 82:151–157.
28. Frisken KW, Dandie GW, Lugowski S, Jordan G. A study of titanium release into body organs following the insertion of single threaded screw implants into the mandibles of sheep. Aust Dent J 2002; 47:214–217.