Journal of
PERIODONTAL RESEARCH

J Periodont Res 2010; 45: 148–152

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2009.01204.x

Short communication

A multiplex immunoassay demonstrates reductions in gingival crevicular fluid cytokines following initial periodontal therapy

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Thunell DH, Tymkiw KD, Johnson GK, Joly S, Burnell KK, Cavanaugh JE, Brogden KA, Guthmiller JM. A multiplex immunoassay demonstrates reductions in gingival crevicular fluid cytokines following initial periodontal therapy. J Periodont Res 2010; 45: 148–152. © 2009 John Wiley & Sons A/S

Background and Objective: Cytokines and chemokines play an important role in the pathogenesis of periodontal diseases. The objective of this study was to quantitatively assess the effect of initial periodontal therapy on gingival crevicular fluid levels of a comprehensive panel of cytokines and chemokines, including several less extensively studied mediators.

Material and Methods: Clinical examinations were performed and gingival crevicular fluid samples obtained from six subjects with generalized severe chronic periodontitis prior to initial periodontal therapy and at re-evaluation (6–8 weeks). Four diseased and two healthy sites were sampled in each subject. Twenty-two gingival crevicular fluid mediators were examined using a multiplex antibody capture and detection platform. Statistical analyses were performed by fitting mixed effects linear models to log-transformed gingival crevicular fluid values.

Results: Gingival crevicular fluid interleukin (IL)-1α and IL-1β were the only cytokines to differ in initially diseased vs. initially healthy sites. Following initial therapy, 13 of the 16 detectable cytokines and chemokines decreased significantly in diseased sites, including IL-1α, IL-1β, IL-2, IL-3, IL-6, IL-7, IL-8, IL-12 (p40), CCL5/regulated on activation, normally T cell expressed and secreted (RANTES), eotaxin, macrophage chemotactic protein-1, macrophage inflammatory protein-1α and interferon-γ. At healthy sites, only three of the 16 mediators were significantly altered following therapy.

Conclusion: This is the first study, to our knowledge, to evaluate such an extensive panel of gingival crevicular fluid mediators within the same sample prior to and following initial therapy. The results confirm that periodontal therapy effectively reduces pro-inflammatory cytokines and chemokines, including less well-described mediators that may be important in initiation and progression of periodontitis. The multiplex assay will prove useful for future gingival crevicular fluid studies.

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Key words: cytokine; chemokine; periodontal therapy; multiplex immunoassay

Accepted for publication December 28, 2008

Chronic inflammation of the periodontium results from complex interactions between bacterial plaque and the host response. Bacteria contribute to hard and soft tissue destruction by producing proteolytic enzymes as well as by directly inducing host proteinases (1,2). In addition, inflammatory mediators produced by the host stimulate release of metalloproteinases and initiate bone resorption. These mediators, many of which can be detected in gingival crevicular fluid, play a major role in the progression of periodontal disease. They include T-helper 1 (Th1) and T-helper 2 (Th2) cytokines, pro-inflammatory cytokines, chemokines, and regulators of T and natural killer cell activity (3–5).

Previous studies have evaluated the effects of periodontal treatment on gingival crevicular fluid mediators; however, because of the small sample volumes and limitations of the assays used, only a few select cytokines were evaluated. Therefore, it is difficult to establish an overall perspective of the relationships between the various types of cytokines and chemokines. This pilot study used a multiplex antibody capture and detection platform to quantitatively assess the effects of initial periodontal therapy on a comprehensive panel of cytokines and chemokines in gingival crevicular fluid.

Material and methods

Subject population

The study population included six subjects with generalized severe chronic periodontitis, aged 40–75 years old, presenting to the Department of Periodontics at the University of Iowa, College of Dentistry. Exclusion criteria included subjects who were pregnant, immunosuppressed, diabetic, exhibited gingival hypertrophy, required premedication, had a history of smoking more than 100 cigarettes in their lifetime, or were taking systemic anti-inflammatory medications. To be enrolled, the subjects could not have taken antibiotics or received periodontal instrumentation in the 6 months prior to the study. Diseased subjects were defined as having multiple sites (over one-third of the dentition) with clinical attachment loss (CAL) ≥ 5 mm. Informed consent was obtained from all subjects according to a protocol approved by the University of Iowa Institutional Review Board.

Clinical procedures

All participants received a comprehensive periodontal examination, including probing depth, recession, CAL, mobility, furcation involvement, bleeding on probing (BOP) and plaque scores. The treatment for all subjects consisted of oral hygiene instruction and two separate sessions of scaling and root planing performed under local anesthesia. A comprehensive periodontal examination was repeated 6–8 weeks following initial periodontal therapy.

Collection of gingival crevicular fluid

Immediately prior to initial periodontal therapy, gingival crevicular fluid was collected from selected sites from the subjects. These included four periodontally diseased (ID) sites (probing depth and CAL ≥ 5 mm, and BOP) and two healthy (IH) sites (probing depth and CAL ≤ 3 mm, no BOP). Six to 8 weeks later, the subjects presented for periodontal re-evaluation. At periodontal re-evaluation, prior to gingival crevicular fluid collection, supragingival plaque was removed, and the site was isolated with cotton rolls and gently air-dried. Gingival crevicular fluid was collected from the previously sampled sites, and designated as RD for re-evaluation diseased sites and RH for re-evaluation healthy sites. A saliva ejector was used to avoid salivary contamination of the samples. Gingival crevicular fluid samples were collected with a paper strip (Periopaper, Amityville, NY, USA) by inserting the strip for 30 s into the gingival crevice until mild resistance was felt. In cases of visible contamination with blood, the strip was discarded and a new site was sampled.

Gingival crevicular fluid volume was immediately determined using a Periotron 8000 (Oraflow Inc., Plainview, NY, USA), which had been calibrated using known volumes of the buffer. Strips from each subject were then placed into 300 μL of a 0.01 M sodium phosphate buffer, pH 7.2, containing 140 mM NaCl and protease inhibitor (Complete Mini; protease inhibitor cocktail tablets; Roche Applied Science, Indianapolis, IN, USA). Following vortexing for 10 s and 20 min of shaking, the strips were removed and the eluates centrifuged for 5 min at 5800 *g* to remove plaque and cellular elements. The samples were frozen at −80°C until further analysis.

Determination of cytokine amounts

Cytokine and chemokine quantities (pg/30 s) were determined using a commercial 22-multiplex fluorescent bead-based immunoassay (Kit 48-011; Millipore, Billerica, MA, USA) and the Luminex 100 IS Instrument (Luminex, Austin, TX, USA). The multiplex kit used was capable of detecting Th1 cytokines [interleukin (IL)-2, IL-12(p70) and interferon (IFN)-γ], Th2 cytokines (IL-3, IL-4, IL-5, IL-10 and IL-13), pro-inflammatory cytokines [IL-1α, IL-1β, IL-6, granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor (TNF)-α, and IL-12(p40)], chemokines [CXCL8/IL-8, CXCL10/interferon-inducible protein (IP)-10, CCL2/macrophage chemotactic protein (MCP)-1, CCL3/macrophage inflammatory protein (MIP)-1α, CCL5/regulated on activation, normally T cell expressed and secreted (RANTES) and CCL11/eotaxin], and regulators of T and natural killer cell activation and proliferation (IL-7 and IL-15).

For the assay, 50 μL aliquots of the gingival crevicular fluid samples in the buffer were incubated with anti-human multi-cytokine beads at 4°C for 18 h. Unbound material was removed by filtration. Twenty-five microlitres of anti-human multi-cytokine biotin reporter was added, and reactions were incubated at room temperature for 1.5 h in the dark. Twenty-five microlitres of streptavidin–phycoerythrin was then added, and the plates were incubated at room temperature for an additional 30 min. Twenty-five microlitres of stop solution was added and the plates (Multiscreen HTS; BV clear plates; Millipore) read in a plate reader

(model 100 IS; Luminex). Concentrations of cytokines in each sample were extrapolated from standards by use of BEADVIEW software (Millipore). All samples were run in duplicate.

Statistical analysis

A log-transformation was applied to the cytokine amounts in subject gingival crevicular fluid samples to account for the positive skewness in the measurements and to make the assumption of normality more defensible. Mixed effects linear models were then fitted to the log-transformed responses using SAS (version 9.1; SAS Institute, Cary, NC, USA). The models included a random subject effect and a random site effect, with sites nested within subjects. The models also included fixed effects for disease status (diseased vs. healthy) and evaluation status (initial vs. re-evaluation), along with a two-way interaction for these variables. A difference was declared statistically significant if the corresponding *p*-value was less than the 0.05 level of significance. Results are reported as quantities of cytokines or chemokines per 30 s (pg/30 s).

Results

Mean gingival crevicular fluid volumes (Table 1) varied and were slightly higher at baseline in the diseased sites (ID) than in the healthy sites (IH) of the periodontitis subjects. At re-evaluation, there was a decrease of gingival crevicular fluid volumes for both the RD and the RH sites (24 and 22%, respectively).

Table 1. Clinical measurements of sites selected for gingival crevicular fluid sampling (means ± SD)

	ID	IH	RD	RH
Parameter	*n* = 24	*n* = 12	*n* = 24	*n* = 12
Probing depth (mm)	5.6 ± 0.71	2.8 ± 0.62	4.0 ± 1.37	2.6 ± 0.90
Recession (mm)	0.6 ± 0.82	0.5 ± 0.80	0.8 ± 1.05	0.7 ± 0.78
CAL (mm)	6.3 ± 1.11	3.3 ± 1.06	4.8 ± 1.74	3.3 ± 1.06
BOP (%)	100	0	42	8
Gingival crevicular fluid volume (μl)	2.1 ± 0.7	1.8 ± 0.8	1.6 ± 0.6	1.4 ± 0.8

Abbreviations: ID, initial diseased; IH, initial healthy; RD, re-evaluation diseased; RH, re-evaluation healthy; CAL, clinical attachment loss; and BOP, bleeding on probing.

Table 2. Cytokine amounts (pg/30 s, means ± SD)

	ID	IH	RD	RH
Th1 cytokines				
IL-2	33 ± 8	35 ± 7	28 ± 6	31 ± 6
IFN-γ	41 ± 21	34 ± 18	28 + 14	28 ± 14
Th2 cytokines				
IL-3	125 ± 73	95 ± 66	87 ± 59	70 ± 80
IL-4	51 ± 44	53 ± 42	48 ± 42	53 ± 54
Pro-inflammatory cytokines				
IL-1α	4070 ± 6269	1888 ± 2365	1922 ± 1870	1003 ± 500
IL-1β	284 ± 369	74 ± 117	95 ± 168	24 ± 24
IL-6	241 ± 324	171 ± 123	126 ± 159	131 ± 114
GM-CSF	48 ± 31	42 ± 31	41 ± 23	60 ± 84
IL-12(p40)	173 ± 94	129 ± 68	99 ± 45	119 ± 51
Chemokines				
IL-8	1826 ± 1169	1205 ± 782	1716 ± 2452	1153 ± 798
IP-10	652 ± 818	747 ± 968	970 ± 1429	3473 ± 8228
MCP-1	25 ± 12	22 ± 15	19 ± 10	22 ± 20
MIP-1α	270 ± 106	234 ± 66	206 ± 64	205 ± 47
RANTES	115 ± 255	58 ± 108	27 ± 19	30 ± 23
Eotaxin	143 ± 81	137 ± 68	129 ± 63	138 ± 69
Regulators of T and NK cell activation and proliferation				
IL-7	84 ± 33	83 ± 27	73 ± 25	77 ± 23

ID, initial diseased; IH, initial healthy; RD, re-evaluation diseased; RH, re-evaluation healthy.

Sixteen of the 22 cytokines evaluated were detectable within the range of the assay and were present in both diseased and healthy sites: IL-2 and IFN-γ (Th1 cytokines); IL-3 and IL-4 (Th2 cytokines); IL-1α, IL-1β, IL-6, GM-CSF and IL-12p40 (pro-inflammatory cytokines); CXCL/IL-8, CXCL10/IP-10, CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES and CCL11/eotaxin (chemokines); and IL-7 (cytokine regulator of T and natural killer cell activation and proliferation). The cytokine mean amounts and standard deviations are presented in Table 2. Six of the mediators evaluated as a part of the panel were not detected in the gingival crevicular fluid, including IL-5, IL-10, IL-12 (p70), IL-13, IL-15 and TNF-α.

Initial evaluation: diseased vs. healthy sites

Interleukin-1α and IL-1β were the only cytokines that varied significantly at baseline between ID and IH sites within the subjects ($p = 0.01$ and 0.0013, respectively) (Table 3). Quantities of IL-1α ranged from 918.0 to 32,580 pg/30 s in the ID sites and from 450.6 to 8,940.0 pg/30 s in the IH sites. Values of IL-1β ranged from 13.9 to 1662.0 pg/30 s in the ID sites and from 11.2 to 412.2 pg/30 s in the IH sites. Quantities of the pro-inflammatory cytokine IL-12 (p40) and IFN-γ were higher in ID sites than in IH sites, approaching statistical significance ($p = 0.08$ and 0.07, respectively).

Re-evaluation: diseased vs. healthy sites

Comparison of the cytokine and chemokine quantities in RD sites vs. RH sites at 6–8 weeks revealed that IL-3 ($p = 0.031$) and IP-10 ($p = 0.045$) quantities were significantly different between these sites (Table 3). While IL-3, a Th2 cytokine, was significantly higher in the RD sites, the chemokine IP-10 was sig-

Table 3. *p*-values of comparisons of log-transformed cytokine amounts

Cytokine	ID vs. IH	RD vs. RH	IH vs. RH	ID vs. RD
Eotaxin	0.51	0.15	0.84	0.0081*
GM-CSF	0.31	0.19	0.52	0.62
IFN-γ	0.07	0.52	0.45	< 0.0001*
IL-12 (p40)	0.08	0.21	0.86	0.0004*
IL-1α	0.01*	0.25	0.36	0.003*
IL-1β	0.0013*	0.14	0.24	0.0002*
IL-2	0.37	0.27	0.13	0.018*
IL-3	0.19	0.03*	0.02*	0.03*
IL-4	0.79	0.08	0.03*	0.37
IL-6	0.5	0.39	0.38	0.0008*
IL-7	0.85	0.22	0.37	0.0097*
IL-8	0.24	0.79	0.99	0.04*
IP-10	0.58	0.04**	0.04**	0.26
MCP-1	0.64	0.34	0.84	0.004*
MIP-1α	0.25	0.83	0.22	0.0005*
RANTES	0.28	0.81	0.41	0.006*

*First category significantly higher than second category ($p < 0.05$).
**First category significantly lower than second category ($p < 0.05$).
ID, initial diseased, IH, initial healthy; RD, re-evaluation diseased; RH, re-evaluation healthy.

nificantly higher in the RH sites. IL-3 values ranged from 53.5 to 232.8 pg/30 s at RD sites compared to 31.7–256.2 in the RH sites. The IP-10 values ranged from 59.6 to 5,976.0 pg/30 s in the RD sites and 61.8–29,280.0 pg/30 s in the IH sites.

Healthy sites: initial evaluation vs. re-evaluation

Quantities of the Th2 cytokines IL-3 ($p = 0.022$), IL-4 ($p = 0.037$), and the chemokine IP-10 ($p = 0.041$) varied significantly between IH and RH measurements (Table 3). Quantities of both IL-3 and IL-4 decreased significantly following initial therapy. Interleukin-3 (IH) ranged from 64.8 to 197.4 pg/30 s and IL-3 (RH) ranged from 31.7 to 256.2 pg/30 s. Interleukin-4 (IH) ranged from 9.8 to 104.4 pg/30 s and IL-4 (RH) ranged from 8.3 to 150.0 pg/30 s. In contrast, the IP-10 response ranged from 48.7 to 3,540.0 pg/30 s in IH sites, which was significantly lower than the IP-10 response in RH sites (61.8–29,280 pg/30 s).

Diseased sites: initial evaluation vs. re-evaluation

The following cytokines were significantly higher in ID sites vs. RD sites: IL-2 and IFN-γ (Th1 cytokines); IL-3 (Th2 cytokine); IL-1α, IL-1β, IL-6 and IL-12p40 (pro-inflammatory cytokines); CXCL/IL-8, CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES and CCL11/eotaxin (chemokines); and IL-7 (cytokine regulator of T and natural killer cell activation and proliferation; Table 3). In contrast, no significant difference was seen for GM-CSF, IL-4 and IP-10 with regard to the ID and RD measurements.

Discussion

A particularly intriguing finding from this study was the similarity in the amounts of cytokines in diseased and healthy sites of periodontitis subjects prior to periodontal therapy. Interestingly, IL-1α and IL-1β were the only cytokines that were significantly higher in diseased sites vs. healthy sites, the latter of which coincides with previous reports showing elevated IL-1β in sites with increased inflammation (6,7). In contrast, many previous studies have reported significant differences in cytokine levels between healthy and diseased sites, with the majority supporting higher levels of cytokines in diseased sites (8–10). Some of these studies, however, compared diseased sites in subjects with periodontal disease to healthy sites in healthy subjects (8,10). The present study compared cytokine levels at healthy and diseased sites within subjects with periodontitis; our findings suggest an overall subject effect on cytokine profiles, which may be a reflection of periodontal status.

The effects of initial therapy on gingival crevicular fluid cytokine levels in subjects with aggressive and moderate to severe chronic periodontitis were assessed by Fokkema *et al*. (11) using an enzyme-linked immunosorbent assay (ELISA). While IL-12 (p70), a Th1 cytokine, increased following initial therapy, IL-1β, IL-6, IL-8, IL-10, IL-12 (p40) and TNF-α remained unchanged (11). In contrast, Gamonal *et al*. (7) found reductions in total amounts of a similar group of cytokines (IL-1β, IL-8, IL-10 and RANTES) at diseased sites following initial therapy in subjects with moderate to advanced periodontitis. Our results support a reduction in pro-inflammatory cytokines and chemokines, including IL-1α, IL-1β, IL-6, IL-8 and RANTES, as well as the Th1 cytokines, IFN-γ and IL-2; a Th2 cytokine, IL-3; and IL-7, a regulator of T and natural killer cell activation and proliferation, as a result of initial therapy. In addition, there were decreases in mediators which have been less extensively studied in gingival crevicular fluid. These included the pro-inflammatory cytokine, IL-12(p40), and several chemokines (eotaxin, MCP-1 and MIP-1α). The RH sites showed very little change in cytokine amounts compared with IH sites, with the exception of three cytokines (IL-3, IL-4 and IP-10). This result was not unexpected, owing to the shallow probing depths and absence of BOP, which suggested stability of the healthy site.

The observed significant elevation of IP-10 at healthy sites after initial therapy is intriguing. Although not significant, this mediator also increased at diseased sites following treatment. IP-10 is a member of the CXC chemokine family, is secreted by a variety of cells (endothelial cells, monocytes, fibroblasts, and keratinocytes) in response to IFN-γ, and is involved in the recruitment of T cells, NK cells, and has the ability to inhibit

angiogenesis (12–15). Interferon-inducible protein-10 has not been extensively studied in periodontal disease; thus its specific role in periodontal disease progression is not fully understood. It has been implicated in periodontal tissue destruction, owing to its presence in gingival crevicular fluid of diseased sites and in diseased tissue samples (8,10). The elevation of IP-10 after therapy was associated with an overall decrease in inflammatory and disease parameters; therefore, it may have a role in wound healing processes rather than reflecting tissue destruction. Our preliminary findings suggest that additional studies are needed to clarify the role of IP-10 in disease and health.

This study clearly demonstrated a reduction in gingival crevicular fluid quantities of an extensive panel of mediators, including several pro-inflammatory cytokines and chemokines, following initial periodontal therapy. In addition, select Th1, Th2 and T and natural killer cell regulatory cytokines were decreased. Our study examined 16 cytokines and chemokines, which led to many tests for comparisons, as summarized in Table 3. Despite the relatively small number of subjects, our analyses uncovered a number of interpretable statistically significant differences. We did not adjust for multiple comparisons, since this would have resulted in very conservative tests, thereby obscuring clinically meaningful differences. In a larger study based on more subjects, such an adjustment would be warranted.

In summary, the findings of this pilot study serve as a basis for future gingival crevicular fluid research. The multiplex bead immunoassay used in this study allows for a more comprehensive assessment of mediators within a single sample and is efficient and cost effective compared with traditional methods. The results obtained with the multiplex bead immunoassay were not compared with other conventional assays, such as an ELISA. However, in a recent study comparing fourteen different immunoassays with four different detection platforms (including a traditional solid-phase sandwich ELISA and immunobead-based assays using the fluidics Luminex fluorometry platform), it was reported that there were interassay disparities as well as significant interlaboratory variation, suggesting that studies using a given assay cannot be directly extrapolated or compared with another assay (16). Irrespective of the assay chosen, one must recognize the technical and biological variables that may impact a given assay and that assay accuracy is also dependent on the cytokine tested (16). Ultimately, the ease, efficiency and expansiveness of multiplex technology may lead to a better understanding of the differences in cytokine profiles in health and disease as well as the impact of periodontal therapy on these mediators.

Acknowledgements

This work was supported by funds from grants RO1 DE13334 (to J.M.G.) and R01 DE014390 (to K.A.B.) from the National Institute of Dental and Craniofacial Research, National Institutes of Health.

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