The Influence of Interleukin Gene Polymorphism on Expression of Interleukin-1β and Tumor Necrosis Factor-α in Periodontal Tissue and Gingival Crevicular Fluid

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**Background:** A specific composite genotype of the polymorphic interleukin-1 (IL-1) gene cluster has recently been associated with severe periodontitis. One polymorphism of the composite periodontitis-associated genotype (PAG) has been functionally linked with expression of high levels of IL-1. The purpose of this study was to test whether gingival crevicular fluid (GCF) levels of IL-1β and tumor necrosis factor-alpha (TNFα), and gingival tissue levels of IL-1α, IL-1β, and TNFα correlate with PAG, and to examine the effect of conservative periodontal therapy on these levels.

**Methods:** Twenty-two adults with moderate to advanced periodontal disease were enrolled. Polymerase chain reaction amplification and restriction enzymes were used to identify specific polymorphisms from peripheral blood samples. GCF samples were collected at baseline and 3 weeks following conservative treatment and analyzed by ELISA for IL-1β and TNFα. An interproximal gingival biopsy was collected at baseline and follow-up and analyzed for IL-1α, IL-1β, and TNFα by ELISA.

**Results:** The genotyping identified 7 as PAG(+) and 15 as PAG(-). The 2 groups were comparable in terms of existing periodontitis and age. In shallow sites (<4 mm), total IL-1β in GCF was 2.5 times higher for PAG(+) patients prior to treatment (P = 0.03), and 2.2 times higher after treatment (P = 0.04), while differences were less apparent in deeper sites. Following treatment, a reduction in IL-1β concentration in GCF was seen for PAG(-) but not for PAG(+) patients. While not statistically significant, a trend was observed in mean tissue levels of IL-1β which were 3.6 times higher in PAG(+) versus PAG(-) patients (P = 0.09).

**Conclusions:** These data suggest that PAG(+) patients may demonstrate phenotypic differences as indicated by elevated levels of IL-1β in GCF. *J Periodontal* 1999;70:567-573.

**KEY WORDS**
Gingival crevicular fluid; cytokines; interleukin-1; periodontitis/therapy; tumor necrosis factor.
Effect of Gene Polymorphism on Expression of IL-1

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as previously described. Briefly, the subject's finger Blood samples were collected and analyzed from all subjects. Institutional Review Board at Columbia Presbyterian Medical Center, and an informed consent was obtained for participation. The study was approved by the same Institutional Review Board.

Inclusion criteria consisted of systemically healthy individuals referred to the Postgraduate Periodontics Clinic at the Columbia University School of Dental and Oral Surgery and referred to the Postgraduate Periodontics Clinic at the Columbia University School of Dental and Oral Surgery for medically necessary treatment. Exclusion criteria included use of antibiotics, pregnancy, lactation, diabetes, HIV infection, bleeding disorders, use of non-steroidal anti-inflammatory drugs, or recent immunosuppressive chemotherapy, and any condition necessitating antibiotic premedication for dental appointments.

MATERIALS AND METHODS

Clinical Examination

Twenty-four systemically healthy individuals referred to the Postgraduate Periodontics Clinic at the Columbia University School of Dental and Oral Surgery were enrolled in the study. All subjects presented with moderate to severe periodontitis. This diagnosis required probing depths of at least 5 mm at 2 sites in each quadrant with radiographic evidence of loss of periodontal bone. The study was approved by the Institutional Review Board at Columbia Presbyterian Medical Center, and an informed consent was obtained from all subjects.

Analysis of Genetic Polymorphisms

Finger-stick blood samples were collected and analyzed as previously described. Briefly, the subject's finger was cleansed with antiseptic wipes and the skin was punctured with a sterile lancet. Finger-stick blood samples were collected on DNAase-free blotting paper and analyzed blind for polymorphisms in the IL-1A gene at position +889 and in the IL-1B gene at position +3953. Alleles of IL-1A (+889) have been demonstrated to be 100% concordant with alleles of IL-1B (+889) (di Giovine and Chaudhary, unpublished observation). IL-1A (+845) was preferred because the sequence offers opportunity of an internal positive control restriction site. All screening methods are polymerase chain reaction-based, have been previously published and have been extensively validated. Reaction conditions and oligonucleotide sequences are as follows:

**IL-1α (+845)**

oligonucleotides:

1. 5'-ATG.GTT.TTA.GAA.ATC.ATC.AAG.CCT.CGA.AGA.AAT.CAA.A-3' (Fnu 4H1 on allele 1: 76 + 29 + 124 bp)
2. 5'-AAT.GAA.AGG.GGA.GGA.TGA.CAG.AAA.TGT.3' (Fnu 4H1 on allele 2: 76 + 153 bp)

**RFLP:**

- Tag I on allele 1: 12 + 85 + 97 bp
- Tag I on allele 2: 12 + 182 bp

**IL-1β (+3953)**

oligonucleotides:

1. 5'-CTC.AGG.TGT.CCT.CGA.AGA.AAT.CAA.A-3' (Fnu 4H1 on allele 1: 76 + 29 + 124 bp)
2. 5'-GCT.TTT.TTG.TCT.GTG.AGC.AGC.AGC.A-3' (Fnu 4H1 on allele 2: 76 + 153 bp)

**RFLP:**

- Tag I on allele 1: 12 + 85 + 97 bp
- Tag I on allele 2: 12 + 182 bp

GCF Collection and Analysis

The baseline evaluation consisted of collection of GCF from the mesiobuccal and mesiopalatal surfaces of premolar and molar teeth from the right and left quadrants in the maxillary arch. Measurement of probing depth was made at these sites to the nearest millimeter. The clinical measurements were recorded at the deepest site within that portion of the tooth using a manual Michigan “O” probe with Williams markings. Prior to collection of clinical measures, teeth were isolated, supragingival plaque was gently removed, and GCF was sampled with precut methylcellulose filter paper strips for 30 seconds then removed to separate microcentrifuge tubes containing 50 µl phosphate buffered saline-Tween 20. Strips were measured for fluid content with a calibrated instrument to define the volume of GCF and stored at -20°C until eluted (maximum 48 hours). Following elution, samples from the mesiobuccal and mesiopalatal strips were combined to form a sample from the mesial surface of each tooth.

Analysis of IL-1β and TNFα in GCF

GCF samples were analyzed blind for IL-1β and TNFα using a commercially available enzyme-linked immunosorbent assay. Both assays are standard sand-
with ELISAs and were performed according to manufacturer’s instructions using human recombinant standards. Results are reported as total amount of cytokine (in pg) per sample, or concentration (pg/µl) by converting fluid analyzer units to µl using calibration curves as described previously.13

Tissue Analysis
After collection of GCF and clinical measures at baseline, an interproximal gingival biopsy was obtained under local anesthesia by an examiner who was unaware of the genotype results. The interdental tissue between the first molar and second premolar was removed by means of a horizontal incision coronal to the alveolar crest and carried from the buccal surface to the lingual/palatal surface. A solid triangular piece of tissue was obtained. A similar sample was taken from the contralateral side of the mouth 3 weeks after treatment. Samples were snap frozen in phosphate buffered saline containing 1% endotoxin-free bovine serum albumin, and stored at -70°C until analyzed. The tissue samples were resuspended in a final volume of 350 µl of RPMI 1640 medium containing 10% of an antiprotease cocktail§ and homogenized on ice using a handheld homogenizer.§ Clarified supernatants were tested for IL-1α, IL-1β, and TNFα content by ELISA as previously described,14 and dsDNA was quantified by fluorescence.15** Results were reported as mean pg/µg dsDNA.

Treatment
Following collection of GCF, clinical parameters, and the tissue sample, subjects received full-mouth root planing and scaling with curets and ultrasonic instruments under local anesthesia for not more than 2 hours in the Postgraduate Periodontics Clinic. The therapy was administered blind as to genotype status. No antibiotics were prescribed following treatment. Three weeks following scaling and root planing, GCF and the contralateral biopsy were collected as above.

Statistical Analysis
Mean group GCF cytokine data for the PAG(+) and PAG(-) groups were calculated by pooling all site values for each genotype group. Intragroup comparisons were made between baseline and follow-up examinations by repeated measures ANOVA. Intergroup comparisons were made between PAG(+) and PAG(-) patients at baseline and after treatment by repeated measures ANOVA. Where probing depth categories were compared, mean patient values for each probing depth category (<4 mm, 4 to 6 mm, >6 mm), were calculated, and group means were then determined. Intergroup comparisons were made by ANOVA at baseline and after treatment. Tissue cytokine values were compared at baseline and follow-up by repeated measures ANOVA. A commercially available statistical analysis software†† was used for data analysis.

RESULTS
Twenty-four patients met entry criteria and were enrolled in the study. Of the 24, 17 (70.8%) were PAG(-) and 7 (29.2%) were PAG(+). There were no differences in the mean ages of the genotype groups. Furthermore, the groups did not differ in severity of existing periodontal disease. Two patients did not complete the study because of failure to comply with study protocol. These patients, both PAG(-), were exited from the study and continued to receive periodontal treatment in the Postgraduate Periodontics Clinic. One severely compromised tooth was extracted when it became symptomatic following scaling and root planing. Consequently, 22 patients (161 surfaces) completed the baseline examination, and 22 patients (160 surfaces) completed the follow-up examination (Table 1).

Table 1.
Mean Age (Years) and Mean Probing Depth (mm) of PAG(+) and PAG(-) Groups

<table>
<thead>
<tr>
<th></th>
<th>PAG(-)</th>
<th>PAG(+)</th>
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<tr>
<td>N</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>Age</td>
<td>45.5*</td>
<td>46.4</td>
</tr>
<tr>
<td>Probing depth</td>
<td>4.7*</td>
<td>4.4</td>
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</table>

*The difference between groups for mean age and mean probing depth was not statistically significant.

Gingival Crevicular Fluid Levels of IL-1β and TNFα (Table 2)
Detectable amounts of crevicular fluid IL-1β were obtained from all samples at baseline (161/161) and all samples at follow-up (160/160). Variation was noted both by site and by individual. TNFα was detected in crevicular fluid in 59% (95/161) of samples at baseline and 49% (78/160) of samples at follow-up. TNFα levels that were below the level of detection (10pg/ml) were assigned a value of zero.

IL-1β in GCF. Mean total IL-1β levels (pg/GCF sample) were compared between PAG groups and are described in Table 2. Total GCF IL-1β for the 2 groups at baseline was not significantly different (P = 0.21). However, when probing depths were considered, significant differences were evident (Fig. 1). In the shallow sites (<4 mm), PAG(+) patients demonstrated 2.5 times more total IL-1β than PAG(-) patients (P = 0.03). Total IL-1β levels were similar between PAG(+) and PAG(-) in the intermediate (4 to 6 mm) and deeper sites (>6 mm).

† Complete, Boehringer, Indianapolis, IN.
§ Omni 2000, Omni International, Warrenton, VA.
** Pico-Green, Molecular Probes, Eugene, OR.
†† Statview, Abacus Concepts, Berkeley, CA.
At the follow-up examination 3 weeks after scaling and root planing, total GCF IL-1ß was slightly greater than what was observed at baseline for both groups. PAG(+) patients showed a trend towards higher total IL-1ß as compared to PAG(-) patients ($P = 0.11$). Again, when stratified by probing depths, significant differences were seen between groups (Fig. 2). In the shallow sites, total IL-1ß for the PAG(+) patients was 2.2 times higher than that for the PAG(-) patients ($P = 0.04$). For the intermediate and deeper sites, no significant differences in IL-1ß levels were noted.

When examined as concentration of IL-1ß (pg/µl of GCF), there was no difference at baseline between the PAG(+) patients and the PAG(-) patients. However, the mean concentration of IL-1ß was significantly reduced following therapy in the PAG(-) but not the PAG(+) patients ($P = 0.004$). When analyzed by probing depths, prior to treatment there was a trend for higher IL-1ß concentrations for the PAG(+) group in sites <4 mm, but no significant differences were noted in other categories. Following treatment, however, the PAG(+) group demonstrated significantly higher concentrations of IL-1ß in shallow ($P = 0.005$) and intermediate ($P = 0.025$) sites.

Total GCF IL-1ß was significantly correlated with IL-1ß GCF concentration in both genotype groups, before ($r = 0.69; P < 0.001$) and after ($r = 0.86; P < 0.001$) treatment.

**Table 2.**

| Mean Values (±SEM) of IL-1ß and TNFα in Gingival Fluid From PAG(+) and PAG(-) Groups |
|-------------------------------|-------------------|---------|-------------------|---------|-------------------|
|                              | Baseline |        | 3 Weeks |        | 3 Weeks |
|                              | PAG(+)  | PAG(-) | $P$     | PAG(+)  | PAG(-) | $P$     |
| GCF Total IL-1ß (pg/sample±SEM) | 173±23  | 141±14 | 0.21    | 208±21  | 169±14 | 0.11    |
| <4 mm                         | 149±27  | 59±10  | 0.03    | 161±35  | 74±11  | 0.04    |
| 4-6 mm                        | 171±32  | 120±14 | 0.23    | 215±26  | 184±17 | 0.33    |
| >6 mm                         | 272±112 | 321±42 | 0.36    | 371±69  | 246±44 | 0.10    |
| GCF [IL-1ß] (pg/ul±SEM)      | 160±16  | 137±11 | 0.23    | 158±14  | 115±8  | 0.004   |
| <4 mm                         | 172±27  | 105±25 | 0.06    | 140±25  | 65±9   | 0.005   |
| 4-6 mm                        | 156±23  | 124±12 | 0.19    | 166±20  | 124±9  | 0.025   |
| >6 mm                         | 223±26  | 145±43 | 0.16    | 178±35  | 151±23 | 0.55    |
| GCF Total TNFα (pg/sample±SEM) | 2.2±0.32 | 2.0±0.29 | 0.78    | 3.1±0.65 | 4.5±0.9 | 0.32    |
| <4 mm                         | 2.1±0.54 | 2.0±0.47 | 0.97    | 1.4±0.45 | 1.7±0.45 | 0.93    |
| 4-6 mm                        | 1.8±0.34 | 1.8±0.42 | 0.92    | 3.0±0.73 | 5.6±1.5 | 0.15    |
| >6 mm                         | 2.6±1.3  | 3.1±1.0 | 0.77    | 10.5±4.6 | 4.8±1.6 | 0.20    |
| GCF [TNF] (pg/ul±SEM)        | 1.8±0.27 | 2.7±0.52 | 0.27    | 2.1±0.40 | 3.2±0.61 | 0.27    |
| <4 mm                         | 2.4±0.60 | 4.2±1.6  | 0.07    | 1.3±0.40 | 1.4±0.38 | 0.94    |
| 4-6 mm                        | 1.5±0.58 | 2.2±0.53 | 0.49    | 2.1±0.54 | 4.0±0.99 | 0.13    |
| >6 mm                         | 1.6±0.77 | 2.3±0.75 | 0.77    | 5.0±2.0  | 2.7±0.89 | 0.45    |

At the follow-up examination 3 weeks after scaling and root planing, total GCF IL-1ß was slightly greater than what was observed at baseline for both groups. PAG(+) patients showed a trend towards higher total IL-1ß as compared to PAG(-) patients ($P = 0.11$). Again, when stratified by probing depths, significant differences were seen between groups (Fig. 2). In the shallow sites, total IL-1ß for the PAG(+) patients was 2.2 times higher than that for the PAG(-) patients ($P = 0.04$). For the intermediate and deeper sites, no significant differences in IL-1ß levels were noted.

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**TNFα in GCF.** TNFα levels in GCF were compared before and after treatment within groups and between groups. There was no difference between PAG(+) and PAG(-) patients for total TNFα or TNFα concentration at baseline. However, total TNFα from the PAG(-) patients doubled 3 weeks after treatment ($P < 0.01$), while total GCF TNFα concentration in the PAG(+) group showed no significant change.

Total TNFα and total IL-1ß in GCF were significantly correlated for the PAG(+) patients at baseline ($r = 0.35; P < 0.02$). This correlation was not seen for the PAG(-) patients ($r = 0.17; P = 0.18$). IL-1ß and TNFα concentra-
Mean tissue cytokine levels are provided in Table 3. The tissue samples from the PAG(+) group demonstrated a 3.6-fold higher mean concentration of IL-1ß at baseline versus PAG(-) patients, and this difference approached but did not reach significance (P = 0.09). Tissue levels of IL-1ß following treatment were much lower in both groups, and these differences were not significant.

Mean IL-1α concentrations in tissue were not significantly different when comparing groups at baseline or after therapy. Treatment did induce a reduction in the concentration of IL-1ß in both groups. The reduction approached significance in the PAG(-) group (P = 0.06). The reduction was not significant in the PAG(+) group due to pronounced variability in IL-1α tissue concentrations in these patients prior to therapy (P = 0.27).

The correlation of tissue levels of IL-1ß and IL-1ß in GCF before treatment was also determined. When all patients were considered, tissue IL-1ß was significantly correlated with total IL-1ß in GCF (r = 0.44; P < 0.05). For the PAG(-) patients, the correlation of tissue IL-1ß and total IL-1ß in GCF was 0.59 (P < 0.02). For PAG(+) patients, the correlation was of similar magnitude (r = 0.53), but this relationship did not reach significance (P = 0.24) since only 7 patients were in this group. TNFα was below the detection threshold of 3 pg/ml in all of the tissue samples.

DISCUSSION

This study demonstrated differences in crevicular fluid levels of the proinflammatory cytokine IL-1ß in patients who are similar in regards to severity of periodontal disease but differ in the composite IL-1 genotype that has been associated with more severe periodontitis. One of the genetic polymorphisms that comprises the periodontitis-associated genotype has been shown to be associated with elevated IL-1 expression in vitro. The data presented here indicate that the composite genotype is associated with increased phenotypic expression of IL-1ß in tissue and crevicular fluid samples from the periodontium.

Whereas bacteria are clearly the initiators of periodontal disease, specific organisms or groups of specific organisms have not fully explained disease severity or the clinical course of disease. Several lines of evidence, including twin studies, have implicated the host genetic influence on the pathogenesis and clinical course of periodontal disease in adults. However, other than the recent association of IL-1 genetic factors with more severe periodontitis, specific genes have not been associated with adult periodontal disease.

Probing depth at a specific site had an important influence on the relationship between genotype and local IL-1ß levels. Although there was a trend towards higher mean GCF IL-1ß in PAG(+) versus PAG(-) patients at baseline and follow-up for both total amount and concentration of this cytokine, the differences between groups in total GCF IL-1ß and IL-1ß concentration were statistically significant when probing depth was considered. Prior to treatment, shallow sites (<4 mm) from PAG(+) patients were found to contain 2.5 times the amount of IL-1ß observed in PAG(-) patients. Furthermore, in the shallow sites, PAG(+) individuals had 40% greater IL-1ß concentration compared to PAG(-) patients. The finding of genotype differences in GCF IL-1 levels in shallow sites is consistent with recent observations in diabetics in which GCF IL-1ß levels were elevated even in sites with minimal to no clinical disease.

It is also of interest that prior to therapy, deep sites did not demonstrate differences by genotype. Untreated sites with deeper probing depths represent a more significant bacterial challenge associated with greater influx of polymorphonuclear leukocytes and macrophages. These 2 cell types have been reported to be the primary source of IL-1ß in the crevicular environment. Hence, increased numbers of these cells would result in increased local levels of proinflammatory cytokines. Histological evaluation of tissue from advanced adult periodontitis lesions reveals an extensive cellular infiltrate that may achieve a local cytokine output which approaches its maximal potential, even in PAG(-) individuals. Deep untreated periodontitis sites may, therefore, not demonstrate differences in IL-1ß regardless of genotype. Further, it is logical to suggest that future studies should consider the expression of IL-1ß in GCF with regard to the presence of important pathogens; i.e., Gram-negative species such as Porphyromonas gingivalis.

This study also showed that the IL-1 genotype may influence IL-1ß levels in the GCF following therapy. The treatment-induced change in the concentration of GCF...
IL-1β in PAG(+) patients was different from what was observed for the PAG(-) patients. After treatment, the concentration of IL-1β in crevicular fluid from PAG(-) patients decreased by a mean of 18.5%, while the concentration in PAG(+) patients did not change. At this post-treatment evaluation, the difference in IL-1β concentration between genotype groups reached significance. Future studies must determine whether the IL-1 genotype-influenced differences in GCF IL-1β levels remain over the long term.

The tissue cytokine patterns were similar to what was observed for GCF. Although not statistically significant, the mean concentration of IL-1β in tissue collected from PAG(+) patients was 3.6 times higher than what was observed for the PAG(-) patients at baseline, and treatment reduced tissue concentrations of IL-1β and IL-1α in both genotype-groups (80% and 69%, respectively, in PAG(+), patients, and 41% and 46%, respectively, in PAG(-) patients).

Thus, the total amount as well as concentration of IL-1β in the GCF and tissues was higher in PAG(+) subjects than in PAG(-) subjects both before and after treatment. It was interesting to observe that differences in GCF levels of IL-1β between PAG groups were more obvious after therapy. This is likely related to a decrease in the contribution of extrinsic variables that control phenotypic expression of IL-1β in the local environment (i.e., magnitude of the subgingival infection). As a result, following scaling and root planing, the contribution of intrinsic variables (i.e., genotype differences) would be a more important determinant of cytokine expression.

Genotype influences on the amount and concentration of IL-1β in GCF may also help to explain conflicting data concerning the effect of therapy on the levels of this cytokine. Future studies of periodontal therapy may need to stratify patients by their innate capacity to produce IL-1β before such studies are undertaken. This may be of special importance in studies that are designed to assess the influence of mechanical or pharmacologic means on IL-1β production before such studies are undertaken. This is likely related to a decrease in the contribution of extrinsic variables that control phenotypic expression of IL-1β in the local environment (i.e., magnitude of the subgingival infection). As a result, following scaling and root planing, the contribution of intrinsic variables (i.e., genotype differences) would be a more important determinant of cytokine expression.

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