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Relationship between the gingival sulcus depth and interleukin-1 isoform concentrations within the adjacent gingival tissue

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Background and Objective: While there is substantial information concerning the concentrations of interleukin-1 isoforms within gingival crevicular fluid, there is little information concerning their concentrations within either normal or diseased gingival tissues. Therefore, the aim of this study was to evaluate the relationship between the concentrations of gingival interleukin-1 isoforms and the adjacent sulcular depth.

Material and Methods: Interdental gingival papillae were excised and grouped based on adjacent pocket depth and the presence of bleeding on probing. Gingiva adjacent to a sulcus of $\leq$ 3 mm without bleeding on probing were classified as 'normal'; gingiva adjacent to a 3-mm sulcus with bleeding on probing were classified as 'diseased-slight'; gingiva adjacent to a 4–6-mm sulcus featuring bleeding on probing were classified as 'diseased-moderate'; and gingiva adjacent to a sulcus of $>$ 6 mm featuring bleeding on probing were classified as 'diseased-severe'. Tissues were solublized and the concentrations of interleukin-1β, interleukin-1α, interleukin-1 receptor antagonist and interleukin-6 were assessed by enzyme-linked immunosorbent assay. Data were compared by factorial analysis of variance, the post-hoc Tukey test and the Pearson's correlation test.

Results: Gingival concentrations of interleukin-6, interleukin-1 receptor antagonist, interleukin-1α- and interleukin-1β were significantly greater at diseased-severe sites than at normal, diseased-slight, or diseased-moderate sites ($p < 0.05$); the gingival concentrations of interleukin-1 receptor antagonist and interleukin-1α were significantly greater at diseased-severe than at diseased-moderate sites ($p < 0.05$). Interleukin-1 receptor antagonist concentrations were significantly correlated with both interleukin-1α and interleukin-1β concentrations. The ratios of concentrations of the interleukin-1 isoforms were different at the various stages of inflammation.

Conclusion: Our data indicated a progressive increase in gingival concentrations of interleukin-1 isoforms with increased adjacent sulcular depth. However, within 'diseased' tissues, the proportional concentrations of interleukin-1α and -β to interleukin-1 receptor antagonist were lowest within diseased-severe tissues.

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Lipopolysaccharides induce the synthesis and release of pro-inflammatory cytokines within the adjacent gingiva. These cytokines may either become incorporated in the gingival crevicular fluid or released into the systemic circulation. There is general agreement that a net accumulation of interleukin-1 isoforms within connective tissues occurs during the pathogenesis of a chronic inflammation (1) and this accumulation is lipopolysaccharide independent (2). There is recent evidence suggesting that specific genetic polymorphisms at the interleukin-1 gene locus might make an individual more susceptible to chronic or aggressive periodontitis (3–9). However, there is some disagreement about the relevance of interleukin-1 polymorphisms in the pathogenesis of periodontal disease (10–12). There are two isoforms of interleukin-1, namely interleukin-1α and interleukin-1β, which bind to the interleukin-1 type 1 receptor (13,14). Interleukin-1β is present in both bound and unbound forms within tissues and serum, while interleukin-1α is usually bound to cellular membranes (14). Both interleukin-1 isoforms stimulate the synthesis of additional interleukin-1 isoforms and interleukin-6 by blocking the signal transducer and activator of transcription 3 signaling pathways (15).

A third isoform of the interleukin-1 family is interleukin-1 receptor antagonist, which blocks the binding of interleukin-1 (α and β) to the interleukin-1 type 1 receptor, thereby preventing the interleukin-1 signal transduction (16–19). Interleukin1 receptor antagonist is present at significantly higher concentrations than interleukin-1β within tissues (20) and possibly regulates the pro-inflammatory response (14,21–25). The concentration of interleukin-1 receptor antagonist within body fluids obtained from nonoral sites has been reported to be 1000-fold higher than the concentration of interleukin-1β, suggesting that interleukin-1 receptor antagonist could be a marker for disease (19,26).

The findings of recent *in vitro* studies on synovial fibroblasts suggest that signs of inflammation result from an imbalance between pro-inflammatory and anti-inflammatory cytokines; in particular, interleukin-6, interleukin-1β and tumor necrosis factor-α (15). A hypothesized mechanism for the regulation of this balance includes interleukin-1 isoforms. The interactions between interleukin-1 and interleukin-6 determine the severity of inflammation and the tissue concentration of interleukin-1β, which has been reported by several authors (22,27–29).

It has been proposed that enhanced expression of interleukin-1β and suppression of interleukin-1 receptor antagonist promotes inflammation and tissue destruction, whereas the reverse suppresses inflammation by inhibiting interleukin-1β accumulation and its stimulatory effects on connective tissue destruction (25,29,30). There is general agreement that either reducing the rate of synthesis of interleukin-1 isoforms or inhibiting their accumulation within connective tissues could be options for either the prevention or treatment of inflammatory diseases, such as rheumatoid arthritis (19,26).Within gingival crevicular fluid, interleukin-1 concentrations have been reported to be higher within sulci adjacent to inflamed gingiva (22,31–44); in contrast, interleukin-1 receptor antagonist concentrations were either equivalent (43) or lower (22,29) at those sites.Interleukin-1 has been reported to be expressed within gingival crevicular fluid during the early stages of gingival inflammation and its expression declined after 7 d (37,45,46). However, another study did not report peak concentrations of interleukin-1 within gingival crevicular fluid until 18 d (34). None of these studies described the role of anti-inflammatory factors in this process and did not include analysis of the adjacent gingiva.

There is also controversy about the relationship between the relative concentrations of interleukin-1 isoforms within both normal and inflamed gingival tissues (20,25). The total concentration of interleukin-1α, interleukin-1β and the interleukin-1/interleukin-1 receptor antagonist ratio, but not the total interleukin-1 receptor antagonist concentration, has been reported to be correlated with periodontal disease and alveolar bone resorption (22). However, the relationship between gingival inflammation and gingival tissue concentrations of interleukin-1 receptor antagonist remains unclear (47).

Thus, previous studies of interleukin-1 and interleukin-1 receptor antagonist in subjects with periodontal disease have either been of gingival crevicular fluid or of gingival samples obtained from a small number of subjects. As interleukin-1α and interleukin-1 receptor antagonist may be bound to tissue components and not available for secretion into either gingival crevicular fluid or the systemic circulation, it would be worthwhile to compare the relative concentrations of these isoforms within healthy and diseased gingiva, as their tissue concentrations may not be accurately represented by gingival crevicular fluid samples. Disparity between gingival and gingival crevicular fluid concentrations of the different interleukin-1 isoforms have diagnostic, and probably therapeutic, implications.

Material and methods

Gingival sample collection procedure

The gingival samples were collected from 110 Hispanic subjects who required extraction of adjacent teeth because of either extensive caries or periodontal disease. These subjects were treated during dental mission trips to various locations within rural regions of the Dominican Republic from 2002 to 2006. Our study was submitted to the Institutional Review Board of the University of Mississippi Medical Center prior to these mission trips and met requirements for exemption from Institutional Review Board committee review. Subjects were excluded from this study if they did not require extraction of adjacent teeth with intact crowns, were < 17 years of age, or had received any medications (other than local anesthetics) prior to the collection of tissue.

Following block anesthesia, the interdental gingival papilla was carefully removed using a scalpel. These tissue samples included the entire

gingival sulcus, junctional epithelium and adjacent connective tissue (48). Prior to the removal of the papilla, the gingival sulcular depth on both its mesial and distal surfaces was measured using a periodontal probe by a single investigator. The gingival sulcular depth that was recorded for each papilla was defined as the greater of those two measurements. Sites featuring bleeding on probing were also recorded. The papilla was placed in a vial containing 70% ethanol (49). One week later, the ethanol was removed from the vial using a pipette and the tissue sample was stored at −80°C.

Tissue preparation

The frozen tissue was thawed, weighed on a microbalance and then placed in a sufficient volume of phosphate-buffered saline to assure the following dilution: 10 mg of tissue/mL of phosphate-buffered saline + protease inhibitor (Sigma Chemical Company, St Louis, MO, USA). The samples were solublized in the phosphate-buffered saline solution by grinding with a mechanical homogenizer, followed by centrifugation at 2000 *g*. The supernatant was used for analysis of biomarkers.

Total protein assay

The total protein concentration within each sample was assessed using a commercial bicinchoinic acid assay kit (Pierce Chemical Company, Rockford, IL, USA). Aliquots from either tissue homogenates or protein standards were added in triplicate to the wells of microtiter plates and incubated with the working solution provided with the kit. The absorbance was read in a microplate spectrophotometer at 570 nm. The total protein concentration was calculated from the standard curve supplied with the kit and was recorded as mg/mL.

Enzyme-linked immunosorbent assay

Cytokine concentrations within each tissue sample were assayed by enzyme-linked immunosorbent assay, using commercial kits (R&D Systems, Minneapolis, MN, USA). Aliquots from either tissue homogenates or cytokine standards were added in duplicate to the wells of microtiter plates for determination of the concentrations of interleukin-1β, interleukin-1α, interleukin-1 receptor antagonist and interleukin-6. The absorbance of each well was read in a microplate spectrophotometer at 450 nm, and the tissue concentration of each cytokine was calculated from the standard curve included with each assay kit. Method and antiserum specificity and plate-to-plate variation controls were employed. Cytokine concentrations were expressed as pg/mg of protein.

Statistical analysis

We grouped gingival samples based on established parameters (50). Gingivae adjacent to a sulcus of ≤ 3 mm without bleeding on probing were classified as 'normal', gingiva adjacent to a 3-mm sulcus with bleeding on probing were classified as 'diseased-slight', gingiva adjacent to a 4–6-mm sulcus featuring bleeding on probing were classified as 'diseased-moderate' and gingiva adjacent to a sulcus of > 6 mm was classified as 'diseased-severe'. Our previous data suggested that our assay methods could detect a 15% difference in gingival cytokine concentrations as a function of sulcular depth (51). Based on previous data, we could assume equal variances in the population. By using $\alpha = 0.05$, we calculated 97% power with sample sizes of 11 in each group. For $\alpha = 0.01$, we also would have substantial power (87%) to determine significant differences in the data between groups containing a minimum of 11 subjects (SAS System 8.1; SAS Institute, Cary, NC, USA).

Statistical analysis of the outcome variables was performed using SPSS v13.0 (SPSS Inc., Chicago, IL, USA). Data were compared by factorial analysis of variance, the post-hoc Tukey test and the Pearson's correlation test. The ratio between either interleukin-1α or interleukin-1β, and interleukin-1 receptor antagonist [activity index (22)] was also calculated for each group. A p-value of < 0.05 was used to indicate significant differences between the outcome variables.

Results

Our Hispanic study population included 50 men and 60 women (total = 110) from the rural population of the Dominican Republic. The mean age of the men was 49.6 ± 19.4 years and the mean age of the women was 45.9 ± 14.4 years (mean ± standard deviation).

Gingival cytokine concentrations

The gingival concentrations of interleukin-6, interleukin-1 receptor antagonist, interleukin-1α and interleukin-1β were significantly higher when adjacent to sulci of ≥ 4 mm (diseased-moderate and diseased-severe sites) than when adjacent to either normal or diseased-slight sites (Table 1, Fig. 1). In addition, the gingival concentrations of interleukin-1 receptor antagonist and interleukin-1α were significantly higher within gingiva adjacent to diseased-severe sites than within gingiva adjacent to diseased-moderate sites (Table 1, Fig. 1). The ratio of interleukin-1 receptor antagonist to interleukin-1α- concentrations was higher within gingiva adjacent to 'diseased' (diseased-slight, diseased-moderate and diseased-severe) sites, than within gingiva adjacent to normal sites. The ratio of interleukin-1 receptor antagonist to interleukin-1β was highest within tissues adjacent to these diseased sites. There was a ratio of almost 2:1 between interleukin-1α + interleukin-1β and interleukin-1 receptor antagonist concentrations at all sites, with the highest ratio (2.5:1) within tissues adjacent to diseased-moderate sites (Table 2).

Correlations between the outcome variables

The gingival interleukin-6 and interleukin-1β concentrations were both significantly correlated with adjacent sulcular depth ($r^2 = 0.247$ and 0.237 respectively, $p < 0.05$). In addition, gingival concentrations of interleukin-1 receptor antagonist were significantly

Table 1. Concentration (mean ± standard error of the mean) of interleukin-6 and interleukin-1 isoforms within gingiva adjacent to either a 'normal' (≤ 3 mm without bleeding on probing) or a 'diseased' (≥ 3 mm + bleeding on probing) gingival sulcus

	Cytokine concentration (pg/mg of protein)			
Sulcus depth	IL-6	IL-1ra	IL-1α	IL-1β
≤ 3 mm 'normal' (n = 26)	10.84 ± 2.03	42.52 ± 5.39	46.12 ± 6.59	40.81 ± 5.97
3 mm 'diseased' (n = 27)	9.10 ± 1.29	44.85 ± 4.22	52.33 ± 5.75	46.08 ± 5.94
4–6 mm 'diseased' (n = 24)	25.04 ± 2.79[a]	71.89 ± 8.18[b]	81.27 ± 8.56[b]	98.81 ± 10.69[b]
>6 mm 'diseased' (n = 33)	30.16 ± 3.89[a]	108.48 ± 12.15[b,c]	123.78 ± 15.15[b,d]	108.69 ± 16.83[b]

[a]Significantly different from 'normal' and ≤ 3 mm 'diseased', $p < 0.05$.
[b]Significantly different from 'normal' and ≤ 3 mm 'diseased', $p < 0.001$.
[c]Significantly different from 4 to 6 mm 'diseased', $p < 0.05$.
[d]Significantly different from 4 to 6 mm 'diseased', $p < 0.001$.
IL, interleukin; IL-1ra, interleukin-1 receptor antagonist.

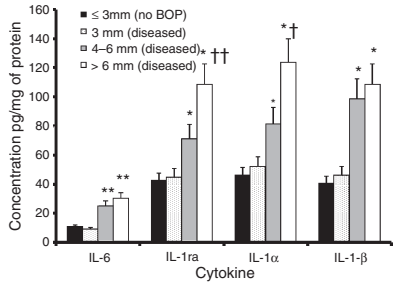

Fig. 1. Concentrations of interleukin-6 (IL-6), interleukin-1β (IL-1β), interleukin-1α (IL-1α) and interleukin-1 receptor antagonist (IL-1ra) (shown as mean ± standard error of the mean) within either 'normal' or 'diseased' gingiva. *Significantly different from 'normal' and ≤ 3 mm 'diseased', $p < 0.001$. **Significantly different from 'normal' and ≤ 3 mm 'diseased', $p < 0.05$. †Significantly different from 4 to 6 mm 'diseased', $p < 0.001$. ††Significantly different from 4 to 6 mm 'diseased', $p < 0.05$. BOP, bleeding on probing.

correlated with both interleukin-1α ($r^2 = 0.997$; $p < 0.001$) and interleukin-1β ($r^2 = 0.729$; $p < 0.001$), and the tissue concentrations of interleukin-1α and interleukin1β were significantly correlated ($r^2 = 0.708$; $p < 0.001$). Interleukin-1 isoform concentrations were negatively, but not significantly, correlated with interleukin-6 concentrations ($r^2 = -0.162$; $p < 0.13$).

Discussion

This is a cross-sectional study of a homogeneous Hispanic population with a high incidence of periodontitis and restricted access to professional care. In addition, these individuals had restricted access to medications that could have modified the host response to periodontal pathogens. Thus, this population had several unique qualities that made them an ideal group for the study of gingival inflammation. Because of the homogeneity of this population, the data had minimal variability.

We have confidence that our grouping of gingival samples appropriately represented normal (sulcus ≤ 3 mm + no bleeding on probing), diseased-slight (sulcus ≤ 3 mm + bleeding on probing), diseased-moderate (sulcus 4–6 mm + bleeding on probing), and diseased-severe (sulcus > 6 mm + bleeding on probing) sites, according to published guidelines (50,52) and the measurement error of the periodontal probe (± 1 mm). The positive correlation between interleukin-6 concentrations taken together with the mean sulcular depth indicated the severity of the inflammation within each of the gingival samples (51,53).

There is significant disagreement concerning the concentration of interleukin-1 isoforms within gingival crevicular fluid obtained from both healthy and diseased sites. Interleukin-1β concentrations within gingival crevicular fluid have been reported to be higher at sites adjacent to inflamed gingiva (22,32,34,37–43,47,54–57), supporting our data obtained from gingival tissues. However, in contrast, there are several studies reporting lower concentrations of interleukin-1β and interleukin-1 receptor antagonist within gingival crevicular fluid obtained from sites adjacent to gingival inflammation (20,22,25,29,47,58). In addition, interleukin-1 receptor antagonist concentrations within gingival crevicular fluid have been reported to be 1000-fold higher at sites of gingival inflammation compared with healthy sites (47,56). Our data differ from the results of previous studies of gingival crevicular fluid, possibly because of binding of the interleukin-1 isoforms to gingival cells and tissue components, making them unavailable for inclusion within the gingival crevicular fluid. However, we do not dispute the role of these isoforms in the pathogenesis of inflammation, but only report differences in their relative concentrations in gingival tissue and gingival crevicular fluid samples. Our data, taken together, suggest that the gingival concentrations of interleukin-1α and interleukin-1β are higher than that of interleukin-1 receptor antagonist in both healthy and diseased tissue and increase in parallel with the gingival sulcular depth during the pathogenesis of gingival inflammation, extending the

Table 2. Ratios of interleukin-1α (IL-1α) and interleukin-1β (IL-1β) and interleukin-1 receptor antagonist (IL-1ra) within gingiva adjacent to either a 'normal' (≤ 3 mm without bleeding on probing) or a 'diseased' (≥ 3 mm + bleeding on probing) gingival sulcus

	Cytokine concentration ratios		
Sulcus depth	IL-1α : IL-1ra	IL-1β : IL-1ra	IL-1α + IL-1β : IL-1ra
≤ 3 mm 'normal' (n = 26)	1.09:1	0.96:1	2.04:1
3 mm 'diseased' (n = 27)	1.17:1	1.03:1	2.19:1
4–6 mm 'diseased' (n = 24)	1.13:1	1.37:1	2.50:1
>6 mm 'diseased' (n = 33)	1.14:1	1:1	2.14:1

previous studies of gingival tissues and gingival crevicular fluid. However, because our data were from a cross-sectional experimental design, we could not determine the relative concentrations of the interleukin-1 isoforms during the early pathogenesis of the gingival inflammation (37,46). The concentrations of the interleukin-1 isoforms within our samples probably represented the equilibrium established within gingiva following the release of interleukin-1 receptor antagonist in response to increasing gingival concentrations of interleukin-1β, which has not previously been described. Previous studies have reported elevated gingival tissue concentrations of interleukin-1 isoforms coincident to periodontal disease, but were of samples obtained only from severely inflamed sites (adjacent sulcus depth > 6 mm) (22,35). Our data support those studies and extend them to report, for the first time, elevated concentrations of interleukin-1 isoforms within both diseased-slight and diseased-moderate sites. We also reported, for the first time, significant correlations between the gingival tissue concentrations of interleukin-1 isoforms at several stages of the pathogenesis of periodontal disease. Our data did not indicate a significant negative correlation between gingival interleukin-1 isoforms and interleukin-6 concentrations, as has been reported during endotoxin challenge in mice (59) and within synovial fibroblasts from rheumatoid arthritis tissues (15). However, our data indicated a trend toward significance, and study of additional gingival tissue samples could have yielded a statistically significant negative correlation between the concentrations of those cytokines.

Thus, we suggest that, within inflamed gingiva, interleukin-1β is probably not overproduced and synthesis of interleukin-1 receptor antagonist impaired, as suggested by studies of inflammation within other tissue types. Our data indicated that the concentrations of gingival interleukin-1 receptor antagonist were not equal to the sum of the concentrations of interleukin-1α and interleukin-1β, suggesting that interleukin-1 receptor antagonist may not be the unique regulator of interleukin-1α and interleukin-1β within gingiva, as suggested by previous studies of inflammation within connective tissue from nonoral sites (60,61). It is possible that the relatively low concentration of interleukin-1 receptor antagonist compared with the sum of the concentrations of interleukin-1α and interleukin-1β represents a progression factor for gingival inflammation. Additional mechanisms for the regulation of interleukin-1 concentrations at sites of inflammation, such as cross-talk between interleukin-1 and interleukin-6 signaling pathways, which have been described in studies of synovial fibroblasts obtained from sites of rheumatoid arthritis (15), must therefore be considered as factors for the progression of periodontal inflammation.

Considering all the results together, we propose that the concentrations of interleukin-1 isoforms are elevated within the gingiva during the pathogenesis of gingivitis and periodontitis. However, the proportion of interleukin-1 receptor antagonist to both interleukin-1α and interleukin-1β within 'diseased' gingiva was lowest at the sites with the most severe inflammation (diseased-severe). These data also explain why the addition of interleukin-1 receptor antagonist to sites of joint inflammation promotes healing (19,26) and supports the further study of interleukin-1 receptor antagonist as a treatment for gingival inflammation.

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