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ABSTRACT

While much research has focused on local and systemic factors contributing to periodontal disease, little is known regarding mechanisms linking these factors. We have previously reported a systemic hyper-inflammatory response to bacterial endotoxin in localized aggressive periodontitis (LAP). The objectives of this study were to delineate cyto/chemokines in gingival crevicular fluid (GCF) and evaluate systemic levels of endotoxin associated with LAP. Clinical parameters, GCF, and peripheral blood were collected from: 34 LAP, 10 healthy siblings, and nine healthy unrelated control individuals. Cyto/chemokines were quantified in GCF, systemic endotoxin levels were quantified in plasma, and correlation analysis was performed among all parameters. Nine mediators were elevated in LAP diseased sites as compared with healthy sites (TNF α , INF γ , IL1 β , IL2, IL6, IL10, IL12p40, GM-CSF, and MIP1 α , $p < 0.001$), while MCP1, IL4, and IL8 were elevated in healthy sites ($p < 0.01$). Four- to five-fold-higher endotoxin levels were detected in LAP plasma compared with that from healthy participants ($p < 0.0001$), which correlated with all clinical parameters and most cyto/chemokines analyzed. In conclusion, higher systemic levels of endotoxin were found in LAP, which correlates with an exacerbated local inflammatory response and clinical signs of disease. (Clinicaltrials.gov number, NCT01330719).

KEY WORDS: aggressive periodontitis, endotoxin, cytokines, chemokines, gingival crevicular fluid, LPS.

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Local Inflammatory Markers and Systemic Endotoxin in Aggressive Periodontitis

INTRODUCTION

Aggressive periodontitis (AgP) is a group of less prevalent, severe, and rapidly progressing forms of periodontitis, characterized by early age onset, initial involvement of molars and incisors, rapid bone destruction, and propensity for familial involvement. Given its less frequent occurrence and difficulties in gathering large populations, knowledge about AgP mechanisms is limited.

Host response plays a critical role in connective and bone tissue destruction in response to bacterial invasion, where cytokines and chemokines are produced in response to bacterial components. Animal and human studies have correlated periodontal destruction and specific cytokines, such as IL2, IL6, IL1 β , and INF- γ in the gingival crevicular fluid (GCF) (Lee *et al.*, 1995; Dutzan *et al.*, 2009).

Similarly, additional studies have provided a correlation between systemic dissemination of bacterial lipopolysaccharide (LPS) and periodontal disease (Pussinen *et al.*, 2004, 2007). Thus, in the context of periodontitis, elevated levels of Gram-negative bacteria can lead to increased levels of LPS in circulation, which, in turn, activate inflammatory mediator production, perpetuating an inflammatory cycle of periodontal destruction.

Little is known regarding the roles of these mediators and the contribution of systemic LPS in AgP. We have identified a cohort of African-American children diagnosed within one clinical setting with very similar patterns of localized AgP (LAP). We previously reported a systemic hyper-inflammatory response to bacterial endotoxin in this cohort (Shaddox *et al.*, 2010). The objectives of this study were to characterize clinical aspects of this population along with local inflammatory profile and systemic LPS levels. Our hypothesis is that individuals with LAP present elevated plasma LPS levels due to local tissue destruction, which, in turn, is associated with elevated levels of cyto/chemokines when compared with those in healthy individuals.

MATERIALS & METHODS

Participant Population and Clinical Measurements

This article is part of a larger study, which has been registered at clinicaltrials.gov (NCT01330719). All participants were recruited from the Leon County Health Department, Tallahassee, Florida (February 2007- November 2009). All data collected were obtained under Institutional Review Board (IRB) informed consent. Inclusion criteria: 5 to 20 yrs old; African-American; and diagnosed with LAP, defined by ≥ 2 teeth presenting pocket depth ≥ 5 mm with bleeding on probing, attachment loss ≥ 2 mm, and radiographic bone

Table 1. Demographic and Clinical Parameters (mean $\pm$ standard deviation)

	LAP (34)	HS (10)	HC (9)
Age	14.04 $\pm$ 4.09	12.56 $\pm$ 5.25	13.33 $\pm$ 4.61
PD all (mm)	3.78 $\pm$ 0.98*	2.12 $\pm$ 0.36	2.21 $\pm$ 0.49
PD pockets (mm)	5.30 $\pm$ 0.67	—	—
CAL all (mm)	0.60 $\pm$ 0.45*	ND	ND
CAL pockets (mm)	3.49 $\pm$ 1.25	—	—
% sites > 4 mm	20.28 $\pm$ 12.93*	0	0
% sites > 7 mm	3.40 $\pm$ 3.59*	0	0
% Bleeding	17.86 $\pm$ 11.75*	4.44 $\pm$ 4.16	1.85 $\pm$ 2.10
% Plaque	49.62 $\pm$ 23.30	65.31 $\pm$ 20.33	39.19 $\pm$ 33.85

LAP = localized aggressive periodontitis; HS = healthy siblings; HC = healthy unrelated control individuals. PD = mean full-mouth probing depth; PD pockets = mean PD in diseased sites; CAL = mean full-mouth clinical attachment loss; CAL pockets = mean CAL in diseased sites. ND = not detected since healthy participants did not present any attachment loss. * $p < 0.001$ LAP vs. healthy siblings and healthy control individuals (ANOVA with Dunn's multiple comparisons).

loss; periodontally healthy participants were age-, sex-, and race-matched. Exclusion criteria: the presence of systemic diseases, conditions, or medications that influence progression and/or clinical characteristics of periodontal disease; having taken antibiotics within the previous 3 months; smokers; or pregnant/lactating women. Complete medical and dental histories and periodontal clinical parameters were collected, including: pocket depth (PD), clinical attachment loss (CAL), bleeding on probing (BoP), visible plaque (P), and radiographic examination. CAL was calculated by PD + GM (gingival margin position). GM was given a negative value when located above the CEJ (cementum-enamel junction). All measurements were performed with the use of a UNC-15 periodontal probe at 6 sites *per* tooth and were recorded with appropriate computer software (Florida Probe, Gainesville, FL, USA).

Gingival Crevicular Fluid (GCF) Sampling

GCF samples were collected from a periodontally diseased site [PD $\geq$ 5 mm, CAL $\geq$ 2 mm, and BoP] as well as from a healthy site [PD $\leq$ 3 mm, no BoP]. Both the diseased and healthy sites were sampled from LAP participants, while a healthy site was sampled from healthy participants. Sites for GCF collection were isolated with cotton rolls, gently air-dried, and supragingival plaque removed. A collection strip (PerioPaper GCF collection strips, Oraflow Inc., Plainview, NY, USA) was inserted into the sites 1 to 2 mm into the sulcus for $\sim$ 10 sec. We measured the volume of GCF (Periotron 8000, Oraflow, Inc.) to obtain a specific range of protein content compatible with Luminex analysis (data not shown). Blood-contaminated samples were discarded. Samples were eluted from the strip in 300 μ L of phosphate-buffered saline (PBS) by centrifugation into an Eppendorf tube. Total protein content of the eluate was determined (BCA Protein Assay, Pierce Thermo Scientific, Rockford, IL, USA). Eluates were stored at -80°C until assays were performed.

Inflammatory Mediator Analysis

We used fluorescence detection kits (Milliplex[®] 29-plex cytochemokine detection kits, Millipore, St. Charles, MO, USA) to detect and quantify 29 cyto/chemokines [IL1 α , IL1 β , IL1 γ , IL2,

IL4, IL5, IL6, IL7, IL8, IL10, IL12(p40), IL12(p70), IL13, IL15, IL17, EGF, Exotaxin, Fractalkine, G-CSF, GM-CSF, IFN γ , IP10, MCP1, MIP1 α , MIP1 β , sCD40L, TGF α , TNF α , and VEGF]. Methodology was followed according to the manufacturer's instructions (Pierce Thermo Scientific). Data were acquired with the use of instrumentation (Luminex[®] 100[™], Millipore) and analyzed with software (Milliplex Analyst[®], Viagene Tech, Carlisle, MA, USA), standard curves, and five-parameter logistics. Data are reported as normalized pg/mL. Normalization to total protein content was obtained according to the formula: normalized pg/mL = [pg/mL $\times$ protein content corrective ratio], where corrective ratio = [lowest protein concentration/protein concentration of sample of interest].

Plasma LPS Levels

One 13 $\times$ 75 mm heparinized vacutainer tube (2.0–4.0 mL) of blood was drawn from all participants. Plasma was separated from red blood cells by centrifugation ($\sim$ 300 $\times$ g for 15 min) and stored at -80°C until analysis was performed. Plasma lipopolysaccharide (LPS) levels were detected and semi-quantified by a chromogenic assay (Endpoint Chromogenic LAL Assay, Lonza, Basel, Switzerland). Endotoxin units/mL were calculated by a standard curve and best-fit linear trend line.

Statistical Analysis

Statistical tests were applied for each inflammatory marker, clinical parameter, and LPS level among the groups. Analysis of variance on ranks (Kruskal-Wallis test) was applied at a significance level of $\alpha = 0.05$. We used Dunn's Multiple Comparisons test to evaluate all pairwise comparisons. In addition, Spearman correlations and forward stepwise regression were run among all variables.

RESULTS

Participant Cohort and Clinical Characteristics

The present study addresses periodontal conditions, local inflammatory profiles, and systemic LPS levels in 34 participants diagnosed with LAP, 10 healthy siblings (HS), and nine

Figure 1. Inflammatory mediator profile in the GCF. GCF was used for cyto/chemokine detection. D/D = LAP participant/diseased site (■), D/H = LAP participant/healthy site (▼). HS/H = healthy sibling/healthy site (○), HC/H = healthy control/healthy site (△). Dot-plots show individual participant GCF values and the median (horizontal line). Values are reported as normalized pg/mL. Any significance found was indicated by a bar on top of each graph connecting the two comparisons. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ (Kruskal-Wallis ANOVA with Dunn's multiple comparisons). Note outlier on siblings group identified by a square. This outlier represents a healthy sibling who developed disease 1 yr following initial examination.

healthy unrelated control individuals (HC). Table 1 presents the demographics and clinical parameters for participants involved in this study. LAP participants presented with localized periodontal pockets ranging from 5 mm to 11 mm, attachment loss ranging from 2 to 9 mm, and radiographic bone loss. All participants with LAP had first molar involvement, while some had additional involvement of incisors. Eleven participants had mixed dentition, and disease was present on primary teeth. The Appendix Fig. is representative of the clinical characteristics of participants with LAP in this population.

GCF Inflammatory Mediator Profiles

Several inflammatory mediators were detected in the GCF of participants (Fig. 1). Cyto/chemokines evaluated but not presented here were not detected at significant levels. Diseased sites from LAP participants presented higher levels of TNF α , INF γ , IL1 β , IL2, IL10, IL12p40, GMCSF, and MIP1 α when compared with levels in their own healthy sites ($p < 0.0001$) as well as in healthy sites of HS and HC ($p < 0.0001$). While IL6 was higher in LAP diseased sites compared with LAP healthy sites ($p = 0.002$), they were not higher than those found in HS or HC healthy sites ($p > 0.05$). Conversely, MCP1 was elevated

in all periodontally healthy sites compared with diseased sites, while IL4 was elevated in LAP and HC healthy sites. Our analysis revealed a single outlier in the HS cohort which presented elevated levels of most mediators analyzed (Fig. 1, boxed data-point). It is of interest to note that this specific site presented disease initiation/breakdown 1 yr after initial examination of healthy periodontium (data not shown).

Plasma LPS Levels and Correlations

Analysis of plasma LPS levels demonstrated a four- to five-fold-higher level of circulating endotoxin in LAP compared with HS and HC (Fig. 2) ($p = 0.0002$). Importantly, plasma LPS levels correlated with multiple clinical parameters of LAP, including mean PD, CAL, % of PD > 4 mm, and BoP ($p < 0.01$). Stepwise regression between LPS and clinical parameters showed that LPS can be predicted by CAL ($p < 0.001$).

In addition, LPS correlated with most cyto/chemokines, except for GMCSF. Negative correlations were found between LPS and MCP1, IL6, and IL8 (see Appendix Table for all parametric correlations). Stepwise regression analysis showed that LPS can be predicted from a linear combination of TNF α ($p = 0.053$) and INF γ ($p < 0.001$); mean PD can be predicted by IL10 ($p < 0.001$);

BoP can be predicted by IL6 ($p = 0.007$) and $\text{INF}\gamma$ ($p = 0.028$); mean CAL can be predicted by IL12p40 ($p < 0.001$); and % sites > 4 mm can be predicted by IL6 ($p < 0.001$) and GM-CSF ($p = 0.042$).

DISCUSSION

The current prevalence of AgP in children and young adults in the US is around 1 to 2% and is estimated to be up to 3 times more prevalent in African-Americans (Albandar and Tinoco, 2002). However, early stages of this disease can go undiagnosed until clear signs of bone destruction are present. If early stages of disease are detected in a young population, treatment can be applied in a more appropriate timeframe, potentially preventing the rapid progression of AgP. More importantly, an understanding of AgP disease mechanisms will also allow for a more appropriate treatment regimen for this disease.

Detection and identification of mediators in the gingival crevicular fluid (GCF) of both healthy and diseased periodontal sites could be important adjuncts to early disease diagnosis as well as provide insight into disease mechanisms. In the present study, we demonstrated elevated levels of at least 9 inflammatory mediators in the GCF of LAP participants' diseased sites compared with their healthy sites, as well as with healthy sites in healthy participants. These results are in agreement with those of other studies reporting higher GCF levels of IL-1 β and GM-CSF in the GCF of individuals with generalized aggressive periodontitis (GAP) when compared with that of periodontally healthy participants (Toker *et al.*, 2008; Teles *et al.*, 2010). In particular, IL1 β increases monocyte cytotoxicity as well as the adhesion of neutrophils, monocytes, T-cells, and B-cells at the site of infection. IL1 β also assists the humoral and adaptive immune response by stimulating B-cell proliferation and T-cell production of IL2, which was also found to be elevated in LAP diseased sites. Although IL10 is often considered an anti-inflammatory cytokine, elevated levels of IL10 in diseased GCF are also responsible for differentiation of cytotoxic T-cells and act as a CD8 $^{+}$ T-cell chemoattractant, promoting an adaptive response capable of local tissue destruction observed in LAP. Collectively, these inflammatory mediators represent a pro-inflammatory response in the local tissues which has the potential to serve as a marker for LAP disease progression. Our methodology for GCF collection differed from those of previous studies in the time of collection. We used 10 sec of collection time (instead of 30 sec as reported by some studies) to avoid saturating the strip, especially in diseased sites. Analysis of our preliminary data has shown that 10 sec would give us enough protein content to be detected by the Luminex analysis without need for dilutions (data not shown).

This study also reports lower levels of 3 inflammatory mediators [MCP1, IL4, and IL8] in the GCF of LAP participants' diseased sites when compared with healthy sites. Interestingly, MCP1 and IL8 also showed negative correlations with plasma levels of LPS. While MCP1 is responsible for monocyte chemotaxis and has been proposed to have a role in osteoclast differentiation, its expression is suppressed by GM-CSF (Kim *et al.*, 2005). Therefore, the elevated levels of GM-CSF in diseased sites found in the present study could

Figure 2. Plasma LPS levels. Plasma was used for the detection of circulating lipopolysaccharide levels (LPS). Localized aggressive periodontitis (LAP), healthy siblings (HS), healthy unrelated control individuals (HC). * $p = 0.0002$, LAP vs. HS and HC (ANOVA with Dunn's multiple comparisons).

explain the reciprocal lower levels of MCP1 in these sites. Similar to findings by Ozmeric *et al.* (1998), the present study showed IL8 levels to be no different or slightly lower in individuals with LAP than in healthy control individuals. IL8 is mainly responsible for neutrophil chemotaxis as well as inducing neutrophil degranulation. Therefore, it is usually expressed at early stages of insult and possibly not during the chronic inflammatory stage seen upon clinical presentation of LAP. IL8 also inhibits the adhesion of leukocytes to activated endothelial cells and therefore possesses some anti-inflammatory activities. Similarly, IL8 plays an important role in the process of wound healing which seems to be absent or dysfunctional in the case of LAP. While Ozmeric *et al.* (1998) suggested that less active IL-8 production in spite of a dense bacterial stimulation in LAP could indicate impaired protection against periodontal infections, it is also possible that the stage of disease during which evaluation was performed and/or wound-healing defects occurred explains the lower levels observed.

Interestingly, GCF from a healthy site of one of the siblings who was initially diagnosed as periodontally healthy presented with mediator levels similar to those of diseased LAP sites. This specific site presented disease breakdown at a later time-point (data not shown). Some of the mediators associated with LAP diseased sites (TNF α , GM-CSF, IL2, IL12p40, IL1 β , $\text{INF}\gamma$, IL6, and IL10) as well as some of those associated with healthy sites (IL8 and MCP1) were elevated. This may suggest that different soluble mediators are associated with different stages of periodontal disease and thus may have the potential to be used as adjuncts to clinical measurements to allow for possible early diagnosis. Longitudinal follow-up of our healthy siblings and unrelated control individuals, as well as healthy sites of LAP participants, will enable us to evaluate the role of GCF mediators in disease initiation and other stages of disease progression.

Periodontal disease has been previously correlated positively with plasma LPS, C-reactive protein concentrations, as well as macrophage cytokine production (Pussinen *et al.*, 2004, 2007). Although the disease in the present study is present in a younger

population and is localized to specific oral sites, elevated systemic levels of endotoxin were observed in LAP, and those levels were strongly correlated with clinical parameters and most elevated cyto/chemokines. Overgrowth of Gram-negative bacteria and periodontal pocket ulceration and inflammation may result in increased release of lipopolysaccharide (LPS) to circulation, which in turn activates host immune cells in the production of inflammatory markers, perpetuating an inflammatory cycle.

Some studies have reported AgP to be associated with polymorphonuclear cell (PMN) abnormalities (Van Dyke *et al.*, 1982, 1988; Genco *et al.*, 1986) or depressed neutrophil chemotaxis (Van Dyke *et al.*, 1985). Other authors have reported elevated presence of pro-inflammatory cytokines, such as TNF α and IL1, in the plasma of individuals with AgP (Shapira *et al.*, 1994), where this elevation is the biological basis for altered neutrophil function. We have reported a systemic hyper-inflammatory response, an elevated release of inflammatory mediators in response to TLR2 and TLR4 stimulation, in our LAP cohort, as well as an attenuated systemic inflammatory response of the healthy siblings (Shaddox *et al.*, 2010). Similarly, we reported a localized high prevalence of MMPs (Alfant *et al.*, 2008) in the same cohort. The present study reports a pro-inflammatory cytokine milieu also in the GCF, which correlates with elevated plasma LPS levels and LAP defining clinical parameters. Analysis of these data, taken together, provides a model in which a localized inflammatory response can lead to local tissue destruction, resulting in systemic release of immune activating agents such as LPS. In populations prone to immune hyper-responsiveness, this systemic release may lead to a perpetuating inflammatory cycle where local inflammation recruits systemically activated immune cells, leading to excessive and rapid local tissue destruction.

Possible limitations of the present study were: (1) collection of 1 GCF strip from a diseased and a healthy site, which means that there could be variations in the degree of inflammation and disease activity in different oral sites, so it would be interesting to evaluate inflammatory patterns in different sites in future studies; and (2) the limited quantity of LAP in primary dentition, which means that as we add more primary dentition disease to this cohort, we will be able to evaluate possible differences in inflammatory patterns and systemic response in primary vs. permanent dentition LAP.

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