Resolution of Crevicular Fluid Leukocyte Activity in Patients Treated for Aggressive Periodontal Disease

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Background: Enhanced neutrophil responses play a critical role in the activation of the innate immune system and causation of aggressive periodontitis (AgP). The hypothesis that comprehensive periodontal treatment expedites resolution of amplified leukocyte activity and facilitates the reconstitution of periodontal health was tested.

Methods: Four different gingival crevicular fluid (GCF) markers from 14 patients were characterized prior to and at 3, 6, 12, 24, and 36 months after periodontal therapy. GCF myeloperoxidase (MPO), beta-N-acetyl-hexosaminidase (beta-NAH), and beta-glucuronidase (beta-G) were determined spectrophotometrically, and cathepsin D (CD) by liquid scintillation counting using [^{14}C] hemoglobin as substrate. The primary outcome was long-term stability of periodontal health.

Results: In untreated AgP, GCF markers were significantly amplified (MPO: 1.9-fold; beta-NAH: 1.3-fold; beta-G: 1.7-fold; CD: 4.7-fold). Following periodontal therapy, the leukocyte activity was significantly dampened (0.3- to 0.5-fold), and paralleled with a sustained improvement of periodontal health (P <0.05). Thereafter and at 3 years, GCF leukocyte responses remained on a physiologic low level compatible to normal immune function.

Conclusions: Comprehensive treatment of AP induces a downregulation of amplified crevicular neutrophil activity. The release of the innate immune system from exacerbating damage elicits a successful reconstitution of long-term periodontal health with no setbacks seen after 3 years. *J Periodontol 2002;73:995-1002.*

KEY WORDS

Gingival crevicular fluid/analysis; periodontal diseases/therapy; neutrophils; immune system/physiopathology.

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It is thought that periodontal inflammation may affect and worsen systemic diseases/conditions such as cardiovascular disease, preterm labor, diabetes mellitus, and other clinical syndromes associated with aberrant leukocyte responses.[1,2] In a revised classification system for periodontal diseases,[3] aggressive periodontitis was redefined to comprise a complex entity of microbial alterations and cellular dysfunctions that differentiate the underlying molecular mechanisms from chronic periodontal disease. Genetic susceptibility, environmental and internal triggers of autoreactivity, and changes in the pathological processes as the disease progresses may account for the severity of the disease.

Excessive neutrophil infiltration is critical to the induction of inflammation of healthy tissues and the amplification of progressing periodontal disease. Several prominent compounds of neutrophil origin have been identified that amplify the inflammatory response including enzymes, superoxide radicals, and reactive oxygen derivates.[4,5] Although anti-inflammatory agents dampen inflammation by inhibiting key proinflammatory mediators, our knowledge of the endogenous mechanisms that govern the resolution of host responses in severe inflammatory conditions is limited.[6]

Gingival crevicular fluid (GCF) contains leukocytes, plasma-proteins, and endproducts of the local cellular and microbial activity, thus contributing to the individual's response to periodontal infection. The myeloperoxidase-hydrogen peroxide-chloride system, which is part of the innate host defense mediated by polymorphonuclear

leukocytes, possesses potent antimicrobial activity.[7] The liberation of the 150-kDa protein myeloperoxidase (MPO) from the azurophilic granules of neutrophils into the extracellular space gives rise to highly reactive oxygen species, including O_2^- and HOCl. Other mediators of homeostasis and tissue inflammation include glycosaminoglycan-degrading glycosidases, glycoside sulfatases, and acid hydrolases from terminal lysosomes of neutrophils.[8,9] The 60-kDA acid lysosomal hydrolase beta-N-acetyl-hexosaminidase (beta-NAH) emanates into GCF during neutrophil phagocytosis. Salivary beta-NAH-glycosidase activities correlate with the degree of inflammation after treatment.[10] Under secretory conditions, the precursor forms of the newly synthesized enzyme will be liberated. During phagocytosis and cellular lysis, the lysosomal beta-N-acetyl-hexosaminidase is present. The 52-kDa lysosomal aspartate-endopeptidase cathepsin D emigrates from the primary azurophilic granules of neutrophils into the extracellular compartment. Cathepsin D (CD) reacts against basement membranes and is functional in a wide variety of tissues during their remodeling or regression and in apoptosis. It is located in fibroblasts and macrophages of the subepithelial connective tissue and in desquamating junctional epithelial cells of the gingival crevice.[11] As a marker of protein hydrolysis and cellular lysis, increasing levels represent the endpoint of tissue-mediated destruction.[12,13] As an important compound of the primary azurophilic granules of leukocytes, the 290-kDa beta-glucuronidase (beta-G) is involved in the catabolism of proteoglycans cleaving non-reducing ends of tetrasaccharides and larger polysaccharides.[14] Beta-G causes cell damage by antigen-induced endocytosis and phagocytosis, formation of immune complexes, and stimulation of complement and apoptosis.[15]

Based on the concept of a functional link between downregulation of leukocyte infiltration and resolution of disease, the hypothesis was tested that in AgP, comprehensive periodontal treatment expedites resolution of amplified polymorphonuclear leukocyte (PMN) recruitment and facilitates the reconstitution of periodontal health.

MATERIALS AND METHODS

Study Population

Fourteen subjects with AgP[3] and evidence of prior attachment loss were recruited from the Department of Periodontology, University of Münster. The patients enrolled into the study (39.6 ± 8.1 years) were screened for past history of periodontitis by radiographic evidence of intrabony defects exceeding more than 50% at the last visit, and probing depths ≥6 mm in at least 8 sites within each quadrant (Table 1). AgP subjects displayed generalized severe periodontal tissue destruction, loss of periodontal support inconsistent with age, persistent gingival inflammation, increasing probing depths, and progressing tooth mobility.[16] They were recruited from referring dentists since control of disease was not achievable in the past. Patients were asked about current and past smoking habits, and packs per year were calculated. Individuals who reported smoking more than 10 cigarettes per day were defined as actual smokers. Patients who had quit smoking for at least 2 years were classified as former smokers. Exclusion criteria included pregnancy, periodontal therapy, or antibiotics in the previous 6 months and any systemic condition that might have affected the progression or treatment of periodontitis and the need for premedication for therapy. Subjects fulfilling the inclusion criteria above were asked to participate in the study and, if they accepted, to sign informed consent documents. Patients were requested to report to the principal investigator any orofacial or medical infection for which antibiotics were administered.

Table 1.

Baseline Demographic and Clinical Characteristics of AgP Subjects

	Females	Males	Total
N subjects	6	8	14
Age at baseline (years)	40.2 ± 7.0	39.3 ± 9.2	39.6 ± 8.1
Number of sites examined	46	62	108
% sites CAL			
<4 mm	0 (0)*	1.6 (1)	0.9 (1)
4-6 mm	21.7 (10)	37.1 (23)	30.6 (33)
>6 mm	78.3 (36)	61.3 (38)	68.5 (74)
% sites GI			
0	0 (0)	1.6 (1)	0.9 (1)
1	6.5 (3)	4.8 (3)	5.6 (6)
2	32.6 (15)	64.5 (40)	50.9 (55)
3	60.9 (28)	29.0 (18)	42.6 (46)

*N in parentheses.

Biochemical Analysis

GCF sampling. In each patient, GCF was harvested at 8 teeth (2 per quadrant) at mesio- or disto-interproximal sites with probing depths (PD) between 6 and 8 mm prior to surgery and at the 3-, 6-, 12-, 24-, and 36-month reexamination visits. Following removal of supragingival plaque with a sterile Gracey 11/12 curet tip, sites to be sampled were isolated with cotton rolls and gently air dried. Then, a paper strip§ was inserted at the entrance of the periodontal pocket for 30 seconds.[17] The GCF volume was determined using an instrument‖ that was calibrated with a quadratic regres-

§ Harco, Tustin, CA.
‖ Harco Periotron 6000, Siemens, Bensheim, Germany.

sion equation curve using serum, 0.9% saline, and 1:1 aliquots of serum and saline. Prior to each periodontal examination, the GCF samples were collected, and the GCF volumes immediately read.‖ The strips were placed into empty 1.5 ml Eppendorf vials and stored at 4°C. Samples visibly contaminated with blood were discarded. The samples were eluted in 160 µl 0.9% sterile saline at pH 7.4 for 10 minutes with constant agitation, and centrifuged at 14,000 g over a 2-minute period. The supernatant was separated into 30 µl fractions¶ and frozen in liquid nitrogen at –196°C.

Recovery and validation. A paper strip was inserted into 4 × 10 µl aliquots of each of the diluted enzymes. The 4 samples per GCF marker were immersed into 160 µl 0.9% sterile saline and vortexed. Strips and liquid were separated by centrifugation (2 minutes, 14,000 g). In the supernatant, the total amount of enzyme activities liberated from the 16 paper strips were determined according to the assay procedures described below and calculated as a percentage of the known enzyme. The mean percent recoveries from the paper strips as relative percent to the corresponding standards were 82.9 ± 18.5% (MPO), 89.1 ± 13.4% (beta-NAH), 91.6 ± 12.4% (beta-G), and 95.2 ± 17.6% (CD).[18] The validation of the enzyme assays was performed with HL-60 promyelocytes.[13]

Myeloperoxidase. Ten µl of the diluted GCF sample was added to 0.1 M citric acid buffer, pH 5.5, containing 0.125% Triton-X-100 solution and 0.1 mM hydrogen peroxide. The oxidation of H_20_2 was performed using 0.8 mM σ-dianisidin as substrate. After 7 minutes of incubation at 21°C, the samples turned brown and the oxidative reaction was stopped by adding glycine/NaOH, pH 10.4. The myeloperoxidase activity of the GCF eluates was determined spectrophotometrically at a wavelength (wv) of 405 nm (reference wv, 650 nm) on uncoated 96-well microtiter plates using a microplate reader.# The total MPO activities were calculated in duplicate in µU GCF sample.

Beta-N-acetyl-hexosaminidase. Ten µl of the diluted GCF sample was eluted in 25 µl 0.9% NaCl, and 0.1 M citric acid buffer, pH 4.6, and 25 µl *p*-nitrophenyl-N-acetyl-beta-D-glusoaminide** were added. The reaction was allowed to proceed at 37°C for 30 minutes and then stopped with 225 µl 0.4 M Glycin-NaOH stopping buffer, pH 10.4. The focused *p*-nitrophenolate was assessed at a wv of 405 nm (reference wv, 492 nm). The total beta-NAH activities were calculated in duplicate in µU GCF sample.

Beta-glucuronidase. Ten µl of the diluted GCF sample was eluted in 25 µl 0.9% NaCl; and 25 µl 4-nitrophenyl-beta-D-glucuronide,†† diluted in 0.2% bovine serum albumin, 0.04% NaN_3, and 0.1 M citric acid buffer, pH 4.6, was added. After incubation at 37°C for 24 hours, the reaction was stopped with 225 µl 0.4 M Glycin-NaOH, pH 10.4. The total beta-G activities were determined spectrophotometrically at a wv of 405 nm (reference wv, 492 nm), computed in duplicate, and expressed in µU GCF sample.

Cathepsin D. The concentration of cathepsin D was determined by liquid scintillation counting using [^{14}C] hemoglobin as substrate. The diluted sample (10 µl) was incubated in a total volume of 200 µl 0.1 M Na-acetate buffer, pH 3.65, with 10 µg [^{14}C] hemoglobin, 50,000 counts per minute (cpm). After 6 hours of incubation at 37°C, the reaction was stopped by adding 0.5 ml 1% (w/v) casein solution and 0.5 ml 25% (w/v) trichloroacetic acid. The samples were centrifuged for 5 minutes at 14,000 × g, and the radioactivity in 1 ml of the supernatant was assessed in a volume of 2 ml scintillation liquid.‡‡ The radioactivity of the supernatant was computed in a liquid scintillation counter as counts per minute, with 1 cpm = 240 pg [^{14}C] hemoglobin. The total cathepsin D activities per 10 µl hemoglobin were determined in duplicate, and the average values were calculated in ng.

Periodontal Examination and Intervention

All patients were monitored at baseline and at 3, 6, 12, 24, and 36 months after periodontal treatment. The clinical examination and periodontal treatment protocols have been described in a previous investigation.[19] The clinical examination included the evaluation of probing depths (PD), clinical attachment levels (CAL), the gingival (GI) and plaque indexes (PI), and gingival crevicular fluid volumes (GCFv). All individuals received antibiotic therapy with 3 × 500 mg amoxicillin§§ and 3 × 250 mg metronidazole‖‖ for 7 consecutive days during scaling and root planing and surgical therapy.

Maintenance

The patients were enrolled in a regularly scheduled periodontal maintenance program. During the first year, subjects were monitored in a 3- to 6-month recall interval, including repeated oral hygiene instructions and a full-mouth tooth cleaning with polishing agents¶¶ according to their individual needs. At each visit, GCF samples were harvested from the initially preselected sites. Thereafter, the clinical parameters were assessed, and additional subgingival instrumentation was performed under local anesthesia at periodontal sites that revealed PD >4 mm and positive bleeding on probing scores. All subjects were regularly screened for caries and examined radiographically to evaluate the crestal bone height and detect early periapical lesions. To achieve a complete rehabilitation of the oral cavity, individuals with needs

¶ Safe Twist, Eppendorf, Hamburg, Germany.
# Behring ELISA Prozessor II, Behringwerke AG, Marburg, Germany.
** Sigma Aldrich Chemie GmbH, Deisenhofen, Germany.
†† Serva, Heidelberg, Germany.
‡‡ Rotiszint, Roth, Karlsruhe, Germany.
§§ Ratiopharm GmbH, Blaubeuren, Germany.
‖‖ Artesan GmbH, Lüchow, Germany.
¶¶ Oralust, Oral-B Laboratories, Kronberg im Taunus, Germany.

for further prosthodontic or implant treatment were referred to a specialist 6 months after periodontal therapy.

Experimental Protocol

At the outset, the full range of periodontal parameters was assessed as described above. Prior to each periodontal examination, we collected the GCF samples from interproximal sites with PD between 6 and 8 mm, and determined the GCF volumes. The reexamination, which included GCF collection and the recording of clinical parameters, was performed for all subjects at 3, 6, 12, 24, and 36 months after completion of active periodontal therapy.

Table 2.

GCF Myeloperoxidase (μU/30 seconds) (oxygen-dependent activity)

Time	N	Mean ± SD	Median	Q1	Q3	Minimum	Maximum	*P**
Baseline	14	6.48 ± 2.56	6.19	3.98	8.52	2.66	10.24	–
3 months	13	3.46 ± 1.40	3.34	2.07	4.73	1.76	5.39	0.002
6 months	13	3.04 ± 1.44	2.80	1.96	3.81	1.43	6.29	0.001
12 months	14	2.69 ± 1.07	2.58	1.76	3.38	1.08	4.96	0.002
24 months	11	2.02 ± 0.67	1.87	1.40	2.80	1.26	3.12	0.003
36 months	13	2.14 ± 0.72	2.02	1.53	2.73	1.12	3.38	0.001

* Wilcoxon signed rank test, compared to baseline; Bonferroni adjustment, $\alpha = 0.05/5 = 0.01$.

Statistical Analysis

Data analysis and statistical tests were performed on a patient level basis. For the biochemical data and clinical parameters, medians, means, and standard deviations were calculated. The GCF parameters were computed as the total amount collected onto the paper strips in 30 seconds, and plotted as medians with the Q1-Q3 quartiles, the minima and the maxima. Wilcoxon signed rank test with Bonferroni adjustment to control for experiment wise error rate was applied to evaluate significant changes of the leukocyte markers for each reexamination compared to baseline, and for long-term changes of the clinical parameters from the outset to the 3-year visit. Statistical significance was determined at an $\alpha = 0.05$.

RESULTS

Oxygen-Dependent Activity

Prior to periodontal therapy, GCF contained 1.9-fold enhanced MPO levels (median = 6.19 versus 3.34 μU/30 seconds after completion of active therapy), suggesting an amplified neutrophil infiltration with an MPO-dependent generation of hypochlorite-modified proteins. There was a significant treatment-induced reduction of MPO indicating a relief from enhanced leukocyte infiltration ($P < 0.05$). During the 3-year period, MPO activities remained significantly different (0.3- to 0.5-fold) from baseline. These observations suggest a persistent downregulation of leukocyte-generated MPO activity after periodontal therapy compatible with normal immune function (Table 2, Fig. 1A).

Matrix Hexosaminide Degradation Activity

Amplified neutrophil infiltration at baseline was associated with beta-NAH levels that were 1.3-fold increased (median = 0.78 versus 0.59 μU/30 seconds at 3 months), compared to normal conditions. During healing and tissue reorganization, a reduction of beta-NAH from exudate leukocytes occurred at 0.5- to 0.8-fold of beta-NAH activity compared to the outset. Notwithstanding, this did not reach significance at any time point ($P < 0.05$). These results provide support for the hypothesis that even in the absence of infection and inflammation after periodontal therapy, a low-level matrix degradation activity occurs in periodontal tissues (Table 3, Fig. 1B).

Exoglycosidic Activity

Beta-G levels were significantly elevated (median = 19.74 versus 10.20 μU/30 seconds after completion of therapy), demonstrating enhanced neutrophil activity in untreated disease conditions. At the post-treatment level, a striking downregulation of neutrophil activity was observed, with beta-G activities inhibited at 0.3- to 0.5-fold of its initial levels that could be maintained over the 3-year trial ($P < 0.05$). These findings demonstrate that upregulated leukocyte infiltration elicits enhanced non-collagenous matrix degradation and exacerbates the vulnerability to lytic tissue damages, but becomes well controlled after periodontal therapy (Table 4, Fig. 1C).

Endoproteolytic Activity

Prior to therapy, cathepsin D was 4.2-fold enhanced (2.70 versus 0.64 μU/30 seconds after completion of active therapy). Periodontal treatment significantly expedited resolution of CD levels ($P < 0.05$). The post-treatment medians of CD were reduced to 0.2-fold compared to baseline, and experienced no changes at any reexamination visit. These results demonstrate that the remission of CD is associated with the resolution of periodontal inflammation accompanied by the reorganization of periodontal tissues (Table 5, Fig. 1D).

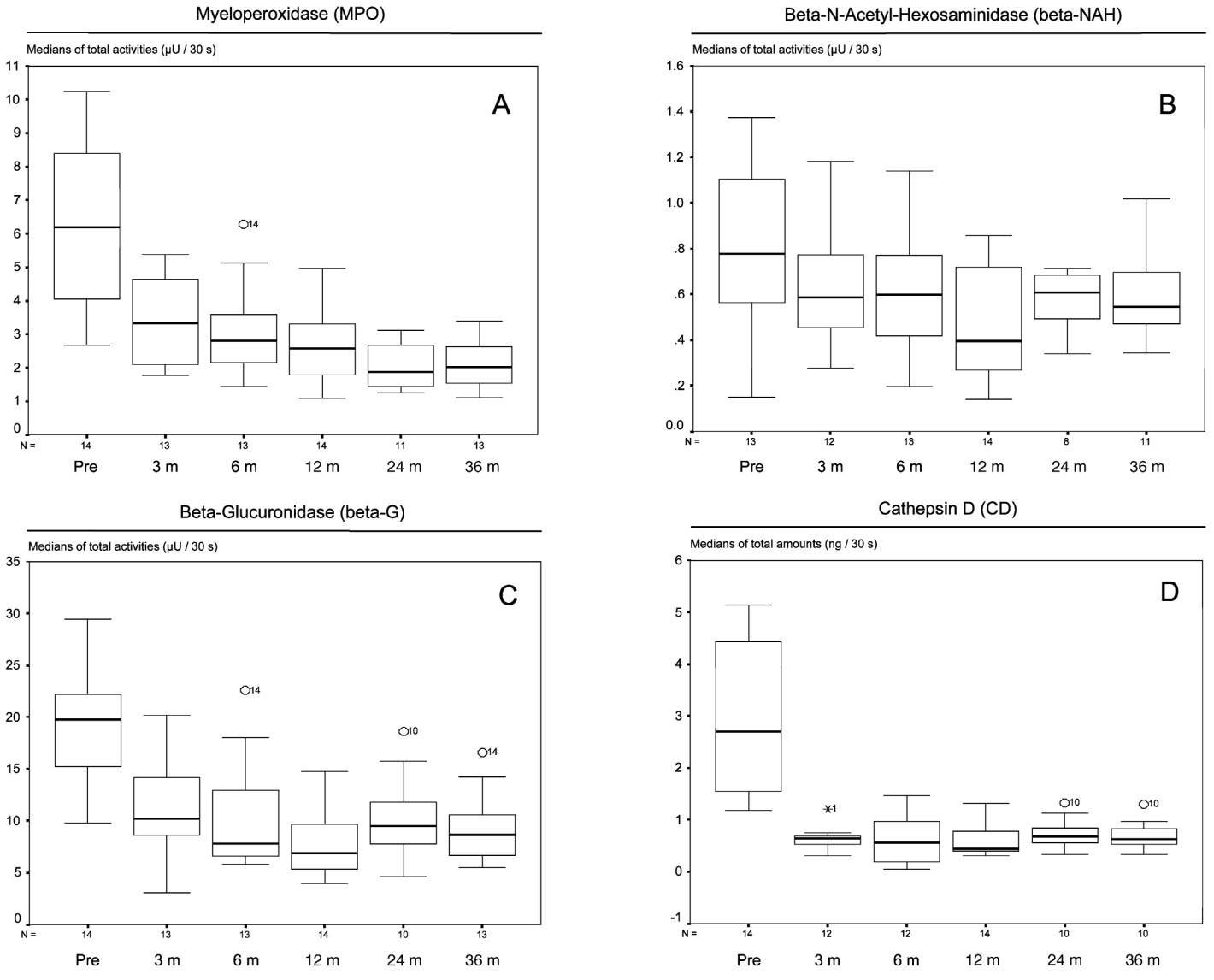

Figure 1.
Box plots for the medians and Q1-Q3 quartiles of leukocyte-generated GCF markers. Lines below and above box plots = minimum-maximum. ***A.*** *Resolution of myeloperoxidase.* ***B.*** *Resolution of beta-N-acetyl-hexosaminidase.* ***C.*** *Resolution of beta-glucuronidase.* ***D.*** *Resolution of cathepsin D.*

Clinical Parameters

All parameters as evidenced by GCFv, PD, and CAL significantly benefited from periodontal therapy, yielding a sustained long-term improvement ($P < 0.05$). During the entire observation period, the PI scores remained on a low level, reflecting patients' sustained motivation for supragingival plaque control (Table 6). The periodontal parameters followed the routes of neutrophil recruitment with no setback after 3 years, suggesting the efficacy of the treatment to resolve the body's challenge from enhanced leukocyte activity.

DISCUSSION

We found elevated levels of MPO, beta-NAH, beta-G, and CD in AgP with evidence of marked tissue infiltration of leukocytes attracted toward the site of infection. Crevicular fluid enzyme activities were significantly decreased upon treatment.

It has been suggested that AgP patients present altered GCF MPO profiles and different MPO isoforms reflecting the PMN stimulation in proportion to the neutrophil granule constituency.[5,20] We identified 1.9-fold enhanced PMN-generated, oxygen-dependent activities promoting excessive periodontal inflammation. The expedited resolution of periodontal disease associated with a change in biochemical profile has been a focus of several studies that acknowledged the counteractive effect of periodontal therapy on MPO in a short-term approach.[21,22] In accordance with other clinical diseases in which leukocyte activities

Table 3.

GCF Beta-N-Acetyl-Hexosaminidase (μU/30 seconds) (matrix hexosaminide degradation)

Time	N	Mean ± SD	Median	Q1	Q3	Minimum	Maximum	P*
Baseline	13	0.79 ± 0.36	0.78	0.48	1.11	0.15	1.37	–
3 months	12	0.62 ± 0.26	0.59	0.42	0.78	0.28	1.18	0.110
6 months	13	0.60 ± 0.29	0.60	0.37	0.84	0.20	1.14	0.028
12 months	14	0.45 ± 0.24	0.40	0.26	0.73	0.14	0.86	0.023
24 months	8	0.58 ± 0.13	0.61	0.47	0.69	0.34	0.71	0.484
36 months	11	0.60 ± 0.20	0.54	0.45	0.73	0.34	1.02	0.182

*Wilcoxon signed rank test, compared to baseline; Bonferroni adjustment, α = 0.05/5 = 0.01.

Table 4.

GCF Beta-Glucuronidase (μU/30 seconds) (exoglycosidic damage)

Time	N	Mean ± SD	Median	Q1	Q3	Minimum	Maximum	P*
Baseline	14	18.99 ± 5.70	19.74	14.66	22.58	9.82	29.44	–
3 months	13	11.47 ± 5.55	10.20	7.19	16.40	3.10	20.21	0.001
6 months	13	10.78 ± 5.41	7.78	6.57	14.64	5.87	22.59	0.003
12 months	14	7.45 ± 3.04	6.91	5.09	9.79	3.99	14.77	0.001
24 months	10	10.18 ± 4.22	9.47	7.47	12.79	4.63	18.64	0.005
36 months	13	9.22 ± 3.32	8.64	6.37	10.72	5.48	16.59	0.001

* Wilcoxon signed rank test, compared to baseline; Bonferroni adjustment, α = 0.05/5 = 0.01.

Table 5.

GCF Cathepsin D (ng/30 seconds) (endoproteolytic degradation)

Time	N	Mean ± SD	Median	Q1	Q3	Minimum	Maximum	P*
Baseline	14	2.99 ± 1.43	2.70	1.51	4.54	1.18	5.14	–
3 months	12	0.63 ± 0.23	0.64	0.52	0.69	0.31	1.21	0.002
6 months	12	0.62 ± 0.47	0.56	0.17	1.06	0.04	1.47	0002
12 months	14	0.60 ± 0.29	0.45	0.39	0.79	0.31	1.31	0.001
24 months	10	0.73 ± 0.30	0.68	0.53	0.91	0.33	1.33	0.005
36 months	10	0.69 ± 0.28	0.63	0.51	0.86	0.33	1.30	0.005

*Wilcoxon signed rank test, compared to baseline; Bonferroni adjustment, α = 0.05/5 = 0.01.

play a pivotal role in healthy tissue inflammation,[4] our findings support the notion that tissue changes observed at sites of excessive inflammation are due to an abnormally enhanced neutrophil-generated, oxygen-dependent metabolism.

Lysosomal glycosidases, in particular hexosaminidase, represent a distinct subset of tissue-degrading enzymes that are activated by proinflammatory stimuli.[8] Prior to therapy, amplified neutrophil infiltration was evidenced by 1.3-fold increased beta-NAH levels, compared to normal conditions. Due to early recolonization with periodontal pathogens, especially *Porphyromonas gingivalis,* initial changes of beta-NAH as response to periodontal therapy experienced a relapse over time. Since beta-NAH is considered to be an outer membrane-associated lipoprotein of *P. gingivalis,*[23,24] unwanted contributions of microbial-originated beta-NAH from the periodontal environment that reinstate and accelerate the neutrophil-generated matrix tissue degradation after therapy and during maintenance may require repeated therapeutic interventions.

Increased beta-G activities in advanced periodontitis represent an intrinsic phenomenon, and appear not to be induced by the microbial load of the oral infection.[25] Beta-G–initiated matrix degradation of dermatan, heparan, and chondroitin sulfate is linked to systemic and subject-based neutrophil responses due to qualitative and quantitative alteration of PMN function in specific periodontal diseases.[26,27] We demonstrated a significant activation (1.7-fold) in untreated AgP. The inhibition of PMN infiltration following treatment and during maintenance resulting in a downregulation of the matrix degradative metabolism agrees with previous findings on GCF beta-G where 140 patients were reexamined after 6 months of periodontal therapy.[28] In our study, the treatment-induced resolution of inflammation statistically dampened the functional PMN responses. However, the beta-G levels in treated AgP displayed a broad range, suggesting single periodontal sites with ongoing neutrophil activation.

In patients with clinical evidence of infection, leuko-

Table 6.

Clinical Parameters at Baseline, and at 3, 6, 12, 24, and 36 Months After Completion of Active Periodontal Therapy

Parameter	Baseline	3 Months	6 Months	12 Months	24 Months	36 Months	Baseline-36 Months	*P**
GCFv								
N	14	13	13	14	10	13	13	
Mean	148.5	101.4	99.9	90.1	90.5	104.3	48.1	0.030
SD	28.2	25.4	35.0	29.7	25.9	37.7	43.0	
Median	151.2	94.9	103.4	94.2	88.9	107.1	41.7	
PD (mm)								
N	14	13	13	14	10	14	14	
Mean	6.3	3.5	3.6	3.7	3.6	4.2	2.1	0.010
SD	1.1	0.9	0.9	1.1	0.8	1.0	1.4	
Median	6.2	3.4	3.5	3.6	3.4	4.1	2.0	
CAL (mm)								
N	13	12	13	14	10	14	13	
Mean	7.4	5.2	5.5	5.4	4.9	5.8	1.6	0.030
SD	1.4	1.2	1.7	1.7	1.5	1.7	1.3	
Median	7.3	5.2	5.6	5.4	4.3	5.8	1.5	
GI								
N	14	13	13	14	10	14	14	
Mean	2.4	0.9	0.8	0.8	0.7	0.8	1.6	0.010
SD	0.4	0.4	0.5	0.5	0.5	0.5	0.7	
Median	2.4	1.0	0.8	0.6	0.6	0.8	1.9	
PI								
N	14	13	13	14	10	14	14	
Mean	0.7	0.5	0.5	0.6	0.6	0.5	0.2	0.636
SD	0.6	0.4	0.4	0.6	0.5	0.4	0.7	
Median	0.5	0.6	0.4	0.4	0.7	0.4	–0.02	

*Wilcoxon signed rank test (baseline to 36 months).

cyte activity is associated with amplified lysosomal activities of cathepsin B and D that recently have been identified as targets for anti-inflammatory agents.[29] CD is linked to PMN exudation, but is more protracted to the endpoint of inflammation where the neutrophils prepare for resolution.[12] In untreated periodontitis, we found a marked increment of CD that reached 4.7-fold difference compared to normal conditions. The release of CD during acute inflammation simultaneously inactivates endogenous proteinase inhibitors allowing uncomplexed, functional active CD to further contribute to ongoing tissue damage.[30] CD activation persistently declined with resolution of inflammation after therapy, and during the 3-year course of periodontal maintenance when tissue destruction is almost controlled. In this view, CD is thought to reflect PMN responses orchestrating finally at the end of inflammation.

The upregulation of functional PMN responses in AgP may be due to an absence or diminution in the endogenous downregulatory or anti-inflammatory mediators generated by this subset of patients. The potential loss of these counterregulatory substances can lead to further amplification of proinflammatory mediators, since they are unopposed by endogenous stop signals for leukocyte recruitment. Given the large number of leukocytes recruited at inflammatory periodontal lesions, an effective treatment of periodontal conditions implicates a downregulation of PMN infiltration that is likely to have a beneficial impact on the prognosis of a number of systemic diseases. The effect between a decrease in PMN responses in periodontal disease and sytemic conditions is based on the hypothesis that *P. gingivalis* elicits leukocyte infiltration, concomitant with elevated PGE_2 levels and upregulated COX-2 expression in infiltrated leukocytes that mediate vasodilation, and increase vascular permeability. This has been documented for murine heart and lung tissues, supporting a potential role for *P. gingivalis* in the evolution of systemic events.[31] Our results stress the observation of a tight regulation of PMN infiltration during inflammation, wound healing and resolution, until homeostasis is achieved.

Within the limitations of the present study, the following conclusions may be drawn: 1) untreated AgP is associated with elevated levels of MPO, beta-NAH, beta-G, and CD that may contribute to promoted loss of periodontal support; 2) periodontal therapy induces a sustained downregulation of leukocyte activity as evidenced by the remission of crevicular GCF markers; and 3) the decreased lysosomal enzyme activities after treatment of AgP can be maintained for up to 3 years.

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REFERENCES

1. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med* 1997;336:973-979.
2. Glurich I, Grossi S, Albini B, et al. Systemic inflammation in cardiovascular and periodontal disease: A comparative study. *Clin Diagn Lab Immunol* 2002;9:425-432.
3. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* 1999;4:1-6.
4. Battino M, Bullon P, Wilson M, Newman H. Oxidative injury and inflammatory periodontal diseases: The challenge of anti-oxidants to free radicals and reactive oxygen species. *Crit Rev Oral Biol Med* 1999;10:458-476.
5. Yamalik N, Çaglayan F, Kilinç K, Kilinç A, Tümer C. The importance of data presentation regarding gingival crevicular fluid myeloperoxidase and elastase-like activity in periodontal disease and health status. *J Periodontol* 2000;71:460-467.
6. Levy BD, Clish CB, Schmidt B, Gronert K, Serhan CN. Lipid mediator class switching during acute inflammation: Signals in resolution. *Nat Immunol* 2001;2:612-619.
7. Ihalin R, Loimaranta V, Lenander-Lumikari M, Tenovuo J. The effects of different (pseudo) halide substrates on peroxidase-mediated killing of *Actinobacillus actinomycetemcomitans*. *J Periodont Res* 1998;33:421-427.
8. Shikhman AR, Brinson DC, Lotz M. Profile of glycosaminoglycan-degrading glycosidases and glycoside sulfatases secreted by human articular chondrocytes in homeostasis and inflammation. *Arthritis Rheum* 2000; 43:1307-1314.
9. Tjelle TE, Brech A, Juvet LK, Griffiths G, Berg T. Isolation and characterization of early endosomes, late endosomes and terminal lysosomes: Their role in protein degradation. *J Cell Sci* 1996;109:2905-2914.
10. Niemen A, Nordlund L, Uitto VJ. The effect of treatment on the activity of salivary proteases and glycosidases in adults with advanced periodontitis. *J Periodontol* 1993; 64:297-301.
11. Ayasaka N, Goto T, Tsukuba T, et al. Immunocytochemical localization of cathepsin D in rat junctional epithelium. *J Dent Res* 1993;72:502-507.
12. Lamster IB. Host-derived enzyme activities in gingival crevicular fluid as markers of periodontal disease susceptibility and activity: Historical perspective, biological significance and clinical implications. In: Johnson NW, ed. *Risk Markers for Oral Diseases: Markers of Disease Susceptibility and Activity. Periodontal Diseases.* London: Cambridge University Press;1991:277-312.
13. Hasilik A. The early and late processing of lysosomal enzymes: Proteolysis and compartmentation. *Experientia* 1992;48:130-151.
14. Layik M, Yamalik N, Caglayan F, Kilinc K, Etikan I, Eratalay K. Analysis of human gingival tissue and gingival crevicular fluid; β-glucuronidase activity in specific periodontal diseases. *J Periodontol* 2000;71:618-624.
15. Pippin DJ, Swafford JR, McCunniff MD. Morphology of azurophil lysosomes in polymorphonuclear leukocytes from humans with rapidly progressive periodontitis. *J Periodont Res* 2000;35:26-32.
16. American Academy of Periodontology. Parameter on aggressive periodontitis. *J Periodontol* 2000;71(Suppl.): 867-869.
17. Brill N. The gingival pocket fluid. Studies of its occurrence, composition, and effect. *Acta Odontol Scand* 1962;20(Suppl. 32):1-115.
18. Nakashima K, Demeurisse C, Cimasoni G. The recovery efficiency of various materials for sampling enzymes and polymorphonuclear leukocytes from gingival crevices. *J Clin Periodontol* 1994;21:479-483.
19. Buchmann R, Hasilik A, Van Dyke TE, Nunn M, Lange DE. PMN responses in chronic periodontal disease: Evaluation of gingival crevicular fluid enzymes and elastase-alpha-1-proteinase inhibitor complex. *J Clin Periodontol* 2002;29:556-565.
20. Miyasaki KT, Nemirovskiy E. Myeloperoxidase isoform activities released by human neutrophils in response to dental and periodontal bacteria. *Oral Microbiol Immunol* 1997;12:27-32.
21. Smith QT, Hinrichs JE, Melynk RS. Gingival crevicular fluid myeloperoxidase at periodontitis sites. *J Periodont Res* 1986;21:45-55.
22. Bartold PM, Narayanan AS. *Biology of the Periodontal Connective Tissues.* Chicago: Quintessence Publishing Co., Inc.; 1998:197-219.
23. Lovatt A, Roberts IS. Cloning and expression in *Escherichia coli* of the nahA gene from *Porphyromonas gingivalis* indicates that beta-N-acetylhexosaminidase is an outer-membrane-associated lipoprotein. *Microbiology* 1994;140:3399-3406.
24. Murty VLN, Piotrowski J, Slomiany A, Slomiany BL. Identification and characterization of N-acetylhexosaminidase from *Porphyromonas gingivalis*. *J Dent Res* 1995;74(Spec. Issue):129(Abstr. 941).
25. Pippin DJ, Cobb CM, Feil P. Increased intracellular levels of lysosomal β-glucuronidase in peripheral blood PMNs from humans with rapidly progressive periodontitis. *J Periodont Res* 1995;30:42-50.
26. Van Dyke TE, Vaikutnam J. Neutrophil function and dysfunction in periodontal disease. *Curr Opin Periodontol* 1994;19-27.
27. Albandar JM, Kingman A, Lamster IB. Crevicular fluid level of β-glucuronidase in relation to clinical periodontal parameters and putative periodontal pathogens in early-onset periodontitis. *J Clin Periodontol* 1998;25:630-639.
28. Lamster IB, Holmes LG, Gross KBW, et al. The relationship of β-glucuronidase activity in gingival crevicular fluid to probing attachment loss in patients with adult periodontitis. Findings from a multicenter study. *J Clin Periodontol* 1995;22:36-44.
29. Meera R, Anand S, Ramesh CV, Puvanakrishnan R. Inhibition of neutrophil derived lysosomal enzymes and reactive oxygen species by a novel tetrapeptide. *Inflamm Res* 1999;48:479-484.
30. Tervahartiala T, Konttinen YT, Ingman T, Häyrinen-Immonen R, Ding Y, Sorsa T. Cathepsin G in gingival tissue and crevicular fluid in adult periodontitis. *J Clin Periodontol* 1996;23:68-75.
31. Pouliot M, Clish CB, Petasis NA, Van Dyke TE, Serhan CN. Lipoxin A(4) analogues inhibit leukocyte recruitment to *Porphyromonas gingivalis:* A role for cyclooxygenase-2 and lipoxins in periodontal disease. *Biochemistry* 2000;39:4761-4768.

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