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PMN responses in chronic periodontal disease: evaluation by gingival crevicular fluid enzymes and elastase-alpha-1-proteinase inhibitor complex

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Abstract

Objectives: In the present trial, the hypothesis was examined that the local PMN responses in untreated and treated chronic periodontitis can be differentiated by gingival crevicular fluid lysosomal enzyme activities and elastase-alpha-1-proteinase inhibitor complex.

Methods: In nine subjects (average age 49.2 ± 7.1 years) with chronic periodontitis, clinical parameters and markers of the PMN-derived inflammatory tissue response in gingival crevicular fluid (GCF) were assessed before and 6 months after surgical periodontal therapy. Myeloperoxidase (MPO), beta-N-acetylhexosaminidase (beta-NAH) and cathepsin D (CD) were analyzed as indicators of the PMN-associated host tissue destruction, and elastase-alpha-1-proteinase inhibitor complex (alpha-1-EPI) as the major serum protein inactivating PMN elastase. The total activities of the lysosomal enzymes MPO and beta-NAH were evaluated spectrophotometrically, the CD levels by liquid scintillation counting with [^{14}C] hemoglobin as substrate, and the total alpha-1-proteinase inhibitor complex using a sandwich-immunoassay.

Results: The clinical parameters revealed a statistical significant decrease at the 6-month reexamination. PD levels dropped from 5.40 to 2.88 mm (change 2.52 ± 1.04 mm), the CAL scores from 6.67 to 4.43 mm (change 2.24 ± 0.77 mm). The 30 s GCF volumes dropped from 129.8 to 68.6, displaying a change of 61.1 ± 18.6, $p \leq 0.05$. The decrease in total MPO, beta-NAH and CD levels (medians: 1.7/0.6 μU MPO, 0.035/0.020 μU beta-NAH, 1.3/0.5 ng CD) following therapy was associated with a significant drop in total GCF amounts of alpha-1-EPI from 76.3 ng at baseline to 52.4 ng after 6 months.

Conclusion: The clinical healing in chronic periodontal disease is associated with a downregulation of the local PMN responses following periodontal therapy. The reorganization of periodontal tissues is characterized by a decrease of lysosomal enzyme activities and the alpha-1-proteinase inhibitor complex in gingival crevicular fluid.

Key words: chronic periodontitis; gingival crevicular fluid; host defense mechanisms; inflammatory tissue response; PMN enzymes

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There is fast-growing evidence that the body's own immune system is challenged by exacerbating damage caused by periodontal disease. New therapeutic agents are being developed to short-circuit or control inflammation caused by periodontal disease; these agents may even lead to new therapies or treatment for other inflammatory conditions such as arthritis and cardiovascular disease (Serhan et al. 2000). Although periodontal disease is thought to be initiated by bacterial plaque, the immune system's response to the event plays the primary role in tissue destruction associated with periodontal disease. Through complex chemical messaging, white blood cells (WBC), of which the most troublesome and abundant are polymorphonuclear leukocytes (PMNs), signal for more and more WBC to be recruited to the bacteria site. The congregation of too many PMNs leads to an inappropriate release of a molecular array of noxious agents intended to fight the bacteria, but the vast quantities that spill over induce inflammation of healthy tissue and encourage the progression of bone loss and periodontal disease (Pouliot et al. 2000).

A question raised by this groundbreaking research is how the inflammatory tissue response of periodontal disease can be diagnosed in a more biological approach rather than being probed mechanically to evaluate the past history of the disease. Inflammatory processes as well as the course and treatment results of certain systemic conditions such as myocardial infarction, stroke, pneumonia and obstetric complications that might be affected by periodontal infections can be easily differentiated by laboratory markers harvested from whole venous blood samples (Voll & Burmester 1994, Page 1998). To apply this diagnostic level of evaluation to periodontal disease, it is reasonable to focus on markers liberated by PMNs. Evasion of neutrophil clearance is a requisite for disease acquisition, with the individual host response playing a primary role in determining the severity of disease (Offenbacher et al. 1993). For diagnostic purposes, once the inflammatory process is established and becomes independent of its primary etiologic factor, the protein levels rather than changes in proteins as a result of therapy become relevant.

Biochemical alteration of gingival crevicular fluid is recognized as part of the individual's response to periodontal infection (Lamster & Grbic 1995). The fluid is easily accessible and contains emigrant leukocytes, circulating plasma proteins, products of local cellular and microbial activity, and metabolites from diseased sites such as the pocket wall and the alveolar bone (Birkedal-Hansen 1995). The database on the variety of GCF compounds in the literature is increasing (Offenbacher et al. 1993, Gustafsson et al. 1995, Adonogianaki et al. 1996, Jin et al. 1999, 2000). However, a systematic approach to establish a collection of GCF data in different patient population groups is missing. In this first paper, we present data on individuals with chronic periodontitis (Armitage 1999) to establish reference levels for further investigations in aggressive periodontitis.

Neutrophils have been implicated as playing a destructive role in the periodontal tissue breakdown process due to high levels of lysosomal enzymes, generation of superoxides and reactive oxygen derivatives (Battino et al. 1999). The 150 kDa protein myeloperoxidase possesses potent antimicrobial activity, and as an indicator of neutrophilic degranulation, has been reported to play a crucial role in tissue injury models (Vakeva et al. 1998). The liberation of myeloperoxidase as a potent injurious oxidative enzyme from the azurophilic granules of neutrophils into the extracellular space gives rise to highly reactive oxygen species, including O_2^- and HOCl (McCulloch 1994). The endproducts become further metabolized in the Krebs cycle to CO_2 and H_2O.

The acid 60 kDaA lysosomal hydrolase beta-N-acetyl-hexosaminidase that enters the gingival crevicular fluid under phagocytotic conditions is recognized to be predominantly of microbial origin. Cloning of the beta-NAH gene in *E. coli* revealed that beta-NAH is an outer-membrane-associated lipoprotein of *P. gingivalis* (Lovatt & Roberts 1994). The salivary beta-NAH-glycosidase activities are considered to correlate with the degree of periodontal inflammation, and to follow periodontal healing after treatment (Niemen et al. 1993). Beta-NAH measurements in gingival crevicular fluid ought to play a role in monitoring the efficacy of periodontal treatment. Extracellular beta-NAH levels are associated with the presence of *Capnocyophaga* sp. (Beighton et al. 1992). While under secretory conditions the precursor forms of the newly synthesized enzyme will be liberated, lyosomal beta-N-acetyl-hexosaminidase is present during phagocytosis and cellular lysis (Lange & Schroeder 1972, Zuehlsdorf et al. 1983).

The lysosomal aspartate-endopeptidase cathepsin D is released as a 52 kDa proteolytic enzyme from lysosomes of human cells, i.e. the primary azurophilic granules of neutrophils, into the extracellular compartment. CD is located in fibroblasts and macrophages of the subepithelial connective tissue and in desquamating junctional epithelial cells of the gingival crevice (Ayasaka et al. 1993). Under lower acidic conditions (pH 3–7), the release of CD, which is inhibited by pepstatin, induces the activation of a variety of enzymes that contribute to the tissue degradation process (Buchmann et al. 1994). However, CD cannot be considered an indicator of the early inflammatory breakdown process. As a marker of protein hydrolysis and cellular lysis, increasing levels represent the endpoint of periodontal tissue destruction (Lamster 1991, Hasilik 1992).

The role of the 53 kDa serine proteinase elastase-alpha-1-proteinase inhibitor complex as the major serum protein inactivating PMN elastase has been previously reported for respiratory injuries in the lung tissues (Yamanouchi et al. 1998). As a synovial liquid marker in rheumatoid arthritis, it serves as an indicator for early inflammatory tissue changes induced by uncomplexed functional elastase from neutrophils (Momohara et al. 1997). If the host is able to produce sufficient alpha-1-proteinase inhibitor to irreversibly bind to the active enzyme center, tissue breakdown will be impeded. Alpha-1-proteinase inhibitor is only available to a limited extent. Its consumption in severe inflammatory conditions might enable the lysosomal enzymes to further degrade periodontal tissues (Buchmann et al. 1995). Even at clinically healthy sites, a lack of alpha-1-proteinase inhibitor complex was reported (Meyle et al. 1992).

In the present trial, an array of four GCF components was assessed to characterize the GCF-associated inflammatory PMN responses in chronic periodontal disease. The hypothesis was examined that the local leukocyte responses in untreated and treated chronic periodontitis can be differentiated by gingival crevicular fluid lys-

osomal enzyme activities and elastase-alpha-1-proteinase inhibitor complex.

Material and Methods

Patients

The data for this study were taken from nine subjects at the Department of Periodontology, University of Münster, with chronic periodontal disease (Armitage 1999) and evidence of prior attachment loss. The average age of the patients (five women and four men) was 49.2 ± 7.1 years at the last visit (age range 40–59 years). Patients selected for entry to the study were screened for periodontal disease encompassing radiographic evidence of intrabony defects exceeding 30% of the root length and probing depths between 6 and 8 mm at eight or more sites within each quadrant. 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. Patients requiring additional antibiotic medications related to other acute infectious diseases during the 6-month observation period had to leave the trial. Table 1 presents the baseline demographic and clinical characteristics of the nine periodontal patients enrolled in the study.

Periodontal examinations

Subjects fulfilling the inclusion criteria above were asked to participate in the study and, if they accepted, to sign informed consent forms. Patients were requested to report to the principal investigator any orofacial or medical infection for which antibiotics were administered. All patients were monitored at baseline and 6 months after periodontal treatment. The probing depths (PD) as distance between the gingival margin and the bottom of the periodontal pocket as well as the clinical attachment levels (CAL) as distance from the cementoenamel junction (CEJ) to the bottom of the periodontal pocket were recorded at four sites per tooth using a straight, rigid periodontal probe (PCP 11, HuFriedy, Chicago, IL, USA) with a 3-3-2-3 mm calibration and a 0.4-mm diameter tip. The PD and CAL measurements (reproducibility ± 1 mm $>95\%$) were performed by the same calibrated examiner. Bleeding on probing (BOP) was recorded dichotomously at four surfaces around a tooth. The presence of supragingival plaque was assessed at four sites per tooth according to the Plaque Index (PI) (Silness & Löe 1964), and was scored from 0 to 3 according to the amount of visible plaque on the individual tooth surface area. The gingival conditions were visually examined at four surfaces per tooth using the Gingival Index (GI) (Löe & Silness 1963), which classifies redness and swelling of the marginal gingiva in degrees of 0 to 3.

GCF sampling

In each patient gingival crevicular fluid (GCF) was harvested from eight teeth in each patient (two per quadrant) at mesio- or disto-interapproximal sites with probing depths between 6 and 8 mm prior to surgery at week -1 and at the 6-month reexamination visit. Following removal of supragingival plaque with a sterile Gracey 11/12 curette tip, we isolated and gently air dried the sites to be sampled with cotton rolls, and inserted a Periopaper strip (Harco, Tustin, CA, USA) at the entrance of the periodontal pocket for 30 s according to Brill (1962). The gingival crevicular fluid volume was determined using the Periotron 6000 (Harco Periotron 304600, Siemens, Bensheim, Germany). The Periotron 6000 was calibrated with a quadratic regression equation curve using serum, 0.9% saline, and 1:1 aliquots of serum and saline (Preshaw et al. 1996). Prior to each periodontal examination, the GCF samples were collected, and the GCF volumes immediately read with the Periotron. The strips were placed in 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 min with constant agitation, and centrifuged at 14 000 *g* over a 2-min-period. The supernatant was separated into 30 µL fractions (Safe Twist, Eppendorf, Hamburg, Germany) and frozen in liquid nitrogen at -196°C.

Periodontal treatment

All individuals were enrolled in an individual oral hygiene program with weekly prophylaxis and repeated motivation and instruction in self-performed oral hygiene. Under local anesthesia (Xylocain Spezial 2%, Astra Chemicals, Sweden) subgingival scaling and root planing (SRP) was performed at sites with PD exceeding 4 mm. At periodontal defects with PD >6 mm, surgical access to the periodontal defects was achieved according to the modified Widman-flap-technique in four sessions. After intrasulcular incision, a mucoperiosteal flap was raised beyond the mucogingival border to get access to the periodontal defect. Following removal of the interradicular inflammatory granulation tissue, the denuded root surfaces were mechanically scaled and root planed, and repeatedly rinsed with 0.1% chlorhexidine digluconate solution (Chlorhexamed fluid, Procter & Gamble, Schwalbach, Germany). No osseous surgery was carried out. Flaps were replaced as close as possible to their initial position to completely cover the periodontal defect and fixed with interdental sutures. No systemic antibiotics were administered. The postsurgical follow-up included a removal of the sutures and careful

Table 1. Clinical characteristics of nine chronic periodontitis patients enrolled in the study and completing the final 6-month reexamination

	$x \pm$ SD
Age (years)	49.2 ± 7.1
Males/females (*n*)	5/4
% smokers	21.2
Number of sites examined	69
Probing depths (PD in mm), baseline	5.4 ± 2.1*
% of sites with PD <4 mm (*n*)	20.0 (*14*)
% of sites with PD 4–6 mm (*n*)	40.5 (*30*)
% of sites with PD >6 mm (*n*)	36.2 (*25*)
Clinical attachment level (CAL in *mm*), baseline	6.7 ± 2.1
% of sites with CAL <4 mm (*n*)	8.7 (*6*)
% of sites with CAL 4–6 mm (*n*)	34.8 (*24*)
% of sites with CAL >6 mm (*n*)	56.5 (*39*)

$x \pm v v \mu$ SD, x = Mean, SD = standard deviation.

Table 2. Classification and origin of 4 selected GCF markers

Enzyme	EC-NR.	Molecular weight(kDa)	Description
Myeloperoxidase	1.11.1.7	150	Oxidoreductase of azurophilic granules
Beta-N-acetylhexosaminidase	3.2.1.30	60	Acid lysosomal hydrolase
Cathepsin D	3.4.23.5	52	Lysosomal aspartate-endopeptidase
Elastase-alpha-1-proteinase inhibitor complex		53	Serine proteinase, competitive elastase-inhibitor

cleaning of the treated periodontal sites 1 week post-therapy. During the first and second postoperative week, a 0.1% chlorhexidine-digluconate solution was administered to the patients to rinse twice daily for 2 min The patients were enrolled in a supportive periodontal maintenance program and monitored on a 3–6-month recall schedule, including repeated oral hygiene instructions and full-mouth toothcleaning according to their individual needs.

Biochemical analysis

Recovery and validation

Into $4 \times 10\,\mu L$ aliquots of each of the diluted enzymes a periopaper was inserted. The four samples per GCF marker were immersed into 160 µL 0.9% sterile saline and vortexed. Strips and liquid were separated by centrifugation (2 min, 14 000 *g*). In the supernatant, the total amount of enzyme activities and the alpha-1-EPI that were liberated from the 16 periopapers were determined according to the assay procedures described below, and calculated as percentage of the known enzyme and alpha-1-EPI-standards. The mean percentage recoveries from the periopaper as a relative percentage of the corresponding standards were $82.9 \pm 18.5\%$ (MPO), $89.1 \pm 13.4\%$ (beta-NAH), $95.2 \pm 17.6\%$ (CD) and $96.4 \pm 14.9\%$ (alpha-1-EPI) (Nakashima et al. 1994). The validation of the enzyme assays was performed with HL-60 promyelocytes (Hasilik 1992). The mean coefficients of variation that were calculated following duplicate measurements of the total GCF enzyme activities and alpha-1-EPI are displayed in Table 4.

Myeloperoxidase

The diluted GCF samples (2×10 µl) were added to 0.1 M citric acid buffer, pH 5.5, containing 0.125% Triton-X-100 solution and 0.1 hydrogen peroxide. The oxidation of H_2O_2 was performed using 0.8 mM ω-dianisidin as substrate. After 7 min 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 (Behring ELISA Prozessor II, Behringwerke AG, Marburg, Germany). The total MPO activities were calculated in duplicate in µU (Table 2).

Beta-N-acetyl-hexosaminidase

The diluted GCF samples 2×10 µL were eluted in 25 µL 0.9% NaCl, and 0.1 M citric acid buffer, pH 4.6. Then, 25 µL p-nitrophenyl-N-acetyl-beta-D-glusoaminide (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) was added. The reaction was allowed to proceed at 37°C for 30 min and then stopped with 225 µL 0.4 M glycine–NaOH stopping buffer, pH 10.4. The hydrolytic β-N-acetyl-glucosaminidase activities were assessed at a wv of 405 nm (reference wv 492 nm). The total beta-NAH activities were calculated in duplicate in µU (Hasilik 1992) (Table 2).

Cathepsin D

Cathepsin D was determined by liquid scintillation counting using [^{14}C] hemoglobin as substrate with a modification of a method previously described by Barrett et al. (1986). The diluted samples were 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 cpm (counts per minute) (Hasilik 1982). After 6 h 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 min at 14 000 *g*, and the radioactivity in 1 mL of the supernatant was assessed in a volume of 2 mL scintillation liquid (Rotiszint, Roth, Karlsruhe, Germany). The radioactivity of the supernatant was computed in a liquid scintillation counter as cpm with 1 cpm = 240 pg [^{14}C] hemoglobin. The cathepsin D activities per 10 µL hemoglobin were determined in duplicate, and the total amount of CD calculated in ng (Table 2).

Elastase-alpha-1-proteinase inhibitor complex

The amount of complexed, functional inactive PMN elastase was determined with the Merck-Immunoassay (Merck KgaA, Darmstadt, Germany). The elastase-alpha-1-proteinase inhibitor complex is detected by two subsequent

Table 3. Baseline and 6-months results of clinical parameters in chronic periodontitis

	Period	Mean	Difference (Baseline − 6 months)	Range	*p*-value
PD (mm)	1*	5.40 ± 2.06**		2.6–8.0	
	2	2.88 ± 1.03	2.52 ± 1.04	1.4–4.3	0.005***
CAL (mm)	1	6.67 ± 2.08		3.7–9.3	
	2	4.43 ± 1.33	2.24 ± 0.77	2.7–6.1	0.005
GI	1	1.74 ± 0.78		0.4–2.8	
	2	1.13 ± 0.43	0.62 ± 0.36	0.4–1.8	0.007
PI	1	1.30 ± 0.86		0.3–2.8	
	2	0.88 ± 0.40	0.41 ± 0.63	0.3–1.6	0.080
BOP	1	0.58 ± 0.19		0.2–0.9	
	2	0.25 ± 0.18	0.33 ± 0.29	0.0–0.6	0.012
GCFV (30 s)	1	129.80 ± 39.40		52.6–183.9	
	2	68.64 ± 20.83	61.16 ± 18.58	27.9–97.1	0.005

*1 = Baseline, 2 = 6 months.

**$x \pm$ SD, x = Mean, SD = standard deviation.

***Wilcoxon Signed-Rank Test.

immunoreactions. In $2 \times 5\,\mu L$ of the diluted samples, the alpha-1-EPI-complex was bound to $20\,\mu L$ of the solid phase fixed anti-PMN-elastase-antibody. After 10 min of incubation at 37°C, 20 µL of the alpha-1-proteinase-antibody-conjugate was added. The alpha-1-EPI-complex was determined at a wv of 492 nm by adding $50\,\mu L$ 4-p-nitrophenylphosphate as substrate, and $150\,\mu L$ of 2 N NaOH as stopping reagent. The total amount of elastase-alpha-1-proteinase inhibitor complex was computed using a calibration curve of four different standards in ng (Table 2).

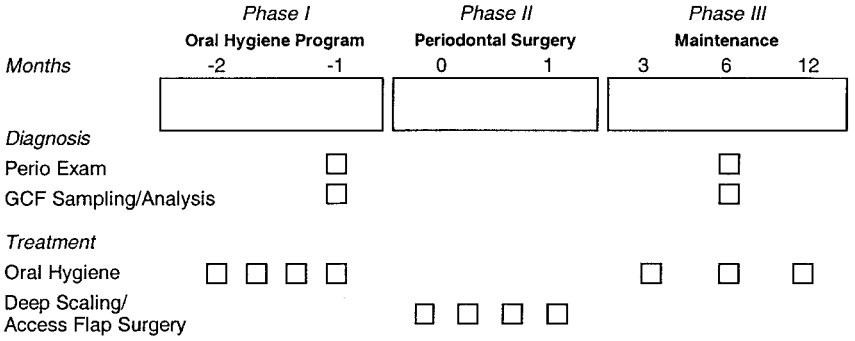

Fig. 1. Experimental protocol of the study displaying the schedule for the clinical and GCF measurements.

Study 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 interapproximal sites with probing depths between 6 and 8 mm, and determined the gingival crevicular fluid volumes. The reexamination that included the GCF collection and the complete recording of the clinical setting was performed for all subjects 6 months after surgical periodontal therapy within the maintenance care (Fig. 1).

Statistical analysis

Data analysis and statistical tests were performed on a patient-level basis using SPSS Base 10.0. The clinical parameters were evaluated using mean values and standard deviations. Significant changes between the pre- and 6-month post-therapy clinical setting and the biochemical data were subjected to the non-parametric Wilcoxon Signed-Rank Test. The GCF parameters were computed as the total amount collected onto the paper strips in 30 s and plotted for both visits as medians, with the Q1 − Q3 quartiles, the minima and the maxima. Statistical significance was determined considering an alpha level of 0.05.

Results

Clinical parameters

Means of clinical measurements before and after surgery periodontal therapy are summarized in Table 3. Between the two appointments, the overall periodontal parameters significantly dropped, representing the effectiveness of the current treatment approach (Wilcoxon-Signed-Rank Test, $p \leq 0.05$). The mean probing depths decreased from 5.40 ± 2.06 to 2.88 ± 1.03 mm (change 2.52 ± 1.04 mm). For the clinical attachment levels, the mean values were 6.67 ± 2.08 at baseline and 4.43 ± 1.33 mm at the 6-month reappointment (change 2.24 ± 0.77 mm). With respect to the fact that the estimated measurement error of rigid periodontal probes is ± 1 mm for PD and CAL measurements (95% confidence), all individuals strongly benefited from mechanical (scaling and root planing) and surgical therapy. At the outset, the gingival index (GI) was 1.74 ± 0.78 and decreased to 1.13 ± 0.43 6 months post-therapy. The plaque index (PI) exhibited moderate scores of 1.30 ± 0.86 and dropped to 0.88 ± 0.40 at the final visit.

Biochemical results

Following periodontal therapy, the medians of the total MPO activity in gingival crevicular fluid displayed a significant decrease from 1.73 µU at baseline to 0.61 µU at the 6-month visit, $p \leq 0.005$. The q3-quartiles at the outset were 3.12 µU and declined at the 6-month reexamination to 1.04 µU; the

Table 4. Gingival crevicular fluid levels of 3 PMN enzymes and elastase-alpha-1-proteinase inhibitor complex in chronic periodontitis at the outset and the 6-month reexamination

	Myeloperoxidase (µU/30 s)		Beta-N-acetyl-hexosaminidase (µU/30 s)		Cathepsin D (ng/30 s)		Elastase-alpha-1-proteinase inhibitor complex (ng/30s)	
Period*	1	2	1	2	1	2	1	2
n	9	9	9	9	9	9	9	9
$x \pm$ sd**	1.97 ± 1.73	0.66 ± 0.54	0.040 ± 0.029	0.026 ± 0.015	1.43 ± 0.69	0.53 ± 0.26	68.15 ± 20.03	46.98 ± 13.33
median	1.73	0.61	0.035	0.020	1.27	0.47	76.26	52.36
q1	0.46	0.17	0.020	0.010	1.04	0.35	52.65	38.50
q3	3.12	1.04	0.063	0.040	2.07	0.80	81.51	56.10
min	0.13	0.05	0.010	0.010	0.32	0.12	24.37	16.86
max	5.59	1.76	0.090	0.050	2.70	0.99	89.20	60.97
CV (%)		5.93		12.33		5.56	10.63	
p-Value***		0.005		0.017		0.005	0.005	

*1 = Baseline, 2 = 6 months.
**$x \pm$ SD, x = Mean, SD = standard deviation.
***Wilcoxon Signed-Rank Test.

maxima dropped from 5.59 to 1.76, representing a recovery from the myeloperoxidase-initiated oxidative tissue injuries. The q1-quartiles of the MPO scores differed only slightly between the two visits, with minimum levels of 0.13 and 0.05 µU (Fig. 2a, Table 4).

The medians of total GCF beta-NAH-activities statistically differed between baseline and the 6-month reappointment. At the outset, the median was 0.035 µU and dropped to 0.020 µU 6 months following therapy, $p \leq 0.017$. The q3-levels decreased from 0.063 to 0.040 µU, while the maxima declined from 0.090 to 0.050 µU. The q1-quartiles dropped from 0.02 to 0.01 µU (Fig. 2b, Table 4). These changes might be therapy-related and due to the release of glycosidase-activities by supragingival plaque bacteria, as further mentioned in the discussion.

The GCF levels of total cathepsin D as a marker of cellular lysis revealed a statistically significant drop with medians of 1.27 ng in untreated individuals, which decreased to 0.47 ng in treated patients, $p \leq 0.005$. The box-drawing in Fig. 2c clearly illustrates this change. The q3-quartiles dropped from 2.07 to 0.80 ng and the q1-quartiles from 1.04 to 0.35 ng indicating that the extracellular release of cathepsin D is strongly reduced due to cell reorganization and tissue remodeling as a benefit of therapy (Fig. 2c, Table 4).

The medians of total alpha-1-EPI in GCF before and after therapy revealed a similar course compared to the GCF enzyme levels. The medians of alpha-1-EPI were 76.26 ng at baseline and decreased to 52.63 ng 6 months following therapy, $p \leq 0.005$. There was also an apparent trend for the q1- and q3-quartiles to exhibit lower scores after periodontal tissue healing. The q3-quartiles decreased from 81.51 to 56.10 ng and the q1-quartiles from 52.65 to 38.50 ng. The maxima were 89.20 ng at the outset and 60.97 ng after 6 months when periodontal tissues were nearly completely rebuilt (Fig. 2d, Table 4).

Discussion

Today, concerted efforts are being focused on the development of natural anti-inflammatory agents, with a beneficial impact on the arrest of inflammatory diseases such as rheumatoid ar-

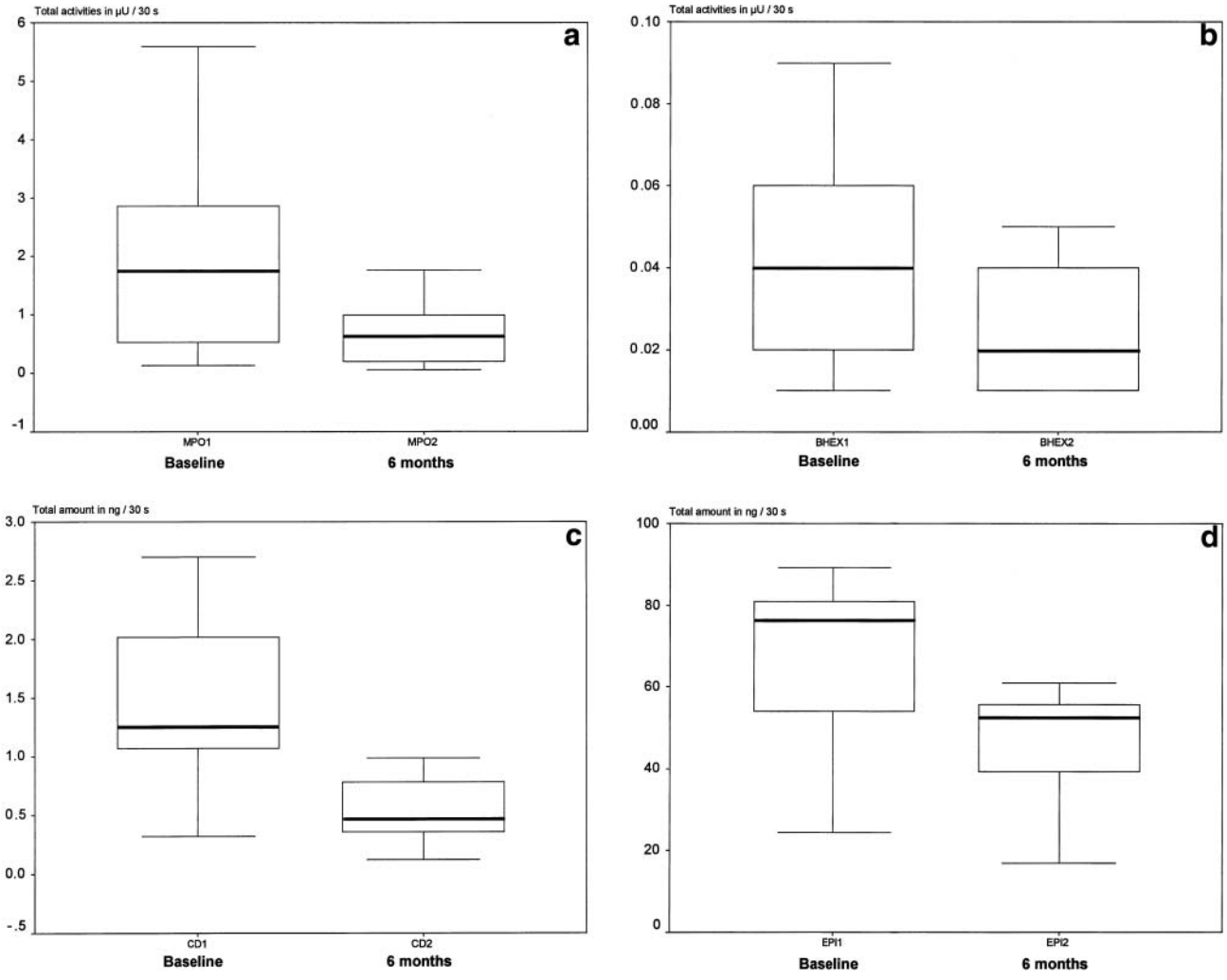

Fig. 2. Box plots for the medians and Q1-Q3-quartiles of *(a)* GCF myeloperoxidase, *(b)* beta-N-acetyl-hexosaminidase, *(c)* cathepsin D, and *(d)* elastase-alpha-1-proteinase inhibitor complex. Lines below and above box plots = Min, Max.

thritis, vascular and periodontal diseases (Pouliot et al. 2000). For the evaluation of the inflammatory tissue response in the oral cavity, chronic periodontal disease represents a controlled and widely accepted condition (Armitage 1996, Persson et al. 1995, Smith et al. 1995). However, before the effect of drug release can be seriously evaluated, protein levels have to be established that can be compared within different periodontal population groups.

Enzymes are well known to characterize a variety of severe and hereditable systemic disorders (Von Figura & Hasilik 1986). In the respiratory system of adults, an imbalance in extracellular release of proteins and free oxygen radicals from granulocytes and proteinase inhibitors has been proposed as one of the major mechanisms of tissue destruction (Wewers et al. 1988). In the oral cavity, myeloperoxidase activities are responsible for oxidative tissue injuries. However, the MPO levels achievable in gingival crevicular fluid are likely to represent the amount of neutrophil infiltration as a highly regulated process. They are associated with extravasation and migration of neutrophils towards the periodontal pocket, the amount of gingival crevicular fluid volume, and the sampling time rather than with the periodontal disease activity itself (Cao & Smith 1989). According to Wolff et al. (1988), we observed a statistically significant decrease in MPO scores following therapy due to pocket shrinkage, reduction of gingival exudate, and the decreased accumulation of neutrophils in the gingival crevice (Smith et al. 1986). In the cross-sectional investigation of Cao & Smith (1989), MPO activities reached 10-fold those in the present study. Recently, intestinal MPO activities following ischemia and reperfusion injury in an animal model yielded levels similar to those of our study (Wada et al. 2001). The usage of different biochemical assays, environmental and nutritional conditions as well as the daytime point of collection are relevant factors when comparing data from different laboratories (Thomas et al. 1991).

Glycosidases are released by bacteria to degrade macromolecules for bacterial growth. *P. gingivalis* is reported to be the major producer of beta-NAH, which hydrolyses protective glycoconjugates within the oral cavity (Beighton et al. 1991, Murty et al. 1995). It is very reasonable that our results demonstrated a striking decrease of GCF beta-NAH-activities in response to surgical periodontal therapy, the rebound of intracrevicular neutrophil accumulation following healing and suppression of *P. gingivalis* levels due to pocket shrinkage. Beighton et al. (1992) presented beta-NAH-levels in untreated periodontitis to be 10-fold higher than in our study. These differences might be explained by the fact that dental and subgingival plaque bacteria were already suppressed following SRP-treatment when the collection of gingival crevicular fluid was performed. The average plaque levels in our patient population group were at a very low level, 0.88–1.3, probably suppressing the conditions for beta-N-acetyl-hexosaminidase-producing bacteria to colonize and grow beneficially. Correlation coefficients between beta-NAH-activities and clinical plaque indices are 0.43 on average (Beighton et al. 1992).

The acidic lysosomal cysteine proteinases cathepsin B, H and L, which participate in intracellular proteolytic degradation processes, are of value in monitoring treatment results rather than detecting early inflammatory tissue changes (Cox & Eley 1992, Eley & Cox 1992). However, as increasing CD-concentrations represent the endpoint of periodontal tissue destruction and serve as a marker of irreversible cellular injury, it is reasonable that the correlation with clinical parameters is weak (Hasilik 1992, Buchmann et al. 1995). The clinical relevance of cathepsin D in life-threatening systemic diseases was recently confirmed by Brandt et al. (1991). In breast cancer therapy, CD is recognized as a prognostic marker for additional radiotherapy to prevent early metastatic events. The data presented in our study displayed a striking decrease of GCF CD-concentrations, from 1.27 to 0.47 ng following periodontal therapy. We suggest that this drop is due to the decreased amount of intracrevicular neutrophils and the tissue remodeling process in treated periodontal conditions where cellular injuries occur less frequently. Although endogenous inhibitors are able to bind to the variety of cathepsins in gingival crevicular fluid, it is of special concern that proteolytic activities of cathepsins are still present in a functional active, uncomplexed form to sustain the inflammatory periodontal disease process (Tervahartiala et al. 1996).

The analysis of the GCF elastase-alpha-1-proteinase inhibitor complex serves as a marker for neutrophilic elastase that is irreversibly bound to the inhibitor complex. Additional granulocyte elastase activities become reversibly blocked by alpha-2-macroglobulin. Due to the instability of the elastase-alpha-2-macroglobulin complex, functional active elastase is consistently available (Galdston et al. 1979, Stone et al. 1983, Giannopoulou et al. 1992, Kennett et al. 1997). These dynamic changes between tissue destruction and protection render the interpretation of antiproteinases as markers of the inflammatory disease process difficult. In plasma, functional free elastase cannot be determined reliably as native uncomplexed alpha-1-proteinase inhibitor complex is circulating and immediately inhibits elastase activities. The average plasma levels in healthy individuals range between 60 and 180 µg/L (Kleesiek et al. 1985) being far lower compared to the amounts that can be collected in gingival crevicular fluid (Adonogianaki et al. 1992, Giannopoulou et al. 1992, Kennett et al. 1995). According to our results, the elastase-alpha-1-proteinase inhibitor complex that represents the available elastase activity in crevicular fluid declined following therapy. The GCF medians in our patient population group were 76.26 ng at periodontal inflamed sites, and 52.36 ng at treated periodontal sites with a long junctional epithelium, where tissue remodeling is a continuous event. Our data of a downregulation of alpha-1-EPI following therapy agree with findings in other reports on chronic periodontitis (Müller et al. 1986, Flemmig et al. 1996). The application of ELISA or Western blot techniques enables a further laser densitometric analysis to differentiate a functional 52 kDa and a degraded 48 kDa elastase-alpha-1-proteinase inhibitor complex in 10^3-fold enhanced concentrations compared to the sandwich-immunoassay (Lee et al. 1997). Depending on the applied method, inhibitor concentrations of elastase-alpha-1-proteinase inhibitor complex in clinically healthy gingiva have been shown to exceed 0.18 µg/µL (Ingman et al. 1994).

The present study examines protein markers in gingival crevicular fluid as indicators of the inflammatory tissue response in chronic periodontal disease to establish levels for reference. Within the limitations

of the study, the following conclusions may be drawn:

- The clinical healing in chronic periodontal disease is associated with a downregulation of the local PMN response following therapy when periodontal tissues become reorganized, as characterized by a decrease in lysosomal enzyme activities and in the alpha-1-proteinase inhibitor complex in gingival crevicular fluid.
- In treated periodontitis where tissue remodeling is almost completed, the amount of alpha-1-proteinase inhibitor complex representing the available GCF elastase is decreased compared to untreated periodontal disease. This may provide further support for the beneficial effect of the current treatment approach.

Unfortunately, no information could be obtained about whether the chronic nature of periodontal disease might be due to the ability of the host to provide sufficient alpha-1-proteinase inhibitor complex binding to elastase to prevent further tissue degradation. Additionally, we have to be aware that standardized laboratory conditions, comparable biochemical methods for analysis and precise patient recruitment and documentation are prerequisites to establish data that might serve as a reliable reference tool. In a further investigation, these findings will be applied to a patient population group with aggressive periodontal disease to support our hypothesis that GCF enzymes are able to distinguish between different forms of periodontal disease.

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Zusammenfassung

Reaktion der PMN bei chronischer Parodontalerkrankung: Evaluierung durch Enzyme des gingivalen Sulkusfluids und des Elastase-Alpha-1-Proteinase-Inhibitor-Komplexes.

Ziele: In der vorliegenden Studie wurde die Hypothese untersucht, dass die lokale PMN-Reaktion von Patienten mit unbehandelter chronischer Parodontitis voneinander unterschieden werden kann durch Untersuchung der lysosomalen Enzymaktivitäten und des Elastase-Alpha-1-Proteinase-Inhibitor-Komplexes des gingivalen Sulkusfluids.

Methoden: Bei 9 Personen (Durchschnittsalter 49,2 ± 7,1 Jahre) mit chronischer Parodontitis wurden vor der Parodontalchirurgie und 6 Monate danach die klinischen Parameter erhoben sowie die Marker der PMN-abhängigen entzündlichen Gewebereaktion aus dem gingivalen Sulkusfluid bestimmt. Myeloperoxidase (MPO), Beta-N-Acetyl-hexosaminidase (beta-NAH) and Cathepsin D (CD) wurden als Indikatoren der PMN-assoziierten Gewebedestruktion. Der Elastase-alpha-1-Proteinase-Inhibitor-Komplex (alpha-1-EPI) wurde als das Hauptserumprotein, welches die PMN-Elastase inaktiviert bestimmt. Die Gesamtaktivitäten der lysosomalen Enzyme MPO und beta-NAH wurden spektrophotometrisch evaluiert, die CD-Titer mittels Liquid-Szintillations-Zählung und [14C]-Hämoglobin als Substrat bestimmt, und der Elastase-Alpha-1-Proteinase-Inhibitor-Komplex unter Verwendung eines Sandwich-Immunoassays gemessen. Die Datenanalyse erfolgte mit SPSS Base 10.0.

Ergebnisse: Bei der 6-Monats-Nachuntersuchung zeigten die klinischen Parameter einen statistisch signifikante Verbesserung. Das Niveau der PD fiel von 5.40 auf 2.88 mm (Veränderung 2.52 ± 1.04 mm), die CAL-Werte gingen von 6.67 auf 4.43 mm zurück (Veränderung 2.24 ± 0.77 mm). Das 30 Sekunden GCF-Volumen fiel von 129.8 to 68.6, und zeigte eine Veränderung von 61.1 ± 18.6, die Reduction beim Gesamt-MPO, beta-NAH und dem CD-Titer (Medianwerte: 1.7/0.6 μU MPO, 0.035/0.020 μU beta-NAH, 1.3/0.5 ng CD) nach der Therapie war verbunden mit einer signifikanten Abnahme bei der Gesamt-GCF-Menge von alpha-1-EPI von 76.3 ng zu Beginn und 52.4 ng nach 6 Monaten.

Schlussfolgerung: Bei chronischer Parodontus ist die klinische Heilung nach Parodontaltherapie verbunden mit einer Reduzierung der lokalen PMN-Reaktion. Die Reorganisation der parodontalen Gewebe ist Charakterisiert durch einen Abfall der Aktivität der lysosomalen Enzyme und des Alpha-1-Proteinase-Inhibitor-Komplexes im gingivalen Sulkusfluid.

Résumé

Réponses des PMN lors de la maladie parodontale chronique : évaluation par les enzymes du fluide gingival et le complexe élastase -alpha-1-inhibiteur de la protéinase.

Objectifs: Dans cette étude, nous avons examiné l'hypothèse selon laquelle les réponses locales des PMN dans les parodontites chroniques traitées et non traitées, pouvaient être différenciées par les activités de l'enzyme lysosomal dans le fluide gingival et par le complexe élastase -alpha-1-inhibiteur de la protéinase .

Méthodes: Chez neuf sujets (moyenne d'âge 49.2 ans ± 7.1) atteints de parodontite chronique, les paramètres cliniques et les marqueurs de la réponse PMN issue de l'inflammation tissulaire dans le fluide gingivale, (GCF) furent notés avant et 6 mois après un traitement parodontal chirurgical. La myeloperoxidase (MPO), la beta-N-acetyl-hexosaminidase (beta-NAH) and la cathepsine D (CD) furent analysées en tant qu'indicateurs de la destruction tissulaire associée aux PMN et le complexe élastase -alpha-1-inhibiteur de la protéinase (alpha-1-EPI) en tant que protéine sérique majeure désactivant l'elastase des PMN. Les activités totales des enzymes lysosomial MPO et beta-NAH ont été évaluées spectrophotométriquement, les niveaux de CD par comptage de scintillation liquide avec l'hémoglobine [14C] comme substrat, et le complexe élastase -alpha-1-inhibiteur de la protéinase total par un test immunologique en sandwich. Les données recueuillies ont été analysées avec SPSS Base 10.0.

Résultats: L'étude des paramètres cliniques montre une diminution significative lors de la visite post traitement (6 mois). Les niveaux de PD tombaient de 5.40 à 2.88 mm (modification de 2.52 ± 1.04 mm), les niveaux d'attache de 6.67 à 4.43 mm (modification de 2.24 ± 0.77 mm). Les volumes de fluide sur 30s tombaient de 129.8 à 68.6, soit une modification de 61.1 ± 18.6, Test de Wilcoxon Signed Rank , p£0.05 . La diminution des niveaux de MPO total , beta-NAH et CD (medians: 1.7/0.6 μU MPO, 0.035/0.020 μU beta-NAH, 1.3/0.5 ng CD) après traitement était associée avec une chute significative des niveaux totaux dans le fluide de alpha-1-EPI de 76.3 ng initialement à 52.4 ng après 6 mois.

Conclusion: La rémission clinique lors de la maladie parodontale chronique est associée avec une sous-régulation de la réponse locale des PMN après traitement parodontal. La réorganisation des tissus parodontaux est caractérisée par une diminution des activités de l'enzyme lysosomal et du complexe élastase -alpha-1-inhibiteur de la protéinase dans le fluide gingival.

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