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Elastase Activity in Gingival Crevicular Fluid of Healthy Volunteers

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Introduction

Elastolytic enzymes play an important role in the destruction of periodontal tissues. Egelberg (1966) described the influence of gingival and periodontal inflammation on the permeability of the dento-alveolar vessels [4]. Gustaffson related periodontal destruction to the activity of polymorphonuclear neutrophils (PMNs) and their products [5]. The neutrophil acts as the first line of defense against invading pathogens and it is the primary cell present of the acute inflammatory response. Neutrophil dysfunction results in progression of disease and early tooth loss, underlining the primary protective role of PMNs. In addition to elastase, collagenase and cathepsin are other components of the primary or azurophilic granules of neutrophils. Damiano (1988) analyzed the enzyme concentration of azurophilic granula and discovered a high substrate specificity [2]. Great quantities of these enzymes are released during the degranulation process or cell lysis. Janoff (1985), Velvart and Fehr (1987) studied the elastolytic proteolysis of collagen, laminin, fibronectin and proteoglycans [6, 9]. Free elastase is rapidly inactivated by a-1- proteinase-inhibitor. Peroxide radicals usually present in the inflammatory exudation react against this inactivation process [7, 8]. Elastase can therefore also be detected in gingival crevicular fluid of clinically healthy periodontal tissues [3]. Longitudinal studies in patients were performed basically by Cox and Eley (1987), demonstrating an increase in elastasolytic activity in gingival crevicular fluid [1]. Elastase can be recovered from gingival crevicular fluid with Periopapers. Qualitative and quantitative laboratory analysis is possible.

Objective

It was the aim of the present study, to establish a micro test system for the detection of elastolytical activity in gingival crevicular fluid. An initial application was tested in experimental gingivitis.

Material and Methods

Calibration of the Periotron 8000®
The Periotron® (Vetter Laborbedarf, Ammerbuch, Germany) was calibrated with double-distilled water and pool serum using a Hamilton® syringe (Hamilton, Darmstadt, Germany) from 0.1 to 0.8 µl in volume increments of 0.1 µl. The arithmetic mean of 10 measures was calculated and the corresponding calibration was plotted.

Development of test and sample buffer
The optimum pH of the assay buffer was found at 8.1. The composition of the buffer used was: Tris-HCl 50 mmol/l (Amersham Life Science, Braunschweig, Germany); sodium chloride 1 mol/l (Merck, Darmstadt, Germany) and Triton-X-100 0.1 % v/v (Sigma, Deisenhofen, Germany). Sample buffer was composed of n-morfolino disodium salt 50 mmol/l (Fluka; Neu-Ulm, Germany); sodium chloride 150 mmol/l (Merck, Darmstadt, Germany); 1.4-dithiotreitol 0.1 mmol/l (Merck, Darmstadt, Germany) and Triton-X-100 0.1 % v/v (Sigma, Deisenhofen, Germany) at pH 5.5. (Amersham Life Science, Braunschweig, Germany); sodium chloride 1 mol/l (Merck, Darmstadt, Germany) and Triton-X-100 0.1 % v/v (Sigma, Deisenhofen, Germany). Sample buffer was composed of n-morfolino disodium salt 50 mmol/l (Fluka; Neu-Ulm, Germany); sodium chloride 150 mmol/l (Merck, Darmstadt, Germany); 1.4-dithiotreitol 0.1 mmol/l (Merck, Darmstadt, Germany) and Triton-X-100 0.1 % v/v (Sigma, Deisenhofen, Germany) at pH 5.5.

Determination of recovery rates
A thin space was created between two cover slips and different sample volumes of human leukocyte elastase (Calbiochem, Bad Soden) with known activities (200, 1600 and 3200 µU/µl) were introduced and absorbed with a Periopaper®. The volume was determined, then eluted in sample buffer and stored at -22 °C for 48h before enzymatical analysis.

Clinical Study
A longitudinal, randomized, double blind experimental gingivitis study was performed in 12 healthy male young volunteers, between 22 - 25 years of age. After their written informed consent, a 18 days pre-experimental phase was started with professional prophylaxis and strict control of oral hygiene (days -18, -14, -7, -4). The following clinical parameters were assessed: a Quigley & Hein plaque index (PLI) and the Saxer & Mühlemann papillary bleeding index (PBI). Two contralateral teeth (tT): 13 and 25 were chosen in the upper jaw as test teeth in all volunteers. On day -1 the use of dental floss was stopped. At baseline, all hygiene procedures were completely ceased for the next 18 days. On day 0, 3, 7, 14 and 18, clinical parameters were assessed and GCF was taken from the mesiobuccal site of both tT, placing two Periopapers® for 20 seconds in the margin of the sulcus. Immediately afterwards, the fluid volume was measured in the Periotron 8000® and the two strips corresponding to each tooth were pooled into an Eppendorf® cup with the respective sample buffer.

Analysis of GCF samples
After a centrifugation at 5000 rpm for 5 min., 10 µl of sample were added to a microtiterplate containing the assay buffer, together with 50 µl of a 10-3 mol/l solution of the fluorogenic substrate MeO-Succ-Ala-Ala-Pro-Val-7-amino-4-methylcoumarin (Bachem Biochemica, Heidelberg, Germany). The plates were covered with a removable film and incubated for 6 h at 25°C.

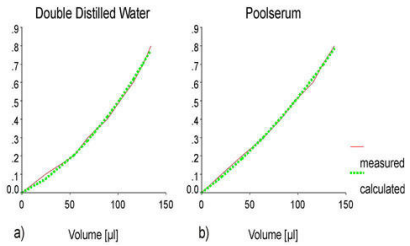

Fig. 1: Calibration of the Periotron 8000
a) r^2double distilled water = 0.999
b r^2poolserum = 0.998 (polinomial regression 3rd degree)

Each sample activity was directly compared to a standard curve of human leukocyte elastase (HLE). Elastolytic activity of the samples was directly calculated using the Biolise® software (SLT-Labinstruments, Crailsheim, Germany). Each sample was analyzed twice.

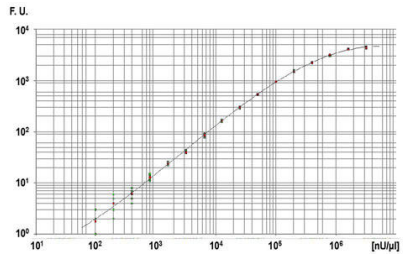

Fig. 2: Standard Curve of the Elastolytic Activity F.U. = Fluoreszenz units - Activity = nU/µl Excitation: lambda = 390 nm; Emission: lambda = 460 nm

The proteolytic cleavage of the polipeptide substrate liberated AMC. The emitted light (lambda = 390 nm) was quantified at 460 nm in the Fluostar®.

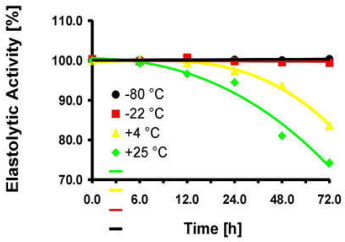

Fig. 3: Sample stability during storage at different temperature

Results

Calibration curves of the Periotron 8000® are shown in Fig. 1. An optimum curve fit was obtained with a polynomial regression calculation (r^2 = 0.999). The fluorogenic substrate MeO-Succ-Ala-Ala-Pro-Val-7-amino-4-metylcoumarin can be used for the diagnosis of elastolytic activity. A standard curve of human leukocyte elastase is shown in Fig. 2.

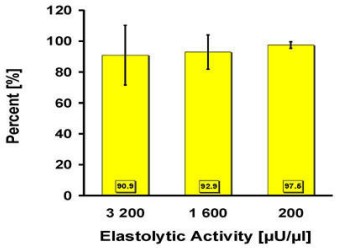

Fig. 4: Mean assay recovery rates (n=50)

The sample buffer allows a storage time up to 48 h (at a pH of 5.5). Better stability of elastolytic activity was observed when samples were kept at -80 °C or at -22 °C (Fig. 3). It is interesting to note that stored at +4 °C, the activity began to decrease after 12 h. After 72 h they presented only 84 % of their initial level.
The recovery rates of this method were about 95 % for all three activities. Activity choice was done in relation to expected clinical activity ranges (Fig. 4).

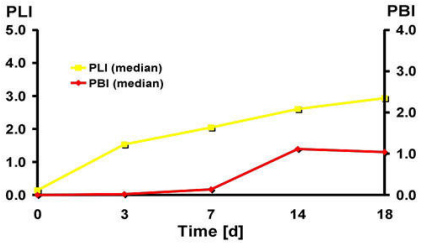

Fig. 5: PLI = plaque index and PBI = papilary bleeding index (during the experimental phase of the study)

Test limit was about 1 µU/µl. However, samples could be recovered from a specific site of the tooth and could be analyzed even in small concentrations of 0.1 µl.
Elastolytical activity was analyzed in all volunteers, even without presenting gingivitis. Fig. 5 shows the median PLI and PBI. At initial examination, both clinical parameters exhibited similarly low scores.
After baseline, PLI increased continuously until day 18 (about 2.8 fold). An increase of the PBI was observed from day 7 to day 18 (about 1.0).
Median elastolytic activities of the test teeth are shown in Fig. 6. The median activity in gingival crevicular fluid was 452 µU/µl at baseline, 708 µU/µl at day 3, 1269 µU/µl at day 7, 1325 µU/µl at day 14 and 1507 µU/µl at day 18.

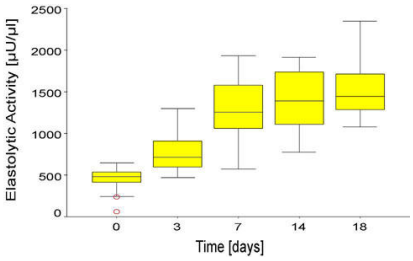

Fig. 6: Changes of elastolytic activity during experimental gingivitis in 12 human volunteers (red = two dropouts)

Discussion and Conclusions

Much effort has been directed since the past decade in the identification of host and bacterial enzymes in crevicular fluid.
Detection of minimal elastolytical activity was possible, even considering that the alkaline assay pH cannot be compared to in vivo pH as found in inflammation. Our assay reflects levels of the total elastolytic activity produced by PMN lysis and dissolved total elastase. The samples were maintained under conditions appropriate for minimizing loss of activity. Our study shows that elastase activity in GCF increases strongly with the development of experimental gingivitis. Although several different assay procedures to measure elastolytic activity have been published, only in a very few of them, the quality of the assay procedure was checked. In our system recovery rates were above 95% and were considered acceptable for use of the assay. The elastolytic activity, that was found in GCF of volunteers, could be due to the different assay procedure.
Further advantages of this method are the possibility to analyze specific sites at single teeth, optimal utilization of the reagents, as well as analysis of up to 80 samples per reading.

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Poster Faksimile: