Eur J Clin Chem Clin Biochem 1997; 35(11):867–871

Effect of an Encapsulated Anti-Elastase Compound on Experimental Gingival Inflammation in the Rat

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Summary: An animal (rat) model of gingival injury ("impaction") induced a gingival inflammatory reaction, which was characterized by a breakdown of gingival collagen and the elastic network, as well as a significant increase of gingival elastase. The present study was conducted to investigate whether ceramides, sphingolipids composed of sphingosine N-acyl-linked to fatty acids, a chemical structure with antielastase properties, could counteract the development of such an inflammatory process.

The ceramides used in these experimental series were extracted from wheat and characterized. The main fatty acids were 16 : 0, 18 : 1, 18 : 2, and the sphingoid moiety was phytosphingosine. Inhibition of elastase by ceramides was demonstrated in vitro and the concentration necessary to inhibit 50% of elastase activity was 41 mg/l using the synthetic substrate methoxysuccinyl-alanine-alanine-proline-valine-*p*-nitroanilide (MeOSuc-AlaAlaProValpNA). However, this anti-elastase activity was not observed in vivo in our animal model of gingival inflammation.

A glycosaminoglycan (Heparin®), recognized as a potent inhibitor of elastase, was entrapped in ceramides. A local treatment of impacted gingivae by encapsulated heparin led to a dose-related decrease of the elastase level in gingival extracts. Encapsulation in ceramides potentiated the effect exerted by heparin alone. This inhibitory effect of encapsulated heparin on elastase suggested a vector effect of these amphipathic molecules.

Introduction

Periodontitis is an infectious periodontal disease with an irregular evolution, characterized by gingival inflammation mainly due to bacteria (1, 2). Clinical criteria such as radiographs, pocket depth and bleeding on probing are needed for periodontitis diagnosis. Some markers such as enzymes[1]) and inflammatory mediators have also been investigated in gingival fluid in order to include biochemical criteria in the diagnosis. These markers include collagenase (3, 4), gelatinase (5, 6), lactoferrin (7), γ-glucuronidase (8), aspartate aminotransferase (9), and elastase (10–14).

The gingival proteinases, collagenase and elastase, have received a great deal of attention during the past few years, since tissue destruction by these enzymes plays a major role in the pathogenesis of periodontitis. In healthy tissues, matrix macromolecules are protected against elastase destruction by natural inhibitors such as α_1-antiprotease, α_2-macroglobulin and α_1-antichymotrypsin (15) which are endogenous regulators of this enzyme activity. However, during inflammatory episodes, polymorphonuclear leukocytes release numerous enzymes and oxidants which can inactivate these natural inhibitors. Therefore, exogenous natural or synthetic antielastase compounds might be efficient in preventing tissue damage.

Elastase is a serine proteinase capable of digesting various components of the extracellular matrix (16), which plays a central role in connective tissue destruction associated with the inflammatory process. It has been demonstrated that several lipidic substances inhibit the serine proteases, pancreatic elastase and plasmin (17, 18). The three-dimensional structure of leukocyte elastase shows an unusual hydrophobic pocket near its active site that can accommodate *cis*-unsaturated long chain fatty acids and their derivatives (19, 20). By binding to this pocket, glycosaminoglycans such as heparin and heparan sulphate may act as strong inhibitors of leukocyte elastase (21).

Ceramides are sphingolipids composed of sphingosine N-acyl linked to fatty acids, conferring an hydrophobic character on the molecule. Owing to their hydrophobic structure, it has been suggested that ceramides extracted from wheat, composed of dehydrophytosphingosine,

[1]) Enzymes:
Neutrophil elastase EC 3.4.21.37;
Neutrophil collagenase EC 3.4.24.34

phytosphingosine, dihydrophytosphingosine and polyunsaturated fatty acids, may inhibit leukocyte elastase in vitro and thus prevent matrix alterations (22).

In previous experiments in the rat (23), we demonstrated that elastic components decreased significantly only when bacteria were added to the mechanical injury. A significant enhancement of gingival elastase level appeared in parallel to the number of invading inflammatory cells, mostly represented by polymorphonuclear leukocytes, which represent the major source of released elastase.

The aim of this work was to investigate whether ceramides, containing or not containing an antielastase compound such as heparin, could counteract the evolution of a gingival-induced inflammation which shows many features of periodontal diseases (23).

Materials and Methods

Reagents

Purified human leukocyte elastase was from Elastin Products Company (St. Louis, MO, USA).

Methoxysuccinyl-$(Ala)_2$-Pro-Val-*p*NA was from Sigma (St. Louis, MO).

Heparin was from Léo Lab. (Paris, Fance).

Ceramides were extracted and purified by INOCOSM (Chatenay-Malabry, France).

Extraction of ceramides from wheat

Wheat grains (Joss variety) were ground to powder and extracted several times with combinations of solvents containing various proportions of methanol, chloroform and acetone (European patent No 91-06-336 PCT/FR92/00182). Triacylglycerols were removed by treating isolated ceramides with acetone. Ceramides were then recrystallized, treated with active charcoal, dried, crushed and micronized.

Characterization of wheat ceramides

Wheat ceramides were characterized by thin layer chromatography, gas chromatography-mass spectrometry and infrared spectroscopy.

Thin-layer chromatography was performed on Silica gel 60 plates (Merck, Darmstadt, Germany) using chloroform/hexane/methanol/acetic acid/water (24 + 14 + 8 + 6 + 0.6 by vol.) as the solvent. The detection reagent was 10 g of copper II sulphate in 100 ml of 80 g/l phosphoric acid solution. Individual ceramides were quantified by densitometry coupled with a Donatec 385/16 computer.

For gas chromatography-mass spectrometry analysis, an HP 5989 A spectrometer was used with an electron energy of 22 eV. Compounds were run on a HP1 column conditioned at 280 °C with a helium (carrier gas) pressure of 15 psi on the column head. The standard Grob-split-splitless injector was used in the splitless mode. Esterified fatty acids from hydrolysed ceramides was performed with a NICOLET SX 730 infrared spectrometer equipped with *Fourier* transformation and a silicon carbide infrared source. Non-hydrolysed ceramides were dissolved in dimethylsulphoxide for infrared spectroscopy.

Determination of solubilities of ceramides in dimethylsulphoxide

Stock solutions of ceramides were prepared in dimethylsulphoxide. Fifty microlitres were withdrawn and added to 100 mmol/l Tris/HCl pH 8.0 containing 0.1 ml/l Triton X-100 and 0.2 g/l sodium azide (NaN_3). The turbidity of the solution was evaluated by laser nephelometry (Behring laser nephelometer, Behring Institute, France) and values were corrected for blanks consisting of buffer containing the same amount of dimethylsulphoxide. Solubility limits corresponded to changes in the slope of the nephelometric curve (24).

Enzyme kinetics using MeOSucAlaAlaProValpNA

Stock solutions of methoxysuccinyl-alanine-alanine-proline-valine-*p*-nitroanilide (MeOSucAlaAlaProVal*p*NA) were prepared in N-methylpyrrolidone and stored in the dark at 4 °C. Human neutrophil elastase activity was determined at 37 °C in thermostated polystyrene cuvettes with 100 mmol/l Tris/HCl pH 8.0 containing 0.1 ml/l Triton X-100 and 0.2 g/l sodium azide (NaN_3). Human neutrophil elastase (16.1 nmol/l) was preincubated for 5 min with 5 to 62.5 mg/l of ceramide. Synthetic substrate (0.025 to 0.2 mmol/l) was then added. The release of *p*-nitroaniline was recorded at 410 nm with a spectrophotometer (Philips PU 8740 UV/Vis). The inhibitory capacity of ceramide was expressed as percentage of inhibition: $\%I = (1\text{-}Vi/Vo) \times 100$ where Vi is the velocity in the presence of the inhibitor and Vo in the absence of the inhibitor, containing the same amount of dimethylsulphoxide.

Experimental gingival inflammation "Impaction"

Male Sprague-Dawley Rats (Depré, Saint-Doulchard, France) weighing 180−200 g were housed at 21−24 °C, given chows and distilled water *ad libitum*. Gingival inflammation was induced using the bacterial strain *Treponema denticola* (IP 6444) as described before (23).

Collection of samples

Animals were euthanized with ether, 10 days after impaction. Gingivae were dissected under a binocular microscope, weighed and placed in sterile plastic microcentrifuge tubes (Eppendorf 0.7 ml) containing 10 µl of phosphate-buffered saline and 0.1 g/l Brij 35, pH = 8, in order to minimize evaporation and protease inhibition (25). These samples were used for elastase assay.

Elastase assays

Gingivae, frozen (−20 °C) after collection, were cut with a cryostat into 10 µm slices and suspended in Tris buffer (100 mmol/l Tris/HCl, NaCl 1 mol/l, Brij 35 0.1 g/l, NaN_3 0.2 g/l, pH = 8). After shaking for 24 h at 4 °C, they were centrifuged, and the supernatant was collected for elastase measurement. Elastase activity was determined using MeOSucAlaAlaProVal*p*NA as substrate.

Briefly, a 125 mmol/l stock solution of the substrate was prepared in N-methylpyrrolidone. Next, 20 µl of this solution were added to 960 µl of buffered solution consisting of 100 mmol/l Tris HCl, 0.1 g/l Brij 35, 0.2 g/l NaN_3, and 20 µl of sample. The mixture was incubated for 24 h at 37 °C and absorbance was recorded at 410 nm in a spectrophotometer (Beckman). Standard curves of substrate hydrolysis were obtained using titrated purified human leukocyte elastase (Elastin Products Company, St. Louis, MO, USA). Results were expressed as ng of active elastase per mg of proteins of the sample.

Pharmacological assay

Rat gingivae impacted with *Treponema denticola* were locally treated with ceramides (prepared with gum arabic and stored in the dark in small sterile flasks at 4 °C) with or without heparin (25×10^3 and 50×10^3 IU/l) every day at the same hour until animal sacrifice. Control animals were not infected.

The level of elastase measured in each biopsy was related to the amount of protein measured by *Lowry*'s technique.

Statistical analysis

One way analysis of variance (Anova from the computer program Statview II) provided an initial estimate of whether the groups were

significantly different. Unpaired *Student*'s t test was calculated in each group between treated and non-treated rats. Results were given as means ± S. E. M. Differences with $p < 0.05$ were considered as significant.

Results

Characterization of wheat ceramides

Table 1 and figure 1 show the composition and the structure of wheat ceramides. The major fatty acids are palmitic acid (16 : 0), oleic acid (18 : 1) and linoleic acid (18 : 2), representing 19, 12 and 53% of total fatty acids, respectively. However the exact composition of the mono-, di-, tri- and polyglycosyl ceramides were not determined. The absence of any absorption when infrared spectroscopy was used suggested that the sphingosine moiety is a phytosphingosine.

Determination of solubility

Figure 2 shows the solubility limit of ceramides which corresponds to a change in the slope of the nephelometric curve. This value was equal to 80 mg/l. Therefore, subsequent enzymatic and pharmacological studies were performed with concentrations below this value.

Inhibition of elastase by ceramide

For testing the inhibition of elastase by wheat ceramides in vitro, we used the specific synthetic substrate MeO-SucAlaAlaProVal*p*NA. Figure 3 represents the percentage of inhibition of elastase by ceramide at concentrations from 5 to 62.5 mg/l. Inhibition of leukocyte elastase increased with the increase of ceramide concentration used (below solubility limits). Previous studies (unpublished data analysed by *Baici*'s equation (26)) showed that this inhibition, hyperbolic and non-competitive for concentrations below 20 mg/l, becomes mixed and linear at higher concentrations.

Tab. 1 Fatty acid composition of wheat ceramides.

Fatty acids	Non-hydroxylated	Hydroxylated
16 : 0	19.04	–
18 : 0	5.10	–
18 : 1	12.30	–
18 : 2	53.22	–
20 : 0	0.61	1.65
20 : 1	1.05	–
22 : 0	2.56	1.88
22 : 1	–	0.47
24 : 0	0.65	–
26 : 0	0.85	–
28 : 0	0.67	–

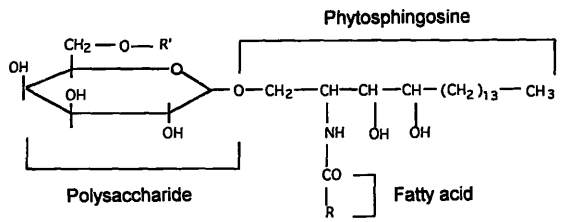

Fig. 1 Fatty acid composition of wheat ceramides.

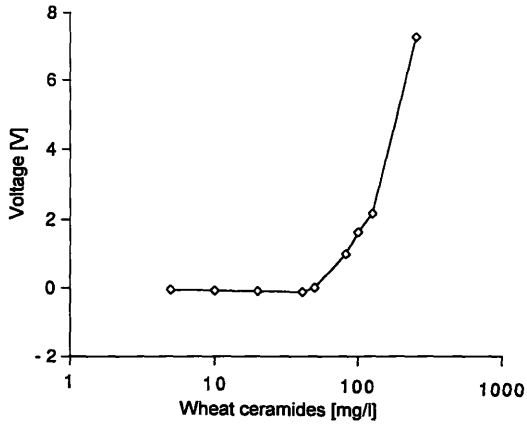

Fig. 2 Limit of solubility of wheat ceramides.

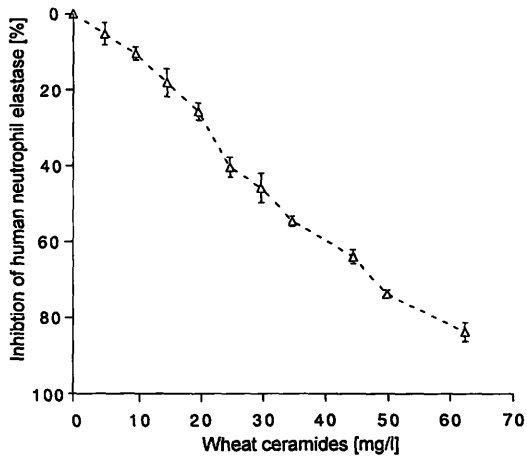

Fig. 3 Inhibition of human neutrophil elastase by wheat ceramides (%).

Pharmacological assay

Figure 4 shows that the elastase level was very low in controls. This value was significantly increased when gingivae were impacted with *Treponema denticola*. Ceramides did not prevent this increase. However, this value decreased significantly in gingivae treated locally with heparin (50 U), and this effect was dose-related (data not shown). This effect was potentiated when heparin was encapsulated in ceramides. Only these data have been reported in figure 4.

Discussion

In the present work we demonstrated that our model of gingival inflammation can be used to test the capacities of some anti-elastase substances to protect and/or to prevent destruction of extracellular matrix macromolecules.

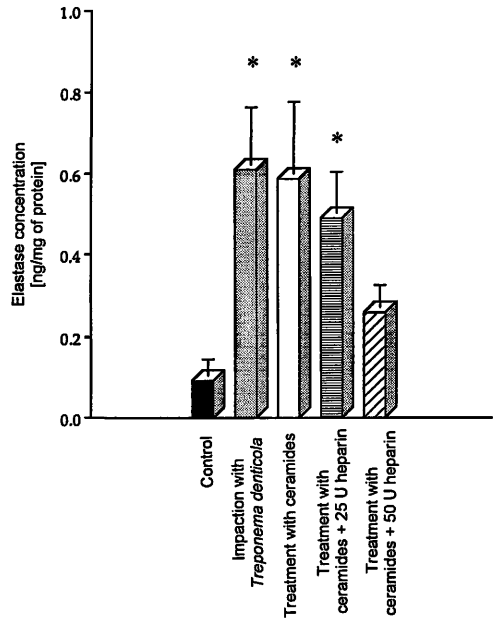

Fig. 4 Amounts of neutrophil elastase (ng/mg of proteins ± SEM) in control gingivae, gingivae impacted with *Treponema denticola*, gingivae impacted with *Treponema denticola* and treated with ceramides alone or ceramides associated to heparin 25 U and heparin 50 U.
* $p < 0.05$ by comparison to control value
number of animals in each group: 6 to 8.

There are a number of biological markers of periodontal diseases but elastase derived from polymorphonuclear leukocytes is certainly the most important one. The increase of elastase in gingival extracts after "impaction" with bacteria parallels the migration of inflammatory cells (essentially polymorphonuclear leukocytes) towards the focus of inflammation. These cells then release their lysosomal content, including enzymes and mediators. This degranulation process is followed by degradation and disorganization of the gingival elastic network, especially when gingival infection occurs (23). These results corroborated the findings of *Bonnaure-Mallet* (27) who showed that pathogens play a pivotal role in the establishment of the disease, and who observed a modification of gingival elastic fibres in patients with severe periodontitis, similar to that observed in impacted animals.

Elastase, derived from polymorphonuclear leukocytes, has a high affinity for lipophilic substances, and an even higher affinity for long-chain unsaturated fatty acids and their derivatives, owing to the presence of an hydrophobic pocket near its active site (19, 20). Oleic acid is the most potent inhibitor, and it is capable of combining with either substrate or enzyme (28).

Recent data indicated that the extended substrate binding domain of leukocyte elastase can accommodate a large variety of hydrophobic lipids (not only fatty acids) like fatty acyl-saccharins (29), polycyclic molecules such as steroidal anti-inflammatory drugs (30) and some cephalosporin derivatives (31). It was speculated that the N-acyl linkage of fatty acid to phytosphingosine confers high hydrophobicity on wheat ceramides, allowing their interaction with the active site of elastase. Besides their physiological properties such as skin hydration, regulation of cellular growth and differentiation (32), we demonstrated in this work that ceramides extracted from wheat possess anti-elastase activity in vitro. The same results were obtained when the inhibitory properties of ceramide were tested on natural substrate (radiolabelled elastin) or on healthy human skin (22).

Heparin and its sulphated derivative, heparan sulphate, are strong inhibitors of leukocyte elastase (21). The same inhibitory activity was demonstrated against both elastase and cathepsin G by N-oleoylheparin (20). It has been demonstrated that heparin fragments such as oleoyl peptide conjugates are efficient in preventing emphysema induced in rodents by intra-tracheal elastase instillation (33, 34).

After "impaction" of gingivae, we observed a significant enhancement of the elastase content of gingival extracts, which parallelled the presence of migratory invading cells (especially polymorphonuclear leukocytes) attracted by various chemoattractants, as previously observed in skin diseases (35). Unlike bifunctional inhibitors, such as oleic acid, the ceramides were unable to bind elastin (22). Protection of the substrate by ceramides therefore consists of inhibition of the enzyme only.

Our experiments showed that local treatment by heparin of impacted gingivae led to a dose related decrease of the elastase level in gingival extract. This inhibiting effect of heparin on elastase was potentiated by association with ceramides, suggesting the possible existence of a vector effect of these amphipathic molecules. A vector effect has already been demonstrated after oral administration of encapsulated superoxide dismutase in liposomes containing ceramides (36, 37).

This preliminary pharmacological assay performed to validate our animal gingival lesion model with a very potent anti-elastase substance opens a large field of investigations on natural or synthetic anti-elastase molecules. Such substances might prevent the destruction of gingival macromolecules which occurs during inflammatory injury and might be useful in therapeutic treatment of periodontal diseases.

Acknowledgements

The authors gratefully thank Mr. *Olivier Muntaner* for his excellent technical assistance.

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Received May 20/July 25, 1997

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