J Periodont Res 2007; 42: 325–330

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2006.00952.x

Neutrophil elastase is involved in the initial destruction of human periodontal ligament

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Ujiie Y, Oida S, Gomi K, Arai T, Fukae M. Neutrophil elastase is involved in the initial destruction of human periodontal ligament. J Periodont Res 2007; 42: 325–330. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard

Background and Objective: It has been reported that noncollagenous proteins may provide mechanical strength to the periodontal ligament. Several proteolytic activities, including that of neutrophil elastase, are reported to increase significantly in periodontal disease. The aim of this study was to investigate the function of neutrophil elastase in the initial destruction of periodontal ligament at early stages of periodontal disease.

Material and Methods: The detection and identification of proteinases in chronic periodontitis and healthy periodontal ligament were examined by zymographic and zymo-Western analysis. The morphological changes of periodontal ligament, digested with or without authentic proteinases, were observed using scanning electron microscopy.

Results: Increases in neutrophil elastase, plasminogen, and matrix metalloproteinase-9 were detected in periodontal ligament from chronic periodontitis, compared with healthy periodontal ligament. Among these proteinases, only neutrophil elastase digested the intact noncollagenous proteins of periodontium. When human healthy periodontal ligament was directly digested by neutrophil elastase in an *in vitro* system, the morphological features were quite similar to that of the periodontal ligament in chronic periodontitis . In healthy periodontal ligament, the collagen fibrils are covered with noncollagenous proteins containing 110 kDa acidic glycoprotein, which was degraded initially by the neutrophil elastase.

Conclusion: It was concluded that neutrophil elastase is involved in the degradation of noncollagenous protein-covered collagen fibrils in the early destructive stages of periodontal disease.

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Key words: chronic periodontitis; interfibrillar substances; neutrophil elastase; periodontal ligament

Accepted for publication August 27, 2006

Periodontal diseases are characterized by inflammation and subsequent loss and/or damage to tooth-supporting tissues. Bacterial plaque and its metabolic products trigger the local infiltration of inflammatory cells and stimulate inflammatory conditions. These infiltrated inflammatory cells and connective tissue cells produce several proteinases, which are associated with the breakdown of extracellular matrices (1). To investigate the mechanisms of initial periodontal destruction, several types of proteinases, namely gelatinases A and B [matrix metalloproteinase (MMP)-2 and -9] (2–4) and other metalloproteinases (MMP-1, MMP-3, MMP-8, MMP-13) (2,5–7), were examined in human healthy and diseased gingiva and periodontal ligament. The results seemed to suggest that in periodontal disease, the destruction of collagen fibers was initially induced by collagenase. In fact, collagen fibers in human periodontal ligament are responsible for maintaining the

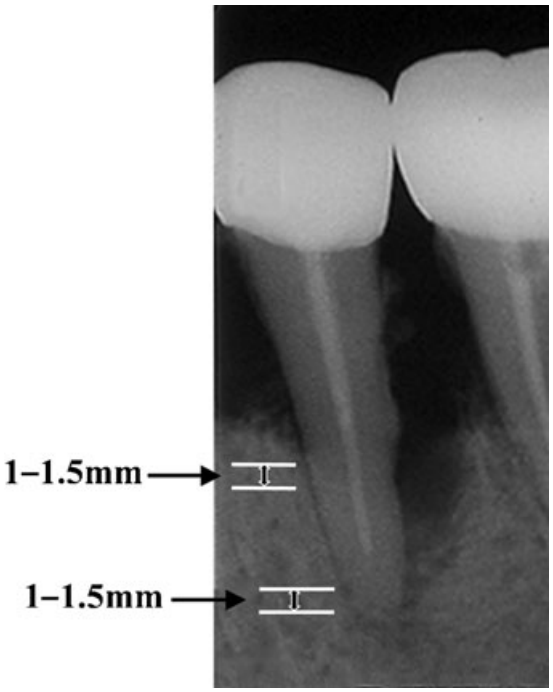

Fig. 1. Radiographic image of a tooth diagnosed as chronic periodontitis. The tooth had pockets deeper than 5 mm, together with severe vertical alveolar bone loss, and the deepest pocket was very close to the root apex. The periodontal ligament sample from a patient with chronic periodontitis was prepared from the periodontal ligament attached to the cementum of extracted teeth, corresponding to the periodontal ligament on the opposite side of the deepest pocket. When preparing the sample using a sharp blade, marked lengths, as shown in the figure, were avoided.

mechanical strength of periodontal ligament, and their degradation advances the destructive process of periodontal disease. However, it has been reported that the degradation of noncollagenous proteins covering the collagen fibrils may decrease the mechanical strength of periodontal ligament without destruction of the collagen fibers (8).

Gingival crevicular fluid plays an important role in oral defense via various mechanisms. The components of gingival crevicular fluid can be characterized as proteins, antibodies, antigens, enzymes, and cellular elements. The cellular elements include bacteria, desquamated epithelial cells, and neutrophils, which migrate through the sulcular epithelium. Neutrophils release neutrophil elastase, the activity of which has been reported to increase significantly in periodontal disease (9–11).

The aim of this study was to investigate the effects of neutrophil elastase in the degradation of noncollagenous proteins, which cover the collagen fibers of periodontal ligament, during the initial stages of periodontal ligament destruction in periodontal disease.

Material and methods

The study protocol was approved by the Ethics Committee of the Institute of Tsurumi University, Yokohama, Japan. All participants were volunteers with no ongoing systemic diseases or infections.

Patients and clinical recordings

All patients had been referred to Tsurumi University Dental Hospital with the diagnosis of chronic periodontitis. They had given informed consent to participate in this experiment. The chronic periodontitis samples were obtained from 40 teeth with chronic periodontitis. These teeth had been extracted from 40 adult patients (16 men, 24 women). Teeth diagnosed with chronic periodontitis, as shown in Fig. 1, in which the periodontal ligament was attached to both cementum and remaining alveolar bone in a site opposite to a deep pocket and appeared normal radiographically, were selected for preparation of the periodontal ligament sample from chronic periodontitis. The control samples, from clinically healthy periodontium, were obtained from 28 teeth extracted for orthodontic treatment from 20 patients (8 men, 12 women). These teeth had no inflammation, deep pockets or radiographic evidence of bone loss.

Tissue preparation

All preparation and extraction procedures were carried out at 4°C or under ice-cold conditions. After the extracted teeth were rinsed in cold saline to wash off the blood, the excess water on their root surfaces was gently absorbed with wrung wet Kimwipe papers. The periodontal ligament tissue retained on the root of the extracted tooth was peeled off using a sharp blade and used as samples. During the preparation of periodontal ligament from patients with chronic periodontitis, 1–1.5 mm of the periodontal ligament from the top portion, and that around the apex of root, were avoided (Fig. 1). Preparation of healthy periodontal ligament was also carried out in a similar manner. The prepared samples were weighed (wet weight) and freeze-dried or stored in deep freezer (−80°C). All experiments described below were performed more than five times.

Detection and identification of proteinases

Each sample (1 mg) was suspended in 50 μL of sample solution (0.01 M Tris-HCl buffer, pH 8.0, containing 1% sodium dodecyl sulphate, 1 mM EDTA, and 25% glycerol) and heated at 100°C for 5 min to obtain maximum proteolytic activity. The supernatant was separated, by centrifugation, into the crude proteinase fraction. The detection and identification of pro-

teinases in the crude proteinase fractions of chronic periodontitis and healthy periodontal ligament samples were performed by zymographic analyses (12) and zymo-Western analyses through Western blotting and zymography (13). As they also contained maximally solubilized proteins, protein profiles were examined by sodium dodecyl sulphate electrophoresis.

For identification of proteinases, the antibodies for human neutrophil elastase (EPC, Owenscville, MO, USA), plasminogen (NOR, Tilburg, the Netherlands) and C-terminal pro-MMP-9 (Sigma, St Louis, MO, USA) were used. After electrophoresis for zymo-Western analyses, the gel was incubated in 0.05 M Tris-HCl buffer (pH 8.0), containing 2 mM $CaCl_2$, for 2 h at 37°C. The gel was then electrotransferred onto a polyvinylidine difluoride membrane. The membrane was treated with blocking serum and immunostained using the avidin-biotin complex (ABC kit, Vector Laboratories, Burlingame, CA, USA). The residual gel was stained by Coomassie Brilliant Blue to detect the proteolytic activities.

Digestion of noncollagenous proteins with proteinases

Noncollagenous proteins of human healthy periodontal ligament were prepared by homogenizing with handy homogenizer (Ikemoto Scientific Technology Co. Ltd, Tokyo, Japan) in 50 mM Tris-HCl buffer (pH 8.0) containing 1 M NaCl and 0.005 M EDTA. After centrifugation, noncollagenous proteins were desalted by ultrafiltration with a YM-1 membrane (Millipore, Bedford, MA, USA) and collected in 50 mM Tris-HCl buffer (pH 8.0). The digestion of noncollagenous proteins was examined by adding commercially available neutrophil elastase (elastase from human leukocyte; EPC), plasminogen (Human Plasma; Athens Research & Technology Inc., Athens, GA, USA), human plasmin (Molecular Innovations Inc., Southfield, MA, USA) and active type MMP-9 (Oncogene, Boston, MA, USA) in 30 μL of 50 mM Tris-HCl (pH 8.0).

Morphology by scanning electron microscopy

The periodontal ligament samples were cut into minute pieces using a sharp blade. They were suspended in 0.05 M Tris-HCl (pH 8.0) and digested by adding the proteinases. After digestion, the residue was fixed in 10% neutral-buffered formalin, dehydrated in a graded ethanol series, critical point dried, and coated with gold (ION COATER IB.3; Eiko, Tokyo, Japan). The morphology of these samples was observed using scanning electron microscopy (JSM5600LV; JEOL, Tokyo, Japan).

Analytical methods

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (14). The gel was stained with Coomassie Brilliant Blue R250 or Stains-all (15). Amino acid sequence analyses were carried out using the Shimazdu protein sequencer PPSQ-23 A (Shimadzu Co., Kyoto, Japan).

Results

Detection and identification of proteinases in the crude proteinase fractions chronic periodontitis periodontal ligament

The protein and proteolytic activity profiles in the crude proteinase fractions of periodontal ligament from patients with chronic periodontitis were compared with those of healthy periodontal ligament, according to their wet weights. Both samples contained mainly α1 and α2 chains of collagen and albumin, whereas the periodontal ligament sample of patients with chronic periodontitis had increased levels of albumin, and α and β globin of hemoglobin. In addition, many acidic glycoproteins, which stained blue with Stains-all, were detected in both samples.

On zymographic analyses, thick caseinolytic activity bands were detected at 30 and 80 kDa in chronic periodontitis and healthy periodontal ligament samples, respectively (Fig. 2). The activity of the 30-kDa band was strong in the periodontal ligament from patients with chronic periodontitis compared with healthy periodontal ligament. On the other hand, the activity of the 80-kDa band from the periodontal ligament of patients with chronic periodontitis was weaker than that of the healthy periodontal ligament. These caseinolytic activities were attributed to serine proteinase, as they were partially inhibited with benzamidine-HCl. Using zymo-Western analyses, the activities of the 30- and 80-kDa bands were determined to be neutrophil elastase and plasminogen, respectively (Fig. 3A,B).

Four gelatinolytic activities were found, at ≈200, 92, 67 and 57 kDa molecular masses, upon addition of Ca ions to both periodontal ligament samples. Among these, the 92-kDa activity was increased in the periodontal ligament from subjects with chronic periodontitis compared with the healthy periodontal ligament, and was determined to be pro-type MMP-9. All gelatinolytic activities were of the metalloproteinase type, which were inhibited with EDTA (Fig. 3C).

Function of the proteolytic activities of the periodontal ligament in chronic periodontitis

When the digestion of noncollagenous proteins of human periodontal ligament was examined with commercially available proteinases, only neutrophil elastase was involved in the degradation of noncollagenous proteins. Although noncollagenous proteins contained many acidic glycoproteins, which stained blue by Stains-all along and Coomassie Brilliant Blue, neutrophil elastase degraded only acidic glycoproteins of ≈110 kDa (Fig. 4A). However, when the neutrophil elastase concentration was high, neutrophil elastase degraded another protein in noncollagenous proteins. The plasminogen and active type MMP-9 did not degrade noncollagenous proteins, whereas plasmin degraded the same protein as neutrophil elastase, although its activity was weak.

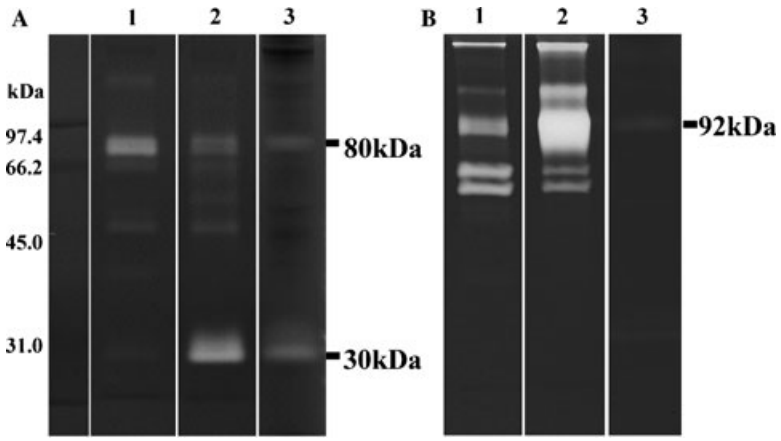

Fig. 2. Zymography of crude proteinase fractions prepared from healthy periodontal ligament and periodontal ligament from patients with chronic periodontitis. The gels contained 0.1% α casein (A) and 0.1% gelatin (B). The proteolytic activities in both samples on zymograms were reproducibly recognized in the samples obtained from 20 patients. The electrophoresed gels were incubated in developing buffer (pH 8.0) with 2 mM Ca at 37°C for 16 h. Numbers on the left, molecular weight standard; lane 1 (A and B), human healthy periodontal ligament; lane 2 (A and B), human periodontal ligament from patients with chronic periodontitis; lane 3 (A and B), human chronic periodontitis sample to which benzamidine-HCl (inhibitor of serine proteinase) (A) and EDTA (inhibitor of metalloproteinase) (B) were added, respectively.

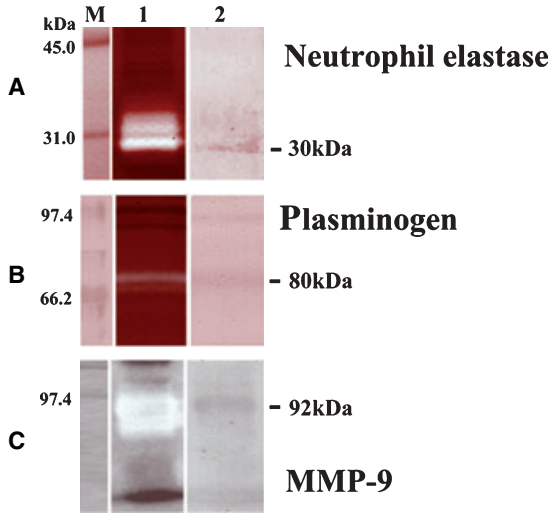

Fig. 3. Zymo-Western analysis of crude proteinase fraction prepared from the periodontal ligament from subjects with chronic periodontitis. The membranes were immunostained with antibodies to neutrophil elastase, plasminogen, and matrix metalloproteinase-9; neutrophil elastase and plasminogen were analyzed on an 8.5% polyacrylamide gel containing 0.1% α casein, and a 4.5% polyacrylamide gel containing 0.1% gelatin was used for matrix metalloproteinase-9. The proteolytic activities were detected by staining with 0.125% Coomassie Brilliant Blue. Numbers on the left represent molecular weight standards; lane 1, proteolytic activities of human chronic periodontitis periodontal ligament; and lane 2, immunodetection using antineutrophil elastase (A), antiplasminogen (B), and anti-matrix metalloproteinase-9 (C). These antibodies were incubated on the membrane transblotted from the electrophoresed gel. The area detected by each antibody corresponded approximately to the proteolytic activity. In the case of plasminogen, an extra immunoreacted band, without proteolytic activity, was found. MMP-9, matrix metalloproteinase-9.

It was demonstrated, by scanning electron microscopy, that the periodontal ligament from patients with chronic periodontitis contained morphologically exposed collagen fibrils, whereas the healthy periodontal ligament did not (Fig. 4B, i and ii). The residue, after extraction of noncollagenous proteins from the healthy periodontal ligament, also revealed the same structure of exposed collagen fibrils (data not shown). When healthy periodontal ligament was digested directly by neutrophil elastase, the structure after digestion revealed exposed collagen fibrils, as in periodontal ligament from subjects with chronic periodontitis (Fig. 4B, iv). The control, incubated by adding buffer alone, did not show any significant morphological changes (Fig. 4B, iii). The digestion of periodontal ligament by MMP-9 and plasmin showed minimal changes of structure.

Discussion

Periodontal disease is characterized by the destruction of extracellular matrix components within the periodontal tissue. The host cell-derived proteinases are mostly involved in the destruction of periodontal tissues.

In the present study, periodontal ligament from chronic periodontitis looked normal radiographically but showed remarkably high neutrophil elastase and MMP-9 activities compared with healthy periodontal ligament. The solubility behavior of noncollagenous proteins from healthy periodontal ligament and the morphological features of periodontal ligament, after extraction of noncollagenous proteins, suggests that healthy periodontal ligament consists of collagen fibrils wrapped in noncollagenous proteins by ionic strength, because it was solubilized with a high concentration of NaCl. As periodontal ligament from subjects with chronic periodontitis demonstrated exposed collagen fibrils, it is suspected that the degradation of noncollagenous proteins by some proteinases causes the characteristic periodontal ligament structure observed in patients with chronic periodontitis. When human

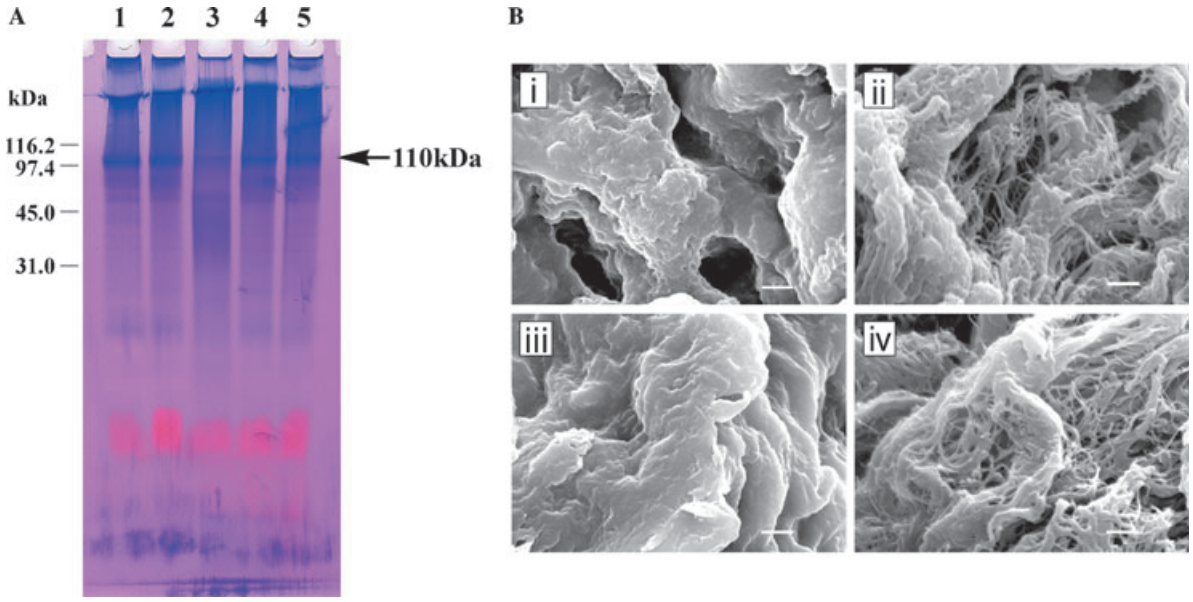

Fig. 4. (A) Electrophoretic patterns of noncollagenous proteins of human healthy periodontal ligament after incubation with commercially available proteinases. The incubation was carried out for 12 h at 37°C and terminated by adding an equal volume of 2% sodium dodecyl sulfate sample solution. The digestions with neutrophil elastase and plasminogen were carried out in a buffer containing 0.002 M EDTA. In the case of matrix metalloproteinase-9, the buffer contained 0.002 M Ca. The ratio of enzyme to noncollagenous proteins of human periodontal ligament was 1 : 300. Electrophoresis was carried out in a 4.5% polyacrylamide gel and the gel was stained with Stains-all. Numbers on the left represent molecular weight standards; lane 1, noncollagenous proteins of healthy periodontal ligament without incubation; lane 2, noncollagenous proteins of healthy periodontal ligament with incubation; lane 3, noncollagenous proteins of healthy periodontal ligament with incubation after adding neutrophil elastase; lane 4, noncollagenous proteins of healthy periodontal ligament with incubation after adding plasminogen; lane 5, noncollagenous proteins of healthy periodontal ligament with incubation after adding matrix metalloproteinase-9. (B) Scanning electron micrographs of human healthy periodontal ligament (i), human periodontal ligament from a patient with chronic periodontitis (ii), human healthy periodontal ligament after incubation without neutrophil elastase (iii) and human healthy periodontal ligament after incubation with neutrophil elastase (iv). The ratio of proteinase to human healthy periodontal ligament was 1 : 200, and the incubation was carried out for 12 h at 37°C. Scale bar = 1 μm.

healthy periodontal ligament was degraded by adding commercially available proteinases, which are usually observed in periodontal ligament from patients with chronic periodontitis, neutrophil elastase markedly degraded noncollagenous proteins of human periodontal ligament, resulting in exposed collagen fibrils. It has been confirmed that only neutrophil elastase activity is detected on the zymogram periodontal ligament from patients with chronic periodontitis when using noncollagenous proteins extracted from porcine healthy periodontal ligament as a substrate (16). Therefore, these results imply that only neutrophil elastase is involved in degradation of the noncollagenous protein of periodontal ligament.

Noncollagenous proteins in periodontal ligament protect collagen fibrils from the action of collagenase. When rat periodontal ligament is digested with bacterial collagenase, it attacks only the collagen fibrils adjacent to the cementum surface (17). The protection of collagen fibrils from collagenase is suspected to be caused by the presence of noncollagenous proteins covering the collagen fibrils. This was confirmed by few changes in morphological features of periodontal ligament after the application of MMP-9 and plasmin.

Noncollagenous proteins of periodontal ligament consist of many acidic glycoproteins, which are stained blue by Stains-all, except for serum-derived proteins (17) and 90-kDa periostin (18). However, there is scant information about these acidic glycoproteins. In healthy periodontal ligament, noncollagenous proteins, wrapping the collagen fibrils as a result of ionic strength, may be responsible for the mechanical strength of periodontal ligament, although its involvement may not be so high. This expectation is a result of the fact that the removal of noncollagenous proteins from rat periodontal ligament causes a reduction in its mechanical strength (8). It was shown that the 110-kDa acidic glycoprotein in the noncollagenous proteins of healthy periodontal ligament was degraded by digestion of neutrophil elastase. As healthy periodontal ligament revealed exposed collagen fibrils after digestion with neutrophil elastase, the 110-kDa acidic glycoprotein may be the key protein of noncollagenous proteins, involved in covering the collagen fibrils, although it is not characterized at present.

The results of the present study strongly suggest that neutrophil ela-

stase, secreted by neutrophilic leukocytes, degrades the noncollagenous proteins which cover the collagen fibers, and is involved in the initial destruction of periodontal ligament before destruction of collagen fibers occurs in the early stages of periodontal disease.

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