Journal of
PERIODONTAL RESEARCH

J Periodont Res 2009; 44: 578–587

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2008.01148.x

The defensive role of lysozyme in human gingiva in inflammatory periodontal disease

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Younes R, Yousfi M, Ghorra C, Khalife S, Igondjo-Tchen-Changotade S, Willig C, Senni K, Charpiot P, Naaman N, Godeau G. The defensive role of lysozyme in human gingiva in inflammatory periodontal disease. J Periodont Res 2009; 44: 578–587. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard

Background and Objective: The presence of lysozyme in human gingiva has not previously been demonstrated. In this study, we looked for evidence for the potential role of lysozyme as a protector of gingival elastic fibres. The objective of this study was also to determine the *ex vivo* susceptibility to hydrolysis of gingival elastic fibres from patients with or without periodontal disease by human leukocyte elastase and by human cathepsin G.

Materials and Methods: Using gingival tissue sections from eight control, 10 gingivitis and 10 periodontitis patients, we evaluated the area fraction occupied by gingival elastic fibres (after selective staining) by the use of automated image analysis. In the *ex vivo* experiments, serial tissue sections from four control, four gingivitis, four young periodontitis and four aged periodontitis patients were submitted to the action of human leukocyte elastase and cathepsin G, after which enzymatic activities were determined by image analysis. Indirect immunodetection of lysozyme was also done on tissue sections for all patients included in this study.

Results: Large variations of the area fraction occupied by elastic fibres were observed in human gingiva from young and aged patients with and without periodontal disease. In control and gingivitis patients, leukocyte elastase and cathepsin G had high comparable elastin solubilizing activities. With young and aged periodontitis patients, the two serine proteinases had weak elastin solubilizing activities. Lysozyme appeared to be present at the periphery of gingival elastic fibres in periodontitis patients.

Conclusion: Lysozyme can be considered an important natural protector of elastic fibres in pathological gingiva.

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Key words: lysozyme; elastic fibre; serine proteinases; periodontal disease

Accepted for publication June 21, 2008

Human gingiva contains macromolecules present in most connective tissues: proteoglycans, glycoproteins, collagens and elastin (1). Human gingiva and human skin have a comparable elastic fibre system, consisting of oxytalan, elaunin and mature elastic fibres (2,3). A few years ago, we determined the morphometric parameters of human gingival elastic fibre network by image analysis and compared it with human skin elastic fibres

in relation to age (4). We reported that the area fraction occupied by skin elastic fibres increased with age, while in the gingiva the area fraction occupied by the elastic fibres did not seem to be significantly modified by age; however, we noted large variations in the area fraction occupied by these fibres in patients of advanced age (50–75 years old) and, concomitantly, great variations in the diameters of these gingival elastic fibres existed. In that study, we also determined the relative frequency histograms of the diameters of the gingival elastic fibres and showed for the 25–49 year age group that the distribution of the gingival elastic fibres appeared Gaussian, while for the 50–75 year age group the relative frequency histogram of the diameters did not show a Gaussian distribution and revealed large variations in the diameters measured.

Gingiva is under constant stress owing to mechanical forces and is susceptible to damage from plaque bacteria and by their products, thus resulting in inflammation (5). During periodontal diseases, the infiltration of inflammatory cell subsets (e.g. lymphocytes, macrophages and polymorphonuclear leukocytes) is well documented (6,7).

Inflammatory cells contain enzymes that can degrade gingival extracellular matrix if released during their functional activity or when the cells degenerate or die (8).

Among these inflammatory cells, polymorphonuclear leukocytes contain leukocyte elastase and cathepsin G in their azurophil granules. These two potent elastolytic serine proteinases have been shown to solubilize *in vitro* human lung elastin (9), as well as skin elastic fibres with *ex vivo* elastolytic tests on human skin tissue sections (10). Furthermore, cathepsin G has been shown to develop not only a significant elastin solubilizing activity by itself but may also potentiate the activity of neutrophil elastase (10).

Interestingly, it has been reported that lysozyme is associated with damaged elastic fibres in many tissues and organs and can prevent the proteolytic degradation of elastin by human leukocyte elastase (11–13). Lysozyme has been found in monocytes and macrophages (14), in neutrophils (15) and in glandular cells (16). Owing to the fact that in the inflamed human gingiva elastic fibres are present in large amounts, we suspected that lysozyme originating from inflammatory cells could prevent elastin degradation by binding to the elastin network.

The aim of the present study was to determine the *ex vivo* susceptibility to hydrolysis by human leukocyte elastase and by cathepsin G of gingival elastic fibres from young and aged patients with periodontitis, or patients with gingivitis, and to compare data with those obtained from the gingiva of healthy patients without periodontal disease. Elastolysis was quantified by computerized morphometric analysis of selectively stained elastic fibres (17). The potential role of lysozyme as protector of gingival elastic fibres was also investigated.

Materials and methods

Patient selection and biopsy material

Twenty-eight human gingival biopsy specimens were collected, as follows.

- Eight healthy patients (four males and four females) between 18 and 55 years of age (mean = 26 years), showing clinically healthy gingiva without bleeding and no evidence of vertical bone resorption or periodontal pockets.
- Ten gingivitis patients (six males and four females) between 22 and 79 years of age (mean = 49 years), clinically diagnosed as such with red colour, swelling of the gingival margin and bleeding corresponding to gingival indexes two and three without vertical bone resorption or periodontal pockets.
- Ten periodontitis patients (six males and four females) aged between 19 and 76 years (mean = 54 years), with no more than one pocket ≥ 5 mm and at least one pocket with ≥ 4 mm loss of attachment.

The groups were classified according to the classification system for periodontal diseases and conditions (18) and based on five different clinical and radiographic indexes, namely: gingival index (GI; 19); plaque index (PI; 20); bleeding on probing; pocket depth (PD) with accompanying clinical attachment loss (CAL) measured using a calibrated Michigan probe with William's markings; and radiographic vertical bone loss (peri-apical X-ray). Measurements were carried out by the same investigator.

Tissue was taken from the attached gingiva of teeth extracted for orthodontic or periodontal reasons. Gingival biopsies were obtained under local anaesthesia, avoiding infiltration into the biopsy site and deformation or compression of the samples.

Among the 28 patients, two control, healthy patients, ascertained clinically and histologically, were selected for elastolysis assays with leukocyte elastase alone and cathepsin G alone to calibrate the enzymatic activities of these two serine proteinases separately. Four other control, healthy patients (two males, two females, aged 18–42 years, mean = 24 years), four gingivitis patients (two males, two females, aged 26–45 years, mean = 36 years), four young periodontitis patients (two males, two females, aged between 19 and 44 years, mean = 32 years) and four aged periodontitis patients (two males, two females, aged between 57 and 68 years, mean = 60 years) were also selected for enzymatic trials with the two serine proteinases separately or with a mixture of the two enzymes.

Inclusion and exclusion criteria

The patients included in this study had no oral or major systemic diseases, nor any overt immunological abnormalities; they were not taking any proprietary medications, had a negative history for hypertension, vascular diseases (which may affect extracellular matrix integrity), diabetes mellitus or acute necrotizing ulcerative gingivitis, and were not pregnant nor had any periodontal therapy within the preceding 6 months. Patients were also excluded in situations in which they: (1) had less than 20 teeth; (2) were currently under orthodontic treatment; or (3) were wearing an ill-fitting prosthesis.

This study had the approval of a local ethical committee. All patients gave their written informed consent according to the Declaration of Helsinki.

Morphological investigations

For morphological investigations, tissue samples were fixed with aldehyde and embedded in paraffin according to routine procedures. Serial tissue sections, 7 μm thick, were cut with a manual microtome and stained with haematoxylin and eosin to assess tissue quality. This staining technique revealed the structural integrity of the gingival tissue and the absence or presence of inflammatory infiltrates in gingiva. A set of tissue sections was stained with (+)catechin-fuchsin (17), thus giving a good contrast between the elastic fibres and the background, which is a prerequisite for automated image analysis. These morphological investigations were done for the 28 patients included in this study.

Image analysis

Computerized morphometric analysis of the gingival elastic fibres was carried out as previously described (4,17). Briefly, the analysis was performed on histological sections observed under a Zeiss standard 14 microscope (Carl Zeiss Group, Oberkochen, Germany) equipped with a video camera.

Black and white images generated by the video camera were converted into 256 grey levels using a Sophretec MVM 600 image memory, transferred to a BFM microcomputer (Sophretec, Levallois-Perret, France), and finally analysed using software for mathematical morphology (21,22). The program calculated the area fraction (Aa%) occupied by the elastic fibres; the Aa% represents the surface of elastic fibres (%) as a function of the tissue area analysed.

The evaluation of elastic fibre diameters was performed semi-automatically on a BIOCOM station (BIOCOM, Les Ulis, France) using the Imagenia 3000 program (BIOCOM). Morphometric analysis was done for all patients included in this study.

Enzymatic digestion

Control experiments – Serial tissue sections, 7 μm thick, from two healthy patients were deparaffinized in three toluene baths, then dehydrated in decreasing ethanol solutions (100, 70, 50, 30 and 10%) and, after two baths of distilled water, dropped into a 0.1 M Tris-HCl buffer (pH 8.0). To appreciate the elastolytic activities of leukocyte elastase and cathepsin G, two conditional experiments were carried out.

A set of three gingival tissue sections from the two healthy patients was overlaid with 40 μL of buffer alone (control experiment), a second set of three gingival tissue sections was overlaid with 40 μL of buffer containing leukocyte elastase (100, 500 or 750 ng of elastase) to calibrate the enzymatic activity of leukocyte elastase alone and a third set of three gingival tissue sections was overlaid with 40 μL of buffer containing cathepsin G (10, 50 or 100 ng of cathepsin G) to calibrate the enzymatic activity of cathepsin G alone. The preparations were then incubated in a moist chamber for 12 h at 37°C, rinsed for 15 s with the cold buffer to stop the reaction, dropped in absolute ethanol for 2 min and stained with (+)catechin-fuchsin. The sections were then mounted in Histolaque (Labo Moderne, Paris, France) and covered with glass coverslips before image analysis as described above.

Elastolysis assays with leukocyte elastase alone, cathepsin G alone or a mixture of the two enzymes— Serial tissue sections (three sections per assay, 7 μm thick) from four control patients, four gingivitis patients, four young periodontitis patients and four aged periodontitis patients were treated as follows. Tissue sections were overlaid with 40 μL of 0.1 M Tris-HCl alone or with buffer containing 100 ng of leukocyte elastase or 10 ng of cathepsin G or 100 ng of leukocyte elastase plus 10 ng of cathepsin G. After 12 h at 37°C in a moist chamber, the enzymatic reaction was stopped by placing the sections into cold buffer, and the tissue sections were submitted to automated analysis after (+)cathechin-fuchsin staining as described above to determine the area fraction (Aa%) occupied by the gingival elastic fibres in each experimental condition. The percentage of gingival elastin solubilized by the leukocyte elastase or by the cathepsin G or by the mixture of these two enzymes was calculated using the following relationship:

$$\frac{\text{Aa\%Control} - \text{Aa\%Assay}}{\text{Aa\%Control}}$$

where Aa% Control is the Aa% with buffer alone and Aa% Assay is the Aa% of enzymatically treated sections.

Human leukocyte elastase and human cathepsin G were purchased from Elastin Product Company (EPC, Owensville, MO, USA).

Indirect immunodetection of lysozyme

For indirect immunodetection of lysozyme, tissue sections were deparaffinized, rehydrated and then rinsed in phosphate-buffered saline and treated with 0.3% H_2O_2 for 20 min to inhibit non-specific staining. The sections were incubated with the primary antibody (anti-human lysozyme from Zymed, San Francisco, CA, USA) at 1:20 dilution, followed by biotinylated anti-mouse immunoglobulins, and finally with streptavidin–peroxydase conjugate (Dakopatts, Glostrup, Denmark). Each incubation was carried out for 30 min at room temperature, followed by three baths of 10 min each in phosphate-buffered saline. The peroxydase activity was revealed using 3,3′-diaminobenzidine tetrahydrochloride with hydrogen peroxyde in buffered solution for 15 min (pH 7.6). Non-specific reactivity of the antibodies was checked by omitting the primary antibody or by using an irrelevant isotype-matched primary antibody. Indirect immunodetection of lysozyme was done for all patients included in this study.

Matrix elastic fibres were also identified by indirect immunofluorescence technique with a primary antibody (goat polyclonal anti-human elastin from Tebu, Le Perray en Yvelines, France) at 1:20 dilution, and appropriate secondary fluorescein-conjugated antibody, at 1:50 dilution. Tissue sections were examined with fluorescent light of 280 nm wavelength.

Statistical analysis

Microsoft Excel 2007 (Microsoft Inc., Redmond, WA, USA) and SPSS 11.5 (SPSS Inc., Chicago, IL, USA) were used for statistical analysis. Analysis was carried out using non-parametric tests, and the subject was used as the unit of measurement. Means and standard deviations of all the quantitative variables (e.g. percentage of area fraction) were calculated in the different stages of periodontal disease and in the control group. Global comparisons of the different means were performed using the Kruskal–Wallis non-parametric test (multiple means comparisons in independent unpaired samples). If significant differences were found ($p < 0.05$), *post hoc* two-group comparisons were assessed using the Mann–Whitney *U*-test (non-parametric means comparisons in two independent unpaired samples), and p values < 0.05 were considered to be statistically significant. Within each group, the non-parametric Wilcoxon ranked sign test for paired data was used to compare the percentage of elastolysis induced by each serine proteinase alone and by the mixture of both proteinases together (here we used paired data analysis because serial tissue sections from each sample were used for enzymatic trials to be compared).

Results

Morphological investigation: organization of gingival elastic fibres

In control, healthy patients, after haematoxylin and eosin staining, no inflammatory infiltrates were observed (not shown). After (+)catechin-fuchsin staining, three types of fibres could be distinguished. Oxytalan fibres were located in the upper part of the gingival connective tissue perpendicular to the epithelium and connected with elaunin fibres organized parallel to the epithelium. In the mid-gingiva, well-defined elastic fibres were present (Fig. 1).

In the four gingivitis patients, moderate inflammatory infiltrates were present (not shown). After (+) catechin-fuchsin staining, the gingival elastic network was comparable to that of healthy patients (not shown). In periodontitis patients, more inflammatory infiltrates were noted (not shown), and considerable changes were observed concerning the elastic fibre network. The oxytalan fibres were rarified and shortened, and the elaunin fibres appeared fragmented. The elastic fibres were fragmented and thickened and showed great variations in their diameters (Fig. 2).

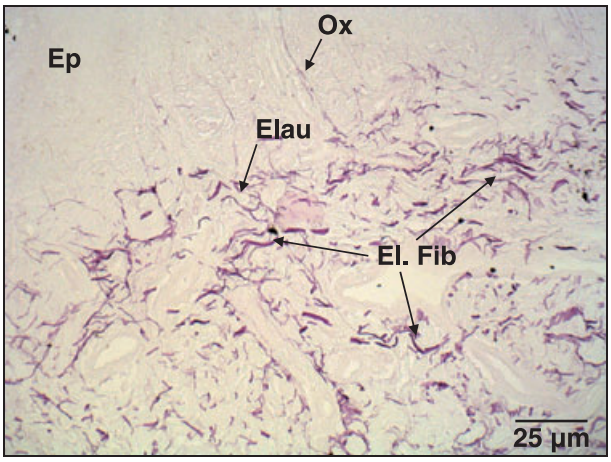

Fig. 1. Histological visualization of the network of gingival elastic fibres in the gingiva of a representative control, healthy donor (aged 23 years). The paraffin-embedded gingival section was stained with (+)catechin-fuchsin. Gingival elastic fibres are well individualized, while the background appears colourless. Gingival pre-elastic fibres were localized at the epithelium–connective tissue interface. Oxytalan fibres were oriented perpendicularly to the epithelium and connected with the elaunin fibres organized parallel to the epithelium. In the mid-gingiva, long and well-defined elastic fibres were present. Abbreviations: Ep, epithelium; Ox, oxytalan fibres; Elau, elaunin fibres; and El. Fib, elastic fibres. Scale bar represents 25 µm.

Morphometric analysis of diameters of gingival elastic fibres in control and periodontitis patients

After staining and image analysis, the gingival fibre diameters were measured for about 100 fibres in control, healthy patients and in patients with periodontitis. The diameters of elastic fibres in the mid-gingiva in control, healthy patients were estimated at

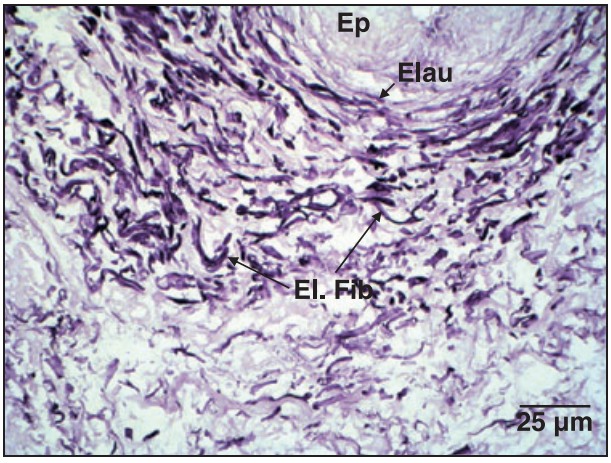

Fig. 2. Histological visualization of the network of gingival elastic fibres in the gingiva of a representative periodontitis patient (aged 65 years). The tissue section was stained with (+)catechin-fuchsin. Pre-elastic fibres were rarified and shortened, and in the mid-gingiva elastic fibres were thickened and fragmented. Abbreviations as in the legend to Fig. 1. Scale bar represents 25 µm.

2.1 ± 0.4 μm and in patients with periodontitis at about 3.6 μm. In control patients, the distribution of gingival elastic fibres appeared Gaussian, while for periodontitis patients the relative frequency histogram of the diameters did not show a Gaussian distribution and revealed large variations in the diameters measured (Fig. 3A,B).

Morphometric analysis of gingival elastic fibres in control, gingivitis and periodontitis patients

The area fraction, Aa%, occupied by gingival elastic fibres evaluated by automated analysis was estimated after staining with (+)catechin-fuschin as 8.8 ± 0.5% for control, healthy patients, 9.2 ± 0.6% for gingivitis patients, 12.3 ± 1% for young periodontitis patients and 14.2 ± 2.8% for aged periodontitis patients.

Degradation of human gingival elastic fibres by human leukocyte elastase or by cathepsin G in control, healthy patients (calibration of the enzymatic activities developed by the two serine proteinases separately) — In control experiments in which tissues sections were incubated only with 0.1 M Tris-HCl buffer, no variation in the intensity of the staining by the polyphenolic dye, (+)catechin-fuchsin, and no qualitative or quantitative variation of area fraction (AA%) determined by automated image analysis were noted when compared with tissue sections submitted only to the staining by (+)catechin-fuchsin.

Only about 20% of elastic fibres were resistant to hydrolysis of human leukocyte elastase for the highest dose of human leukocyte elastase (750 ng); about 40 and 85% were resistant to hydrolysis with 500 and 100 ng of human leukocyte elastase, respectively.

Likewise, the action of human cathepsin G on the human gingiva elastic fibres after incubation of tissue sections with increasing amounts of cathepsin G, estimated by image analysis, showed that about 35% of elastic fibres were resistant to hydrolysis for the highest dose of cathepsin G (100 ng), about 60 and 90% were resistant to hydrolysis with 50 and 10 ng of cathepsin G, respectively.

Comparison of the elastolytic activities of leukocyte elastase and cathepsin G on gingival elastic fibres

When gingival tissue sections from control, healthy patients were overlaid with 100 ng of human leukocyte elastase, about 15% of elastic fibres were solubilized (percentage of residual fibres, 85 ± 6%); with gingival tissue sections from gingivitis patients, about 16% of elastic fibres were solubilized (percentage of residual fibres, 84 ± 5%); with gingival tissue sections from young periodontitis patients, 10% of elastic fibres were solubilized (percentage of residual fibres, 90 ± 5%); and with gingival tissue sections from aged periodontitis patients, about 5% of elastic fibres were solubilized (percentage of residual fibres, 95 ± 2%). The four means were not statistically different (Kruskal–Wallis test, $p > 0.05$; see Fig. 4). Since no statistical differences were found between means of control and gingivitis groups (Mann–Whitney *U*-test, $p > 0.05$), nor between young and aged periodontitis groups, control and gingivitis groups were pooled, as

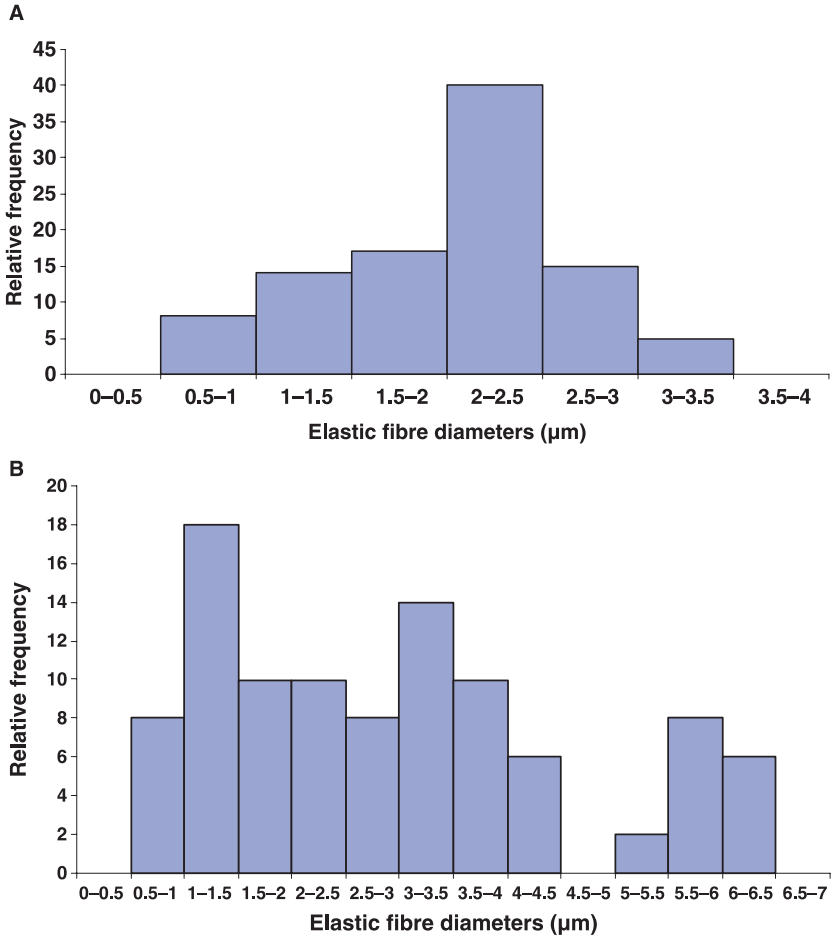

Fig. 3. Distribution of gingival elastic fibre diameters in control and periodontitis patients. (A) Relative frequency histogram of the diameters of gingival elastic fibres in control, healthy patients. The diameter of elastic fibres in the mid-gingiva was estimated as 2.1 ± 0.4 μm. The distribution appears Gaussian. (B) Relative frequency histogram of the diameters of gingival elastic fibres in periodontitis patients. The average diameter of elastic fibres in the mid-gingiva was estimated as about 3.6 μm. The distribution does not show a Gaussian distribution and reveals large variations in the diameters measured.

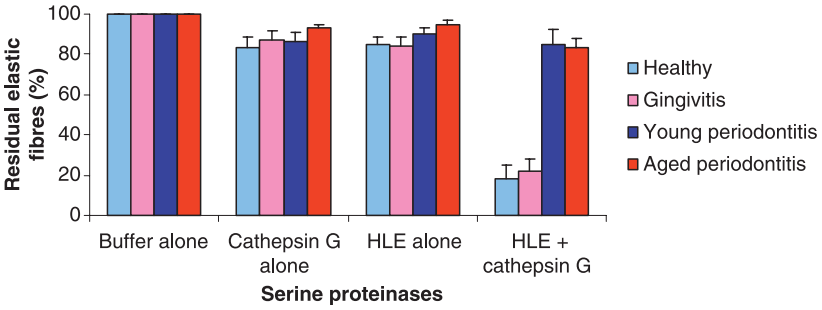

Fig. 4. Percentage of serine proteinase-mediated elastolysis. With 100 ng of human leukocyte elastase (HLE) or 10 ng of cathepsin G alone overlaid on gingival tissue sections from healthy control or gingivitis patients these two serine proteinases have a comparable solubilizing activity. For young periodontitis patients and aged periodontitis patients, HLE and cathepsin G have weak elastin solubilizing activities. When gingival tissue sections were overlaid with 100 ng leukocyte elastase plus 10 ng of cathepsin G, about 80% of elastic fibres were solubilized in control, healthy patients and in gingivitis patients, while only about 16 and 17% of elastic fibres were solubilized in young and aged periodontitis patients, respectively. On the ordinate, 100% is the control value obtained when the specimens were incubated with buffer alone.

were the two periodontitis groups. The decrease in the percentage of residual fibres between the control/gingivitis pooled groups and the periodontitis groups (aged and young) is not statistically significant (Mann–Whitney *U*-test, $p > 0.05$).

When gingival tissue sections were overlaid with cathepsin G (10 ng), about 13% of elastic fibres were solubilized for control patients (percentage of residual elastic fibres, 87 ± 4%), about 17% of elastic fibres were solubilized for gingivitis patients (percentage of residual fibres, 83 ± 5%), about 14% of elastic fibres were solubilized for young periodontitis patients (percentage of residual fibres, 86 ± 3%) and about 7% were solubilized for aged periodontitis patients (percentage of residual fibres, 93 ± 2%). These four means were not statistically different (Kruskal–Wallis test, $p > 0.05$; see Fig. 4). Since no statistical differences were found between means of control and gingivitis groups (Mann–Whitney *U*-test, $p > 0.05$), nor between young and aged periodontitis groups, control and gingivitis groups were pooled, as were the two periodontitis groups. Regarding the cathepsin G elastolysis, no statistically significant variations (Mann–Whitney *U*-test, $p > 0.05$) were found in the percentage of residual elastic fibres between the control/gingivitis pooled group and the periodontitis groups (aged and young).

When gingival tissue sections were overlaid with 40 µL of buffer containing 100 ng of leukocyte elastase and 10 ng of cathepsin G, about 82% of elastic fibres were solubilized in control, healthy patients (percentage of residual fibres, 18 ± 7%), about 78% of elastic fibres were solubilized in gingivitis patients (percentage of residual fibres, 22 ± 6%), 16% of elastic fibres were solubilized in young periodontitis patients (percentage of residual fibres, 84 ± 8%) and 17% of elastic fibres were solubilized in aged periodontitis patients (percentage of residual fibres, 83 ± 4%). These four means were statistically different (Kruskal–Wallis test, $p < 0.001$; see Fig. 4). Since no statistical differences were found between means of control and gingivitis groups (Mann–Whitney *U*-test, $p > 0.05$), nor between young and aged periodontitis groups (Mann–Whitney *U*-test, $p > 0.05$), control and gingivitis groups were pooled, as

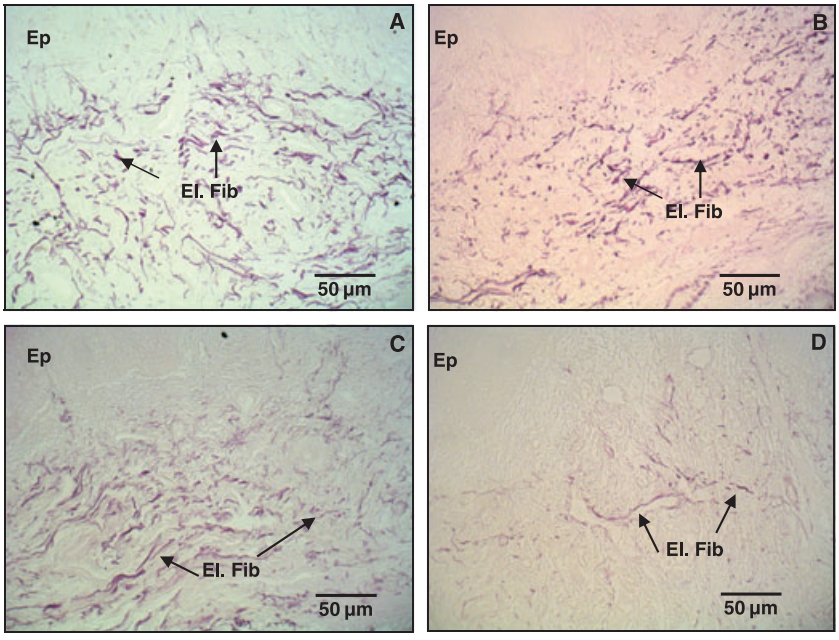

Fig. 5. Comparison of the effects of human leukocyte elastase, cathepsin G and a mixture of the two proteinases on human gingival elastic fibres in a control, healthy donor aged 37 years. (A) Tissue section overlaid with 40 µL of buffer alone (control experiment). (B) Tissue section overlaid with 100 ng of leukocyte elastase in 40 µl of buffer. (C) Tissue section overlaid with 10 ng of cathepsin G in 40 µL of buffer. (D) Tissue section overlaid with 100 ng of leukocyte elastase plus 10 ng of cathepsin G in 40 µL of buffer. When gingival tissue sections were overlaid with leukocyte elastase alone (B) or cathepsin G alone (C), the elastic fibres were degraded by these proteinases, but with the mixture of the two enzymes (D) a major degradation was observed when compared with the control experiment with buffer alone (A). Abbreviations as in the legend to Fig. 1. Scale bar represents 50 µm.

were the two periodontitis groups. Statistically significant variations were found in the percentages of residual elastic fibres between the control/gingivitis pooled groups and the periodontitis groups (aged and young; Mann–Whitney *U*-test, $p < 0.000003$).

The activity of the mixture of the two proteinases was about twofold higher than the sum of activities of the individual enzymes in the control/gingivitis pooled patients, and the difference is statistically significant (mixture vs. cathepsin G alone, Wilcoxon ranked sign test, $p = 0.000001$; and mixture vs. human leukocyte elastase alone, Wilcoxon ranked sign test, $p = 0.000001$). In contrast, no stimulation of enzymatic activity was found for periodontitis patients (mixture vs. cathepsin G alone, Wilcoxon ranked sign test, $p > 0.05$; and mixture vs. HLE alone, Wilcoxon ranked sign test, $p > 0.05$; see Fig. 4).

Figures 5 and 6 show histological sections to illustrate the action of leukocyte elastase, cathepsin G and leukocyte elastase plus cathepsin G on gingival elastic fibres in control, gingivitis and periodontitis patients.

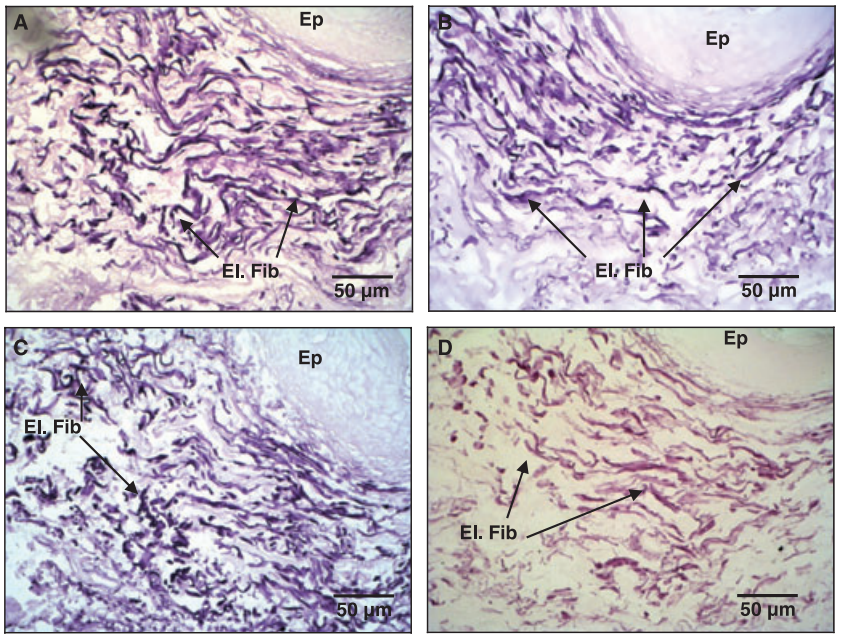

Fig. 6. Comparison of the effects of human leukocyte elastase, cathepsin G and a mixture of the two proteinases on human gingival elastic fibres in a periodontitis patient aged 57 years. (A) Tissue section overlaid with 40 µL of buffer alone (control experiment). (B) Tissue section overlaid with 100 ng of leukocyte elastase in 40 µL of buffer. (C) Tissue section overlaid with 10 ng of cathepsin G in 40 µL of buffer. (D) Tissue section overlaid with 100 ng of leukocyte elastase plus 10 ng of cathepsin G in 40 µL of buffer. When the gingival tissue sections were overlaid with leukocyte elastase alone (B) or with cathepsin G alone (C), very few elastic fibres were sensitive to hydrolysis and, furthermore, with the mixture of the two proteinases (D) few elastic fibres were affected when compared with the control experiment with buffer alone (A). Abbreviations as in the legend to Fig. 1. Scale bar represents 50 µm.

Indirect immunodetection of lysozyme and elastic fibres in gingival tissue sections

Immunofluorescence with polyclonal antibody to elastin showed marked fluorescence labelling on elongated elastic fibres in the gingiva from a periodontitis patient aged 44 years (Fig. 7A). After indirect immunoperoxidase detection of lysozyme in the same patient, a clear peroxidase activity, showing the presence of lysozyme (Fig. 7B), was detected at the periphery of the immunodetected elastic fibres (Fig. 7A).

In gingival tissue sections from control, healthy donors and from gingivitis patients, no peroxidase activity was detected by the indirect immunoperoxidase technique (not shown).

Owing to the autofluorescence property of elastic fibres, the association between lysozyme and gingival elastic fibres was clearly visible when tissue sections were observed under a microscope with fluorescent light and photonic light at the same time. The elastic fibres appeared fluorescent and lysozyme appeared as brown labelling underlying the elastic fibres, thus demonstrating that lysozyme binds to elastic fibres (see Fig. 7C).

No peroxidase activity was detected in control experiments in which the primary antibody was omitted or when irrelevant secondary antibody was used (not shown).

Discussion

The results reported in this study regarding gingival elastic fibres are based on the validity of image analysis for assessing the elastin content of tissues. This was demonstrated by Uitto and co-workers (23), who reported that the area fraction occupied by human dermal elastic fibres was significantly correlated with the desmosine content determined by radioimmunoassay. Furthermore, this technique was considered to be fairly accurate and reproducible. (+)Catechin-fuchsin staining can also be considered to be the stain of choice for the detection and quantitative estimation of tissue elastic fibres. This stain distinguishes the elastic fibres from other macromolecular components and yields a fairly colourless background, which is a prerequisite for automated analysis (17). Furthermore, we demonstrated some years ago, after immunostaining using human monoclonal antibody to elastin and the (+)catechin-fuchsin method for serial human tissue sections, that the elastic fibres revealed were superimposable and that no significant variations were observed after quantification of elastic fibres by automated image analysis between immunostaining and (+) catechin-fuchsin staining (24).

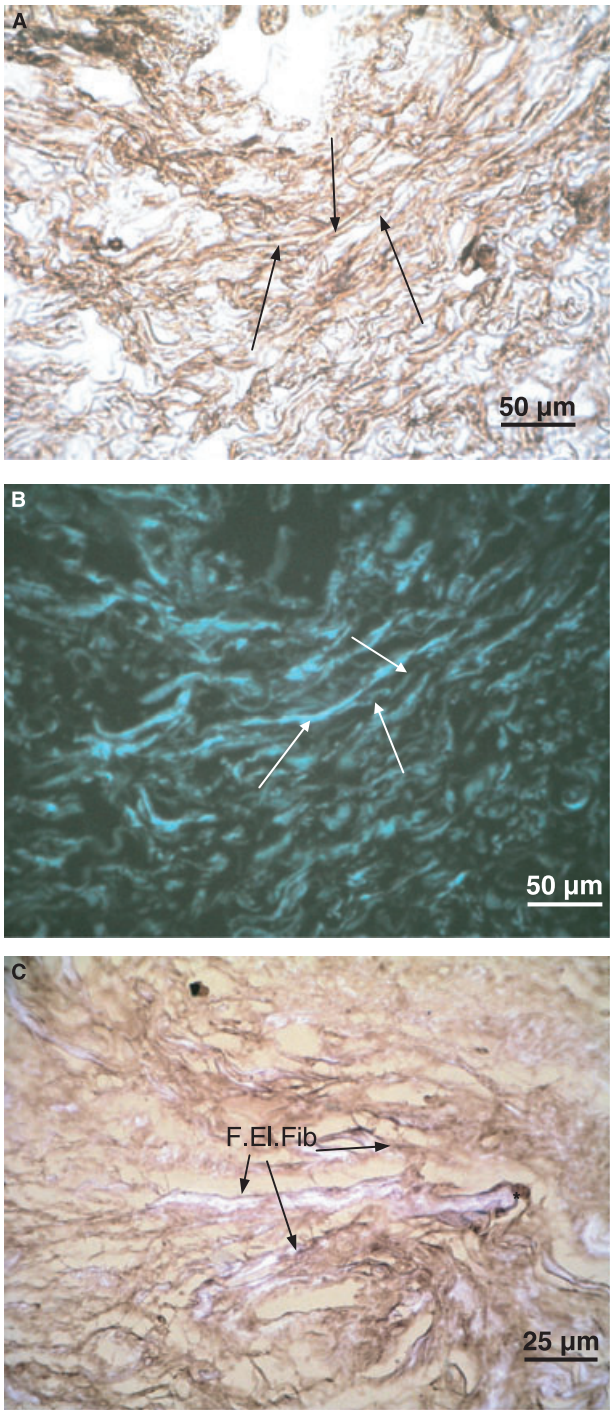

Fig. 7. Indirect immunodetection of lysozyme and elastic fibres in gingiva from a periodontitis patient aged 44 years. (A) Indirect immunofluorescence of gingival elastic fibres. Prominent fluorescence is associated with numerous fibres. White arrows indicate elastic fibres. Scale bar represents 50 µm. (B) Indirect immunoperoxidase detection of lysozyme. The brown pattern of peroxidase activity observed indicates the presence of lysozyme at the periphery of the elastic fibres immunodetected in (A) Black arrows inidcate elastic fibres. Scale bar represents 50 µm. (C) The tissue section was observed under a Zeiss axioplan microscope with fluorescent light and conventional light simultaneously. The elastic fibres appear fluorescent, and the brown labelling resulting from the detection of the peroxidase activity which underlines the elastic fibres shows that lysozyme is bound to the elastic fibres. Abbreviation: F.El.Fib, fluorescent elastic fibres. Asterisk indicates peroxidase activity. Scale bar represents 25 µm.

Our results concerning the evaluation by image analysis of the elastic fibre network in human gingiva showed large variations of the area fraction (Aa%) occupied by these fibres and their diameters from healthy patients to aged periodontitis patients. These results confirmed those we published some years ago, when we determined the morphometric parameters of the human gingival elastic fibres and human skin elastic fibres in relation to age (4). An interesting finding is the large distribution of the elastic fibre diameters shown in advanced pathological gingiva. This intriguing observation raises questions about the molecular assembling of gingival elastic fibres. Since elastin is extremely stable and its turnover is slow, it has been considered that elastin lasts the entire lifespan of the host (23). Therefore, the thickening of gingival elastic fibres cannot be attributed only to continuous deposition of elastin. Gingiva is submitted to several inflammatory episodes and, as reported by numerous authors, during periodontitis the inflammation becomes uncontrolled, leading to the destruction of soft tissue and bone (25,26).

Among inflammatory cells present in diseased gingiva, polymorphonuclear leukocytes contain two serine proteinases, namely human leukocyte elastase and cathepsin G, with elastolytic activities (27), so we suspected that thick elastic fibres present in the gingiva of periodontitis patients could be resistant to both proteinases. With *ex vivo* experiments on control and gingivitis patients, we demonstrated that cathepsin G and leukocyte elastase have a significant and comparable elastin solubilizing activity on elastic fibres; these results being consistent with those we reported on the elastolytic activities of leukocyte elastase and cathepsin G on human dermal elastic fibres (10).

Furthermore, with gingival tissue sections from control and gingivitis patients, we found that the elastolytic activity of the mixture of the two proteinases was about twofold higher than the sum of activities of the individual enzymes. This stimulation factor of two is of the same order of magnitude

(×1.9) as that observed with human skin sections (10).

With gingival tissue sections from periodontitis patients, leukocyte elastase and cathepsin G had weak elastin solubilizing activities. Furthermore, no stimulation of enzymatic activity was evidenced with these patients with the mixture of the two serine proteinases compared with control, healthy and gingivitis patients. These results suggest that in periodontitis, gingival elastic fibres were appreciably resistant to serine proteinases.

By indirect immunodetection, we demonstrated the presence of lysozyme at the periphery of gingival elastic fibres in young and aged periodontitis patients and its absence in gingiva from healthy or gingivitis patients. Thus, the increase in the diameters of elastic fibres in patients with periodontitis could be due in part to deposition of lysozyme on elastic fibres. Furthermore, the deposition is not related to the age of the patients but to the pathological status of the gingiva.

Lysozyme has been shown to be associated with damaged elastic fibres in many tissues and organs, such as lung (12,28), skin (29) and vessels (30). Lysozyme has also been shown to prevent the proteolytic degradation of elastin by human leukocyte elastase, pancreatic elastase, thermolysin and *Pseudomonas* elastase (11). We have recently found, in *ex vivo* experiments with human actinic skin tissue sections, that lysozyme binds to elastin and limits elastin degradation by human leukocyte elastase (13). Therefore, our results with gingiva from periodontitis patients strongly suggest that the limited degradation of gingival elastic fibres that we found in *ex vivo* experiments with leukocyte elastase and cathepsin G is due to lysozyme deposition on the elastic fibres. As reported by Park *et al.* (11), lysozyme does not function by directly inhibiting the activity of the proteinase but, by binding to elastin, it prevents the protease from interacting with the elastin substrate in ways that normally favour proteolysis. In the present study, we demonstrated, with indirect immunodetection, that lysozyme was associated with gingival elastic fibres in periodontitis patients, while control, healthy patients were lacking in lysozyme. Incubation of gingival sections with leukocyte elastase, cathepsin G or a mixture of the two enzymes resulted in an important decrease in the area fraction occupied by elastic fibres in control patients (more than 80%), while only a slight decrease was found in periodontitis patients ($<20\%$).

Lysozyme was reported by Jacquot *et al.* (31) to be present at a high concentration in the airway secretion from patients with chronic pulmonary disease, and was considered to be an anti-bacterial agent involved in the pulmonary defence mechanism. These authors showed that human airway lysozyme was cleaved by *Pseudomonas aeruginosa* elastase but not by human leukocyte elastase, and that the cleavage of lysozyme by *Pseudomonas* elastase was accompanied by parallel losses of its bacteriolytic activity and its immunoreactive property. Thus, we hypothesize that the presence of lysozyme in human pathological gingiva could be a host-defence mechanism against micro-organisms (the exact clinical impact of lysozyme in pathological gingiva should be elucidated). By preventing degradation of elastin, lysozyme can be considered a natural protector of elastic fibres.

Acknowledgements

This study was funded by St Joseph and Paris Descartes Universities.

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