Collagenase in the Human Periodontal Ligament

Paul Christner†

Collagenase activity was demonstrated by direct incubation to be present in human periodontal ligament. This activity was found in only one of two populations of ligament, i.e., those ligaments taken from teeth in which their attachment site was at least 2.5 mm apical to the cementoenamel junction. The collagenase was demonstrated to be of host origin because its degraded collagen into 3/4 and 1/4 chain fragments characteristic of mammalian collagenases. The enzyme was shown to be inhibited in the presence of EDTA and to have a pH optimum of 7.5.

Over 50% of the soft connective tissue of the periodontium has been found to be collagenous and anhydrous cementum of human teeth has been reported to be over 90% collagenous. The destruction of collagen in the periodontal ligament is reported to be associated with periodontal disease and collagenase (bacterial or mammalian) is the only enzyme known to degrade native collagen at neutral pH. The mechanism of bacterial collagenase is quite different from that of mammalian or enase. Bacterial collagenases, the most common one having been isolated from Clostridial species, degrade native triple helical collagen into small molecular peptides. The site of cleavage is at the amino side of most glycine residues. Mammalian collagenase attacks the native collagen helix at only one point, 9% of the distance from the amino terminal end, producing 3/4 and 1/4 triple helical pieces. These pieces are not stable as triple helices at normal body temperature, 37°C and, therefore, dissociate. These dissociated collagenous peptides are then susceptible to many general proteases.

Many workers have published reports on mammalian collagenases. These collagenases are all similar in action and have been found in tissues associated with chronic inflammatory disease such as chronic rheumatoid arthritis where breakdown of collagen occurs. They have been reported associated also with invasive tumors and with keratocysts of the jaw. Further, collagenases have been found in normal tissue such as skin, gingiva, and alveolar bone. The origin of these collagenases has not been identified in all cases; however, fibroblasts, polymorphonuclear leukocytes, and macrophages have been shown to contain collagenase. It has been suggested that the collagenase found in certain white blood cells in the areas of inflammation may be a causative agent in the pathogenesis of a chronic inflammatory disease such as rheumatoid arthritis.

Periodontal disease is usually a chronic inflammatory disease. Several laboratories have reported low levels of collagenolytic activity associated with the human gingiva. Generally, the reports have shown a positive correlation between enzyme activity and the degree of inflammation. In one report, however, there was found lower levels of collagenase activity in the inflamed gingiva than in the controls.

Evidence is reported here which suggests that active collagenase is present in human periodontal ligament. This collagenase activity is not found in all specimens examined, but confined to ligaments derived from teeth where the periodontal ligament attachment site to the tooth is significantly apical to the level of the cementoenamel junction.

MATERIALS AND METHODS

Ligaments. Periodontal ligaments were obtained from patients undergoing extractions at the Oral Surgery Clinic, University of Pennsylvania Dental School. The extracted teeth were immediately chilled and subsequently frozen at -20°C. After collection of a suitable number, the teeth were separated into two groups: (I) those with loss of cervical portion of the ligament and (II) those with normally attached ligament. Teeth were determined to have apical movement of the ligament if the ligament were attached at all points more than 2.5 mm apical to the cementoenamel junction. Teeth were determined to have normally attached ligament if the attachment site were not recessed at any point on the tooth more than 0.5 mm apical to the cementoenamel junction. Teeth which did not fit into either of these groups were not used. After separation of the teeth, the ligaments were scraped into cold, distilled water and lyophilized.

Enzymes. Trypsin and bacterial collagenase from Cl. histolyticum were of the highest purity available from...
Worthington Biochemical Co., Freehold, N.J. The collagenase was further purified by DEAE column chromatography and isoelectric focusing according to Myoshi and Rosenbloom, who showed that the final preparation to be free of nonspecific protease activity. Mammalian collagenase isolated and purified from human skin was given by Dr. John Jeffreys.

Isotopes. Uniformly labeled 14C and 2.3 3H proline were obtained from New England Nuclear, Boston, Mass.

Chemicals. Soybean trypsin inhibitor was purchased from Sigma Chemical Corp., St. Louis, MO. Other chemicals were of reagent grade.

Ligament Incubations. Incubations were carried out in 1 ml of 0.05 M CaCl2, 0.2 M Tris-HCl pH 7.7 unless otherwise noted in the text. All incubations were performed in duplicate using at least three different batches of ligaments.

Sodium Dodecyl Sulfate (SDS) Polycrylamide Gel Electrophoresis. Five percent SDS polycrylamide disc gels were prepared and run essentially according to Weber and Osborn. Samples for electrophoresis were boiled for 2 min in 1% SDS, 0.1% β-mercaptoethanol in 0.05 M PO4 buffer pH 7.0, and then applied to the polycrylamide gels. After electrophoresis, gels containing radiolabeled material were cut into 1.5 mm slices, dissolved with 30% H2O2 at 55°C overnight, suspended in New England Nuclear Formula 950 A and counted in a Packard liquid scintillation counter. Gels containing microgram amounts of proteins were stained with 0.25% coomassie blue for 30 minutes and then de-stained in methanol-acetic acid-H2O (50:75:875) overnight. Densitometer tracings of these gels were made at 620 nm with a Joyce Loebel Spectrophotometer.

Preparation of a Collagen 3/4 a Chain Peptide. 3H and 32C collagen were prepared by incubating chick tendon fibroblasts with 3H or 32C proline as previously described. The medium from each incubation was taken and digested with one mg/ml of pepsin for 16 hours at 4°C at a pH of 4. The resultant digests were dialyzed against 0.2 M Tris pH 7.7 with 0.05 M CaCl2. The 3H proline labeled retentate was used as an 3H proline labeled collagen standard. The 32C proline labeled retentate was further digested with 2 µg/ml of mammalian skin collagenase at 37°C for 2 hours and used as a 32C collagen α chain standard.

DETAILS OF STUDY AND RESULTS

The first objective of this work was to determine if there was any collagenase present in the periodontal ligament. To accomplish this, pooled ligaments (ligaments not yet separated into Group I or II) which had been obtained from human teeth were incubated as follows: One milligram of pooled ligament was suspended in 1 ml of 0.05 M CaCl2, 0.2 M Tris pH 7.7 and shaken at 37°C for up to 18 hours. For incubation times of more than 6 hours one drop of toluene was added to prevent bacterial growth. The incubations were stopped by making the mixture 1.0% in sodium dodecyl sulphate (SDS) and 0.1% in β-mercaptoethanol and boiling for 2 minutes. The samples were then electrophoresed on SDS polycrylamide gels. The results shown in Figure 1 indicated that there was significant degradation of all the peaks by 6 hours and almost complete digestion by 18 hours. Type I collagen standards were also electrophoresed on separate gels; the positions to which these collagens migrated were superimposed on the ligament densitometer tracings (see arrows). It can be seen that the collagen migrated to the same position as the higher molecular weight ligament peaks. This suggests that collagenous proteins were being degraded in the ligament. It was next decided to determine if this degradation was found in all ligament types or only in a selected population.

Because periodontal disease is almost always associated with loss of ligament in association with bone loss, this degradation was divided into two groups: those in...
which the attachment site had receded apically (Group I) and those which were normally attached to the tooth at the cementoenamel junction (Group II). One milliliter of these two classes of ligaments was then incubated 1, 2, 4, 6 and 24 hours at 37°C in 0.05 M CaCl₂, 0.2 M Tris pH 7.7. Following the incubation, SDS solubilized material was electrophoresed on 5% polyacrylamide gels. The results shown in Figure 2 established that the SDS solubilized proteins from the normally attached ligament (Group II) zero time incubation were primarily proteins which co-migrated with the β, α1, and α2 chains of collagen (arrows on Fig. 2 again show that migration of collagen standards). The apically moved ligament (Group I) zero time incubation showed the majority of its protein to be smaller than a chain size. However, this ligament also had significant amounts of protein which migrated in the position of β, α1, and α2 chains. When these ligaments were incubated for 2 to 24 hours the SDS soluble protein of the apically recessed ligament completely degraded within 2 hours, whereas the SDS soluble protein from the normally attached ligament was not destroyed in the same amount of time (Fig. 2). In fact no degradation of Group II ligament could be demonstrated even when the incubation was carried out for 24 hours (not shown). This digestion of only Group I ligament suggested that an active collagenase was associated only with apically positioned ligament specimens.

Because the normally attached ligament when incubated for up to 24 hours still showed protein peaks in the position of β and α chains, it was possible to use this ligament as a control to determine which protein peaks on the SDS gels were susceptible to a known collagenase. Therefore, the normally attached ligament was incubated with isoelectrically purified bacterial collagenase. Yoshiba and Rosenbloom have shown this preparation to be free of nonspecific proteases and to specifically attack collagen. The results of this incubation are shown in Figure 3. It was found that the peaks in the β and α chain positions were susceptible to collagenase as well as the higher molecular weight peaks (presumably γ chains or higher molecular weight aggregates). In addition, the two smaller molecular weight specimens labeled A1 and A2 (presumably α1 and α2-β chains) were degraded by the purified bacterial collagenase. These results taken with those in Figures 1 and 2 were very suggestive that there was an enzyme found only in apically recessed (Group I) ligament which degraded collagen. To further demonstrate this point it was decided to use an exogenous collagenous substrate and determine if it could be degraded.

Therefore, embryonic chick tendon fibroblasts were prepared. They have been shown to synthesize large amounts of collagen. The cells were labeled with H proline and the media was subsequently digested with peptic, an enzyme which destroys most other proteins except collagen. This procedure has been shown to produce a homogeneous labeled collagenous substrate consisting of triple helical native α chains. This substrate was then used to test for the presence of collagenase in the Group I and II ligaments. One milligram of normally attached or apically positioned ligament was incubated with 10,000 cpm's of labeled H collagen prepared as described in Materials and Methods. The incubation was carried out at 22°C overnight. The low temperature was chosen because the products of digestion of mammalian collagenase ⅓ and ⅔ α chains would remain triple helical at this temperature and be resistant to degradation by any other general proteases present in Group I or II ligament. It, therefore, would be possible to recover these ⅓ and ⅔ pieces and demonstrate their existence on SDS gels. Therefore after the overnight incubation, digestion was terminated by the addition of SDS and β-mercaptoethanol. The samples were then electrophoresed on 5% SDS polyacrylamide gels (Fig. 4). It can be seen that the labeled chick tendon collagen after being mixed with
normally attached ligament still electrophoresed as whole ω1 and ω2 chains. However, the 3H collagen when mixed with apically positioned ligament and electrophoresed, showed significant conversion from whole ω1 and ω2 chains to two smaller peptides which electrophoresed in a position indicative of ω1 and ω2 ¾ pieces (the digestion products of mammalian collagenase).

It was next of interest to verify the identity of the new peaks seen in Figure 4 produced from the incubation of 3H proline labeled collagen with Group I ligament. Therefore, 14C proline labeled collagen was prepared essentially as 3H proline labeled collagen described above. The 14C collagen was then digested with 2 µg of purified human skin collagenase at 37°C for 4 hours. Conditions which have been shown to completely degrade the triple helical collagen molecule into 1/4, 1/4 fragments. This collagenase is a well characterized enzyme which has been shown to be a true mammalian collagenase. The ¾ and 1/4 14C labeled collagenous pieces were then used as standards to determine if the 3H labeled collagenous digestion products resulting from the digestion with Group I ligament were indeed truly 1/4 ω1 and ω2 chains. Ten thousand cpm of the 3H and 14C labeled collagen digestion products were mixed and then electrophoresed on SDS gels and the results are shown in Figure 5. It can be seen that the 3H proline ¾ ω1 and ω2 bands co-migrated with the 14C material digested with purified skin collagenase. This indicated that apically displaced ligament contained an active mammalian collagenase. This collagenase is capable of digesting exogenous as well as endogenous collagen.

It was decided to further characterize the collagenase found in Group I ligament to determine if it had additional similarities to other reported mammalian collagenases. Therefore, several protease inhibitors were tested in an incubation system similar to that described in the legend to Figure 4. N-ethyl maleamide, an inhibitor of thiol proteases, EDTA (ethylenediaminetetraacetate), an inhibitor of metal proteases and phenylmethylsulfonylflouride (PMSF), an inhibitor of serine proteases, were each added to an incubation mixture of Group I ligament and 3H labeled collagen. The results showed that degradation was prevented only when 0.05 M EDTA was added. Addition of excess Ca++ (0.1 M) overcame this loss of collagenolytic activity. The results are shown in Figures 6a, b, and c (For clarity of presentation the results of the incubation in the presence of PMSF are not shown). Other mammalian collagenases have been shown to be Ca++ requiring metal proteases and these results agree with published reports for other mammalian collagenases.

The optimum pH at which Group I collagenase degrades collagen was also determined. Four milligrams of ligament were mixed with 20,000 cpm's of labeled collagen and incubated at a pH ranging from 5 to 10 for 6 hours at 22°C. The reactions were terminated by the addition of SDS, and β-mercaptoethanol and the samples then electrophoresed on 5% SDS polyacrylam-
ide gels. The relative activity of the collagenase was determined by summing the counts under the $\% a_1$ and $\% a_2$ peaks and expressing them as a fraction of the total under the whole $a$ chain and $\% a$ chain peaks.

The fraction degraded was thus determined for each pH and is graphed in Figure 7. The optimum pH of 7.5 was in agreement with the pH optimum reported for other mammalian collagensases. In addition to characterizing the Group I collagenase, it was decided to determine more completely whether there was any collagenase activity in Group II ligament.

Many authors have reported the enhancement of collagenase activity after a sample was treated with trypsin. It has been suggested that this treatment either converts an inactive proenzyme to an active enzyme by cleavage of part of the molecule similar to other zymogen conversions or that the trypsin digests an inhibitor which is noncovalently bound to the collagenase. It was reasoned that there might be an inactive (latent) collagenase in Group II ligament which could be made active with trypsin treatment. Therefore, Group II ligament was preincubated with several concentrations of trypsin at 37°C for 60 minutes. $^3$H labeled embryonic chick tendon collagen was added with soybean trypsin inhibitor to the ligament and the incubation was continued at 22°C for 18 hours. The resultant digests were electrophoresed on SDS gels. The results (Fig. 8) showed there was no significant cleavage of the labeled collagen even after trypsin activation.

Figure 7. pH curve of Group II ligament collagenase. Four mg of Group II ligament was incubated with 20,000 cpm of $^3$H-proline labeled collagen for 6 hours at 22°C. Na acetate buffer was used for the incubations at pH 6 or below. Incubation buffer was used for the incubations at pH 7.0 to 9.0, and NaOH-glycine was used for the incubations performed at a pH above 9.0. The incubations were terminated as described in the legend to Figure 1 and electrophoresed on 3% polyacrylamide gels. The method of calculating the percentage hydrolyzed was described in the text.

Figure 8. Activation of Group II ligament collagenase. One mg of Group II ligament was incubated in 1 ml of incubation buffer with 1, 5, 10, 25, 50, or 100 $\mu$g of trypsin. The incubation was carried out for 60 minutes at 37°C. A 2-fold molar excess of soybean trypsin inhibitor was then added and the incubation continued at 37°C overnight with the addition of 10,000 cpm of $^3$H-proline labeled collagen. Each incubation was terminated as described in the legend to Figure 1 and electrophoresed on 3% SDS polyacrylamide gels. Only the results of the activation with 50 $\mu$g of trypsin are shown for clarity; however, the results were similar for all the samples.
DISCUSSION

Many have reported the presence of collagenase in the human gingiva. The studies generally found a positive correlation between the amount of collagenase activity and the severity of inflammation. Normal gingiva usually has been shown to have low levels of active collagenase. Fullmer et al. have reported the purification of a host collagenase isolated from human gingival tissue.

The work presented here is the first report of a collagenase found in the human periodontal ligament. The collagenase was determined to be of host origin producing the characteristic 23-kDa cleavage products indicative of a mammalian collagenase. It was shown to be a metal requiring protease as are other collagenases and to have a pH optimum of 7.5 which agrees with the published literature. The presence of the collagenase was not found in all periodontal ligaments.

The ligaments in the study were divided into two groups: apically positioned, Group I, and normally attached, Group II. It was assumed that the Group I ligaments would more closely represent ligaments from patients with past or present periodontal disease and that Group II would more closely represent ligaments from patients without periodontal disease. This assumption was based on the fact that recession of the periodontal ligament accompanies periodontal disease. It was felt that the most likely cause of the large apical movement found in the Group I ligaments was periodontal disease. However, the author is aware that other factors also can cause periodontal ligament loss and it is therefore not possible at this point to know whether the blood flow is completely accurate. In any case, it is interesting that collagenase was found only in apically positioned periodontal ligament.

The method used to demonstrate the collagenase activity in this paper was different than that used by others. In most mammalian systems, direct examination of the tissue failed to show any collagenase activity and the tissues had to be maintained in culture which allowed the collagenase to be isolated from the culture fluid. One inference from this data of others was that in normal and even in inflamed tissue there was enough inhibitor present to prevent the expression of the host collagenase activity. This hypothesis may explain why no collagenase activity could be demonstrated in Group II ligament even with trypsin activation (Fig. 8) although collagenous peptides which migrated in the position of a1 and a2 \( \pi \) peptides were found (Fig. 3). An endogenous tissue inhibitor may prevent expression of any Group II ligament collagenase on direct tissue incubation. The reason for the large amount of collagenase activity in Group I ligament may be due to the destruction of an inhibitor collagenase balance.

In periodontal disease bacterial plaque may produce many proteolytic enzymes and other toxic substances. These toxic substances cause white blood cells to migrate into the inflamed area. Polymorphonuclear leukocytes and macrophages have been shown to infiltrate the ligament. These white blood cells also contain many proteolytic enzymes. Fibroblasts in the affected ligament have been shown to undergo morphologic changes and to ingest collagen. Any of these cell sources or plaque could produce enough proteases to destroy an inhibitor of collagenase. Alternatively, the above mentioned host cells could respond to the plaque and produce much more collagenase in the ligament either by migrating there (white blood cells) or by increasing their rate of biosynthesis and secretion.

Experiments are in progress to identify the cells producing collagenase in the periodontal ligament and gingiva and to measure their rate of synthesis of this protein. The mechanism of activation of ligament collagenase will also be studied.

ACKNOWLEDGEMENT

The author wishes to thank Miss Denise Damato for excellent technical assistance.

REFERENCES

Collagenase in the Human Periodontal Ligament


Send reprint requests to: Dr. Paul J. Christner, Department of Histology, School of Dental Medicine, and Center for Oral Health Research, University of Pennsylvania, Philadelphia, PA 19104.