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Collagen breakdown by gingival collagenase and elastase

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Summary. The granule fraction of human polymorphonuclear leucocytes (PMNs), the concentrated product of gingival washing from 2 human volunteers and the culture fluid of samples of human gingiva were incubated with neutral salt soluble collagen from rat skin and the patterns of collagen degradation were studied by SDS polyacrylamide gel electrophoresis. Collagenase from human gingiva cleaved the collagen molecules in a fashion similar to that of the PMN granule fraction. Collagen was also attacked by elastase from human PMNs and, to a lesser extent, by elastase from the gingival washings.

Inflammation of the gingiva is accompanied by an increased migration of polymorphonuclear leucocytes (PMNs) toward the oral cavity through the gingival sulcus[3-5]. These cells discharge their endogenous enzymes in the gingival environment, either by phagocytosis or upon death[6]. Previous work from our laboratory has shown that inflamed gingivae contain more free lysosomal enzymes[7]. During experimental gingivitis, obtained by suppression of toothbrushing in human volunteers, free collagenase and elastase were found to increase in the washings obtained from marginal gingiva[8].

The aim of the present investigation was to clarify further the mode of action of gingival collagenase and elastase upon collagen molecules by using the technique recently described by Murphy et al.[9].

Materials and methods. The granule fraction of human PMNs was prepared by dextran sedimentation and differential centrifugation from 400 ml of heparinized human blood[10,11]. The granules were suspended in 2 ml of 0.05 M Tris-HCl buffer, pH 7.6, containing 0.2 M NaCl and 5 mM $CaCl_2$. They were frozen and thawed 10 times, centrifuged at 30,000 × g for 40 min and the supernatant used as a crude enzyme solution.

Gingival washings were collected from 2 male subjects, 25 and 34 years old and with no visible clinical signs of inflammation, by circulating 5 ml of physiological saline for 15 min along the buccal and palatal marginal regions, using acrylic individual appliances connected to a peristaltic pump[12]. After centrifugation of the product of washing at 200 × g for 15 min, the supernatant was concentrated 40 times by dialysis under vacuum for several hours[10].

Samples of marginal gingiva were obtained from patients during periodontal therapy. After being cut into cubes of approximately 1 mm^3, the fragments were cultured in Eagle MEM medium[13] supplemented with 100 units/ml of penicillin G.potassium and 100 µg/ml of dihydrostreptomycin sulfate for up to 10 days in an atmosphere of 5% CO_2 and 95% O_2, with daily changes of the medium. Collagenase was assayed in the medium after ammonium sulfate fractionation[14].

Neutral salt-soluble collagen from rat skin[8-15] was incubated at 25 °C for 6–20 h with samples of the 3 preparations, i.e. granule fraction of PMNs, concentrated gingival washing and culture fluid of gingiva. The reaction mixture consisted of 50 µl of the collagen solution, 50 µl of the enzyme solution and 50 µl of 0.05 M Tris-HCl buffer, pH 7.6, containing 0.2 M NaCl and 5 mM $CaCl_2$. In some instances, the enzymes were inhibited by adding either

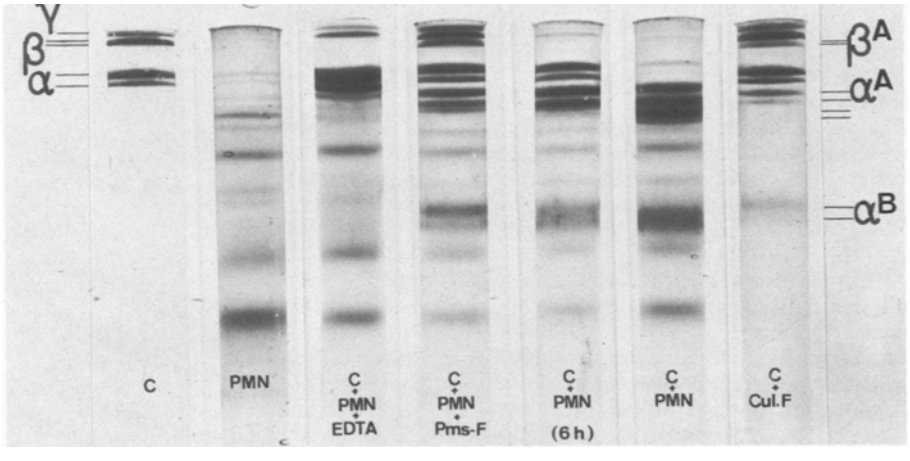

Fig. 1. SDS-polyacrylamide gel electrophoresis of collagen (C) digestion products. Aliquots of PMN granule fractions, alone (C+PMN) or in the presence of EDTA (C+PMN+EDTA) or Pms-F Pms-F (C+PMN+Pms-F), and culture fluid of human gingiva (C+Cul F) were incubated with rat skin collagen, as described in 'materials and methods'. The incubation time was 20 h, except for 1 of the assays (6 h). Native collagen (C) and granule fraction of PMN (PMN) in the absence of collagen are included as references for intact γ-, β- and α-chains of collagen and for bands of foreign proteins.

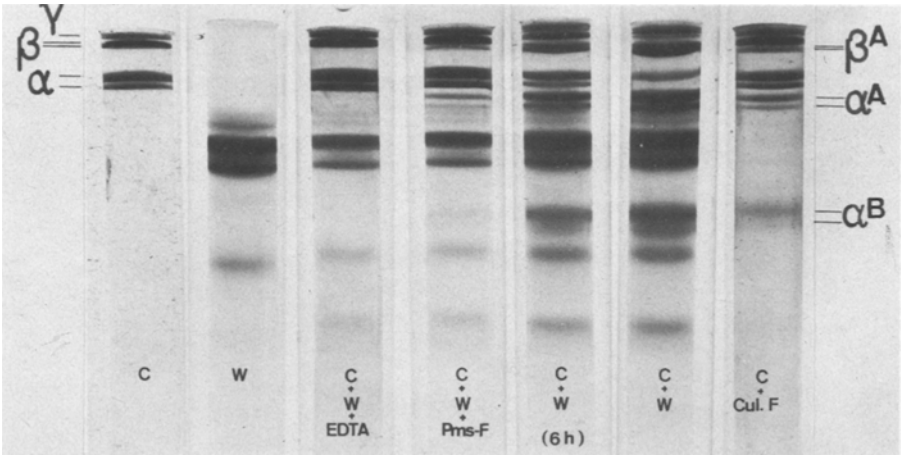

Fig. 2. SDS-polyacrylamide gel electrophoresis of collagen digestion products. Aliquots of concentrated gingival washings, alone (C+W) or in the presence of EDTA (C+W+EDTA) or Pms-F (C+W+Pms-F) and culture fluid of human gingiva (C+Cul.F) were incubated with rat skin collagen, as described in the 'material and methods'. The incubation time was 20 h, except for 1 of the assays (6 h). Native collagen (C) and concentrated gingival washing (W) in the absence of collagen are included as references for intact γ-, β- and α-chains of collagen and for bands of foreign proteins.

50 µl of 80 mM disodium ethylenediaminetetraacetic acid (EDTA)[8] or 50 µl of 8 mM phenylmethanesulphonylfluoride (Pms-F)[14] to the reaction mixtures instead of the Tris-HCl buffer. The mixture of 150 µl was then made up to a total volume of 200 µl with 0.05 M Tris-HCl buffer. After incubation, 100 µl of 0.1 M sodium phosphate buffer, pH 7.2, containing 8 M urea together with 2% mercaptoethanol[17] were added to 100 µl aliquots of the reaction mixture. After heating at 100 °C for 3 min, 20 µl of 0.05% malachite green and 20 µl of glycerol were added. A 50 µl aliquot of the mixture was then subjected to electrophoresis in 7% polyacrylamide gel as described by Kowashi et al.[8].

Results. When collagen was incubated with either PMN granule extracts or culture fluids of human gingiva, the ¾ (α^A) fragments of the collagen molecule and the ¼ fragments (α^B), which are typical for mammalian collagenase action, were found. These breakdown products were not formed in the presence of EDTA, which is an inhibitor of collagenase, a metallo-enzyme. The γ- and β-bands, representing trimers and dimers of the α-chains, had already disappeared after an incubation time of 6 h with the PMN granule extract, confirming the presence of elastase. After 20 h, a weak α-band and corresponding α^A- and α^B-fragments were formed, which is an indication of collagenase action. When inhibiting collagenase by EDTA, the γ- and β-bands were weakened, whereas the α-band was strongly increased, which is again evidence of elastase action. γ- and β-bands and their ¾ fragments, however, are visible upon inhibition of elastase with Pms-F.

The patterns of collagen degradation by concentrated human gingival washings are presented in figure 2: they are similar to those obtained with the granule extracts and show the presence in the washings of active collagenase. However, the persistence of the γ- and β-bands, even after long incubation times, and the modest increase of the α-band when inhibiting collagenase by EDTA, indicate a low concentration of free elastase in our gingival washings.

Discussion. The electrophoretic patterns presented confirm the presence of mammalian collagenase in the gingival sulcus and in the culture fluid of human gingiva. This enzyme cleaved collagen molecules into typical ¾ and ¼ fragments, similarly to the collagenase from the granules of PMNs.

As for elastase, the pattern of collagenolysis obtained with the granular fraction of human PMNs confirmed that the enzyme is responsible for the conversion of γ- and β- to α-chains, probably by attacking the non-helical region of the collagen molecules, such as described by Starkey and Barrett with porcine articular cartilage[21]. Its activity was found to be very low in the washings of sound gingivae, which seems to confirm that the enzyme is probably bound to physiological inhibitors in healthy gingival sulci[8].

In contrast to collagenase, no elastase activity on collagen could be shown in the culture fluid of gingiva; it is probable that the main source of collagenase from the gingival explants are the fibroblasts[22], whereas these cells do not contain elastase[23]. The presence of physiological inhibitors of elastase in the culture fluid should also be considered.

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