Collagenase in Sjögren's syndrome

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Abstract

Objective—To study collagenase production in labial salivary glands in patients with Sjögren's syndrome (SS).

Methods—Collagenases were localised in labial salivary glands by immunohistochemistry. Collagenase activity against triple helical type I collagen monomers in stimulated saliva was measured using sodium dodecyl sulphate polyacrylamide gel electrophoresis and laser densitometry; tissue inhibitor metalloproteinase (TIMP) was measured by enzyme linked immunosorbent assay.

Results—Cells containing collagenase of matrix metalloproteinase (MMP)-1 type were more frequent and more intensely staining in SS than in healthy glands. Only SS saliva contained functional enzyme ($11.7 (6.8) \times 10^{-6}$ IU/l). Cells containing MMP-8 type neutrophil collagenase were not found in situ, which was in accordance with sialochemical findings/doxycycline inhibition studies. TIMP was found in both SS and normal saliva.

Conclusions—Fibroblast, but not neutrophil type, collagenase is synthesised, secreted, and subsequently activated, but is not inhibited by TIMP in labial salivary glands or saliva in SS. Collagenase may destroy glandular and salivary duct tissue and perturb factors influencing the morphogenetic extracellular matrix.

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Sjögren's syndrome (SS), an autoimmune disease of unknown aetiology, is characterised by keratoconjunctivitis sicca and xerostomia as a result of decreased lachrymal and salivary secretion caused by destruction of the glands by an as yet unknown mechanism.¹ The main extracellular structural proteins in the lachrymal and salivary glands are type I and III collagen, which are highly susceptible to degradation by specific interstitial collagenases from fibroblasts, matrix metalloproteinase-1 (MMP-1), and granulocytes (MMP-8).^{2,3} Interstitial collagens provide mechanical support and maintain structural integrity, both of which are lost by treatment with high concentrations of collagenases.⁴ In addition, interstitial collagens at the epithelium-mesenchyme interface provide a substrate for epithelial cell-matrix interactions, which govern embryonic morphogenesis and the continuous renewal of tubulo-alveolar salivary glands.^{5,6} Loss of structural support, collagenase

mediated alterations in the morphogenetic properties of interstitial collagens, or both, might therefore explain the sialectasis, acinar atrophy, and loss of secretory parenchyma which lead to the sicca symptoms characteristic of SS.

This study investigated the presence, cellular source and types of collagenases in affected glands and assessed their secretion and state of activation in saliva in patients with SS.

Patients and methods

PURIFICATION OF TYPE I SOLUBLE COLLAGEN

Purification of soluble type I collagen was by the method of Miller and Rhodes,⁷ from rat tail tendon. Type I collagen contains approximately 13.6% of hydroxyproline and this was used to calculate the collagen content in the sample studied according to the equation:

$$C_0 = \frac{\Delta A_{\text{imp}}}{\Delta A_{\text{std}}} \cdot (1/0.136)$$

PATIENTS AND SAMPLES

We studied eight patients with SS and six healthy controls. Diagnosis of SS was according to the Copenhagen criteria.¹ Five of the patients had primary SS and three patients had the secondary form of the disease: one patient had underlying rheumatoid arthritis (RA) and two had Reiter's syndrome. All subjects gave their informed consent. Six to eight labial salivary glands (LSG) were obtained from each subject by biopsy (taken under infiltration anaesthesia by blunt dissection), embedded in Tissue-Tek OCT compound, and snap frozen in liquid nitrogen. Stimulated saliva was collected over five minutes from each patient by the same clinician at the same time of the day and under identical conditions. Immediately after the collection of the saliva the samples were centrifuged at 1000 g for five minutes and the supernatants were frozen at -70°C until analysed.

IMMUNOHISTOCHEMISTRY

Antisera to fibroblast type MMP-1 collagenase and neutrophil type MMP-8 collagenase were produced and characterised as described previously.^{8,9} Sections were stained using the avidin-biotin peroxidase complex (ABC).¹⁰ To control for method specificity, we compared results from omission of primary antiserum, use of normal rabbit serum (diluted 1:100–1:400), use of irrelevant antisera (rabbit anti-

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gastrin diluted 1:2000, or rabbit anti-pancreastatin diluted 1:4000) or omission of one of the subsequent steps in the ABC method.

ENZYME LINKED IMMUNOSORBENT ASSAY
TIMP was measured by ELISA according to the method of Günther *et al.*¹¹

DETERMINATION OF TOTAL AND AUTOACTIVE COLLAGENOLYTIC ACTIVITY

Two saliva samples were preincubated in parallel for 20 minutes at 22°C in the presence or absence of 1 mmol/l phenylmercuric chloride (PMC) dissolved in ethanol. After preincubation with PMC, soluble native type I collagen was added to a final concentration of 1.5 µmol/l before incubation for 12–24 hours at 22°C and measurement of collagenase activity using the method of Turto *et al.*¹²

The degradation was quantified densitometrically with an LKB Ultrascan Laser Densitometer model 2202. The value representing the degraded αA-collagen chains was multiplied by 4/3 and its proportion of the total collagen in the sample used as a measure of collagenase activity. Results were converted to International Enzyme Units per gram of protein present in the sample, using the equation:

$$A = \frac{10^4 \cdot \rho_{su} \cdot V_{su} \cdot p}{M_r \cdot t \cdot \rho_{pr} \cdot V_{sa}}$$

where ρ_{su} is concentration of collagen in the stock substrate solution, V_{su} is the volume of substrate solution, p is percent degradation of the substrate, M_r is molar mass of the substrate, t is incubation time in minutes, V_{sa} is volume of the sample, ρ_{pr} is concentration of protein in the sample, and A is the enzyme activity in the sample.

Figure 1 Staining of fibroblast collagenase (MMP-1) in a labial salivary gland from a patient with Sjögren's syndrome. Duct epithelial cells stain strongly (small arrows), whereas the lymphocytes in the periductal infiltrate are MMP-1 negative (arrow heads). However, some fibroblast- and macrophage-like periductal mononuclear cells are MMP-1 positive (arrows). Note that the basal parts of the acinar cells (acini are marked with asterisk) are MMP-1 positive. Avidin-biotin peroxidase complex staining, haematoxylin counterstain.

DOXYCYCLINE INHIBITION STUDIES
Purified MMP-1 and MMP-8, and saliva obtained from patients with SS were analysed for their collagenase type in the doxycycline inhibition test described in detail elsewhere.¹³

STATISTICAL METHODS
Results are expressed as mean (SD). The significance of the differences between mean values was tested with Wilcoxon's rank sum test.

Results

IMMUNOHISTOCHEMISTRY
In LSGs from patients with SS, MMP-1 was mainly localised in salivary duct epithelial cells (fig 1). Normal LSGs from healthy controls stained in a topologically identical pattern, although collagenase containing cells were fewer and more weakly staining (not shown). In both diseased and normal LSGs, few MMP-8 positive neutrophils were found, located either intravascularly or in the tissue interstitium (not shown).

SALIVARY COLLAGENASE AND TIMP
Collagenolytic activity, assessed after activating PMC pretreatment, was 11.7 (6.8) × 10⁻⁶ IU/l (2.1 (1.2) × 10⁻⁶ IU/g of protein) in stimulated Sjögren saliva. There was no collagenase activity in the control salivas ($p < 0.01$) (fig 2).

Measurement of collagenase in saliva obtained from patients with SS, in the presence and absence of PMC, showed that 76.1 (13.9)% of the total collagenase was in an endogenously active form.

Doxycycline IC₅₀ values for MMP-1 and MMP-8 were 280 µmol/l and 26 µmol/l, respectively. The type of collagenase present in the sample was assessed by incubation in the presence of 0, 100 and 600 µmol/l doxycycline.¹³ Doxycycline inhibition studies showed that approximately 95% of all collagenase in saliva in SS was of the MMP-1 type (data not shown).

Many of the samples from both patients with SS and healthy controls contained TIMP-1 ($p > 0.05$) (fig 3).

Figure 2 Total collagenase activity, measured after pretreatment with phenylmercuric chloride, in saliva from patients with Sjögren's syndrome (SS) and healthy controls (C).

Figure 3 Tissue inhibitor of matrix metalloproteinase (TIMP-1) in saliva from patients with Sjögren's syndrome (SS) and in healthy controls (C). The values below the broken line are less than the limit of detection of the TIMP-1 ELISA (15 ng/ml).

Discussion

The present study has demonstrated the presence of collagenase in salivary glands in SS. The collagenase gene is activated by several cytokines, including interleukin-1, platelet derived growth factor, tumour necrosis factor α and epidermal growth factor, all produced at inflammatory sites, as in SS. The induction of collagenase in focal adenitis therefore could be explained as a cytokine mediated effect.

Immunostaining revealed a distinct topological pattern of collagenase expression localised predominantly in salivary duct epithelial cells. This complemented the sialochemical findings which demonstrated increased amounts of collagenase in saliva in SS. Collagenase in cells other than neutrophils is not stored but is released into the extracellular space shortly after synthesis.¹⁴ Thus both sialochemical and immunohistochemical findings suggest that collagenase synthesis and secretion are increased in salivary glands in SS.

Collagenase exists as two separate molecules coded by two different genes, namely the fibroblast type MMP-1 and the neutrophil type MMP-8 collagenase genes.^{2,3} The enzymes differ not only in molecular size and substrate specificity, but also in pathways for activation and inhibition, cellular origin, and immunoreactivity. Our immunohistochemical findings with MMP-1 and MMP-8 specific antisera suggest that the collagenase in SS is of the MMP-1 type, because MMP-8 positive neutrophils were observed only occasionally intravascularly and in the tissue interstitium. This impression was further confirmed by doxycycline inhibition studies in which the 50% inhibitory concentration values for MMP-1 and MMP-8 collagenases were 280 $\mu\text{mol/l}$ and 26 $\mu\text{mol/l}$, respectively.¹³ Measurement of collagenase in the presence of 0, 100 or 600 $\mu\text{mol/l}$ doxycycline, used to determine the cellular source of secreted collagenases,¹³ confirmed the immunohistochemical results in suggesting that the collagenase (to 95%) relevant to SS is the MMP-1 type (in contrast to, for example, the collagenase derived from gingival pockets in adult periodontitis or patients with reactive arthritis). In this context, it is interesting to note that chemically modified tetracyclines,

which lack the dimethylamino group attached to the fourth carbon of the A ring of tetracycline, retain their anticollagenase action in spite of a loss of antimicrobial potency.

Collagenase is synthesised as a latent proenzyme, in which the fourth coordination site of the active site zinc is bound to the thiol group of Cys⁷³. This proenzyme can be activated by several different pathways, all of which release the active site zinc from the thiol group of Cys⁷³ by the so-called cysteine switch mechanism.¹⁵ Collagenase activity was therefore measured in the presence and absence of PMC, which is a hydrophobic compound seeking the substrate binding pocket of collagenase and which, because of its organomercurial content, reacts with the thiol group of Cys⁷³. PMC is an activator of both fibroblast type MMP-1 collagenase and neutrophil collagenase, MMP-8. A high proportion (76.1 (13.9) %) of the total collagenase in saliva from patients with SS was found to be active in the absence of PMC. This suggests not only that collagenase is synthesised and secreted, but also that the latent proenzyme has been converted in vivo into an active enzyme species, capable of degrading collagen. In SS this collagenolytic capacity seems to exceed the protection afforded by TIMP-1.

Because collagenase has a key role in initiating collagenolysis, MMP-1 may be a major factor responsible for the degradation of salivary glands in Sjögren's syndrome. Many adhesion proteins are involved in normal developmental processes in addition to their role during regenerative processes in wounds, malignancies, and inflammation. These events are accompanied by processing of the extracellular matrix, for example expression of tissue heterogeneity and processing by matrix proteinases. Interstitial collagens are important components of the extracellular matrix and experimental work has suggested that collagenolysis severely compromises the normal unipodial branching pattern of developing salivary glands.^{5,6} It is therefore possible that enhanced collagenolysis in inflamed LSGs in SS compromises the continuing remodelling and restructuring which is normally guided by the collagenous extracellular matrix and is necessitated by apoptosis and the accelerated cell death associated with focal adenitis.

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