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 locally increased 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) x 10^5 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. 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). Interstitial collagens provide mechanical support and maintain structural integrity, both of which are lost by treatment with high concentrations of collagens. 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.

Loss of structural support, collagenase mediated alterations in the morphogenetic properties of interstitial collagens, or both, might therefore explain the sialo, 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 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:

\[ C_0 = \frac{\Delta A_{400}}{\Delta A_{100}} \times (100-136) \]

**PATIENTS AND SAMPLES**

We studied eight patients with SS and six healthy controls. Diagnosis of SS was according to the Copenhagen criteria. 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. Sections were stained using the avidin-biotin peroxidase complex (ABC). To control for method specificity, we compared results from omission of primary antisera, use of normal rabbit serum (diluted 1:100–1:400), use of irrelevant antisera (rabbit anti-
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.13

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.13

The degradation was quantified densito-
metrically 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:

$$A = \frac{10^4 \cdot \rho_m \cdot V_e \cdot p}{M_r \cdot t \cdot \rho_p \cdot V_s}$$

where $\rho_m$ is concentration of collagen in the
stock substrate solution, $V_e$ 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, $V_s$ is volume
of the sample, $\rho_p$ is concentration of protein in
the sample, and $A$ is the enzyme activity in the
sample.

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 sig-
nificance 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
endothelial 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 doxy-
cycline.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 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 (arrows heads). However,
some fibroblast- and macrophage-like periductal mononuclear cells are MMP-1 positive
(arrow). 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.

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. 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. 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 μmol/l and 26 μmol/l, respectively.

Measurement of collagenase in the presence of 0, 100 or 600 μmol/l doxycycline, used to determine the cellular source of secreted collagenases, 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 anticollegenase 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 Cys17. This proenzyme can be activated by several different pathways, all of which release the active site zinc from the thiol group of Cys17 by the so-called cysteine switch mechanism. 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 Cys17. 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. Intrastitial collagen is important components of the extracellular matrix and experimental work has suggested that collagenase activity compromises the normal unipodial branching pattern of developing salivary glands. 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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