Increased Acinar Damage of Salivary Glands of Patients With Sjögren's Syndrome Is Paralleled by Simultaneous Imbalance of Matrix Metalloproteinase 3/Tissue Inhibitor of Metalloproteinases 1 and Matrix Metalloproteinase 9/Tissue Inhibitor of Metalloproteinases 1 Ratios

Paola Pérez,[1] Yoon-Jeoung Kwon,[1] Cecilia Alliende,[1] Lisette Leyton,[1] Sergio Aguilera,[2] Claudio Molina,[3] Cecilia Labra,[1] Marcela Julio,[1] Cecilia Leyton,[1] and María-Julieta González[1]

Objective. **Previous findings in labial salivary glands (LSGs) from patients with Sjögren's syndrome (SS) suggest that increased activity and expression of matrix metalloproteinase 9 (MMP-9) and MMP-3 trigger the destruction of acinar structures in these glands. Tissue inhibitors of matrix metalloproteinases (TIMPs) tightly control MMP activity, and TIMP expression is an important modulator of effects attributed to MMPs. This study was undertaken to investigate the correlation between the balance of MMPs/TIMPs in the LSGs of SS patients and the degree of inflammatory infiltration and acinar structure integrity.**

Methods. **Three groups of SS patients classified according to focus score and residual tissue were studied. The expression of MMP-2, MMP-3, MMP-9, TIMP-1, and TIMP-2 was examined at the messenger RNA and protein levels. The ratio of MMP/TIMP ex-** **pression (R value) was calculated. Focus score and acinar structure were evaluated by histologic analysis.**

Results. **In SS patients the MMP-3/TIMP-1 ratio was higher than 1 and the MMP-9/TIMP-1 ratio was much higher than 1 whereas the MMP-2/TIMP-2 ratio nearly equaled 1, suggesting elevated proteolytic activity due mainly to MMP-9. R values were independent of the focus score of inflammatory cells, but correlated well with the dramatic changes observed in morphologic integrity of acini, as revealed mainly by the lack of nuclear polarity. Acinar changes were more evident when R values for both MMP-9/TIMP-1 and MMP-3/ TIMP-1 were higher.**

Conclusion. **This study provides evidence that an altered balance between MMPs and their inhibitors is associated with acinar damage. Since salivary gland acinar cells express both MMPs and TIMPs, these cells may play an important role in extracellular matrix destruction and in the LSG pathophysiology in SS.**

[1]Paola Pérez, BSc, Yoon-Jeoung Kwon, BSc, Cecilia Alliende, BSc, Lisette Leyton, PhD, Cecilia Labra, BSc, Marcela Julio, BSc, Cecilia Leyton, MSc, María-Julieta González, MSc: University of Chile, Santiago, Chile; [2]Sergio Aguilera, MD: Andrés Bello University, Santiago, Chile; [3]Claudio Molina, DDS, MSc: Mayor University, Santiago, Chile.

Address correspondence and reprint requests to María-Julieta González, MSc, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Casilla 70061, Santiago 7, Chile. E-mail: jgonzale@med.uchile.cl.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that play a key role in tissue-remodeling events under both normal and pathologic conditions (1). Their expression is regulated by growth factors, cytokines, and hormones, as well as by interactions with extracellular matrix (ECM) proteins (1). Endogenous inhibitors, such as tissue inhibitors of matrix metalloproteinases (TIMPs), also exist to counterbalance MMP activity (2).

To date, 4 TIMP family members have been described. TIMPs 1, 2, and 4 are secreted as soluble proteins, whereas TIMP-3 is associated with matrix components as an insoluble protein (3). Noncovalent,

1:1 stoichiometric binding of each TIMP to the catalytic site of the activated forms of secreted MMPs leads to the MMPs' inhibition (3). All TIMPs inhibit active MMPs with relatively low selectivity. However, it has been reported that TIMP-1 binds to and delays the activation of latent MMP-9 and MMP-3 pro forms, while TIMP-2 binds to and regulates activation of proMMP-2 (4). MMP activity is tightly controlled by TIMPs, and the resulting equilibrium regulates integrity of the ECM and, in so doing, plays a key role in a wide range of physiologic processes (1).

As a consequence, imbalances in MMP/TIMP expression ratios have been implicated in various patho-logic conditions, including cardiovascular diseases, cancer, and autoimmune diseases (5–7). In this context, it is interesting to note that patients with severe and active Sjögren's syndrome (SS), a chronic autoimmune disorder that causes alterations in the structure and function of exocrine glands, express elevated levels of MMP-3 and MMP-9 in acinar and ductal cells from labial salivary glands (LSGs). These changes are closely associated with destruction of the basal lamina of acini and ducts, as well as degradation of the major interstitial structural proteins of the ECM (8,9). Additionally, high MMP-9 activity correlates with structural and functional changes of the gland in these patients, whereas MMP-2 activity does not differ from that in controls (8,9). In other structures of LSGs that lack both MMP-3 and MMP-9, such as blood vessels, the integrity of the basal lamina is maintained (8,9). In contrast, in acini and ducts, where expression and activity of these MMPs is high, the basal lamina is completely disorganized, suggesting a localized role of MMPs in the cells that produce them (10). Furthermore, studies have shown that the activity of MMP-9 is elevated in saliva of SS patients (11).

When transformed cell lines derived from salivary glands are treated with cytokines (tumor necrosis factor α, interleukin-1β, interferon-γ), MMP messenger RNA (mRNA) and protein levels, as well as gelatinolytic activity, are highly stimulated (12,13). Levels of the same cytokines are strongly increased locally in salivary glands of patients with SS (14).

Expression of MMPs is mainly transcriptionally regulated. Hence, MMP protein levels correlate well with mRNA expression (15). In the present study, the molecular mechanisms underlying salivary gland remodeling processes in the LSGs of patients with primary SS were characterized. For this purpose, levels of mRNA for both MMPs and TIMPs were quantified, and correlations between the MMP/TIMP ratios obtained and both the quantity of inflammatory cells present in LSG and the morphologic integrity of the residual gland parenchyma were calculated.

We found that in LSGs from patients with SS, both MMP-9/TIMP-1 and MMP-3/TIMP-1 ratios exceeded 1, suggesting elevated levels of proteolytic activity. The latter was independent of the focus score of inflammatory cells but correlated well with dramatic changes in the morphology of acini, manifested mainly as a loss in nuclear polarity. Since these endopeptidases are reportedly produced by acinar and ductal cells in LSGs, our findings suggest that these cells play a crucial role in ECM remodeling processes and in the pathophysiology of SS.

PATIENTS AND METHODS

Patients with primary SS. The SS patients (n = 16) were diagnosed according to the American–European consensus criteria (16). They were evaluated for keratoconjunctivitis sicca, xerostomia, and the presence of anti-La and anti-Ro autoantibodies. MMP and TIMP expression levels were measured in LSGs of SS patients with different degrees of infiltrating cells and enough gland tissue to perform these measurements. Thus, patients were classified into 3 groups according to focus score and quantity of remaining gland tissue, as follows: group 1 = focus score of 1–2 and 80–90% residual parenchyma (n = 6; age range 37–62 years [mean 51]); group 2 = focus score of 3–4 and 60–70% residual parenchyma (n = 6; age range of 35–56 years [mean 51]); group 3 = focus score >4 and 40–50% residual parenchyma (n = 4; age range 34–68 years [mean 52]).

One control group consisted of 10 subjects (age range 29–49 years [mean 43]) who did not fulfill criteria for primary SS and were negative for rheumatoid factor, antinuclear antibody, and antibodies to Ro and La. Only a mild nonspecific chronic sialadenitis was found on lip biopsy of these patients (Chisholm and Mason grade 1) (17). A second control group (n = 4; age range 34–68 years [mean 52]) had moderate or severe nonspecific chronic sialadenitis.

Biopsies. LSGs obtained as described by Daniels (18) were snap-frozen in liquid nitrogen and stored at −80°C until processed (see below). Informed consent was obtained from all subjects before LSG biopsy was performed. The protocols utilized in this study were approved by the Ethics Committee of the Faculty of Medicine, University of Chile.

Total RNA extraction. Total RNA was extracted from LSGs, using the RNeasy Mini Kit according to the instructions of the manufacturer (Qiagen, Valencia, CA). DNA contamination was controlled in a polymerase chain reaction (PCR) experiment omitting the reverse transcriptase (RT). RNA concentration and quality were determined by measuring absorbance at 260 and 280 nm. All samples used in these studies had absorbance at 260 nm:absorbance at 280 nm ratios between 1.9 and 2.3. RNA integrity and size distribution were controlled by electrophoresis of total RNA (1 μg) on 1%

denaturating agarose gels followed by staining with ethidium bromide.

RT-PCR. Total RNA was reverse-transcribed to complementary DNA (cDNA) using oligo primers and RT (Superscript II; Invitrogen, San Diego, CA). Complementary DNA products were amplified in PCR mixtures containing 4% (volume/volume) of the appropriate cDNA dilution (600, 300, and 60 ng of total RNA), 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.4 μM oligonucleotides, and 1.25 units Taq polymerase (Roche, Mannheim, Germany). The PCRs were conducted with a PTC-100 thermocycler (MJ Research, Waltham, MA). Amplification was performed in sequential cycles including 1.5 minutes of denaturation at 94°C, 2 minutes of primer annealing at the specified temperature, and 2 minutes of extension at 72°C. The following primers/conditions were applied: for MMP-2 5′-GGTTCATTTGGCGGACTGCG-3′ (sense), 5′-TTCTCCAGGCCCGGTGTATC-3′(antisense) (28 cycles were performed at an annealing temperature of 55°C, yielding a 670-bp product); for MMP-3 5′-GTTCCGCCTGTCTCAAGATG-3′ (sense), 5′-TTGGAGGGAAACCTAGGGGTG-3′ (antisense) (32 cycles were performed at an annealing temperature of 50°C, yielding a 406-bp product); for MMP-9 5′-A-GACCTGAGAACCAATCT-3′ (sense), 5′-GGCACTGAGG-AATGATCTAA-3′ (antisense) (35 cycles were performed at an annealing temperature of 50°C, yielding a 1,146-bp product); for TIMP-1 5′-ATACCAGCGTTATGAGATCAAG-3′ (sense), 5′-GTTTGCAGGGGATGGATAAACA-3′ (antisense) (30 cycles were performed at an annealing temperature of 50°C, yielding a 314-bp product); for TIMP-2 5′-TT-CAAAGGGCCTGAGAAGGATA-3′ (sense), 5′-ACAGG-AGCCGTCACTTCTCT-3′ (antisense) (28 cycles were performed at an annealing temperature of 50°C, yielding a 368-bp product); and for GAPDH 5′-ACA ACAGCCTCA-AGATCATCA-3′ (sense), 5′-GTCCAGGGGTCTTACT-CC-3′ (antisense) (25 cycles were performed at an annealing temperature of 50°C, yielding a 670-bp product). Conditions were chosen such that all cDNA were analyzed in the exponential phase of the amplification curve.

PCR product characterization. All RT-PCR products were analyzed by 1% agarose gel electrophoresis in TAE buffer (40 mM Tris [pH 8.0], 40 mM acetic acid, 2 mM EDTA) followed by ethidium bromide staining. PCR products were analyzed densitometrically, and values obtained were normalized to those obtained for GAPDH. Results shown were averaged from 3 sets of independent experiments. In all cases, the identity of the amplified cDNA sequence was verified by direct sequencing.

Western blotting. LSGs were homogenized in RIPA buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.5% deoxycholate, 1% Nonidet P40) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml each of leupeptin, aprotinin, and benzamidine) at a ratio of 1:5 (weight/volume) at 4°C.

The detergent-soluble supernatants were recovered after spinning at 800g for 5 minutes at 4°C and stored at –70°C for further analysis. The Bradford method or the Pierce bicinchoninic acid method was used to determine protein concentrations in the extracts. Samples with equal amounts of total protein were separated on SDS–8% or 10% polyacrylamide gels for MMP-9 and TIMP-1, respectively, and electro-

transferred to nitrocellulose (Bio-Rad, Hercules, CA) for 15 hours at 60 mA, 4°C for MMP-9 or 3 hours at 200mA, 4°C for TIMP-1. Blots were blocked with 10% milk powder in Tris buffered saline–Tween (TBST) (10 mM Tris HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20) for 2 hours for MMP-9 and overnight for TIMP-1. Membranes were then incubated with anti-human MMP-9 or TIMP-1 antibodies for 2 hours (Oncogene Research Products, Cambridge, MA). After 5 washes in TBST, blots were incubated with horseradish peroxidase–conjugated anti-mouse antibodies (for MMP-9) or anti-goat antibodies (for TIMP-1) (Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature and washed 5 times in TBST. Horseradish peroxidase–conjugated antibodies detected using an enhanced chemiluminescence Western blotting detection system according to the instructions of the manufacturer (Amersham International, Tokyo, Japan).

Histologic and immunohistochemical analysis. Samples were fixed in 1% paraformaldehyde or Bouin's fixative for 6 hours and paraffin embedded. Sections were then deparaffinized, rehydrated, and digested for 10 minutes with 0.1% trypsin in 10 mM Tris HCl (pH 7.8), for detection of MMP-9. Endogenous peroxidase activity was quenched with 0.3% H$_2$O$_2$ in methanol, and nonspecific binding was blocked with 2% bovine serum albumin. Sections were incubated with a monoclonal antibody against MMP-9 (R&D Systems, Minneapolis, MN) followed by incubation with a biotinylated secondary antibody and further incubation with a streptavidin–peroxidase conjugate (Dako, Carpinteria CA). The reaction was developed with 3′,3′-diaminobenzidine (Sigma, St. Louis, MO). Preimmune controls were also included. Mayer's hematoxylin was used as a counterstain. TIMP-1 was detected in cryosections fixed sequentially in acetone at –20°C for 10 minutes and paraformaldehyde at 4°C for 15 minutes. The conditions for blocking were similar to those described above. Sections were incubated with a polyclonal antibody against TIMP-1 (Santa Cruz Biotechnology, Santa Cruz, CA), after which the same procedures as those described for MMP-9 were carried out.

Statistical analysis. Normalized data were processed to calculate mean values and relative standard deviations. To evaluate possible fluctuations in the MMP/TIMP mRNA balance between SS patient samples and control patient samples, the results were expressed using the following formula (subsequently referred to as the R value): R = ([MMP$_{patient}$/mean MMP$_{controls}$]/[TIMP$_{patient}$/mean TIMP$_{controls}$]). This formula takes into account variations in the expression of both the enzyme (MMP) and its inhibitor (TIMP), standardized in each case to the average levels detected in controls. The Mann-Whitney U test was used to assess the significance of differences in R values.

RESULTS

Relative levels of MMP and TIMP mRNA expression in primary SS. In all cases, the primers chosen were appropriate and generated specific single amplicons, with no additional bands, as indicated in Figures 1A and B. To determine the relative amount of mRNA for each gene, densitometric values obtained were normalized to
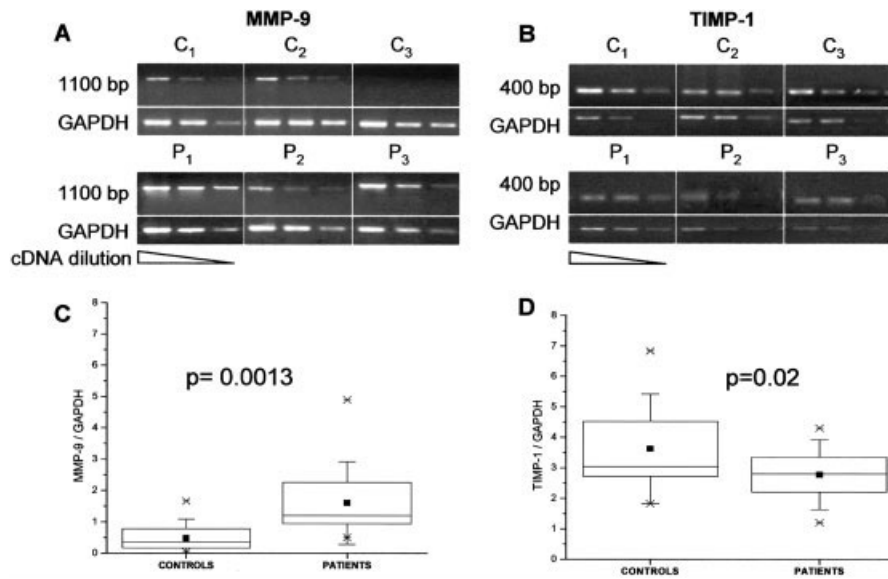
Figure 1. Reverse transcriptase–polymerase chain reaction (RT-PCR) studies of the levels of mRNA for matrix metalloproteinase 9 (MMP-9) and tissue inhibitor of metalloproteinases 1 (TIMP-1) in labial salivary gland specimens from Sjögren's syndrome (SS) patients (P) and controls (C). Total RNA was reverse transcribed to cDNA and amplified by RT-PCR as described in Patients and Methods. PCR products were resolved by electrophoresis on 1% agarose gels. For each subject, 3 dilutions were used (600, 300, and 60 ng of total RNA). **A**, MMP-9 shows a single band at 1,100 bp. **B**, TIMP-1 is observed as a band at 400 bp. Representative samples from 3 SS patients and 3 controls are shown. **C** and **D**, Quantitative densitometric analysis of both amplicons, normalized to GAPDH values, indicating the statistical significance of the difference between SS patients and controls. Data are representative of 3 independent experiments and are presented as box plots, where boxes represent the 25th to 75th percentiles, lines within the boxes represent the median, solid squares within the boxes represent the mean, and lines outside the boxes represent the SD. Asterisks indicate outliers.

GAPDH data. As shown in Table 1, no changes in relative expression levels of mRNA were found for MMP-2 ($P = 0.3$) or TIMP-2 ($P = 0.2$) when SS patients were compared with controls. Levels of mRNA for MMP-3 were 1.5-fold greater in the SS patient group than in controls, but this increase was not statistically significant ($P = 0.09$). Nine of 16 SS patients had higher values than the mean value in control subjects. For MMP-9, relative mRNA expression levels differed significantly between the 2 groups ($P = 0.0013$), and the mean value was 2.4-fold higher in patients than in controls (Table 1 and Figures 1A and C). Conversely,

Table 1. Relative levels of mRNA for MMPs and TIMPs*

Gene	Relative level of mRNA in controls		Relative level of mRNA in SS patients		
	Mean ± SD	Range	Mean ± SD	Range	P†
MMP-2	1.3 ± 0.6	0.3–2.7	1.4 ± 0.97	0.2–2.7	0.3
MMP-3	1.5 ± 0.3	1.2–2.0	2.2 ± 1.1	0.3–4.7	0.09
MMP-9	0.5 ± 0.6	0.01–1.7	1.2 ± 0.6	0.5–4.9	0.0013
TIMP-1	3.6 ± 1.8	1.6–6.8	2.9 ± 1.1	1.2–4.3	0.02
TIMP-2	1.7 ± 0.7	0.5–2.6	1.6 ± 0.8	0.8–3.6	0.2

* MMPs = matrix metalloproteinases; TIMPs = tissue inhibitors of metalloproteinases; SS = Sjögren's syndrome.
† By Mann-Whitney U test.
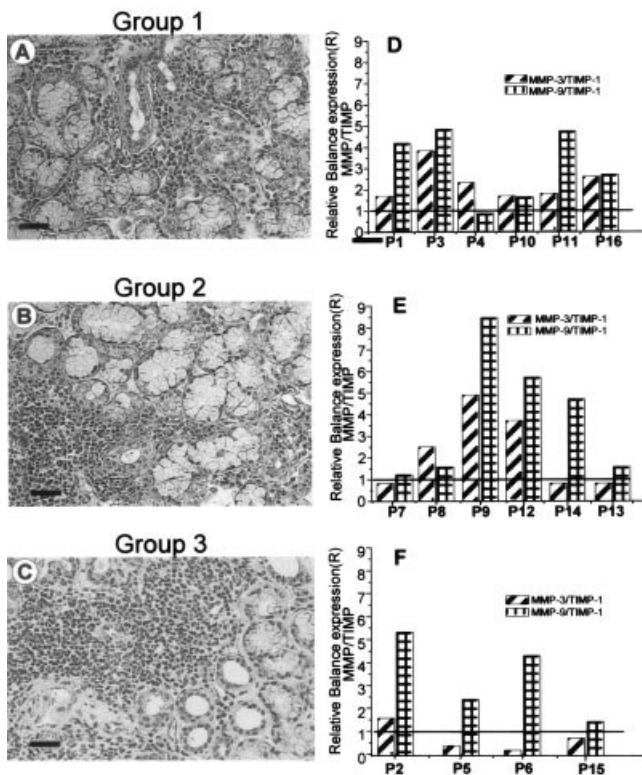
Figure 2. MMP/TIMP ratios (R values) and focus scores. **A, B,** and **C,** Representative sections of labial salivary glands from 3 groups of SS patients classified as indicated in Patients and Methods. Bars = 50 μm. **D**, **E,** and **F,** Graphs indicating the R values for MMP-3/TIMP-1 and MMP-9/TIMP-1 in patients in group 1, group 2, and group 3, respectively. Horizontal lines (R values of 1) correspond to the expected physiologic balance between MMP and TIMP. The data suggest no correlation between R values and focus scores. See Figure 1 for definitions.

relative expression levels of TIMP-1 mRNA were 1.2-fold lower in SS patients than in controls ($P = 0.02$) (Table 1 and Figures 1B and D).

Determination of MMP/TIMP mRNA ratios and their relationship to morphologic integrity. In a previous report we described high gelatinolytic activity occurring in close association with severe glandular damage in SS (9). In a study using T cells from patients with tropical spastic paraparesis (19), increased expression of MMPs was followed by increased expression of TIMPs, thus maintaining a balanced ratio between the 2 proteins. In the present study we evaluated the possibility that fluctuations in MMP/TIMP ratios may be associated with disease. The R value reflecting the MMP-2/TIMP-2, MMP-9/TIMP-1, and MMP-3/TIMP-1 ratio in each SS patient was determined (see Patients and Methods). This value takes into account variations in the
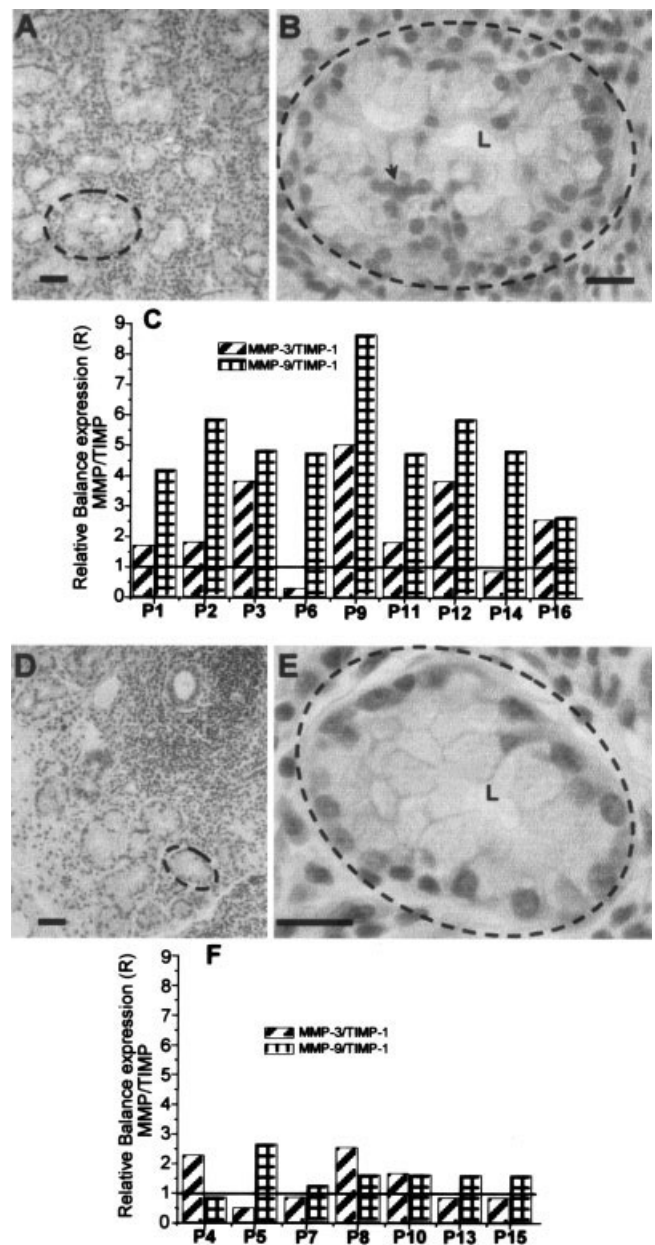
Figure 3. MMP/TIMP ratios (R values) and acinar morphologic integrity. Representative sections of labial salivary glands (LSGs) from SS patients are shown, with the circled regions in **A** and **D** enlarged in **B** and **E,** respectively (bars = 50 μm in **A** and **D** and 20 μm in **B** and **E**). The SS LSG shown in **A** and **B** and the one shown in **D** and **E** had similar inflammatory infiltration but different R values. The patient whose morphologic results are shown in **A** and **B** is from the group of patients whose R values for MMP-3/TIMP-1 and MMP-9/TIMP-1 are shown in **C**; the patient whose morphologic results are shown in **D** and **E** is from the group of patients whose R values for MMP-3/TIMP-1 and MMP-9/TIMP-1 are shown in **F**. Notable differences in acinar morphology were observed, with more pronounced acinar alterations in patients with higher R values. **Arrow** indicates the stained nucleus. **L** = lumen (see Figure 1 for other definitions).

Table 2. Relative levels of latent MMP-9, active MMP-9, and TIMP-1 proteins*

	Protein expression level in control		Protein expression level in SS patients		
	Mean ± SD	Range	Mean ± SD	Range	P†
Latent MMP-9	0.04 ± 0.03	0.03–0.08	0.11 ± 0.06	0.03–0.2	0.02
Active MMP-9	0.05 ± 0.03	0.03–0.09	0.16 ± 0.14	0.02–0.48	0.02
Total MMP-9	0.11 ± 0.04	0.03–0.13	0.34 ± 0.12	0.23–0.58	0.03
TIMP-1	0.12 ± 0.11	0.03–0.24	0.07 ± 0.07	0.01–0.24	0.3

* See Table 1 for definitions.
† By Mann-Whitney U test.

expression of both the enzyme and its specific inhibitor and, as such, is an indicator of an imbalance between MMP (degradation of ECM) and TIMP (deposition of ECM).

R values for MMP-2/TIMP-2 ranged from 0.8 to 1.3 (mean 1) in SS patients (n = 16), and were not significantly different from R values calculated for control subjects (P = 0.7). For MMP-3/TIMP-1, 9 of 16 SS patients had R values that exceeded 1 (mean 1.5), indicating alterations in MMP-3/TIMP-1 ratios in SS patients. Nevertheless, this value was not significantly higher than the value in controls (P = 0.2). For MMP-9/TIMP-1, 15 of 16 SS patients had R values that exceeded 1 (mean 3.7; P = 0.02 versus controls), indicative of an imbalance in the expression of this enzyme/inhibitor pair in patients.

Since MMP/TIMP balance is a critical determinant of net proteolytic activity and is generally considered a prerequisite for MMP involvement in tissue damage, we investigated whether the R values obtained paralleled glandular damage. Correlations of R values with both the degree of inflammatory infiltration and acinar integrity in LSG were calculated for the SS patients, grouped according to focus score and residual parenchyma. No correlation was found between R values and focus scores (Figure 2). However, SS patients with high R values for MMP-3/TIMP-1 and MMP-9/TIMP-1 exhibited dramatic alterations in acinar integrity, with loss of nuclear polarity being the most frequent change observed (Figures 3A–C). In contrast, SS patients with R values of ~1 had normal acini despite the proximity of inflammatory cells (Figures 3D–F).

Evaluation of protein levels of MMP-9 and TIMP-1. Differential expression of mRNA for MMP-9 and TIMP-1 was further confirmed by Western blot analysis of protein levels of gland extracts from SS patients and controls Under reducing conditions, the antibody against MMP-9 recognized a band of 98 kd and 2 bands of 86 and 83 kd, corresponding to the latent and active forms of MMP-9, respectively. The densitometric values were normalized to β-actin. As shown in Table 2 and Figures 4A and B, MMP-9 protein expression was ~3-fold higher in SS patients than in controls (P = 0.03). In contrast, among the 16 SS patients examined, greatly reduced levels of TIMP-1 were observed in some (n = 5; represented by patients 14 and 16 in Figure 4D), slightly lower levels were found in half (n = 8; represented by patients 12 and 15 in Figure 4D), and TIMP-1 levels similar to those seen in control subjects were detected in the remaining 3 (represented by patients 11 and 13 in Figure 4D). As a consequence, despite the trend toward low TIMP-1 protein levels in SS patients, the latter was not significantly different from findings in controls (P = 0.3). Data obtained by Western blotting were corroborated by immunohistochemical analysis. MMP-9 levels were increased in acini and ducts of LSGs of SS patients, independent of the presence of inflammatory foci (Figure 4C). Immunohistochemical analysis of TIMP-1 revealed an intense immunoreaction in acini, while TIMP-1 was almost undetectable in ducts of LSGs from controls. In contrast, in SS patients little TIMP-1 was expressed in the whole gland (Figure 4F). These results were confirmed by Western blot analysis (Figures 4D and E).

DISCUSSION

MMPs are secreted as latent pro forms that require N-terminal truncation for activation. Members of the TIMP family inhibit MMPs. Several studies analyzing their expression, protein levels, and gelatinolytic activity in salivary glands, saliva, and tears (13,20,21) suggest that MMPs, particularly MMP-9, are involved in the development of SS. Given that TIMPs represent the major regulators of MMP activity, we conducted the current study to investigate alterations in MMPs in relation to their respective TIMPs in LSGs of SS patients, by estimating MMP/TIMP ratios. These
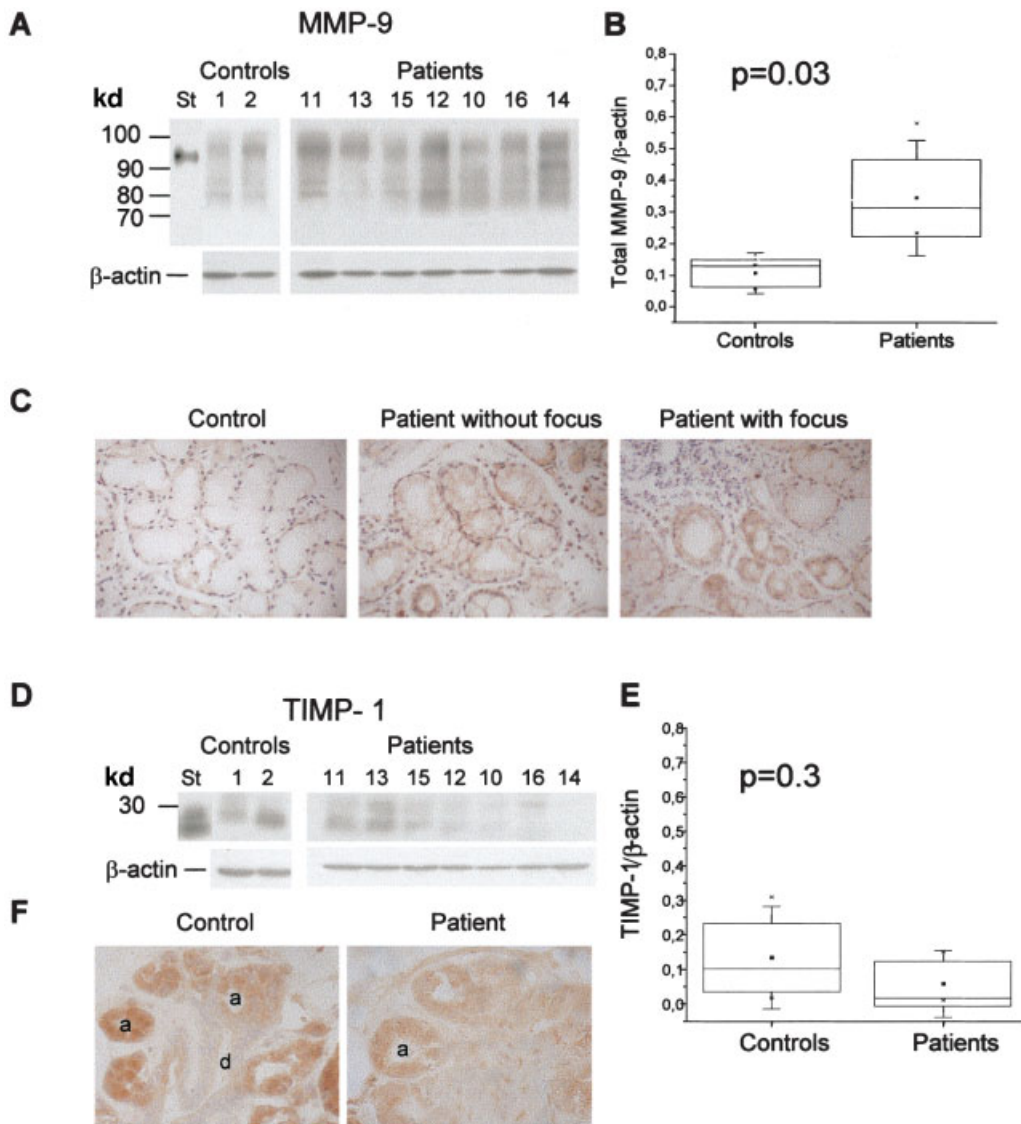
Figure 4. Expression of MMP-9 and TIMP-1 proteins in extracts and sections from labial salivary glands of SS patients and controls. The samples were probed separately by immunoblotting or immunohistochemistry with mouse anti–MMP-9 or goat anti–TIMP-1 antibodies, and reactivity was revealed by chemiluminescence or diaminobenzidine staining, respectively. In the immunoblots, β-actin was used as a loading control. **A,** Western blot analysis for MMP-9 in controls and SS patients, showing 3 bands of 98, 86, and 83 kd. **B,** Quantitative densitometric analysis, showing relative expression levels of total MMP-9 normalized to β-actin. **C,** MMP-9 immunohistochemistry analysis of a control gland section and 2 areas of the same gland from an SS patient, at a site far from and a site close to the focus of inflammatory cells. **D,** Western blot analysis for TIMP-1. **E,** Quantitative densitometric analysis, showing relative expression levels of TIMP-1 normalized to β-actin. **F,** TIMP-1 immunohistochemistry analysis of gland sections from a control and an SS patient. **a** = acini; **d** = ducts. Prestained molecular weight standards of 100, 90, 80, and 70 kd are shown in **A** (Protein Ladder; Gibco BRL Life Technologies, Gaithersburg, MD). Standard (St) corresponds to pure proMMP-9 (92 kd) in **A** and to pure TIMP-1 (28 kd) in **D**. Data in **B** and **E** are representative of 3 independent experiments and are presented as box plots, where boxes represent the 25th to 75th percentiles, lines within the boxes represent the median, solid squares within the boxes represent the mean, and lines outside the boxes represent the SD. Asterisks indicate outliers. See Figure 1 for other definitions.

ratios were compared with morphologic findings of acinar damage and inflammatory infiltration of the gland.

A ratio of MMP/TIMP expression favoring proteolytic activity was observed in many of the SS patients, with R values greatly exceeding 1 for MMP-9/TIMP-1

and exceeding 1 for MMP-3/TIMP-1 (Figure 2), while R values for MMP-2/TIMP-2 remained close to 1. R values did not correlate with the focus score and, interestingly, among SS patients with similar quantities of inflammatory cells infiltrating the gland tissue, very different R values were determined (Figure 2). In contrast, a strong correlation between high R values and marked histologic alterations of the glandular acini of SS patients was observed. Notably, patients with high R values for MMP-3/TIMP-1 and MMP-9/TIMP-1 exhibited the most severe morphologic changes in LSG acini (Figure 3). MMP protein levels also correlated well with mRNA expression (Figure 4), whereas, although both TIMP-1 mRNA and TIMP-1 protein levels appeared to be low in SS patients, protein levels analyzed by Western blotting were not significantly different from those in controls. The tendency toward low TIMP-1 levels was observed in 13 of the 16 SS patients studied; however, only 5 of them had clearly low levels, while 8 showed slightly reduced levels of TIMP-1 protein and 3 had values similar to those in control subjects (Figure 4). The latter could explain the lack of statistical significance of these results. The trend toward low TIMP-1 protein levels was corroborated by the results of immunohistochemical analysis, in which low TIMP-1 reactivity was also seen in SS patients. Therefore, the low protein expression correlated with TIMP-1 mRNA levels.

The balance between the levels of activated enzymes and free TIMPs determines overall MMP activity. Thus, maintenance of this equilibrium is essential, and any disturbance in this balance likely results in tissue damage by increased proteolysis, as observed in this study. This is an important finding, since the reasons underlying the loss of structural integrity of acini from salivary glands of SS patients have been poorly understood.

The formation of a ternary proMMP-9–TIMP-1–MMP-3 complex in vitro has been described as an intermediate stage in the activation of proMMP-9. Excess MMP-3 weakens the interaction between proMMP-9 and TIMP-1, leading to proMMP-9 processing and further activation (22). The authors of that report also evaluated the action of MMP-3 on proMMP-9 by SDS–polyacrylamide gel electrophoresis under reducing conditions and found 3 bands of 92, 84, and 82 kd, with the first corresponding to the MMP-9 latent form and the others to active forms (22). In the present study, the gland extracts from controls and SS patients showed 3 bands of 98, 86, and 83 kd, suggesting that proMMP-9 might be activated through formation of the above-mentioned ternary complex.

A number of purified proteases, including trypsin, chymase, MMP-2, trypsin 2, plasmin, and MMP-3, have been reported to activate proMMP-9 in vitro (22). However, based on in vitro kinetic and catalytic parameters, MMP-3 appears to be the most efficient activator of proMMP-9 (22). Nevertheless, MMP-3 is also produced as a zymogen and requires activation to convert the latent form of MMP-9 into an active enzyme. Our previous studies have demonstrated high levels of expression of MMP-9 and MMP-3 in LSGs of SS patients, with high MMP-9 activity in patients with the most severe pathologic manifestations (9). Relative protein levels of MMP-3 could not be evaluated here with the techniques available. Thus, we were not able to determine whether this might represent a plausible mechanism by which MMP-9 is activated at different stages of the disease.

An alternative proposed mechanism for activation of proMMP-9 is by human trypsin 2, a serine protease present in acinar cells (21,23). We postulate that this enzyme may be important when the level of active MMP-3 in the LSG of SS patients is low. However, digestion of proMMP-9 with human trypsin 2 produced a unique peptide of 77 kd (23). Since no such band was detected in our experiments (Figure 4), it is unlikely that trypsin 2 activates MMP-9. As discussed in previous reports, other enzymes with serine-arginine protease activity have been detected, but predominantly in patients in whom MMP activity is reduced, perhaps due to advanced progression of the disease (9). Thus, the exact mechanism by which proMMP-9 is activated remains to be defined. Future experiments will explore the nature of the proteases involved.

Recent studies demonstrated increased MMP-9/TIMP-1 ratios in whole mixed saliva from patients with primary SS (24), similar to our findings in individual acini. Given that whole saliva extracts were used, the cellular origin of MMP-9 and TIMP-1 was unclear, and since mucositis occurs frequently in SS, such chronic inflammation may be expected to favor the appearance of enzymes secreted from a variety of cells. Our data provide evidence in support of the notion that the imbalanced enzyme/inhibitor ratio detected in saliva reflected events occurring in the salivary gland. However, to demonstrate this directly, other possible sources of these proteins in saliva would have to be evaluated.

Finally, the altered ratio of MMP/TIMP mRNA expression described herein suggests that regulation of the genes occurs either at the transcriptional level or by changes in mRNA stability. The regulation of MMP genes in normal tissues has yet to be thoroughly exam-

ined, but available evidence suggests that MMP family members are expressed as a complex in a highly individualized tissue-specific manner. Inducible MMPs (MMP-1, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, and MMP-13) contain 1 or more activator protein 1 (AP-1)–binding site(s) in their promoter regions whereas the promoter regions of constitutively expressed MMPs (MMP-2 and MMP-11) do not contain AP-1–binding sites, suggesting that AP-1 may be important in controlling the expression of inducible MMPs while different transcription factors regulate the expression of MMP-2 and MMP-11 (25–28). Additionally, several factors, such as cytokines, oxidative stress, and growth factors, modulate MMP expression at the transcriptional level (27,29), thereby contributing to the complexity of the issue (27,29). These factors, in particular, cytokines, are present in salivary glands of SS patients, and are likely to modulate MMP transcription (14).

To date, few reports have focused on the induction of MMPs in SS (12,13,20), although it has been suggested that a better understanding of the molecular mechanism regulating induction and repression of specific MMPs or TIMPs may provide valuable information for developing novel therapeutic approaches (27). Inducers seem to also have a strong effect on the stability of MMP and TIMP mRNA, with a few authors reporting modifications of the half-life of these mRNA (30,31). However, there is a lack of evidence for changes in the stability of MMP and TIMP mRNA in acinar or ductal cells.

The data reported here, together with our previous observations (8,9), are the first to indicate that increased MMP-3 and MMP-9 expression, as well as activity derived from exocrine epithelial cells of LSGs, is one of the causes of acinar destruction in SS. The current results suggest that substantial increases in MMP expression in the diseased LSG may be potentiated by moderate decreases in TIMPs. This observed imbalance correlates strongly with the morphologic integrity of acini and the extent of ECM remodeling in LSGs of patients with SS.

ACKNOWLEDGMENTS

REFERENCES

1. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 2001;17:463–516.
2. Woessner JF Jr. MMPs and TIMPs: an historical perspective. Mol Biotechnol 2002;22:33–49.
3. Nagase H, Brew K. Engineering of tissue inhibitor of metalloproteinases mutants as potential therapeutics. Arthritis Res 2002;4 Suppl 3:S51–61.
4. Murphy G, Willenbrock F. Tissue inhibitors of matrix metalloendopeptidases. Methods Enzymol 1995;248:496–510.
5. Konttinen YT, Ainola M, Valleala H, Ma J, Ida H, Mandelin J, et al. Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis. Ann Rheum Dis 1999;58:691–7.
6. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002;2:161–74.
7. Watanabe N, Ikeda U. Matrix metalloproteinases and atherosclerosis. Curr Atheroscler Rep 2004;6:112–20.
8. Goicovich E, Molina C, Perez P, Aguilera S, Fernandez J, Olea N, et al. Enhanced degradation of proteins of the basal lamina and stroma by matrix metalloproteinases from the salivary glands of Sjögren's syndrome patients: correlation with reduced structural integrity of acini and ducts. Arthritis Rheum 2003; 48:2573–84.
9. Perez P, Goicovich E, Alliende C, Aguilera S, Leyton C, Molina C, et al. Differential expression of matrix metalloproteinases in labial salivary glands of patients with primary Sjögren's syndrome: mechanisms of exocrine parenchyma destruction. Arthritis Rheum 2000;43:2807–17.
10. Gonzalez MJ, Molina C, Alliende C, Perez P, Goicovich E. Matriz extracelular de glándulas salivares en el síndrome de Sjögren. In: Anaya JM, Ramos M, Garcia M, editors. Síndrome de Sjögren. Medellin: Corporación para Investigaciones Biológicas; 2001. p. 113–30.
11. Hanemaaijer R, Visser H, Konttinen YT, Koolwijk P, Verheijen JH. A novel and simple immunocapture assay for determination of gelatinase-B (MMP-9) activities in biological fluids: saliva from patients with Sjogren's syndrome contain increased latent and active gelatinase-B levels. Matrix Biol 1998;17:657–65.
12. Azuma M, Motegi K, Aota K, Hayashi Y, Sato M. Role of cytokines in the destruction of acinar structure in Sjogren's syndrome salivary glands. Lab Invest 1997;77:269–80.
13. Wu AJ, Lafrenie RM, Park C, Apinhasmit W, Chen ZJ, Birkedal-Hansen H, et al. Modulation of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) by interferon-γ in a human salivary gland cell line. J Cell Physiol 1997;171:117–24.
14. Fox PC, Brennan M, di Sun P. Cytokine expression in human labial minor salivary gland epithelial cells in health and disease. Arch Oral Biol 1999;44 Suppl 1:S49–52.
15. Matrisian LM. Matrix metalloproteinase gene expression. Ann N Y Acad Sci 1994;732:42–50.
16. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al. Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002;61:554–8.
17. Chisholm DM, Mason DK. Labial salivary gland biopsy in Sjogren's disease. J Clin Pathol 1968;21:656–60.
18. Daniels TE. Labial salivary gland biopsy in Sjögren's syndrome: assessment as a diagnostic criterion in 362 suspected cases. Arthritis Rheum 1984;27:147–56.
19. Kettlun AM, Cartier L, Garcia L, Collados L, Vasquez F, Ramirez E, et al. TIMPs and MMPs expression in CSF from patients with TSP/HAM. Life Sci. 2003;72:2863–76.
20. Azuma M, Aota K, Tamatani T, Motegi K, Yamashita T, Ashida Y, et al. Suppression of tumor necrosis factor α–induced matrix metalloproteinase 9 production in human salivary gland acinar cells by cepharanthine occurs via down-regulation of nuclear factor κB: a possible therapeutic agent for preventing the destruc-

tion of the acinar structure in the salivary glands of Sjögren's syndrome patients. Arthritis Rheum 2002;46:1585–94.

21. Konttinen YT, Halinen S, Hanemaaijer R, Sorsa T, Hietanen J, Ceponis A, et al. Matrix metalloproteinase (MMP)-9 type IV collagenase/gelatinase implicated in the pathogenesis of Sjogren's syndrome. Matrix Biol 1998;17:335–47.

22. Ogata Y, Itoh Y, Nagase H. Steps involved in activation of the pro-matrix metalloproteinase 9 (progelatinase B)-tissue inhibitor of metalloproteinases-1 complex by 4-aminophenylmercuric acetate and proteinases. J Biol Chem 1995;270:18506–11.

23. Sorsa T, Salo T, Koivunen E, Tyynela J, Konttinen YT, Bergmann U, et al. Activation of type IV procollagenases by human tumor-associated trypsin-2. J Biol Chem 1997;272:21067–74.

24. Asatsuma M, Ito S, Watanabe M, Takeishi H, Nomura S, Wada Y, et al. Increase in the ratio of matrix metalloproteinase-9 to tissue inhibitor of metalloproteinase-1 in saliva from patients with primary Sjogren's syndrome. Clin Chim Acta 2004;345:99–104.

25. Ma Z, Qin H, Benveniste EN. Transcriptional suppression of matrix metalloproteinase-9 gene expression by IFN-γ and IFN-β: critical role of STAT-1α. J Immunol 2001;167:5150–9.

26. Ma Z, Shah RC, Chang MJ, Benveniste EN. Coordination of cell signaling, chromatin remodeling, histone modifications, and regu-lator recruitment in human matrix metalloproteinase 9 gene transcription. Mol Cell Biol 2004;24:5496–509.

27. Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T. Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem 2003;253:269–85.

28. Fini ME, Cook JF, Mohan R, Brinckerhoff CE. Regulation of matrix metalloproteinase gene expression. In: Parks WC, Mecham RP, editors. Matrix metalloproteinases. London: Academic Press; 1998. p. 300–56.

29. Vincenti MP, Brinckerhoff CE. Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors. Arthritis Res 2002;4:157–64.

30. Vincenti MP. The matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) genes: transcriptional and posttranscriptional regulation, signal transduction and cell-type-specific expression. Methods Mol Biol 2001;151:121–48.

31. Akool el-S, Kleinert H, Hamada FM, Abdelwahab MH, Forstermann U, Pfeilschifter J, et al. Nitric oxide increases the decay of matrix metalloproteinase 9 mRNA by inhibiting the expression of mRNA-stabilizing factor HuR. Mol Cell Biol 2003;23:4901–16.