Abstract

**Background**—Excess tissue matrix accumulates in systemic sclerosis (SSc), accounting for both visceral and dermal fibrosis. It is suggested that decreased serum levels of matrix metalloproteinases (MMPs) or increased levels of tissue inhibitors of matrix metalloproteinases (TIMPs) may account for this matrix accumulation.

**Objective**—To measure serum levels of tissue inhibitors of metalloproteinases, TIMP-1, TIMP-2, and collagenase-1 (MMP-1), in patients with diffuse cutaneous systemic sclerosis (dcSSc), limited cutaneous systemic sclerosis (lcSSc), primary Raynaud’s phenomenon (RP), and in normal controls.

**Methods**—Serum samples from patients with dcSSc (n=83), lcSSc (n=87), RP (n=50), and normal controls (n=98) were analysed using enzyme linked immunosorbent assays (ELISAs) for total TIMP-1, TIMP-2, and MMP-1. Results from each assay were analysed by the Kruskal-Wallis test. Dunn’s multiple comparison post-test was then applied between groups.

**Results**—TIMP-1 levels were significantly raised in dcSSc and lcSSc groups compared with the RP group and normal controls (p<0.01 to p<0.001). In the dcSSc group, TIMP-1 levels were significantly higher in early disease (<2 years) than in late stage disease (>4 years) (p<0.05). This was not found for the lcSSc group. Serum TIMP-2 and MMP-1 levels in dcSSc and lcSSc did not differ significantly from those in normal controls. Increased levels of TIMPs were not convincingly associated with organ disease. No assay result correlated with autoantibody status (anti-topoisomerase 1 (anti-Scl-70), anticentromere antibody, or anti-RNA polymerase). No significant differences in serum TIMP-1, TIMP-2, or MMP-1 levels were shown in the RP group compared with normal controls.

**Conclusions**—Raised TIMP-1 levels in the SSc groups support the hypothesis that matrix accumulation occurs in SSc at least in part owing to decreased degradation. Moreover, the variation in TIMP-1 levels between the early and late disease stages of dcSSc seems to reflect the early progressive course of dermal fibrosis seen clinically. The expected reduction in serum MMP-1 levels in the SSc groups was not found. This suggests that tissue matrix accumulation is due to increased inhibitors rather than to decreased MMPs. (Ann Rheum Dis 2001;60:846–851)

Fibroblasts play an important part in the metabolism of extracellular matrix and connective tissues within the skin. They produce enzymes that belong to a family of zinc dependent endopeptidases—matrix metalloproteinases (MMPs), which collectively can digest all matrix components. The first enzyme in this family—interstitial collagenase-1 (MMP-1), has been widely studied and can degrade fibrillar collagen at neutral pH. Fibroblasts also produce specific inhibitors of MMPs, known as tissue inhibitors of matrix metalloproteinases (TIMPs). These act locally specifically to block active MMPs and prevent matrix digestion. Four TIMPs have been described; TIMP-1 and TIMP-2 have been the most widely studied and are produced in soluble forms. TIMP-1 is enhanced by growth factors—for example, transforming growth factor β, whereas TIMP-2 is thought to be constitutively expressed. TIMP-3 is associated with the extracellular matrix and its expression seems to be inducible.1 It is widely believed that the balance between MMP and TIMP levels governs connective tissue homeostasis.

Systemic sclerosis (SSc) is a multisystem disease affecting the skin, dermal blood vessels, and internal organs. It is characterised pathologically by the overproduction of connective tissue—notably, collagen.2 It embraces a clinical spectrum, which is divided into diffuse cutaneous systemic sclerosis (dcSSc): widespread skin thickening occurs, and limited cutaneous systemic sclerosis (lcSSc): skin thickening is limited to the face and distal extremities. Lung disease is common in dcSSc and is often complicated by pulmonary fibrosis in the presence of anti-topoisomerase 1 (anti-Scl-70).

Raynaud’s phenomenon (RP) is an episodic event characterised by pallor, cyanosis, suffocation and/or pain in the peripheries in response to cold stress. It occurs as an initial complaint in 90% of patients with SSc;3 though most patients who have RP (3–10% of the adult population)4 do not have SSc or any associated connective tissue disease, but have primary RP. Excess matrix/collagen deposition does not occur in primary RP.
Timp-1, Timp-2, and Mmp-1 in systemic sclerosis and Raynaud’s phenomenon

In both forms of SSc the observed excess of collagenous extracellular matrix seems to occur as a result of both increased synthesis and decreased degradation of matrix components. Increased collagen mRNA expression has been demonstrated by in situ hybridisation in affected skin, and increased collagen production occurs in cultured SSc fibroblasts. Cultured SSc fibroblasts also show decreased amounts of matrix-degrading collagenase-1 (MMP-1) both with mRNA expression and enzyme production. Stromelysin (MMP-3) production, another member of the MMP family, is also decreased in SSc fibroblasts in comparison with controls. Moreover, local MMP inhibitors—TIMPs—are increased—that is, TIMP-1, TIMP-2, and also TIMP-3, whose mRNA expression is raised in scleroderma fibroblasts both in culture and by in situ hybridisation.

It is suggested that the pathological matrix accumulation that occurs in SSc partly results from excess levels of TIMPs, or decreased levels of MMPs. This study tests this hypothesis by examining serum levels of these moieties in dCSSc, lCSSc, RP, and normal controls. As far as we know this is the first study to look at serum MMP-1 levels, and the first to assay both serum TIMP-1 and TIMP-2 levels in patients with SSc and RP.

Methods

Patient classification

Patients attending the scleroderma unit at the Royal Free Hospital, London, who fulfilled the American College of Rheumatology preliminary criteria for SSc were divided into dCSSc and lCSSc groups. Other patients were classified as having primary RP without evidence of SSC. The dCSSc, lCSSc, RP, and normal groups comprised 83, 87, and 80 patients, respectively. A further group of 98 controls were obtained from healthy blood donors.

Sample collection

Whole blood was collected during routine outpatient procedures. Samples were centrifuged within three hours of collection at 10 000 rpm for 10 minutes. Serum samples were then split into 2 ml samples and stored frozen at −70°C.

Materials and equipment

 Peroxidase conjugated donkey antisheep IgG was obtained from Jackson ImmunoResearch Laboratories, West Grove, PA 19390; normal mouse serum from Dako A/S, 42.DK-2600 Glostrup, Denmark; fetal calf serum (FCS) from Gibco, Paisley, Scotland; protease free bovine serum albumin (BSA) from Sigma, Steinheim Germany; and o-phenylenediamine (OPD) buffer capsules and tablets from Sigma. Anti-TIMP-1 coating and detecting antibody were supplied by Dr Gillian Murphy, University of East Anglia, UK. Coating antibody MAC93 and sheep anti-TIMP-2 antibody were as previously described. 96 well plates were read on a Dynatech Laboratories microplate reader and a Labsystems plate reader.

Total TIMP-1 assay

An enzyme linked immunosorbent assay (ELISA) detecting total TIMP-1, including free TIMP-1 and TIMP-1 bound to MMPs, was performed using a method that has previously been reported. Sensitivity of detection of the assay is 1.4 ng/ml and the inter- and intra-assay coefficients of variation are 10.4–13.7% and 8.8–9.7%, respectively. There is no cross reactivity of this assay with TIMP-2.

Total TIMP-2 assay

The TIMP-2 ELISA was prepared using antibodies supplied by Dr Gillian Murphy, University of East Anglia. Sensitivity of this method is ~2 ng/ml, with a between-assay precision of 10.6% CV.

Nunc Immuno Maxisorb microtitre plates were coated with 200 ml/well 2 mg/ml MAC 93 in 50 mM bicarbonate buffer, pH 9.6, covered with a plate sealer, and left overnight at room temperature. The following morning the antibody was aspirated and the plates then blocked with 250 ml 0.2% protease free BSA in phosphate buffered saline (PBS). The blocking buffer was aspirated after a few minutes and a further 250 ml added to each well. Plates were covered with plate seals and left for a minimum of one hour at room temperature, then stored at 4°C.

Standard concentrations were prepared in advance and stored frozen in aliquots at −70°C. Standards were diluted in base buffer from a 180 mg/ml stock TIMP-2, giving final concentrations of 351, 234, 117, 78, 50, 26, 8.7 ng/ml TIMP-2. Once thawed, standards were kept at 4°C for a maximum of one week.

Table 1  Serum TIMP-1, TIMP-2, and MMP-1 levels in patients with diffuse cutaneous systemic sclerosis (dCSSc), limited cutaneous SSc (lCSSc), Raynaud’s phenomenon (RP), and in normal controls

<table>
<thead>
<tr>
<th></th>
<th>dCSSc</th>
<th>lCSSc</th>
<th>RP</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP-1</td>
<td>403.5</td>
<td>353.8</td>
<td>305.3</td>
<td>275.0</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>(314.0–517.1)</td>
<td>(312.4–469.2)</td>
<td>(259.4–396.4)</td>
<td>(229.5–348.0)</td>
</tr>
<tr>
<td>Number</td>
<td>83</td>
<td>87</td>
<td>80</td>
<td>98</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>187.0</td>
<td>177.0</td>
<td>141.5</td>
<td>165.5</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>(145.0–208.0)</td>
<td>(138.0–193.0)</td>
<td>(113.5–178.5)</td>
<td>(141.5–190.5)</td>
</tr>
<tr>
<td>Number</td>
<td>83</td>
<td>87</td>
<td>80</td>
<td>92</td>
</tr>
<tr>
<td>MMP-1*</td>
<td>3.530</td>
<td>3.010</td>
<td>2.390</td>
<td>2.380</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>(1.455–7.275)</td>
<td>(1.505–7.060)</td>
<td>(1.000–4.010)</td>
<td>(1.640–3.780)</td>
</tr>
<tr>
<td>Number</td>
<td>82</td>
<td>87</td>
<td>77</td>
<td>97</td>
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</tbody>
</table>

*MMP-1 assay results shown were analysed after assigning a minimum assay result of 1 ng/ml.
Blocked ELISA plates were washed three times with base buffer. Sample or standard (10 ml) was added to each well followed by 200 ml/well of assay buffer (base buffer plus 1% normal mouse serum, 10% FCS, 1% protease free BSA). The plates were left overnight at 4°C, washed with the base buffer, and 200 ml/well, 10 mg/ml sheep antihuman-TIMP-2 antibody added. After one hour at room temperature, plates were washed and developed using 200 ml/well OPD. The reaction was stopped with 50 ml/well 2 M H2SO4 and the absorbance read at 492 nm with a Labsystems plate reader.

Figure 1 Serum TIMP-1 level in diffuse cutaneous systemic sclerosis (dcSSc), limited cutaneous systemic sclerosis (lcSSc), Raynaud’s phenomenon (RP), and normal groups. Box plots with upper and lower bars showing the data range, and upper, middle, and lower lines in the box showing 75th, 50th (median), and 25th centiles respectively. Kruskal-Wallis test across all four groups p<0.0001. Only significant Dunn’s multiple comparison post-test p values between groups shown.

Figure 2 Serum TIMP-2 level in diffuse cutaneous systemic sclerosis (dcSSc), limited cutaneous systemic sclerosis (lcSSc), Raynaud’s phenomenon (RP), and normal groups. Box plots with upper and lower bars showing the data range, and upper, middle, and lower lines in the box showing 75th, 50th (median), and 25th centiles respectively. Kruskal-Wallis test across all four groups p<0.0001. Only significant Dunn’s multiple comparison post-test p values between groups shown.

TOTAL COLLAGENASE-1 MMP-1 ASSAY
The ELISA used to detect collagenase-1, measures both proenzyme and active enzyme, as well as enzyme in complex with TIMP. The limit of detection of this assay is 5 ng/ml. The technique used has been slightly modified from that previously published.16 In this study, plates were coated with 2 mg/ml RRU-CL1 antibody (not 1 mg/ml) and samples were diluted fourfold in protein diluent (PBS containing 0.05% Tween 20 and 0.5 mg/ml BSA). Of the results obtained by this assay in this study, 18% were below the lowest standard used in the ELISA of 5 ng/ml, but above background. For consistency and comparability between results such values were assigned a minimum value of 1 ng/ml.

STATISTICAL ANALYSIS
Statistical analysis was performed using GraphPad Prism 2.01. The ELISA data did not fit a Gaussian distribution despite attempts at log transformation. Non-parametric tests were therefore used. To avoid errors inherent in repeated application of Mann-Whitney U tests, the Kruskal-Wallis test was performed. The Kruskal-Wallis test was used to make simultaneous comparison of the assay data from each of the four groups of subjects, to determine if there was significant variation in the medians of the groups analysed. If this achieved 95% significance, Dunn’s multiple comparison post-test was then used to compare the assay results of one group with another. As previously mentioned, 18% of the MMP-1 assay results were assigned the minimum assay value of 1 ng/ml. Because of this, any significant differences observed in the MMP-1 assay results...
were further confirmed by $\chi^2$ analysis; categorising the data as detectable or undetectable.

The demographic data from each of the four subject groups were analysed with Student’s $t$ test or Spearman’s rank correlation where appropriate.

**Results**

Table 1 and figs 1–3 show the results from the TIMP-1, TIMP-2, and MMP-1 ELISAs. The results from the TIMP-1 assay demonstrate that serum levels in the dcSSc and lcSSc groups were significantly higher than in the RP group and in normal controls. The TIMP-1 levels in the dcSSc group were higher than in the lcSSc group; however, this did not reach statistical significance. The RP group had higher TIMP-1 levels than the normal control group, but again, this observation was not statistically significant. Serum TIMP-2 levels in the dcSSc and lcSSc groups differed significantly from the RP group but did not differ from the normal controls. The RP group had lower TIMP-2 levels than the controls, though this was not statistically significant. Serum MMP-1 levels did not differ significantly between either of the SSc groups or between SSc groups and normal controls. There were significantly higher MMP-1 levels in SSc groups than in the RP group. However, MMP-1 levels in the RP group were not significantly different from normal controls.

**ASSAY RESULTS AND DISEASE DURATION**

Significant correlations between serum TIMP-1 and MMP-1 levels and disease duration were found in both SSc groups. For the dcSSc group, the correlation between disease duration and serum TIMP-1 gave an $r$ of $-0.33$ ($p<0.005$), and for MMP-1 $r=-0.24$ ($p<0.05$). For the lcSSc group the correlation between disease duration and TIMP-1 gave an $r=0.30$ ($p<0.01$), and for MMP-1 $r=0.22$ ($p<0.05$). When the patients with dcSSc were split into groups with early disease (<2 years) and late disease (>4 years) (as has been done elsewhere), a significant drop in serum TIMP-1 levels in late compared with early disease was seen (table 2 and fig 4). No similar relation was found for the lcSSc group.

**ASSAY RESULTS, ANTIBODY STATUS, AND VISCERAL ORGAN INVOLVEMENT**

Assay results from patients with SSc with or without internal organ damage were compared (data not shown). For the lcSSc group, it appears that those with any organ involvement have significantly raised serum TIMP-1 levels ($p<0.01$), whereas those with renal disease have increased TIMP-2 levels ($p<0.05$).

No significant relationship was found between TIMP-1, TIMP-2, or MMP-1 assay
results and the presence or absence of antitopoisomerase 1 antibody (anti-Scl-70), anticentromere antibody, or anti-RNA polymerase antibody status.

GROUP DEMOGRAPHY

Table 3 shows the demographic and clinical data for each of the four groups. The four groups differed significantly in sex and age. An examination of the effect of sex on TIMP-1, TIMP-2, and MMP-1 serum levels in the controls showed no significant differences between men and women. Similarly, no correlation was found between age and TIMP-1 or TIMP-2, though a weak correlation between age and serum MMP-1 level \((r=-0.35, p=0.03)\) was found in the female controls.

**Discussion**

The raised levels of serum TIMP-1 found in the dcSSc and lcSSc groups support the hypothesis that there is decreased matrix breakdown in SSc and agree with findings from cultured fibroblasts. As might be expected TIMP-1 levels in the RP group were not significantly different from those of normal controls. We did not find any significant difference in TIMP-1 levels between the dcSSc and lcSSc groups as reported in a previous Japanese study by Kikuchi et al. This discrepancy may be due to the small number of patients enrolled in the Japanese study—namely, 22 patients with lcSSc, compared with 87 patients in this study. Other possible differences include the racial origins of the study groups and differences in the TIMP-1 assays used.

Serum TIMP-2 levels in both SSc groups are not significantly raised compared with normal controls. Levels of TIMP-2 in the SSc groups are, however, significantly higher than in the RP group, and although the RP group has decreased levels of TIMP-2 compared with normal controls, this is not statistically significant. Our findings contradict those of Yazawa et al, whose study of 128 Japanese patients with SSc concluded that serum TIMP-2 levels were significantly higher in SSc than in the controls. In this latter study the higher levels of serum TIMP-2 were restricted to a small proportion of the patients with SSc (23%, 29/128) and, possibly, these patients biased the results. Furthermore, Yazawa et al used parametric methods of statistical analysis to compare non-parametric serum TIMP-2 data and this may explain why their findings are different. Our results suggest that the serum TIMP-2 level in patients with SSc is not significantly different from that in controls. This supports findings that TIMP-2 is not regulated and agrees with cultured SSc fibroblast studies, in which TIMP-2 mRNA levels were no different from controls.

Serum MMP-1 levels in this study are low. Similarly, low levels have been reported for serum MMP-1 in other conditions and in controls. These low levels may reflect tissue or cell binding of MMP-1, preventing its release into serum. At the beginning of this study we predicted that MMP-1 levels would be decreased in SSc. However, there is no significant difference in serum MMP-1 levels in either of the SSc groups in comparison with normal controls. Studies in cultured SSc fibroblasts show reduced MMP-1 production on western blot analysis and decreased bioassay activity. Published reports of MMP-1 and cultured SSc fibroblasts, however, are not entirely consistent. One paper showed that cultured fibroblasts from patients with “early stage” SSc (that is, <1 year) demonstrated increased MMP-1 mRNA expression while cultured fibroblasts from patients with “mid-stage” SSc (that is, 2–4 years) had decreased MMP-1 mRNA expression. In any event differences between in vitro and in vivo results are perhaps not surprising as MMP-1 detected in serum may come from connective tissues sources other than dermal fibroblasts. In view of the significantly raised TIMP-1 levels shown, net collagenolytic activity may be determined more by the level of inhibitors present, rather than by reduced MMP production.
For both the SSc groups there is a significant correlation of disease duration with TIMP-1 and MMP-1 assay results (with p values between <0.05 and <0.005, see above). Although the magnitude of r values is low (0.22–0.33), this suggest that these elements vary progressively throughout the disease course. It is also interesting to note that r values are negative for both these moieties in dcSSc, suggesting a decrease over time, but positive in lcSSc, suggesting an increase. One might speculate that this reflects the differential course of dermal fibrosis seen clinically in each of the subtypes of SSc. This is shown further by dividing the dcSSc group into patients with early and late stage disease (table 2, fig 4).

These results agree with TIMP-1 expression data from cultured SSc fibroblasts, and are consistent with clinical observations that early stage dcSSc is characterised by more progressive skin and visceral fibrosis than late stage dcSSc. It is also significant that a similar relation is not apparent for the lcSSc group, as dermal and visceral fibrosis tends to be milder, and varies little between the <2 year and >4 year stages compared here.

Our results do not show any relationship between TIMP-1, TIMP-2, or MMP-1 and anti-topoisomerase 1 (anti-Scl-70), anticientromere antibody, or anti-RNA polymerase antibody status, and there is no relation between our assay results and organ involvement in dcSSc. These results differ from those of Kikuchi et al, who found that serum TIMP-1 levels were associated with anti-Scl-70 positive status and pulmonary disease in Japanese patients with dcSSc. This discrepancy may partly be due to the smaller number of patients in this latter study—that is, 40 patients with dcSSc, compared with 83 patients in this study, as well as both clinical and racial differences in the dcSSc cohorts studied. Such differences are apparent in the higher proportion of patients with dcSSc with anti-Scl-70 antibodies in the Japanese study—namely, 53% (21/40), compared with 19% (16/83) for this study. For the lcSSc group there does appear to be some association between TIMP-1 levels and involvement of any organ, and between TIMP-2 and renal disease. However, in view of the multiple tests involved, despite our efforts to avoid such errors, it is best to view these observations with caution. Further studies are needed to confirm these findings.

Our SSc, RP, and control groups differ in age and sex, but this should not significantly affect the interpretation of our results. We are unable to show a relation between sex and the moieties measured in our normal control group, and equally there is no correlation between age and serum TIMP-1 and TIMP-2 levels. The weak correlation found between serum MMP-1 and age in female controls is unlikely to be a robust finding, and other observers have not found any correlation between serum MMP-1 or TIMP-1 levels and age or sex. This study confirms the importance of serum TIMP-1 in SSc. The role of serum TIMP-2 and MMP-1, however, appears less certain. Although this is only a limited study of the many moieties involved in matrix metabolism, the results suggest that matrix accumulation in SSc is likely to be due to the presence of excess TIMPs rather than decreased MMPs.

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