Plasma TGFβ in systemic sclerosis: a cross-sectional study

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Abstract
Objectives—To determine whether the active 25 kDa form of the fibrogenic cytokine transforming growth factor β (TGFβ) can be detected in plasma from patients with systemic sclerosis and to examine the relationship between plasma TGFβ and clinical markers of disease severity and serum concentrations of the aminoterminal peptide of type III procollagen (PIIINP) (a laboratory marker of the fibrotic process).

Methods—A cross-sectional study was made of 39 patients with systemic sclerosis (11 diffuse and 28 limited), nine patients with primary Raynaud’s disease and 60 healthy controls. TGFβ1 and TGFβ2 were measured by enzyme linked immunosorbent assay (ELISA) (sensitivity 100 pg/ml) and PIIINP by radioimmunoassay.

Results—TGFβ1 was detected in plasma from six of 39 patients with systemic sclerosis but not in any patient with primary Raynaud’s disease or healthy controls. TGFβ2 was not detected in plasma from patients or controls. No clear relationship was demonstrated between TGFβ1, clinical features or PIIINP concentrations.

Conclusions—The 25 kDa form of TGFβ1 can be detected in the plasma of some patients with systemic sclerosis. This provides limited support for the hypothesis that this cytokine plays a role in the pathogenesis of this disease. However, longitudinal studies, particularly in early diffuse disease, are required to clarify the relationship between circulating TGFβ1 and disease activity.

Patients and methods

PATIENTS AND CONTROLS

The study population consisted of 39 patients with systemic sclerosis (all fulfilling the preliminary American Rheumatism Association criteria for diagnosis and classification), with organ involvement defined according to Medsger. Demographic data were as follows:

- **Diffuse systemic sclerosis**: 11 patients (three male, eight female); median age 48 (range 36–53) years.
- **Limited systemic sclerosis**: 28 patients (two male, 26 female); median age 46 (range 29–71) years.

Median disease duration was 8 years (range 9 months to 28 years; only two patients had disease duration less than 2 years). Twenty one patients had evidence of oesophageal involvement, nine had interstitial pulmonary disease, three had cardiac involvement, and two had controlled renal involvement. Two patients with diffuse disease were receiving interferon alfa therapy and two other patients had received interferon therapy in the preceding 12 months. Seven patients were receiving corticosteroids (six less than 10 mg daily and one 30 mg daily). Two patients were being treated, in addition to corticosteroids, with D-penicillamine and one with cyclophosphamide.
Control groups consisted of nine patients with primary Raynaud's disease (two male, seven female; median age 42, range 33–65 years) and 60 healthy volunteers (26 male, 34 female; median age 37, range 23–61 years).

TGFβ1 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

The 25 kDa form of TGFβ1 was measured with a double antibody ELISA using an enhanced chemiluminescence detection system (Amerlite, Kodak UK). MicroFLUOR plates were coated with a monoclonal antibody capable of recognising isofoms 1–3 of TGFβ (Genzyme) at a concentration of 2-5 μg/ml in carbonate/bicarbonate buffer, pH 9.6. After blocking with 0.1% bovine serum albumin-phosphate buffered saline–0.05% Tween, samples were added in duplicate to the plates at one fifth dilution in the blocking buffer and incubated overnight at 4°C. After washing, bound TGFβ1 was detected with a chicken anti-TGFβ1 (R and D Systems) at 2 μg/ml (incubation four hours at 4°C). After further washing, peroxidase conjugated rabbit anti-chicken IgG was added to each well in 1/2000 dilution (two hours, 4°C). After washing, the plates were developed with the Amerlite signal reagent, as directed by the manufacturer, and read on an Amerlite plate reader. A standard curve was included on each plate using doubling dilutions of pure recombinant TGFβ1 (R and D Systems). Data were processed using Graph software (Microsoft). Values for unknowns were read against the standard curve and expressed as pg/ml.

The sensitivity of the assay was 100 pg/ml. The inter-assay coefficient of variation ranged from 4.7% at the mid point of the standard curve to 15% and 8% at the upper and lower ends of the working range, respectively. The intra-assay variation was 4.8%.

Preliminary experiments using normal plasma and serum spiked with recombinant TGFβ1 showed that unacceptable quenching of the Amerlite signal occurred in serum but not plasma. This effect was most marked towards the detection limit of the assay: at 100 pg/ml of added TGFβ1, the chemiluminescence signal obtained from TGFβ1 in assay buffer was reduced by 52% in serum, but by only 15% in plasma. Subsequent assays were therefore performed only on plasma.

The chicken antibody used in this assay detects only the 25 kDa active form of TGFβ1 and not the latent high molecular weight TGFβ (TGFβ bound to a carrier protein) which is present in large amounts in platelets. This has important implications for plasma and serum assay of TGFβ1: assays capable of detecting latent TGFβ would be prone to error because of detection of TGFβ released from platelets during separation, storage, or, in the case of serum, during clotting. Although previous studies have shown that latent TGFβ is present in normal plasma, no TGFβ could be detected in normal plasma using our assay, even when blood was stored unseparated at room temperature for up to six hours. All TGFβ1 assays in this study were performed on plasma obtained from blood collected into potassium EDTA anticoagulant. Samples were centrifuged at 900 g within two hours of collection and plasma stored at −70°C until required for assay.

TGFβ2 ELISA

TGFβ2 was assayed using a commercial ELISA kit (Amersham). Limit of detection using this kit is 62.5 pg/ml. Samples were prepared as for the TGFβ1 assay; like that assay, the TGFβ2 ELISA detects only the 25 kDa form of TGFβ2, but not latent or complexed forms. The control group for the TGFβ2 assay comprised 20 healthy controls randomly selected from the larger control group used in the TGFβ1 study.

PROCOLLAGEN III AMINOPEPTIDE RADIOIMMUNOASSAY

Serum procollagen III aminoacid (PIIINP) was measured by competitive single antibody radioimmunoassay according to published methodology, using the Orion diagnostics PIIINP RIA kit (marketed by Pharmacia in the UK). The antibody used in this assay recognises only the high molecular weight PIIINP fragment released from type III procollagen, and not degradation products of PIIINP. The assay has previously been used to study collagen synthesis in systemic sclerosis. Since normal ranges for this assay are already well characterised, the control group consisted of 17 randomly selected healthy individuals only. The median PIIINP obtained in this group (2.4 μg/ml, range 2.2–4.76 μg/ml) agrees with that provided by the kit manufacturer.

Results

TGFβ1 was detected in plasma from six of 39 patients with systemic sclerosis (median 1550 pg/ml, range 610–2800 pg/ml), but was not detected in patients with primary Raynaud’s disease or healthy controls (fig 1). The probability of detecting TGFβ1 in the plasma from six patients in the systemic sclerosis group is chance, but none in the 60 healthy control subjects, is 0.0029 (Fisher’s exact test).

No clear relationship was detected between the pattern of cutaneous/visceral involvement and the presence of TGFβ1, but two of the
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We have demonstrated (n = 28), Raynaud’s disease (n = 9) and healthy controls (n = 17). Dotted line indicates the median value for each group. Preliminary analysis using the Kruskal-Wallis test suggested subgroup analysis was appropriate. Between group analysis using the Mann-Whitney U test (two-tailed) showed significant differences in median PIIINP between limited systemic sclerosis vs controls (p = 0.0018) and Raynaud’s (p = 0.0045) and diffuse systemic sclerosis vs controls (0.0027) and Raynaud’s (p = 0.0049). Differences between diffuse and limited systemic sclerosis and Raynaud and controls did not achieve statistical significance.

Discussion

We have demonstrated that the biologically active 25 kDa form of TGFβ1 is present in the plasma of a minority of patients with systemic sclerosis. These findings complement recent demonstrations of circulating TGFβ in other disease processes associated with abnormal deposition of fibrous tissue: lung and liver fibrosis after marrow transplantation, chronic vascular rejection of renal allografts and diabetes mellitus (B Coups, P Brenchley, unpublished data). There is, however, no simple relationship between the presence of circulating TGFβ1 and the extent of tissue fibrosis in the patients with systemic sclerosis (although there are indications that TGFβ1 is perhaps more likely to be detected in patients with interstitial lung disease). This superficially argues against a direct role for TGFβ1 in the pathogenesis of tissue sclerosis in this disease, but may reflect difficulties in assessing the fibrotic process in systemic sclerosis; active fibrosis is difficult to detect clinically at a single assessment and laboratory measures such as PIIINP may reflect connective tissue breakdown in addition to de novo collagen synthesis (although collagen breakdown is not increased in systemic sclerosis). It is also possible that active TGFβ1 may be only intermittently present in the circulation in systemic sclerosis; the plasma half life of the active form of TGFβ is approximately three minutes. Active TGFβ is thought to be generated from latent TGFβ at sites of tissue injury by a combination of proteolysis and low pH. Active TGFβ is then rapidly cleared by mechanisms which include binding to α1 macroglobulin, the small proteoglycan, decorin, and possibly soluble TGFβ receptors, followed by clearance via the liver. TGFβ would be detectable in the circulation only if gross overproduction occurred or if clearance mechanisms were impaired. Assay of the latent and complexed forms of TGFβ would be required to clarify this question.

Difficulties in assessing active fibrosis in systemic sclerosis and possible fluctuations in plasma TGFβ imply that the relationship between circulating TGFβ and disease activity will be clarified only by longitudinal studies. The majority of patients in the study reported here had well established disease and many were taking potentially disease modifying and TGFβ suppressing drugs: interferon alfa reduces TGFβ expression in fibrotic liver disease and corticosteroids inhibit transcription of TGFβ genes. The effects of cyclophosphamide and penicillamine on TGFβ production are unknown, although the myelosuppressive action of cyclophosphamide might be expected indirectly to reduce TGFβ production by monocytes. Future prospective studies might usefully concentrate upon the subgroup of patients with early or rapidly progressive diffuse disease.

Circulating TGFβ2 was not detected in plasma from any patient or control. This is perhaps surprising, as there is some evidence to suggest that TGFβ2 may be more important in the pathogenesis of systemic sclerosis than TGFβ1: it has been reported that expression of TGFβ2 mRNA and protein is increased in most scleroderma skin biopsies, whereas TGFβ1 is found in only a minority. It should be noted, however, that tissue expression of TGFβ does not necessarily indicate local biological activity; tissue TGFβ may be substantially in latent form (there is some evidence to suggest that TGFβ may be sequestered in
high concentrations in certain forms of extra-
cellular matrix \(^{28}\)). Tissue analysis of the co-
distribution of TGF\(\beta\), the TGF\(\beta\) latency
associated peptide (cleaved from TGF\(\beta\) to
create active TGF\(\beta\)) and matrix molecules such
as decorin (which binds and inactivates
TGF\(\beta\)) should clarify this question.

The TGF\(\beta\)1 and TGF\(\beta\)2 assays used in this study
have a limit of detection of 60–100 pg/ml. It is possible that significant increases in
TGF\(\beta\) concentration occur in the systemic
sclerosis group at values below this limit;
studies in small numbers of patients using a
receptor binding assay suggested a trend
greater concentrations of TGF\(\beta\) in systemic
sclerosis (between 40 and 130 pg/ml).\(^{32}\) Our
findings are in conflict with those of Higley et al.,\(^{29}\) who reported no significant difference in
plasma concentrations of TGF\(\beta\)1 between
systemic sclerosis patients and controls, using
an ELISA assay with a limit of detection for
the active form of TGF\(\beta\)1 of approximately 1 ng/
ml (10 times less sensitive than the assay used
here). However, they also reported detectable
(>1 ng/ml) TGF\(\beta\)1 in 26% of healthy controls
and 75% of patients with Raynaud’s
phenomenon. The presence of the active form
of TGF\(\beta\)1 in healthy peripheral blood in
centres well within the biologically
active range would not be anticipated from
previous studies.\(^{19,23}\) This perhaps suggests
the possibility that the ELISA assay used by
Higley’s group\(^ {13}\) was not truly specific for
the active 25 kDa form of TGF\(\beta\)1, but perhaps
also detected larger molecular weight forms
of TGF\(\beta\)1 which are present in blood from
healthy subjects in ng/ml concentrations.\(^ {19,21}\)

The cellular origin of the TGF\(\beta\) detected in
this study requires consideration. Previous
studies have emphasised fibroblast TGF\(\beta\)
production; less attention has been given to
the platelet and macrophage, potentially
greater sources of TGF\(\beta\). Platelets contain large
amounts of stored latent TGF\(\beta\).\(^ {30}\) Since
platelet physiology is disturbed in systemic
sclerosis,\(^ {30}\) and endothelial damage (which
potentially upregulates TGF\(\beta\) \(\alpha\) activation)
occur early in the disease,\(^ {31}\) there would seem
to be a strong case for further examination of
platelet TGF\(\beta\)1 storage and release in systemic
sclerosis.

As indicated above, microvascular abnormal-
ities are prominent in systemic sclerosis.\(^ {30,31}\)
Although TGF\(\beta\) has hitherto been considered
only as a mediator of fibrosis, it also has actions
which might contribute to the abnormal
vascular phenomena in systemic sclerosis: it is
known to induce the synthesis of endothelin,\( ^{2}\)
a potent vasoconstrictor implicated in systemic
sclerosis, and to reduce synthesis of nitric
oxide, a potent endothelium derived vaso-
dilator (A Roberts 1993, personal communi-
cation). Recent evidence suggests that reduced
nitric oxide synthesis might contribute to the
vasospasm of Raynaud’s phenomenon.\(^ {34}\)

It is unlikely that TGF\(\beta\) is the only cytokine
involved in mediating tissue sclerosis in
systemic sclerosis. Other fibrogenic cytokines
have been demonstrated in sclerodermatous
tissue; in particular, platelet derived growth
factor (PDGF), the PDGF receptor, and gene
products indicating signalling through the
PDGF receptor have been demonstrated in
sclerodermatous skin.\(^ {34}\) It seems more likely
that, as in normal wound healing, a network of
growth factors controls the deposition of
fibrous tissue. However, it may be that TGF\(\beta\)
plays a central role in this network (analogous
to the central role which may be played by
tumour necrosis factor \(\alpha\) in macrophage
mediated inflammation);\(^ {35}\) neutralisation of
TGF\(\beta\) activity seems to inhibit fibrosis
potently.\(^ {36,37}\) TGF\(\beta\) also has the capacity to
induce its own synthesis.\(^ {38}\) It has been
suggested that an abnormal positive feedback
mechanism for TGF\(\beta\) production might be of
major importance in pathological tissue
fibrosis.\(^ {3}\)

The effects of inhibiting TGF\(\beta\) activity have
potential therapeutic implication in systemic
sclerosis. It has been demonstrated in
laboratory animals that neutralisation of TGF\(\beta\)
activity inhibits tissue fibrosis in the lung,
kidney and skin.\(^ {36,37}\) Inhibition of TGF\(\beta\)
activity in man, using monoclonal antibodies
or analogues of neutralising molecules such as
decorin, may have widespread application in
diseases characterised by pathological fibrosis.

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